Inhibition of Release of Neurotransmitters from Rat Dorsal Root Ganglia by a Novel Conjugate of a Clostridium botulinum Toxin A Endopeptidase Fragment and Erythrina cristagalli Lectin*

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Michael J. Duggan†, Conrad P. Quinn‡, John A. Chaddock§, John R. Purkiss‡, Frances C. G. Alexander‡, Sarah Doward‡, Sarah J. Fooks‡, Lorna M. Friis‡, Yper H. J. Hall‡, Elizabeth R. Kirby‡, Nicola Leeds‡, Hilary J. Moulsdale‡, Anthony Dickenson**, G. Mark Green**, Wahida Rahman**, Rie Suzuki**, Clifford C. Shone‡, and Keith A. Foster‡

From the †Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom and the ‡University College London, University College, Gower Street, London WC1E 6BT, United Kingdom

Clostridial neurotoxins potently and specifically inhibit neurotransmitter release in defined cell types. Here we report that a catalytically active derivative (termed LH N/A) of the type A neurotoxin from Clostridium botulinum has been coupled to a lectin obtained from Erythrina cristagalli to form a novel conjugate. This conjugate exhibits an in vitro selectivity for nociceptive afferents compared with the anatomically adjacent spinal neurons, as assessed using in vivo primary neuronal culture systems to measure inhibition of release of neurotransmitters. Chemical conjugates prepared between E. cristagalli lectin and either natively sourced LH N/A or recombinant LH N/A purified from Escherichia coli are assessed, and equivalence of the recombinant material is demonstrated. Furthermore, the dependence of inhibition of neurotransmitter release on the cleavage of SNAP-25 is demonstrated through the use of an endopeptidase-deficient LH N/A conjugate variant. The duration of action of inhibition of neurotransmitter release by the conjugate in vitro is assessed and is comparable with that observed with Clostridium botulinum neurotoxin. Finally, in vivo electrophysiology shows that these in vitro actions have biological relevance in that sensory transmission from nociceptive afferents through the spinal cord is significantly attenuated. These data demonstrate that the potent endopeptidase activity of clostridial neurotoxins can be selectively retargeted to cells of interest and that inhibition of release of neurotransmitters from a neuronal population of therapeutic relevance to the treatment of pain can be achieved.

The clostridial neurotoxin (CNT)† family includes tetanus toxin (TeNT), produced by Clostridium tetani, and the seven antigenically distinct botulinum neurotoxins produced from strains of Clostridium botulinum (BoNTs). These proteins are responsible for the conditions of tetanus and botulism, respectively, that develop as a direct result of inhibition of Ca2+-dependent neurotransmitter release, a mechanism of action common to all the CNTs. In the case of BoNTs, intoxication of the neuromuscular junction is thought to occur in at least three phases: an initial binding phase, an internalization phase, and finally a neurotransmitter blockade phase (1).

All CNTs have a similar structure and consist of a heavy chain (~100 kDa) covalently joined to a light chain (~50 kDa) by a single disulfide bond. Proteolytic cleavage of the heavy chain of C. botulinum neurotoxin type A (BoNT/A) generates two fragments of ~50 kDa each. The C-terminal domain (Hc) is required for target cell binding, with the N-terminal domain (HN) being proposed to be involved in intracellular membrane translocation (2). Under conditions in which the disulfide bond between the light and heavy chains is maintained, trypsin cleavage results in a 100-kDa species termed LH N/A (light chain plus N-terminal heavy chain domain) representing a catalytically active, non-cell binding, non-toxic derivative of BoNT/A. In addition to obtaining LH N/A from BoNT/A, we have recently reported that LH N/A can be expressed and purified from a heterologous expression host (3).

It is proposed that CNTs bind to their target cell by a combination of specific high affinity binding events, possibly involving more than one ganglioside and glycoprotein component (4). The proposal that BoNT/B binds to synaptotagmin and the gangliosides GT1b and GD1a (5) and that BoNT/A and BoNT/E may also bind to synaptotagmin (6) has supported this concept. Having accomplished the first cell intoxication stage of binding, CNTs require mechanisms to facilitate internalization into, and intracellular routing within, the target cell. Although the definitive mechanisms remain unclear, the role of an acidic compartment has been proposed (7) in common with a number of other bacterial protein toxins (8). It is proposed that it is the role of the Hc domain to facilitate translocation of the endopeptidase into the cytosol, and the successful retargeting of functional LH N/A into model cell lines (9) has excluded the obligatory requirement of the Hc domain for intracellular trafficking mechanisms.

