IVth International Symposium on Neurotoxins in Neurobiology

University of Bath, Bath, UK
19th-23rd September, 1993

Meeting Report

IVth International Symposium on Neurotoxins in Neurobiology

Bath, U.K., 19th - 23rd September 1993

The Forth International Symposium on Neurotoxins in Neurobiology was held at the University of Bath, U.K. from September 19th to 23rd. This series of symposia has its origins in the interests of neuroscientists in Latin America in exploring the use of natural neuroactive agents as tools for neuroscience research, and as potential lead compounds for the development of novel pharmaceuticals and agrochemicals. The Latin American origin of the initiative reflects the fact that the South American continent contains the largest areas of tropical vegetation in the world, covering a diversity of plant and animal species. Environmental constraints (terrain, climate etc.) have led to the evolution of organisms with unique complements of specific and selective neuroactive compounds. Latin American scientists dedicated to natural product research can create a bridge between this enormous and largely untapped natural wealth and the international scientific community. This has been attempted through this series of symposia: the latest symposium was the first of the series to be held outside Latin America but was characterised by a strong Latin American presence, both among speakers and participants.

MAMBA TOXINS

The wealth of neuroactive substances found in nature is even greater than the diversity of organisms producing them, since one species may elaborate a variety of neurotoxins. This is illustrated by looking at the venom of the green mamba (*Dendroaspis augusticeps*). This is now well known as the source of α-dendrotoxin (αDTX), a specific blocker of certain

voltage-gated K^+ channels (Ref. 1). In the brain intracerebral injection of αDTX causes convulsions by precipitating neurotransmitter release (GIACINTO BAGETTA, University of Rome). With higher doses, seizures are followed by neuronal loss, implicating the release of glutamate as a possible mediator in this action of αDTX. But NMDA antagonists, used at concentrations that are protective in other models of degeneration, do not prevent this cell loss. Thus αDTX is seen as a novel tool for exploring mechanisms of neuronal degeneration. In addition to αDTX, Dendroaspis venom contains the first natural peptide inhibitor of acetylcholinesterase (AChE) to have been described (FEDERICO DAJAS, Instituto de Investigaciones Biológicas, Montevideo). This peptide, fasciculin, inhibits mammalian AChE with a K_i value of 1nM, whereas micromolar concentrations are required to inhibit avian and invertebrate forms of AChE, and butyryl-cholinesterase. Thus fasciculin displays equisite potency and selectivity, characteristics of so many natural products. Also unique to mamba venoms (thus far) are protein toxins directed against the muscarinic acetylcholine receptor (EVERT KARLSSON, Biomedical Centre, Uppsala; DIANA JERUSALINSKY, University of Buenos Aires). These toxins, from green mamba named MT1....MT4, are potent agonists, with a preference for the M1 subtype of muscarinic receptor. Why should the snake produce this array of toxins targeted at different components of the synapse? Karlsson suggested that inhibition of AChE and K^+ channels, together with activation of muscarinic receptors would provide a synergistic potentiation of cholinergic transmission, except that mamba venom also contains short α-neurotoxins that block nicotinic receptors!

All of these toxins appear to share a common evolutionary origin, and conform to the same basic structure: each consists of about 65 amino acids held together by 4 disulphide bonds. This heterogeneity of structurally homologous toxins poses a problem for the isolation of pure proteins. The way forward is the cloning and expression of single toxin genes to allow purification of the recombinant protein. This has been accomplished for αDTX from black mamba (*D. polylepsis*; LEONARD SMITH, Fort Detrick, Maryland) and for erabutoxin-a, a short chain curaremimetic toxin from the sea snake *Laticauda semifasciata* (ANDRE MENEZ, CEA, Gif-sur-Yvette). The 65 amino acid, four disulphide bond structure is

conserved between species of snakes, and determination of the X-ray crystal structure of the recombinant protein by Menez has shown that the polypeptide has a flat, "three finger" structure. Site directed mutagenesis has identified important residues for nicotinic receptor binding and blockade.

CONUS TOXINS

This theme of functional diversity with structural homology recurred when the conotoxins were discussed (BALDOMERO OLIVERA, University of Utah, Salt Lake City). Each species of *Conus* contains in its venom 50-200 different peptides directed at different macro molecular targets (Ref 2). These include presynaptic Ca^{2+} channel blockers (Ω conotoxins), competitive antagonists of postsynaptic nicotinic receptors (αconotoxins), nicotinic receptor channel blockers (psi-conotoxins), Na^+ channel activators (δ conotoxins), Na^+ channel blockers, selective for channels in skeletal muscle (μ conotoxins), K^+ channel blockers (k conotoxins) and NMDA receptor blockers (conantokins). The conotoxins are small peptides, 10-30 amino acids in length. There is little sequence identity between the various peptides except in the arrangement of cysteine residues (and in the disulphide crosslinks that form as a consequence). Thus, the peptides exhibit a highly conserved structural framework composed of multiple disulphide crosslinks which makes them conformationally rigid. The conantokins, however, are stabilised by an unusual post-translational carboxylation of glutamate residues to give carboxyglutamates, which stabilise an α-helical conformation of these peptides in the presence of Ca^{2+}.

