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Retargeted Clostridial Endopeptidases: Inhibition of Nociceptive Neurotransmitter Release In Vitro, and Antinociceptive Activity in In Vivo Models of Pain

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Abstract: Clostridial neurotoxins potently and specifically inhibit neurotransmitter release in defined cell types. Previously reported data have demonstrated that the catalytically active LH_N endopeptidase fragment of botulinum neurotoxin type A (termed LH_N/A) can be retargeted to a range of cell types in vitro to lead to inhibition of secretion of a range of transmitters. Here, we report the synthesis of endopeptidase conjugates with in vitro selectivity for nociceptive afferents compared to spinal neurons. Chemical conjugates prepared between *Erythrina cristagalli* lectin and LH_N/A are assessed in vitro and in in vivo models of pain. 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. The duration of action of inhibition of neurotransmitter release by the conjugate in vitro is also assessed and is comparable to that observed with *Clostridium botulinum* neurotoxin. Selectivity of targeting and therapeutic potential have been confirmed by in vivo electrophysiology studies. Furthermore, the analgesic properties of the conjugate have been assessed in in vivo models of pain and extended duration effects observed. These data provide proof of principle for the concept of retargeted clostridial endopeptidases as novel analgesics. © 2004 Movement Disorder Society

Key words: *Clostridium botulinum*; pain; lectin; antinociception

The clostridial neurotoxin (CNT) family includes tetanus toxin, 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 Ca^{2+}-dependent neurotransmitter release. In the case of BoNTs, intoxication of the neuromuscular junction is thought to occur in at least three phases: an initial binding phase, an internalisation phase, and finally a neurotransmitter blockade phase.[1]

All CNTs have a similar structure and consist of a heavy chain (HC; ~100 kDa) covalently joined to a light chain (LC; ~50 kDa) by a single disulfide bond. The HC consists of two domains of approximately 50 kDa each; the C-terminal domain (H_C) is required for target cell binding, with the N-terminal domain (H_N) being proposed to be involved in intracellular membrane translocation.[2] It has been reported that a 100-kDa species termed LH_N/A (representative of the LC and H_N domains of type A neurotoxin coupled by a disulfide bond) can be prepared by proteolytic cleavage of BoNT/A[3] or purified from a heterologous expression host.[4] We have reported previously the replacement of the H_C domain with a variety of ligands

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and retargeting of the LH_N/A fragment into a range of neuronal and non-neuronal cells.[5,6]

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. As a model system, we have chosen to study the effects of selectively targeting the endopeptidase domain to nociceptive afferents. 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 several transmitters (including glutamate, substance P, and calcitonin gene related peptide) from synaptic vesicles.[7] We reported previously that release of substance P from a rat embryonic dorsal root ganglia (eDRG) neuronal culture system (an in vitro system representative of nociceptive afferents) is sensitive to inhibition by BoNT/A,[8] indicating that release of substance P is SNARE (*s*oluble *N*-ethylmaleimide-sensitive factor-*a*ttachment protein-*re*ceptor) -mediated. We theorised 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. To target nociceptive neurons, it was observed that galactose-containing carbohydrates are selectively present on nociceptive neurons in the central and peripheral nervous system relative to other neurons.[9,10] In addition, our own experiments with fluorescent-labelled lectins identified *Erythrina cristagalli* lectin (ECL) as a suitable ligand for targeting LH_N/A endopeptidase to nociceptive afferents.

Here, we report on the ability of retargeted LH_N/A to achieve cell selective inhibition of secretion, and in vivo efficacy in a variety of pain models. These observations indicate the potential future therapeutic use of retargeted clostridial endopeptidases, and specifically in this instance for the treatment of pain.