Once the CNT (or fragment) has gained access to the cytosol, the proteolytic light chains specifically hydrolyze key components of the SNARE protein complex (10) required for synaptic vesicle docking, fusion, and neurotransmitter release. In the case of BoNT/A and BoNT/E the substrate is synaptosome-
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EXPERIMENTAL PROCEDURES

Synthesis and Purification of LH\textsubscript{A}ECL Conjugates—Recombinant and “native” (i.e. prepared by trypsin treatment of BoNT/A) LH\textsubscript{A}ECL were prepared as described elsewhere (3). Derivatization of ECL (Sigma; reconstituted to 10 mg/ml in phosphate-buffered saline) and LH\textsubscript{A}ECL (5 mg/ml in phosphate-buffered saline) was based on methodology described previously (9). Briefly, ~2 reactive leaving groups were introduced into LH\textsubscript{A}ECL and a single sulfhydryl group was introduced into ECL, in both cases by reaction with N-succinimidyl-3-(2-pyridyldithio) propionate. Initial fractionalization of the conjugate mixture was performed by size exclusion chromatography (Superose-12, or Superdex G-200 depending on the scale of conjugation). Application of high molecular weight material to immobilized lactose (Sigma) isolated functional conjugate, which was eluted by the addition of 0.3 M lactose. Spectroscopically eluted fractions were dialyzed extensively against phosphate-buffered saline to remove lactose.

Construction and Purification of recLH\textsubscript{A}(A/H227T)—To create an LH\textsubscript{A}ECL variant defective in endopeptidase activity, a single amino acid mutation at position 227 of the light chain sequence was performed by introduction of the codon for tyrosine (TAC) in place of histidine (CAC). Mutagenesis was performed by overlap polymerase chain reaction using a mutagenic primer pair of CATACGCGGCACTACCGGGCTCATCG and CGATGACGGGCGTAGATGAGCTCGTGTG. A single silent SacI site was introduced to aid screening of mutants. The integrity of the entire LH\textsubscript{A}(A/H227T) DNA sequence was confirmed by sequencing. Mutated DNA was transformed into Escherichia coli TG1, and expression and purification procedures followed essentially as described previously (3).

Characterization of LH\textsubscript{A}ECL Conjugates—SDS-PAGE and Western blot analyses were performed by standard protocols (Novex). Assay of the ability of recLH\textsubscript{A}, recLH\textsubscript{A}(A/H227T), and conjugates to cleave SNAP-25 \textit{in vitro} was performed essentially as previously described (23).

In Vitro Primary Neuronal Culture—Primary neuronal cultures of eDRG and eSCN were established using modifications of existing protocols (24–26). Briefly, dorsal root ganglia and spinal cord neurons were harvested from 15-day-old fetal Sprague-Dawley rats. For culture of eDRG, dissociated cells were plated onto 24-well plates coated with Matrigel at a density of 1 × 10\textsuperscript{6} cells/well. One day postplating the cells were treated with 10 μM cytosine β-d-arabinofuranoside for 48 h. Cells were maintained in Dulbecco’s minimal essential medium supplemented with 5% heat-inactivated fetal bovine serum, 5 mM L-glutamine, 0.4% d-glucose, 40 ng/ml corticosterone, 20 ng/ml triiodothyronine, 0.15% (w/v) sodium bicarbonate, and 2% N1 supplement. Cultures were maintained for 3 weeks at 37°C in 95% air/5% CO\textsubscript{2} before addition of test materials.