Given that there are some 500 species of cone snails, there are many hundreds of conus toxins yet to be "discovered" (Fig. 1). For example, the mode of action of the "King Kong peptide" (a δ-conotoxin) that makes small lobsters behave as though they were dominant has still to be established but this particular toxin must have great potential in Western society as an assertiveness drug!

ION CHANNEL TOXINS

The consensus among diverse organisms must be that ion channels make the best targets if you want to disable your prey or your enemies. Various scorpion toxins block αDTX-sensitive and Ca^{2+}-activated K^+ channels (ALAN HARVEY, University of Strathclyde, Glasgow), toxins from the tropical sponge *Agelas conifera* act on Na^+ channels (GLADYS ESCALONA DE MOTTA, University of Puerto Rico, Rio Piedras) and polyamine toxins from spiders and wasps target ligand gated ion channels, especially glutamate receptors which mediate neuromuscular transmission in the invertebrate prey of these species (PETER USHERWOOD, University of Nottingham; IAN BLAGBROUGH, University of Bath).

OSVALDO UCHITEL (Facultad de Medicina, Universidad de Buenos Aires) described the application of Ca^{2+}-channel-specific toxins in elucidating which types of Ca^{2+} channel may mediate neurotransmitter release in different preparations. At the mouse neuromuscular junction, funnel web spider toxins (which preferentially block P-type channels) prevent presynaptic calcium currents and transmitter release, whereas the N-type channel blocker Ω conotoxin and the L-type channel-specific drug dihydropyridine had little effect. In contrast both P- and N-type Ca^{2+} channels appear to play a role at the frog neuromuscular junction, based on toxin sensitivities.

CLOSTRIDIAL TOXINS

The most potent neurotoxins known, the botulinum toxins, are directed at a quite different set of targets, whose elucidation in the last year is radically changing our understanding of exocytosis. These bacterial toxins comprise a family of structurally homologous proteins, designated A-G, indicating that they derive from a common ancestor (MICHEL POPOFF, Institut Pasteur, Paris). They consist of a disulphide linked heavy and light chain, and induce a flaccid paralysis by blockade of acetylcholine release at the neuromuscular junction. The structurally related tetanus toxin causes a spastic paralysis because it is retrogradely

transported to the spinal cord where it blocks the release of GABA and glycine. The toxins act via a 3 stage process, analogous to other bacterial toxins: (i) binding, (ii) internalisation, (iii) enzyme action.

OLIVER DOLLY (Imperial College, London) showed that [^{125}I]botulinum toxin type A (BoNT A) binds through its heavy chain to specific ecto-acceptors present on cholinergic terminals. A putative acceptor protein for BoNT A has been cloned from *Torpedo* electric organ (C. SOLSONA, University of Barcelona): the 757 amino acid protein has a single putative membrane spanning segment and no homology with any known protein. The inhibition of GABA and glycine release by tetanus toxin reflects its specificity for a different acceptor, localised on these nerve terminals. Dolly described the appearance of gold particles in endocytic vesicles following incubation of tissue with gold labelled BoNT A. This internalisation of the toxin may involve the ability of the heavy chains to form channels in the membrane of endocytic vesicles (FRANK LEBEDA, USAMRIID, Frederick, Maryland), which could facilitate delivery of the light chain of the toxin to the cytoplasm. The light chain possesses the inhibitory capability and can inhibit the release of all neurotransmitters if it is delivered intracellularly. The light chains have a conserved Zn^{2+} binding motif and they possess endoprotease activity: Zn^{2+}-protease inhibitors (e.g. phosphoamidon) or Zn^{2+}-chelators (ROBERT SHERIDAN, USAMRICD, Maryland) delay the onset of neuromuscular blockade by BoNT. In 1992, the synaptic vesicle protein synaptobrevin was identified as the substrate for BoNT B and tetanus toxin, (Ref. 3) but synaptobrevin is not cleaved by BoNT A. It was very timely that the substrate for BoNT A was reported in Nature (Ref. 4) just 10 days before this meeting: the target for BoNT A appears to be SNAP-25 (synaptosomal associated protein of Mr 25K), a protein associated with the plasma membrane and now directly implicated in exocytosis. Another presynaptic plasma membrane protein, syntaxin, is reputedly the target for BoNT C (Ref. 5): the substrates for these clostridial toxins are illustrated schematically in Fig. 2.

* * *

Thus some very nasty neurotoxins are powerful tools for the molecular dissection of synaptic transmission, providing potent and selective probes for neurotransmitter receptors, ion channels and for the complex molecular machinery of exocytosis. The bounty of Nature is awesome and wonderful! There is no doubt that the field of neurotoxins can provide many rich pickings for numerous symposia to come.

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S. Wonnacott, University of Bath, Bath, U.K.