MATERIALS AND METHODS

Synthesis, purification, and characterisation of LH_N/A-ECL conjugates LH_N/A-ECL was prepared according to methodology reported elsewhere.[11] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses were performed by standard protocols (Novex). Assessment of the ability of recombinant LH_N/A (*rec*LH_N/A), *rec*$LH_N/A(H_{227}Y)$, and conjugates to cleave SNAP-25 (synaptosomal-associated protein of Mr = 25 kDa) in vitro was performed essentially as previously described.[12]

In Vitro Primary Neuronal Culture

Primary neuronal cultures of eDRG and embryonic spinal cord neuron (eSCN) were established using a previously reported procedure,[11] which is a modification of earlier procedures.[13–15]

In Vitro Assessment of Neurotransmitter Release and SNAP-25 Cleavage

Release of substance P from eDRG was assessed by enzyme-linked immunosorbent assay as described previously.[11] Release of glutamate from eDRG was assessed as described previously.[16] Release of transmitter from eSCN was determined essentially as described previously.[14] Determination of the ratio of cleaved SNAP-25 to uncleaved SNAP-25 in eDRG after exposure to conjugate material was assessed as described previously.[17]

In Vivo Assessment

For assessment of conjugates in an in vivo electrophysiology model, test material was applied intrathecally using methodology previously described.[18,19] For assessment of conjugates in an acute thermal pain model, 5 ml of test material was applied intrathecally using methodology previously described[20] and latencies were assessed as the time taken to elicit hindpaw flick when placed on a metal hot plate at 55°C. A cut-off of 30 seconds was used.

RESULTS

Purification and Characterisation of LH_N/A-ECL Conjugate

Conjugation of ECL to the native LH_N/A (nLH_N/A) and the $recLH_N/A$ fragment was performed essentially as previously described for a wheat germ agglutinin–LH_N/A conjugate.[5] Purification of the conjugate was achieved by a two-step process, involving size-exclusion chromatography to remove unconjugated ECL followed by affinity chromatography (immobilised lactose) to remove unconjugated LH_N/A. The final product is a heterogeneous mixture in which the predominant species has a mass of approximately 220 kDa. The functionality of both the sugar-binding domain and the endopeptidase were confirmed by lactose binding and in vitro SNAP-25 cleavage assay,[12] respectively. Additionally, the mouse lethality assay[21] suggested that the conjugate material has an improved toxicity profile of the order of 1×10^7 compared to BoNT/A.

Inhibition of Neurotransmitter Release In Vitro

Because in vitro DRG cultures include neuronal populations representative of primary nociceptive afferents and that SCN cultures are exquisitely sensitive to BoNT