In the case of eSCN, dissociated cells were plated onto 12-well plates coated with poly-l-lysine at a density of 2 × 10\textsuperscript{6} cells/well. After 1 week, the cells were treated with 35 μg/ml uridine and 15 μg/ml 2-fluoro 5'-deoxyuridine and grown in minimal essential medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 2 mM L-glucose, 40 ng/ml corticosterone, 20 ng/ml triiodothyronine, 0.15% (w/v) sodium bicarbonate, and 2% N1 supplement. Cultures were maintained for 3 weeks at 37°C in 90% air/10% CO\textsubscript{2} before addition of test materials.

In Vitro Assessment of Neurotransmitter Release and SNAP-25 Cleavage—Release of substance P from eDRG was assessed by enzyme-linked immunosorbent assay. Briefly, eDRG cells were washed twice with low potassium-balanced salt solution (BSS: 5 mM KCl, 137 mM NaCl, 1.2 mM MgCl\textsubscript{2}, 5 mM glucose, 0.44 mM KH\textsubscript{2}PO\textsubscript{4}, pH 7.4, 2 mM CaCl\textsubscript{2}). Basal samples were obtained by incubating each well for 5 min with 1 ml of low potassium BSS. After removal of this buffer, the cells were stimulated to release by incubation with 1 ml of high potassium BSS (100 mM free potassium). Diluted samples were assessed for substance P content. Substance P immunoactivity was measured using Substance P Enzyme Immunoassay Kits (Cayman Chemical Company or R&D Systems) according to manufacturers’ instructions. Substance P is expressed in pg/ml relative to a standard substance P curve run in parallel.

Release of glutamate from eDRG was as described previously (27). Briefly, eDRG were exposed to 2–5 μM/ml (1 mM/ml) of \textsuperscript{[3H]}-l-glutamate for 80 min at 37°C, prior to extensive washing in low potassium BSS. Cells were stimulated to release by the addition of 100 associated protein-25 (SNAP-25), whereas the vesicle-associated membrane protein and syntaxin families of proteins are substrates for neurotoxin types B, D, F, G, and type C, respectively (11, 12). It has been demonstrated that cleavage of these components of the SNARE complex by CNTs results in inhibition of transmitter release from a variety of neuronal cell systems. It is also known that formation of the SNARE complex is a universal mechanism of vesicle fusion and secretion, not limited to neuronal cell types. To circumvent the limited availability of the requisite toxin receptor(s) on the target cell of interest, we have previously reported the replacement of the H\textsubscript{C} domain with a variety of ligands and retargeting of the LH\textsubscript{A}ECL fragment into a range of neuronal and non-neuronal cells (9, 13).

In this study we have further developed the technology of retargeting clostridial endopeptidases with a view to endowing the conjugate with an ability to selectively target cells of potential therapeutic interest relative to anatomically and functionally closely related cells. Furthermore, we have investigated aspects of the duration of action of retargeted endopeptidases because a number of reports (14, 15) highlight the significant longevity of action of BoNT/A when used therapeutically. As a model system we have chosen to study the effects of selectively targeting the endopeptidase domain to nociceptive afferents. \textit{In vivo}, the role of nociceptive afferents is to sense noxious stimuli at the periphery and to transmit this information to the central nervous system where it is perceived as pain. Transmission of this signal is dependent on release of a number of neurotransmitters (including glutamate, substance P, and calcitonin gene-related peptide) from synaptic vesicles (16). Though these transmitters are found in the same terminals of small-diameter primary afferents, glutamate is released from a different population of synaptic vesicles to the neuropeptides (substance P and calcitonin gene-related peptide) (17). We have previously reported that release of substance P from a rat embryonic dorsal root ganglia (eDRG) neuronal culture system (an \textit{in vitro} system representative of nociceptive afferents) is sensitive to inhibition by BoNT/A (18), indicating that release of substance P is SNARE-mediated. We theorized that if clostridial endopeptidases could be selectively targeted to the nociceptive afferents in preference to anatomically adjacent neurons inhibition of the transmission of noxious stimuli may be specifically prevented.

In the search for suitable targeting ligands we observed that lectins (non-immunoglobulin proteins that recognize and bind carbohydrates) (19, 20) had the potential to selectively bind to extracellular moieties and thus be used to differentiate between cell types. It has been reported that galactose-containing carbohydrates are selectively present on nociceptive neurons in the central and peripheral nervous system relative to other neurons (21, 22). From these reports, and experiments to identify binding of fluorescent-labeled lectins, we identified Erythrina cristagalli lectin (ECL) as a suitable ligand for selectively targeting LH\textsubscript{A}ECL endopeptidase to nociceptive afferents.