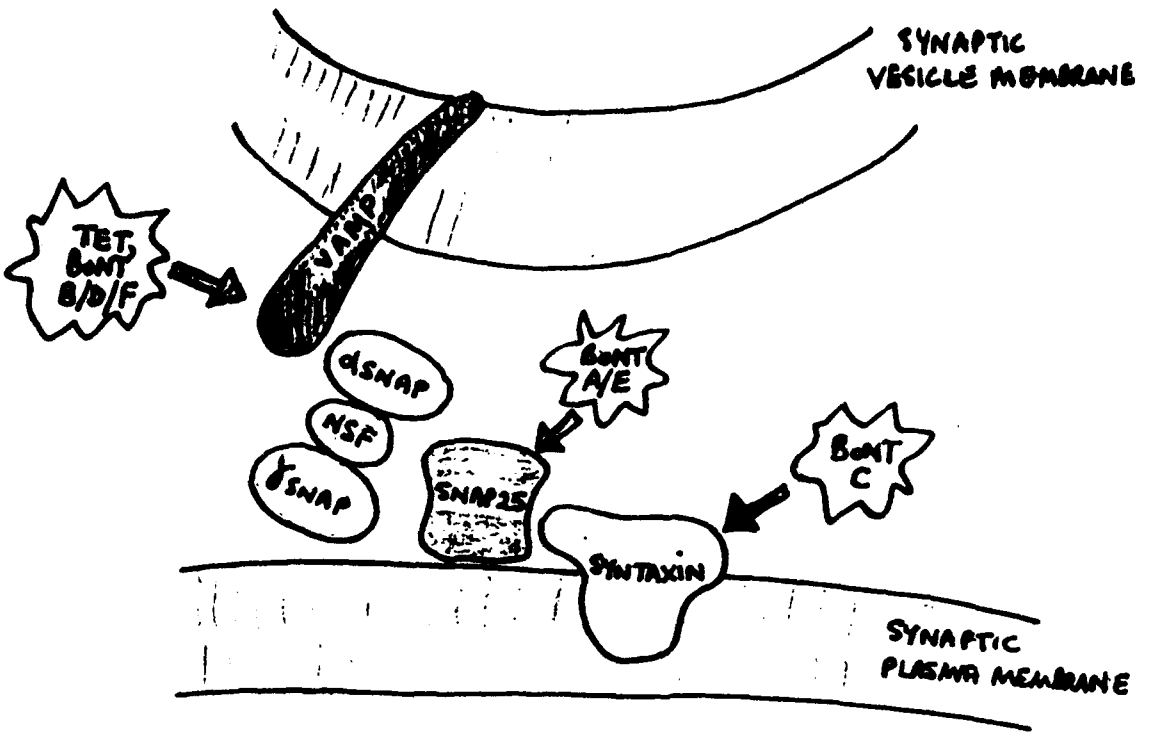
F. Dajas, Instituto de Investigaciones Biológicas, Montevideo, Uruguay.

Figure Legends

Fig. 1. The cone snail pharmacologist (courtesy of B. Olivera).

Fig. 2. Schematic view of the targets for clostridial toxins at the active zone. BoNT A and E selectively cleave SNAP25 (synaptosomal-associated protein M_r 25K)[4], a protein associated with the intracellular face of the plasma membrane. BoNT B, D and F, as well as Tetanus Toxin, target the synaptic vesicle membrane protein VAMP/synaptobrevin[3]. The substrate for BoNT C is reputedly syntaxin[5], an intrinsic protein of the presynaptic plasma membrane implicated in the docking of synaptic vesicles at the active zone. These 3 target proteins have been found in a complex with the soluble proteins NSF (N-ethyl-maleimide-sensitive factor) and α-and γ-SNAPs (soluble NSF attachment proteins); together these proteins may constitute the docking/fusion complex.


Rx


SYNAPTIC
VESICLE MEMBRANE
TET
BoNT
B/D/F
VAMP
αSNAP
NSF
γSNAP
BoNT
A/E
SNAP25
BoNT
C
SYNTAXIN
SYNAPTIC
PLASMA MEMBRANE

IVth International Symposium on Neurotoxins in Neurobiology

Bath, England, 19 - 23 September 1993

Welcome to the IVth International Symposium on Neurotoxins in Neuroscience. This series of Symposia has its origins in the interests of neuroscientists in Latin America in exploring the use of natural neuroactive agents as tools for neuroscience research and as potential lead compounds for the development of novel pharmaceuticals and agrochemicals. In particular, colleagues in Uruguay, Argentina, Brazil and Chile were responsible for planning and organising the previous three Symposia, the last of which was held in Piriapolis, Uruguay, in 1991. This group has grown into the organisation called LANBIO which is concerned with the development of natural neuroactive compounds originating in Latin America and with ensuring that any commercial exploitation of such agents brings benefits to local scientists. LANBIO has provided some support for this meeting. Previous Symposia have been generously supported by the International Foundation for Science (IFS) and the International Programme in Chemical Sciences (IPICS) and both organisations have provided support for this Symposium. In addition, generous financial help has been provided by the European Research Office of the US Army and by the International Society for Neurochemistry (ISN).

Previous Symposia have been characterised by an atmosphere of friendly informality that is conducive to extensive discussion and debate. The organising committee sincerely hopes that this tradition will continue in the IVth Symposium and urges speakers to ensure that ample time is left for thorough discussion of their presentations.