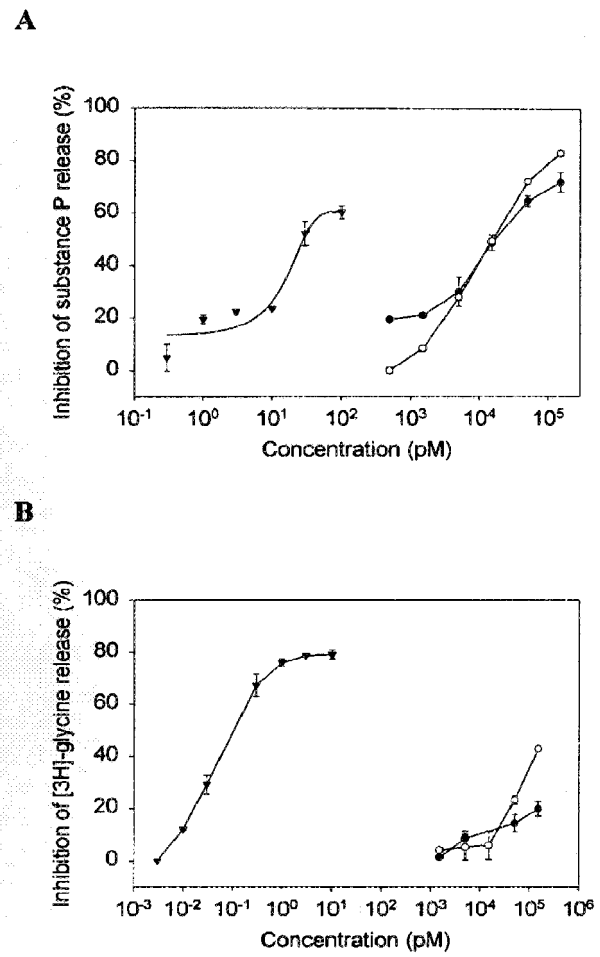

FIG. 1. Inhibition of neurotransmitter release from cultured neuronal cells in vitro. **A,B:** Embryonic dorsal root ganglia (eDRG; A) and spinal cord neuron (SCN; B) were exposed for 3 days to a range of concentrations of nLH_N/A-ECL (open circles), *rec*LH_N/A-ECL (filled circles), and botulinum neurotoxin (triangles). After this time, the ability of the cells to release substance P (eDRG) or glycine (embryonic SCN) was assessed. Results are expressed as percentage inhibition compared with untreated controls. Each concentration was assessed in triplicate, and for each treatment, the dose–response curve is representative of at least three experiments. Each point shown is the mean of at least three determinations ± SEM.

holotoxin, and both these cell types have been shown previously to be susceptible to BoNT,[8,22] the in vitro system represents an ideal screen for efficacy of retargeted endopeptidases.

Figure 1 indicates the comparative effectiveness of nLH_N/A-ECL, *rec*LH_N/A-ECL, and BoNT in their ability to inhibit release of substance P from eDRG (Fig. 1A) and glycine from eSCN (Fig. 1B) after 3 days exposure. The IC_{50}s (concentration causing 50% inhibition) 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 nLH_N/A-ECL, *rec*LH_N/A-ECL, and BoNT/A, respectively. These data, therefore, confirm the equivalence of the two conjugated ECL products. It was not possible to calculate the IC_{50} for LH_N/A-treated cells due to the lack of effect, even at high concentration. In addition, the effect of ECL-targeted conjugates on the release of the fast neurotransmitter glutamate from eDRG was also determined. After application of 10 μg/ml nLH_N/A-ECL to eDRG for 3 days, 83.3 ± 9.1% (n = 3) inhibition of glutamate release was observed, compared to 11.4 ± 1.7% inhibition by LH_N/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. Thus, the ECL-targeted LH_N/A endopeptidase has a different in vitro profile of activity to BoNT/A.

Duration of action of ECL-targeted endopeptidase conjugates in vitro was assessed in the eDRG model. A total of 40 μg/ml nLH_N/A-ECL or 40 μg/ml *rec*LH_N/A-ECL was applied to eDRG for 16 hours before removal and assay of substance P release at specific intervals up to 24 days post-application, at which time significant effects were still observed (Fig. 2). 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.[8]

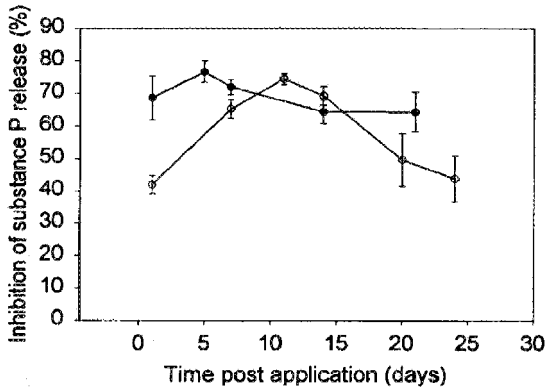

FIG. 2. Inhibition of substance P release from embryonic dorsal root ganglia (eDRG) over extended periods. The eDRG were exposed for 16 hours to 40 μg/ml each of nLH_N/A-ECL (open circles) and *rec*LH_N/A-ECL (filled circles). After this time, the solution was removed, the cells washed, and the medium replaced. The ability of the cells to release substance P over the subsequent 24 days was assessed. Results are expressed as percentage inhibition compared with untreated controls. Each point shown is the mean of at least three determinations ± SEM. The data are representative of three experiments.