Here we report that LH\textsubscript{A}ECL conjugate binds to, internalizes into, and inhibits stimulated neurotransmitter release from cultured eDRG neuronal cell types in preference to spinal cord neurons. Through the use of an endopeptidase-deficient mutant of LH\textsubscript{A}ECL, in both cases by reaction with N-succinimidyl-3-(2-pyridyldithio)propionate.
mM KCl for 3 min. Released glutamate was identified following separation of L-[3Hglutamate from non-methylated L-[3Hglutamine by ion exchange chromatography (Dowex-1).

Release of transmitter from eSCN was determined essentially as described previously (25). Briefly, eSCN cells were washed with BSS and then loaded with [3H]glucose for 30 min prior to wash and then removal of basal and stimulated (using 56 mM K+ solution) samples. Cells were lysed by addition of 250 μl of 2 M acetic acid/1% trifieroacetate, and a sample was used to determine total counts from which % release could be calculated. The basal, stimulated, and cell lysate readings were determined by liquid scintillation counting of the cleared supernatant. The basal, stimulated, and cell lysate readings were determined by liquid scintillation counting of the cleared supernatant collected from each treatment. Determination of the ratio of cleaved SNAP-25 to uncleaved SNAP-25 in eDRG following exposure to conjugate material was assessed as described previously (28).

In Vivo Electrophysiology Model—Test material was applied intrathecally using methodology previously described (29, 30). Briefly, to assess the acute effects of test material on the responses of spinal cord neurons to stimulation of C- and A-fibers, 100 μl of LH/A-ECL (4.5 μg/ml) was applied to the exposed spinal cord of halothane/nitrous oxide anesthetized rats. Rats were maintained at 37 °C in a state of areflexia under 1.5–1.8% halothane. Extracellular recordings of convergent dorsal horn neurons were made with parylene-coated tungsten electrodes descended through the spinal cord (mean depth of recording neurons was 700 μm from the surface of the cord). The responses of neurons to transcutaneous electrical stimulation (2-ms wide pulses) of the center of the receptive field were recorded. Responses elicited by a train of 16 stimuli at three times the stimulation threshold for C-fibers were quantified and followed for up to 8 h following spinal application of test material.

In a separate group of animals, assessment of electrophysiological response 24 h after application of conjugate was performed by modification of the above procedure. 10 μl of a 4.5 μg/ml solution of LH/A-ECL was applied by intrathecal injection between lumbar sections L4-L5. Animals were allowed to recover, and then analysis of neuronal activity was made at 24 h postapplication, at which time 10 neurons from a single animal were assessed for response to transcutaneous electrical stimulation as described above. Recordings from 10 neurons from an untreated animal were used to establish control response.

Mouse Toxicity—The ability of botulinum toxin and conjugate mate-rial to induce paralysis in mice (20 g) was tested by an intraperitoneal injection of 0.5 ml of test sample in gelatin-phosphate buffer (1%/w/v) Na2HPO4, 0.2%/w/v) gelatin, pH 6.5–6.6, using methodology reported previously (31).

RESULTS

Purification and Characterization of LH/A-ECL Conjugate—To prepare a conjugate capable of selective targeting of eDRG neurons, data were obtained for the binding of a range of fluorescein isothiocyanate-labeled lectins to eDRG and eSCN. Data obtained for the lectin obtained from the seeds of ECL demonstrated the most appropriate pattern of eDRG localization (data not shown).