International Organising Committee

F. Dajas	Montevideo - Chairman
O. Dolly	London
A. Harvey	Glasgow
G. Lunt	Bath
J. Medina	Buenos Aires
R. Silveira	Montevideo
K. Tipton	Dublin

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S. Wonnacott	Bath
Giovanna Pinto	Secretarial Support

The IVth International Symposium has received generous support from:

The British Council
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Pfizer Central Research
The Royal Society
Smith Kline Beecham
The Speywood Laboratory
Tocris Neuramin
Wyeth Research (UK) Ltd
Zeneca (ICI Agrochemicals)

Domestic Matters - IMPORTANT - Please read

All sessions take place in Lecture Theatre 5 West 2.1 - this is beyond the banks and shop area of the University and should be signposted NEUROTOXINS

Breakfast, lunch and some dinners are in the Refectory which is on the lowest floor of building 2 WEST - about half way along the main pedestrian walkway adjacent to the steps that lead down to the lake. Again it is well signposted!

POSTERS - these will be on display throughout the meeting in the PHARMACOLOGY LABS along the corridor adjacent to the lecture theatre and directly off the coffee and tea areas.

PLEASE PUT UP YOUR POSTER AS SOON AS YOU CAN - SUNDAY AFTERNOON WOULD BE BEST! Posters should be fixed with Velcro tabs NOT pins - we will provide the tabs.

There is a frequent bus service to Bath - No 18 - goes from Car Park F which is at the opposite end of the University from the lecture theatre - just keep walking along the pedestrian walkway keeping on the right hand side - you cannot miss the bus stop!

Banks on campus close 2.30 pm - banks in town close at 4.30 pm.

Any problems of any kind - look for George Lunt or Ian Blagbrough or Susan Wonnacott.

There are no tickets for any of the meals associated with the meeting, therefore it is important that you wear your badge at all times. If you do not wear a badge there is a strong possibility that the catering staff will be reluctant to serve you!

IVTH INTERNATIONAL SYMPOSIUM: NEUROTOXINS IN NEUROBIOLOGY

Monday 20th Novel Neurotoxins

Chairperson: Professor George G. Lunt

9.00 am Welcome to Bath and to the IVth International Symposium

9.15 am Professor Edson X. Albuquerque, Baltimore
Methyllycaconitine, mecamylamine, dihydro-β-erythroidine and physostigmine: a novel approach to the characterisation of nicotinic receptors.

10.00 am Professor Federico Dajas, Montevideo
Comparative acetylcholinesterase inhibitor potency of fasciculin in the CNS

10.45 am Coffee

11.15 am Professor Giacinto Bagetta, Rome
ζ-Dendrotoxin produces seizures and brain damage in rats

11.45 am Dr. Susan Wonnacott, Bath
Anatoxin-a and methyllycaconitine: novel neurotoxins for SAR studies on nicotinic subtypes

12.30 pm Lunch + Poster viewing

Chairperson: Professor F. Dajas

2.15 pm Dr. Diana A. Jerusalinsky, Buenos Aires
Muscarinic toxins from the venom of dendroaspis snakes with agonist-like actions.

3.00 pm Dr. Alejandro C. Paladini, Buenos Aires
Characteristics and properties of benzodiazepines and other ligands for the central benzodiazepine receptor of common occurrence in natural sources.

3.45 pm Dr. David B. Sattelle, Cambridge
Actions of neurotoxins (bungarotoxins, neosurugatoxin, lophotoxin) on invertebrate nicotinic receptors

4.30 pm **TEA AND POSTER SESSION - authors (of posters 26-38) present**

6.00 - 7.00 pm Dinner followed by a University Reception from the Vice-Chancellor, Professor V. D. VandeLinde.

Tuesday 21st **Toxin Structure, Function and Site of Action**

8.55 am	**Chairperson: Dr. Ian S. Blagbrough**
9.00 am	Professor Evert Karlsson, Uppsala
	Protein toxins against muscarinic acetylcholine receptors
9.45 am	Professor Alan L. Harvey, Glasgow
	Structure-activity studies on scorpion toxins that block potassium channels
10.30 am	Coffee
11.00 am	Professor André Ménez, Gif sur Yvette
	Recognition sites of snake curaremimetic toxins and acetylcholine receptors
11.45 am	Dr. Leonard Smith, Fort Detrick, U.S.A.
	Cloning and expression of mamba toxins
12.30 pm	Lunch + Poster viewing
2.00 pm	Professor P J. Barrantes, Bahia Blanca
	Stable heterologous expression of nicotinic acetylcholine receptor in clonal cell lines: α-bungarotoxin binding and channel gating properties
2.45 pm	Professor Marcus V. Gomez, Belo Horizonte
	Effects of scorpion venom tityustoxin on central nervous system
3.30 pm	Dr. Osvaldo Uchitel, Buenos Aires
	Characterisation of calcium channels involved in transmitter release at the mammalian neuromuscular junction
4.15 pm	**TEA AND POSTER SESSION - authors (of posters 39-50) present**
6.45 pm	Dinner, followed by a tasting of English wines and cheeses