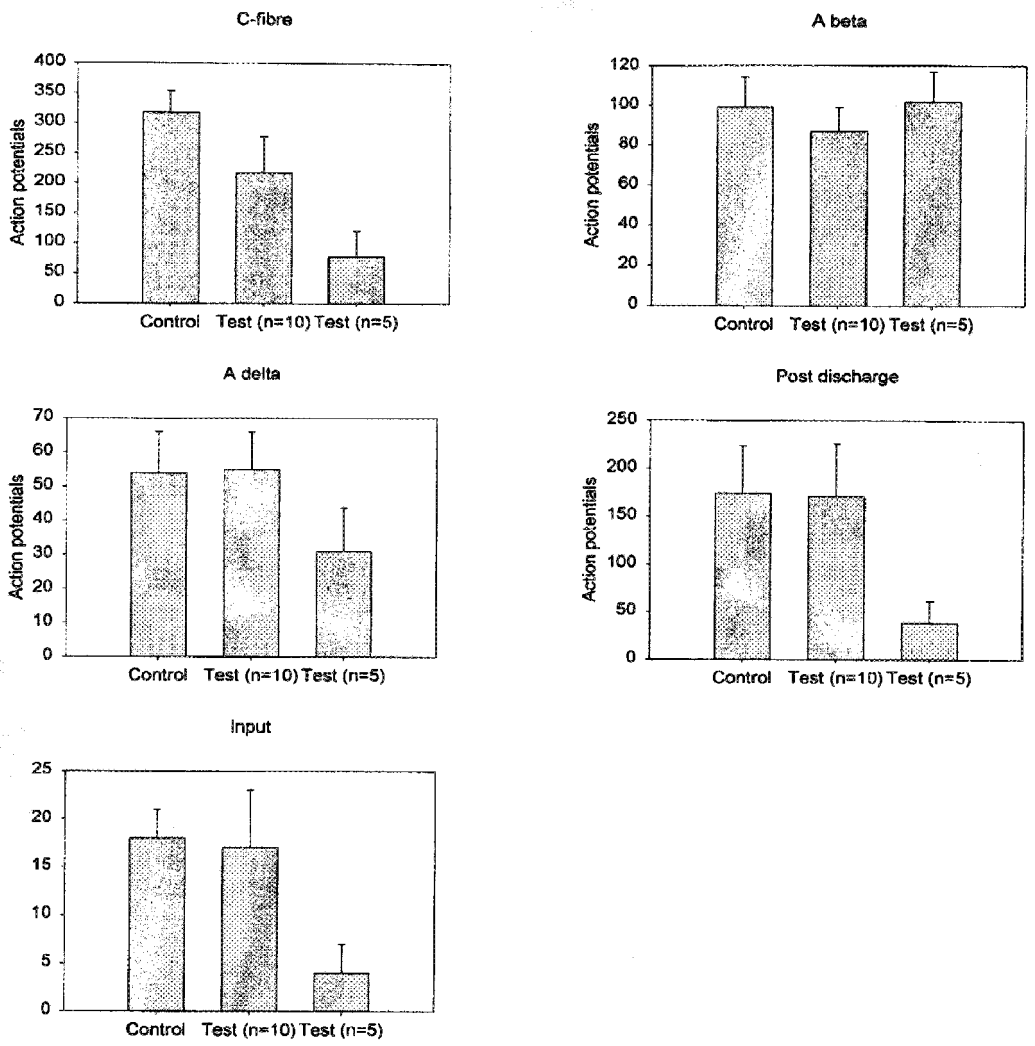

FIG. 3. Inhibition of C-fibre function as assessed by in vivo electrophysiology. LH_N/A-ECL (10 μl of a 4.5 μg/μl solution) was injected into the intrathecal space of an anaesthetised rat between lumbar sections L4 and L5. Animals were allowed to recover, and analysis of neuronal activity was made at 24 hours postapplication, at which time 10 neurons from a single animal were assessed for response to transcutaneous electrical stimulation as described in the Materials and Methods section. 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-fibre, A-β fibre, A-δ fibre responses, along with an assessment of postdischarge and input. See Results section for explanation of terms.

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%.