Conjugation of ECL to the nLH/A (native LH/A) and recLH/A (recombinant LH/A) fragment was performed essentially as previously described for a wheat germ agglutinin-LH/A conjugate (9), resulting in a conjugation efficiency of 15.9 ± 4.3% (mean yield of ~5 mg of conjugate from 8 mg of LH/A and 25 mg of ECL). There were no significant differences between the derivatization rates or final conjugate yield when nLH/A, recLH/A, or recLH/A (H227Y) was used for conjugate synthesis. Purification of the conjugate was achieved by a two-step process, involving size-exclusion chromatography to remove unconjugated ECL followed by affinity chromatography (immobilized lactose) to remove unconjugated LH/A. When analyzed by SDS-PAGE (Fig. 1), conjugated species of ~160 kDa was the major component of the final material, representative of a single LH/A endopeptidase covalently coupled to two ECL monomers. The species of ~160 kDa apparent in lane 6 of Fig. 1 represents ECL monomers that have been liberated from the conjugate in the presence of SDS, thus the final molecular mass of the predominant conjugate species is ~200 kDa. However, it is clear from SDS-PAGE and size exclusion data that there is a range of conjugate species of varying ECL:LH/A ratios present in the final purified mixture. Analysis of the conjugate material by native PAGE is suggestive of predominant conjugate species of 200 and 400 kDa (data not shown).

In addition to the functionality of the ECL domain, as assessed by lactose binding, it was necessary to confirm that the endopeptidase activity of the conjugate was not adversely affected by the derivatization/conjugation process. By utilizing an in vitro SNAP-25 cleavage assay (23), it was possible to demonstrate that the catalytic activity of LH/A was maintained in the context of the conjugate. The concentration of material to result in 50% cleavage of SNAP-25 (EC50) was estimated to be 8.3 ± 1.7 pm, 5.1 ± 1.6 pm, 12.8 ± 7.8 pm, and 7.5 ± 2.2 pm for recLH/A, nLH/A, recLH/A-ECL, and nLH/A-ECL, respectively. It was not possible to estimate an EC50 for recLH/A (H227Y) and recLH/A (H227Y)-ECL due to insufficient cleavage of SNAP-25; however, data indicated that the endopeptidase activity of the recLH/A (H227Y) species was reduced ~300-fold compared with recLH/A. This is in reasonable agreement with the data previously reported for this H227Y mutation in the light chain of BoNT/A (32).

The toxicity of the conjugate compared with BoNT/A was assessed in the standard measure of toxicity for botulinum toxins, the mouse lethality assay. The mouse lethality assay is highly sensitive for BoNT, with a detection limit of only 5 pg (33). Injection of 50 μg of recLH/A-ECL or nLH/A-ECL conjugate did not result in any mouse deaths. Therefore the conjugate material has an improved toxicity profile of the order of 1 × 107, even though full endopeptidase activity is retained. This presumably reflects the differing neuronal selectivity of the two agents.

Inhibition of Neurotransmitter Release in Vitro—In vitro DRG cultures obtained from embryonic rat tissue include neuronal populations representative of primary nociceptive afferents, and by measuring the release of appropriate neurotransmitters, effects of agents on this neuronal population can be assessed. The close proximity of SCN to the DRG in vivo and the fact that SCN are exquisitely sensitive to BoNT holotoxin make the SCN system an appropriate control for the effects of inappropriately targeted endopeptidase. In addition, the outcome of treating both these cell types with BoNT has been reported previously (18, 34).

BoNT/A, nLH/A-ECL, recLH/A-ECL, recLH/A (H227Y)-
Inhibition of Neurotransmitter Release

ECL, and unconjugated control materials were applied to eDRG and eSCN 3 days prior to assay of neurotransmitter release (substance P, and for some assays glutamate, from eDRG; glycine from eSCN). Fig. 2 indicates the comparative effectiveness of nLHN/A-ECL, recLHN/A-ECL, and BoNT in their ability to inhibit release of substance P from eDRG (Fig. 2A) and glycine from eSCN (Fig. 2B). The IC_{50} values for inhibition of substance P were 17.5 ± 5.5 nM (n = 8), 17.5 ± 2.5 nM (n = 12), and 5.6 ± 0.93 pm (n = 4) for eDRG treated with nLHN/A-ECL, recLHN/A-ECL, and BoNT/A, respectively. These data, therefore, confirm the equivalence of the two conjugated ECL products. By comparison, it was not possible to calculate the IC_{50} for nLHN/A-, recLHN/A(H227Y)-, or recLHN/A(H227Y)-ECL-treated cells due to the lack of effect even at high concentration. In all cases, the dose response observed for neurotransmitter release was in good agreement with the cleavage of SNAP-25. For example, cleavage of SNAP-25 (and inhibition of substance P release) from 30 μg/ml nLHN/A-ECL or recLHN/A-ECL-treated eDRG was determined to be 90.2 ± 6.0% (80.9 ± 4.3%) and 74.8 ± 6.5% (76.9 ± 2.2%), respectively.