Wednesday 22nd **Neurotoxins affecting transmitter release**

8.25 am	**Chairperson: Dr. Susan Wonnacott**
8.30 am	Professor J. Oliver Dolly, London
	Botulinum and tetanus toxins: multifunctional probes for investigating protein trafficking and neurotransmitter release
9.15 am	Dr. Michel Popoff, Paris
	Organisation of the botulinum neurotoxin complex genes in Clostridium botulinum c and genetic transfer in C. botulinum
10.00 am	Dr. Irina Surkova, London
	Latrotoxin and exocytosis
10.30 am	Coffee
11.15 am	Dr. John Middlebrook, Fort Detrick
	Development of a molecularly engineered vaccine for botulinum toxin
12.00 pm	Dr. Michael Adler, Fort Detrick
	Antagonism of botulinum toxin-induced muscle weakness by 3,4-diamino-pyridine in rat diaphragm preparations
12.20 pm	Dr. Frank Lebeda, Fort Detrick
	Topographic models for the transmembrane channels formed by botulinum toxin
12.40 pm	Dr. Robert Sheridan, Maryland
	Interactions between Zn chelators and botulinum neurotoxins at the neuromuscular junction
1.00 pm	Lunch
2.30 pm	Tours of Bath are available at no extra cost! Options are a bus tour of the City and surroundings - with a guide and commentary or a conducted walking tour of the City's historic sites accompanied by a Mayor's guide. Details will be given on Monday.
6.15 pm	Coach departs from 6 East for Civic Reception.
7.00 pm	Civic Reception, Roman Baths
8.15 pm	Conference Banquet, Bath Guildhall. Including presentation of two Tocris Neuramin poster prizes
10.45 pm	Coach returns to University.

Thursday 23rd **Neurotoxins and ion channels**

8.55 am	**Chairperson: Professor Alan L. Harvey**
9.00 am	Professor Baldomero M. Olivera, Salt Lake City
	Conotoxins: peptide ligands for receptors and ion channels
9.45 am	Professor Keith F. Tipton, Dublin
	Small Neurotoxins: glutamate analogues and nitric oxide
10.30 am	Coffee
11.00 am	Dr. Ian S. Blagbrough, Bath
	New natural products and their potent synthetic analogues: potentiators and antagonists of receptor operated ion channels
11.45 am	Professor Peter N. R. Usherwood, Nottingham
	Site and mode of action of polyamine amides toxins - a 10 year checkup
12.30 pm	Concluding remarks and future Symposia
1.00 pm	Lunch and Final Reminiscences - Delegates depart

Speakers' Abstracts

1 - 25

METHYLLYCACONITINE, MECAMYLAMINE, DIHYDRO-β-ERYTHROIDINE, AND PHYSOSTIGMINE: A NOVEL APPROACH TO THE CHARACTERIZATION OF FUNCTIONAL NICOTINIC ACETYLCHOLINE RECEPTORS (nAChR).

Albuquerque, E.X., Alkondon, M., Pereira, E.F.R., Castro, N.G., and Maelicke, A. Dept Pharmacol. Exp. Ther., Sch. Med. Univ. of Maryland, Baltimore, MD, USA 21201; Lab. Mol. Pharmacol., "Inst. Biofísica Carlos Chagas Filho", Federal Univ. Rio de Janeiro, Rio de Janeiro, RJ, Brazil; Institute of Physiological Chemistry and Pathobiochemistry, Johannes-Gutenberg University Medical School, Duesbergweg 6, Mainz, FRG.

The snake toxin α-bungarotoxin (α-BGT) played a key role in the characterization of muscle and muscle-type nAChRs (1,2). This toxin allowed the isolation and purification of these receptors, thus leading to the amino acid sequencing and molecular cloning of the receptor. It was not until recently that neuronal nAChRs were seen to be more diverse than muscle nAChRs (3). Although biochemical studies have demonstrated the presence of α-BGT binding sites throughout the brain, they also showed that these sites did not overlap those labeled by [^{3}H]nicotine (4). For several years, the pseudo-irreversible nature of receptor block by α-BGT and the slow onset of α-BGT action hampered the correlation in the brain of α-BGT sites with functional neuronal nAChRs. The discovery of methyllycaconitine (MLA), an alkaloid extracted from the seeds of *Delphinium brownii*, was extremely helpful for the characterization of neuronal nAChRs (5). It was shown biochemically that MLA can bind to neuronal nAChRs and that this alkaloid can antagonize the binding of α-BGT to neurons. In addition, it was proven electrophysiologically that hippocampal neurons express α-BGT-sensitive nAChRs and that MLA could block specifically, potently, and reversibly these receptors (6). In cultured hippocampal neurons, the blockade by MLA of nicotinic whole-cell current was found to occur with an IC_{50} of about 150 pM (6). In about 95% of the hippocampal neurons studied, nicotinic responses were sensitive to the competitive antagonist α-BGT or MLA, whereas in 5% the responses were seen to be insensitive to either antagonist (7,8). Instead, some of these responses could be blocked by mecamylamine and other could be blocked more efficiently by dihydro-β-erythroidine (DHβE, 7). Thus, MLA, mecamylamine, and DHβE could be used to reinforce clues from the kinetic properties of the nicotinic currents elicited in hippocampal neurons and the sensitivity of the responses to different agonists to demonstrate that these neurons can express at least three different nAChR types. It is noteworthy that functional expression studies have shown that fast-desensitizing, MLA sensitive nicotinic currents can be evoked in oocytes expressing the α7 neuronal nAChR subunit (9). Also, our recent work on mouse fibroblasts stably expressing the combination of α4 and β2 nAChR subunits has demonstrated that whereas DHβE blocks nicotinic currents evoked in these cells, MLA or α-BGT does not affect these currents (10). Thus, based on the findings obtained in hippocampal neurons and expression systems, it has been suggested that the MLA-sensitive receptor expressed in the neurons bears the α7 subunit, whereas the DHβE-sensitive nAChR may be composed of the subunit combination α4β2 (7). Although muscle nAChRs are well-characterized pharmacologically and physiologically, and the neuronal nAChRs have been the subject of intense research, recent studies have shown the presence on these receptors of a binding site different from that for ACh through which receptor activation may be modulated (11). It has been demonstrated electrophysiologically that physostigmine can activate single-channel currents in isolated frog muscle fibers, cultured hippocampal neurons, and mouse fibroblasts expressing the α4β2 neuronal nAChR (10-15). Although such an agonist effect of physostigmine is unaffected by competitive nicotinic antagonists, it can be blocked by the nAChR-specific antibody FK1 (10,11,13-15). In addition, measurements of physostigmine-activated ion flux into *Torpedo* electroplax vesicles showed that the agonist effect of this carbamate can be observed after the nAChR had been desensitized by high concentrations of ACh (14). Thus, it was suggested that physostigmine activates muscle or neuronal nAChRs via a site that is distinct from that for ACh. By means of indirect immunofluorescence studies using FK1, we have shown that the residue Lys-125 of the nAChR α subunits, which is highly conserved in the receptor α subunits sequenced so far, is an important feature of the physostigmine-binding site (16). It is most likely that an endogenous agent may exist that could be recognized by this site on nAChRs and thereby modulate receptor activation. In fact, in the late 70's the same hypothesis was made based on the findings that the effect of perhydrohistrionicotoxin (H_{12}HTX) on EPCs is dependent upon whether ACh is applied exogenously or released into the synaptic cleft from the nerve terminal (17). Thus, MLA, DHβE, mecamylamine, and physostigmine consitute a group of pharmacological tools that will certainly open new avenues to increase our understanding of the properties of nAChRs and the involvement of these receptors in physiological and pathological conditions.