Inhibition of Neuronal Activity In Vivo

Using methodology previously described[18,19], the recording of single dorsal horn neuronal activity provided a powerful means of testing the effects of agents on sensory transmission from peripheral sensory afferents through the spinal cord. After application of the conjugate directly onto the exposed spinal cord, nLH_N/A-ECL

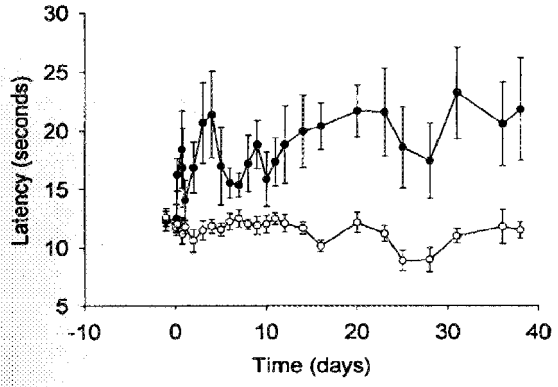

FIG. 4. Antinociceptive effect of LH_N/A-ECL in a model of thermal pain. LH_N/A-ECL (5 μl of a 5.0 μg/ μl solution) was injected into the intrathecal space of an anaesthetised mouse between lumbar sections L1 and L3. Animals were allowed to recover for 2 hours before analysis of paw withdrawal latency. Data represent mean withdrawal latency for *rec*LH_N/A-ECL (filled circles) treated animals (n = 5) and phosphate buffered saline (open circles) control animals (n = 8).

exerted no effect over the first 5 hours. Over the next few hours, however, there was a progressive decrease in C-fibre–evoked activity, suggesting that the conjugate selectively inhibits noxious-evoked activity with a slow onset of action. *rec*LH_N/A-ECL had similar effects, which were not related to the presence of ligand alone. When electrophysiological recordings were made in a zone that had received LH_N/A-ECL 24 hours earlier, inhibition of C-fibre–evoked responses were observed (Fig. 3).

When assessed in a behavioural model of pain, a single application of 25 μg of LH_N/A-ECL into the intrathecal space of a mouse was demonstrated to effect long-term analgesia (Fig. 4). Compared to an intrathecal dose of 1 mg of morphine, which had a duration of action of less than 1 day, the LH_N/A-ECL–dependent increase in the latency of response was maintained at more than 30 days postapplication. The combination of in vivo electrophysiology and behavioural data are strong indicators of the in vivo efficacy of the conjugated material.

DISCUSSION

This work has taken the activity of retargeting clostridial endopeptidases reported previously[5,6] toward a more therapeutic goal. In this instance, the LH_N/A fragment has been selectively targeted to primary nociceptive afferents. Inhibition of release of neurotransmitter is a mechanism of action that is fundamental to nociception and, because it is known that nociception depends on the release of multiple neurotransmitters and that is well-suited to inhibition by a therapeutic that affects general processes such as vesicular release. We have demonstrated that LH_N/A-ECL is able to inhibit the release of both the fast neurotransmitter glutamate in addition to the neuropeptide substance P, and thus confirmed the ability to block release from multiple vesicle populations.

One of the most striking aspects of intoxication with the botulinum neurotoxins is their extended duration of action. In clinical use, this feature results in prolonged relief of symptoms for the patient, with therapeutic benefit often lasting many months. Conversely, classic analgesics such as morphine are highly effective in the short-term, but the antinociceptive effects rapidly decrease such that there is a continual need for resupply of the analgesic. This report has demonstrated that, within the limits of the in vitro eDRG assay, the duration of action of conjugates was equivalent to that of holotoxin. This finding 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. In both the electrophysiology study, and more dramatically in the mouse hot plate model, extended duration of action is observed. Thus, the retargeted endopeptidase molecule has retained the potential to effect vesicular release over extended periods in models of a clinical situation that is currently lacking in methods to effect extended analgesia.

The in vitro and in vivo data described in this report demonstrate that LH_N/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, LH_N/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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