In addition to the assessment of the inhibition of release of substance P, the effect of ECL-targeted conjugates on the release of the fast neurotransmitter glutamate from eDRG was also determined. Following application of 10 μg/ml nLHN/A-ECL to eDRG for 3 days, 83.3 ± 9.1% (n = 3) inhibition of glutamate release was observed, compared with 11.4 ± 1.7% inhibition by LHN/A alone.

In the eSCN model, the IC_{50} for inhibition of glycine by BoNT/A is 0.03 ± 0.01 pm (n = 3), whereas the IC_{50} for inhibition of glycine release by LH N/A-ECL conjugates could not be calculated due to the low effect (mean inhibition of release of 17.04 ± 2.1% (recLHN/A-ECL) and 40.94 ± 2.36% (nLHN/A-CL) at the maximum concentration used (30 μg/ml)). The ratio of IC_{50} data for inhibition of release for eSCN compared with eDRG neuron for BoNT/A-treated cells is 0.005:1. Due to the low effect of LH N/A-ECL conjugates in the eSCN model, it was not possible to accurately calculate such a ratio for LH N/A-ECL; however, it is estimated to be in the order of at least 6.9:1.

Duration of action of ECL-targeted endopeptidase conjugates in vitro was assessed in the eDRG model. 40 μg/ml nLHN/A-ECL or 40 μg/ml recLHN/A-ECL was applied to eDRG for 16 h prior to removal and assay of substance P release at specific intervals up to 24 days postapplication (Fig. 3). Maximal inhibition of substance P release was achieved after ~10 days (71 and 68.2% for nLHN/A-ECL and recLHN/A-ECL, respectively) with significant effects still observed to the end point of each assay. In a parallel series of experiments, effects of BoNT/A were also maintained to the end point of the assay (data not shown). The data therefore indicate that retargeted LH N/A does retain extended duration of effect in in vitro cell models akin to that of the holotoxin. The data also demonstrate the equivalence of the recombinant LH N/A and native LH N/A in this respect.

In addition to establishing the selectivity and duration of action of LH N/A-ECL, the in vitro cell culture systems have provided data regarding the mechanism of action of the conjugates. In the first instance, use of the endopeptidase-deficient ECL conjugate, recLHN/A(H227Y)-ECL, has confirmed that inhibition of neurotransmitter release is dependent on cleavage of SNAP-25. Second, delivery of the conjugate has been shown to be ECL ligand-mediated because both release and SNAP-25 cleavage were decreased in the presence of increasing ECL ligand. At the maximum concentration of competing ligand assessed, 100-fold molar excess, inhibition of substance P release by LH N/A-ECL was reduced from 62.7 to 11%. Third, none of the conjugates demonstrated a cytotoxic effect toward the cells, even after prolonged exposure.

Inhibition of Neuronal Activity in Vivo—The recording of single dorsal horn neuronal activity provides a powerful means of testing the effects of agents on sensory transmission from peripheral sensory afferents through the spinal cord. Following transcutaneous electrical stimulation above C-fiber thresholds, activity due to Aβ- (non-noxious), Aδ-, and C-fibers (noxious) can be separated on the basis of latency. The responses of the recorded neurons to afferent stimuli involve the release of...
glutamate from Aβ-, Aδ-, and C-fibers and an additional contribution of peptides such as substance P and calcitonin gene-related peptide from C-fibers (possibly also Aβ-fibers). Activation of the N-methyl-D-aspartate receptor for glutamate then causes an increase in excitability of the neurons, which is manifested as the postdischarge (firing over the normal C-fiber-induced level) (30). Analysis of these responses allows the input (a measure of transmission from the presynaptic afferent terminal onto the spinal postsynaptic neuron) and postdischarge (measure of postsynaptic hyperexcitability) to be extracted.