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COMPARATIVE ACETYLCHOLINESTERASE INHIBITOR POTENCY OF FASCICULIN IN THE CENTRAL NERVOUS SYSTEM

Federico Dajas

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Fasciculin (FAS), was the first natural peptide inhibitor of acetylcholinesterase (AChE) described. At present, the value of FAS lies on its use as a tool to gain knowledge of the AChE enzyme and to define AChE functions in different nervous cholinergic structures, mainly in the central nervous system (CNS).
Chemical modifications of the AChE molecule and testing of the remaining FAS inhibitory capacity is a way to provide clues about the site of FAS interaction with the enzyme. This approach , combined with the study of the comparative inhibitory profile of FAS and other AChE inhibitors, has confirmed that FAS acts on the peripheral anionic site of the enzyme.
FAS shows a unique pharmacological profile in the CNS, specific for different cholinergic nuclei. A comparative study of the inhibitory capacity of FAS and other AChE inhibitors undertaken in the rat striatum, in vivo and in vitro, showed that, in vitro, FAS is a hundred times more powerful than BW-284c51 (BW) in equimolar doses. In vivo, FAS is three and two times more potent than physostigmine and BW, respectively. The study of the cholinergic control of dopamine release by microdialysis and push-pull cannulae procedures have shown that changes in AChE levels of activity do not modify the release of dopamine in the striatum with a wide range of AChE inhibition (35-95%) and inhibitors (Paroxon, BW, FAS). It is concluded that the effects on the AChE inhibition are seen, in vivo, only after the release of acetylcholine has been triggered by an active input and not in basal conditions.
Studies with FAS fragments, for example, with a loop including the 15-40 aminoacid sequence, did not show significant effects neither in vivo nor in vitro.

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α-DENDROTOXIN PRODUCES SEIZURES AND BRAIN DAMAGE IN RATS

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α-Dendrotoxin (DTx) increases neuronal excitability by blocking certain fast-activating, voltage-dependent K^+ channels (Stansfeld et al., 1987). In the CNS DTx potentiates spontaneous synaptic transmission and facilitates the release of neurotransmitters (see Dolly, 1991); these effects may explain its potent convulsant action. It is well known that seizures are often associated with excitotoxicity. Therefore, we have studied the epileptogenic and neuropathological characteristics of brain from rats stereotaxically injected with this neurotoxin. A unilateral injection of 35 pmol of DTx into the CA_1 hippocampal area or the dentate gyrus (DG) produced motor and ECoG seizures, followed at 24 h by multifocal brain damage. Significant neuronal loss was observed in the CA_1, CA_3, and CA_4 pyramidal layers and DG granule cell layer, ipsilateral to the site of injection. A dose of 3.5 pmol of DTx produced seizures but failed to produce significant neuronal loss. Injection of DTx (35 pmol) into the amygdala produced seizures after a latency period of 50 min but significant neuronal loss was not evident. 4-Aminopyridine (100 nmol), injected into the CA_1 area elicited motor and ECoG changes similar to those of DTx; no brain damage was observed at 24 h. Systemic pretreatment with antagonists of the NMDA receptor complex (MK801 or CGP37849) did not afford any protection against the effects induced by DTx microinfused into the CA_1 area. A monolateral surgical lesion to the Schaffer collaterals, through which the CA1 pyramids receive excitatory inputs from the CA3 area, and systemic administration of NBQX, a non-NMDA receptor antagonist, failed to prevent seizures and damage to the CA1 neurones but minimized the neuronal loss observed in distant areas to the site of DTx injection. In conclusion, the present experiments suggest that DTx may be an useful neurobiological tool to study mechanisms of seizures and neuronal cell death.