Following application of the conjugate directly onto the exposed spinal cord, nLHN/A-ECL exerted no effect over the first 5 h. Over the next few hours, however, there was a progressive decrease in both the C-fiber-evoked activity and the postdischarge of the recorded neurons suggesting that the conjugate selectively inhibits noxious-evoked activity with a slow onset of action. recLH8/A-ECL had similar effects. This suggests a mode of action at presynaptic sites that is selective for noxious-evoked activity, which was confirmed by the finding that the conjugate reduced the total input into the recorded neuron. Contrary to the relatively clear response to addition of conjugate material, ECL alone had a predominantly facilitatory effect on the C-fiber-evoked response and postdischarge, and again no effect on Aβ-fiber-evoked responses.

A final in vivo study was devised to investigate the potential actions of LH8/A-ECL over a longer time course than was possible in the acute studies. LH8/A-ECL was injected into the intrathecal space of an anesthetized rat at the L4-L5 segment, and the animal was allowed to recover and was then prepared for electrophysiological recordings from this segment 24 h later. A total of 10 randomly selected neurons were recorded in the treated animal, and 10 cells in a normal un.injected animal were used as controls. The responses measured are presented in Fig. 4. After 24 h, data from the 10 neurons recorded indicated reduction in C-fiber-evoked responses; the C-fiber-evoked response was reduced by 31.8%, whereas Aβ-fiber was reduced by only 12.1% and Aδ-fiber remained unchanged. Postdischarge and input were unchanged. Of these 10 neurons when the recordings made from those (5) closest to the zone of the spinal cord corresponding to the site of injection were analyzed, the cells here showed strongly inhibited responses. In this zone, C-fiber activity was reduced by 75.8% and postdischarge by 78.2% as was input (77.8%). The Aδ-fiber-evoked activity was reduced by 42.3%, whereas the Aβ-fiber-evoked responses were unchanged.

**DISCUSSION**

This report represents the first attempt to selectively target the endopeptidase activity of clostridial neurotoxins to specific cell types. Previous reports (9, 13) have outlined the feasibility of endopeptidase retargeting, described some of the properties of such novel conjugates, and proposed that such an approach may be used to generate novel tools. The focus of the data presented in this report, however, has been to engineer a retargeted clostridial endopeptidase conjugate to have selectivity for nociceptive afferent neurons, with little effect on neighbor-

**FIG. 4.** Inhibition of C-fiber function as assessed by in vivo electrophysiology. LH8/A-ECL (10 μl of a 4.5 μg/μl) was injected into the intrathecal space of an anesthetized rat between lumbar sections L4-L5. Animals were allowed to recover, and analysis of neuronal activity was made at 24 h postapplication, at which time 10 neurons from a single animal were assessed for response to transcutaneous electrical stimulation as described under “Experimental Procedures.” Recordings from 10 neurons from an untreated animal were used to establish control response. Data for all 10 neurons (Test {n = 10}) in the experimentally treated animal and selected data for 5 neurons (Test {n = 5}) that were within a strongly inhibited zone, are presented. Data were obtained for C-fiber, Aβ-fiber, Aδ-fiber responses, along with an assessment of postdischarge and input. See “Results” for explanation of terms.
ing neuronal populations. This has obvious potential in applying the technology therapeutically.

An initial aspect of this work was to identify ligands that had greater selectivity for the target primary nociceptive sensory afferents than surrounding cells. From this analysis it was established that lectins that recognized terminal galactosyl residues were one such category of ligand and the lectin ECL was chosen for this model study. When assessed in the eDRG and eSCN in vitro models, the IC50 for BoNT/A-dependent inhibition of transmitter release from eDRG is 187-fold greater than that for inhibition of release from eSCN, whereas the equivalent IC50 ratio for the LH3/A-ECL conjugate has been estimated to be at least 0.15. Thus the conjugate has a projected improvement of over 10^3 compared with BoNT/A in in vivo terms of selectively targeting nociceptive neurons relative to adjacent spinal cord neurons. Utilizing an endopeptidase conjugate containing wheat germ agglutinin, a glucosyl-binding lectin, that has been reported previously (9), the IC50 for inhibition of transmitter release from eDRG and eSCN were determined to be 0.32 ± 0.05 μg/ml and 0.06 ± 0.01 μg/ml, a DRG:SCN ratio of 5.3. Again, this is significantly different from the data described for ECL-based conjugates and confirms that galactosyl lectins are preferred lectin ligands for this application.

As anticipated, the use of an endopeptidase-deficient LH3/A mutant (recLH3/A(H227Y)) confirmed that conjugate-dependent inhibition of neurotransmitter release is a result of SNAP-25 cleavage rather than nonspecific ligand-mediated effects. The equivalence of cleavage and inhibition data further support this.

The majority of the data presented in this report regarding inhibition of neurosecretion from eDRG neuronal cultures is for release of substance P. This is an important neurotransmitter of nociception and is representative of the neuropeptide transmitters that are released by primary nociceptive afferent neurons. However, the observation that LH3/A-ECL is able to inhibit the release of glutamate (also important in nociceptive transmission) from eDRG neuronal cultures as effectively as it does substance P is therefore important as it demonstrates that the conjugate is inhibiting secretion from all the vesicle populations in the target neuron. This ability to block secretion from multiple vesicle populations in a given target neuron is particularly important in situations like nociception, where it is known that multiple neurotransmitters are involved in the sensation of pain. One of the most striking aspects of intoxication with the botulinum neurotoxins is their extended duration of action. In clinical use this results in prolonged relief of symptoms for the patient, with therapeutic benefit often lasting many months. The data obtained from the in vitro eDRG assay clearly demonstrate that the duration of action of conjugates was equivalent, within the limits of the assay, to that seen with the holotoxin. These in vitro results demonstrate that clostridial endopeptidase delivered into a cell by a novel binding ligand retains the property of prolonged inhibition of secretion. This would indicate that the extended duration of action is a property of the endopeptidase activity of the neurotoxin and that it is independent of the cellular delivery mechanism. The results are also supportive of the potential for longevity of effect in vivo. The observation that reduction of C-fiber activity by LH3/A-ECL was observed 24 h after intrathecal application is indicative that the extended duration of action observed in vitro is indeed being realized in vivo.

Though the in vitro data are critical to understanding the mechanism of action of the conjugate, a key observation from this report is that the in vivo electrophysiological data confirmed the in vitro conclusions. Both the recombinant and the native versions of the conjugate showed activity at the level of the dorsal horn of the spinal cord. The marked effects on input and the C-fiber-evoked responses suggest a main site of action for LH3/A-ECL on transmission from the presynaptic peripheral nociceptive sensory neuron. The observations are therefore consistent with LH3/A-ECL inhibiting release of neurotransmitters from the terminals of primary nociceptive afferents. The effects of the conjugate on postdischarge is likely to be indirect due to the reduction in transmitter release secondarily reducing the neuronal excitability. The in vivo results thus show that the conjugate acts to selectively inhibit nociceptive-evoked activity, sparing β-fiber firing which, under normal conditions, conveys non-noxious activity. This is supportive of the view that the selective targeting of the endopeptidase activity of BoNT/A results in selective blocking of primary nociceptive transmission in the spinal cord. The ligand alone had no inhibitory effects on the primary nociceptive afferents, but did modify the electrophysiological response recorded following peripheral afferent stimulation. This is perhaps not surprising given that ECL was selected for its ability to selectively bind to these neurons and is further evidence for its selective interaction with primary nociceptive afferents at the spinal level in vivo. The effects were not consistent with inhibition of neurotransmission, unlike those of the LH3/A-ECL conjugates.

The in vitro and in vivo data described in this report demonstrate that LH3/A-ECL is a novel agent designed to selectively deliver the endopeptidase activity of BoNT/A to primary nociceptive afferent neurons. The retargeted conjugate displays properties in terms of selectivity, duration of action, and lack of cytotoxicity, which are supportive of the ability to produce agents based upon retargeted clostridial neurotoxin endopeptidase with therapeutic potential. Specifically, LH3/A-ECL display properties, both in vitro and in vivo, which are consistent with the application of this approach to the development of novel analgesic agents with extended duration of action.

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