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ANATOXIN-A AND METHYLLYCACONITINE: NOVEL NEUROTOXINS FOR SAR STUDIES ON nAChR SUBTYPES.

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Despite the vast knowledge about nicotinic acetylcholine receptor (nAChR) structure and function, ligand recognition remains poorly understood. The receptor binding site for agonists and competitive antagonists is ill-defined. The nicotinic pharmacophore that describes ligand requirements has not significantly advanced from the studies of Beer & Reich[1], which predicted a hydrogen bond acceptor (carbonyl oxygen of acetylcholine) and a group suitable for electrostatic interaction (alkylammonium moiety) separated by a distance of 5.9Å. The emergence of a family of nAChR proteins in the nervous system calls for renewed efforts to determine the structural requirements for ligand recognition and receptor activation or blockade, in order to elucidate and exploit therapeutically the different requirements of the nAChR subtypes.

Anatoxin-a, a product of the cyanobacterium Anaebaena flos aqua, is the most potent agonist so far characterised at the majority of subtypes of nAChR[2]. Anatoxin-a lends itself to SAR studies because it has a semi-rigid skeleton and is amenable to synthetic chemistry. Analysis of an extensive series of analogues has lead to the following conclusions:

- The carbonyl and secondary amine functions are crucial for activity and potency;
- The Trans orientation of the acetyl sidechain is the preferred conformation. This has implications for the three dimensional shape of the ligand and suggests that simple computation of the Beers & Reich distance is inadequate in predicting nicotinic potency.
- Whereas the nitrogen and carbonyl carbon do not tolerate the addition of substituents, extension of the methyl group by methylation does not diminish potency, suggesting that this group projects into a substantial space in the binding pocket.

Methyllycaconitine (MLA) is the product of Delphinium sp. MLA is a potent competitive nicotinic antagonist that is highly selective for the subset of neuronal nAChR that are sensitive to αBungarotoxin[3]. This sensitivity extends to insect nAChR (that also bind αbungarotoxin), suggesting homology between these two nAChR and predicting an evolutionary relationship. MLA is a complex norditerpenoid alkaloid, structurally related to the sodium channel ligand aconitine. Chemical modification of MLA is aimed at defining the structural basis of its potency and its subtype selectivity, that will in turn be instructive about the recognition site on the nAChR itself.

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MUSCARINIC TOXINS FROM THE VENOM OF DENDROASPIS SNAKES WITH AGONIST-LIKE ACTIONS

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The venom of Elapid snakes from the gender Dendroaspis contains proteins with activity at muscarinic acetylcholine receptors (MAChR), called Muscarinic Toxins (MTX). MTX1 and 2, isolated from Dendroaspis angusticeps venom, inhibit the specific binding of the muscarinic antagonists ^{3}H-QNB[1] and ^{3}H-pirenzepine to synaptosomal membranes and slices of rat brain[2]. In agreement with this data, ^{125}I-MTX2 binding to cerebral membranes and slices is displaced either by atropine or pirenzepine[2]. Similar activity has been found in protein fractions isolated from Dendroaspis viridis and polylepsis venoms[3]. All these toxins are devoid of anticholinesterase activity, do not inhibit specific binding of radioligands to adrenergic, gabaergic or benzodiazepine receptors and competitively inhibit the binding of the M_1 MAChR antagonist ^{3}H-pirenzepine[3].

MTX2 was able to enhance carbachol-stimulated phosphatidylinositol turnover in rat cerebral cortex[3]. Taking into account that a major component of the cholinergic stimulation of PI turnover is activated by a M_1 subtype of receptor[4], the biochemical and pharmacological studies suggest that at least MTX2 behaves as a specific ligand for the M[1]MAChR subtype.

As it has previously been shown, the immediate post-training injection of muscarinic antagonists into the dorsal hippocampus of rats produces amnesia, while agonists improve retention of an inhibitory avoidance task[5]. The putative M_1 muscarinic ligand MTX2 was injected into the dorsal hippocampus of rats after a step-down inhibitory avoidance training session. 24 hours later, in the test session, a dose-dependent memory improvement was detected. With the joint infusion of the toxin and the antagonist scopolamine, the facilitatory effect disappeared.

Hence, MTX2 shows muscarinic agonist-like actions, which are probably mediated by the M_1 subtype of MAChR. This is the first protein reported to act as a muscarinic ligand with agonist-like behaviour.

Further studies will reveal the specific pharmacology of the putative MTXs. They might reflect a common ancestral gene that may have evolved expressing proteins with high degree of homology, like MTX1 and 2.

These and other proteins would have specialised, acting on specific target molecules which subserve related functions. The same fraction of proteins from Dendroaspis angusticeps venom also contains the K channel blocker dendrotoxin and the anticholinesterasic fasciculin. All these toxins seem to contribute for a long lasting cholinomimetic effect on the synaptic junction.

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CHARACTERISTICS AND PROPERTIES OF BENZODIAZEPINES, AND OTHER LIGANDS FOR THE CENTRAL BENZODIAZEPINE RECEPTOR, OF COMMON OCCURRENCE IN NATURAL SOURCES

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For many years benzodiazepines were considered non-natural heterocycles until the discovery, by De Blas and his collaborators in 1985, of their presence in brain.

Since then, a wealth of new data confirmed De Blas' results and we, together with several research groups, have found different benzodiazepines and benzodiazepine-like substances in human, animal and plant sources. These evidences including in vitro and in vivo experiments supporting the biosynthesis of benzodiazepines in brain, will be discussed.

Our experiments with plant materials following, in part, ethnopharmacological clues, has led to the discovery that several flavonoids have medium to high affinity for the central benzodiazepine receptor and that they display an interesting array of pharmacological properties. These facts prompted the launching of an intensive effort to detect more members, natural or synthetic, of this new family of neuroactive compounds.

A progress report of this research will also be presented.

ACTIONS OF NEUROTOXINS (BUNGAROTOXINS, NEOSURUGATOXIN, LOPHOTOXIN) ON INVERTEBRATE NICOTINIC ACETYLCHOLINE RECEPTORS

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The nervous systems of insects are rich in nicotinic acetylcholine receptors (nAChRs) that are blocked by both α-bungarotoxin[1] and κ-bungarotoxin[2]. A functional locust (*Schistocerca gregaria*) nAChR expressed in *Xenopus* oocytes using messenger RNA transcribed from a cloned α-subunit cDNA is also blocked by both toxins[3]. A comparison of amino acid sequences in the toxin binding regions of invertebrate and vertebrate receptors suggests a role for the proline residues close to the acetylcholine binding site in determining the actions of these snake toxins. Nematode (*Ascaris suum*) nAChRs, though largely insensitive to α-bungarotoxin, are blocked by neosurugatoxin (NSTX) the Japanese ivory mollusc toxin, a potent blocker of vertebrate ganglionic nicotinic acetylcholine receptors. Hitherto, NSTX has been found to block only neuronal nAChRs that are insensitive to α-bungarotoxin. Here we provide evidence that NSTX blocks non-competitively an α-bungarotoxin-sensitive nAChR on an identified insect neurone at concentrations of 10nM and above[4]. The coral toxin, lophotoxin, and its analogue bipinnatin B are potent blockers of insect nAChRs[5] and all other nAChRs tested to date. Studies on *Ascaris suum* show that lophotoxin fails to block the nematode nAChR. Lophotoxin binds to Tyr 190, and it is of interest that Fleming et al[6] have shown the presence of a proline at position 190 in the *Caenorhabditis elegans* nAChR sequence which may account for the insensitivity to lophotoxin observed in nematodes.

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PROTEIN TOXINS AGAINST MUSCARINIC ACETYLCHOLINE RECEPTORS

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A large number of protein toxins from snakes and Conus are known that bind to the nicotinic acetylcholine receptor, but so far protein toxins against the muscarinic receptor have been found only in mambas, African snakes of genus Dendroaspis. Four toxins have been isolated from the green mamba D. angusticeps (MT1, .. MT4) and two (MTα, MTβ) from the black mamba D. polylepis (MT = muscarinic toxin). The toxins have 65 or 66 amino acid residues and four disulphides (mol. wts. ≈ 7,500).

The specificity of green mamba toxin MT1 for subtypes of muscarinic receptors was studied. The binding of ^{3}H-AF-DX 384 (highest affinity for receptors of subtype M2) to homogenates of porcine heart muscle (≈ 100 % M2) was inhibited with a Ki (Kd) of 14 μM. Inhibition of the binding to synaptosomal membranes from pig brain proceeded in two steps. The binding decreased by 37 % in the first step which approximately corresponds to the content of M1 or M4 receptors in brain. A Ki of 32 nM was obtained from the first step. The second step was similar to the inhibition to heart receptors. Pirenzepine inhibited the binding of ^{125}I-MT1 to brain receptors with a Ki of 8 nM, a value much closer to Kd for M1 (16-18 nM) than to Kd for M4 (120 nM). It was concluded that pirenzepine displaced the toxin from M1 receptors. ^{125}I-MT1 bound to brain receptors with a Kd of 20 nM and a Hill coefficient of 1.0 (one toxin binds per receptor). Thus, MT1 has about 500 times higher affinity for M1 (Kd = 26 nM, average value) than for M2 (Kd = 14 μM).

Black mamba toxin MTβ started inhibiting the binding of ^{3}H-NMS (N-methylscopolamine)(same affinity for all subtypes) to heart and brain receptors at about 0.1 μM. MT1 is different, the first effect on the NMS binding to brain was seen at 1 nM and to heart at about 1 μM.

In stripped guinea-pig ileum MT1 (670 nM) produced contraction and MT β (670 nM) first contraction and then relaxation. The effects were inhibited by atropine.

Amino acid sequences of toxins MT1, MT2, MT3 and MTβ are known. They are homologous to a large number of other snake toxins. The greatest homology is with the synergistic type toxins of mamba venoms.

STRUCTURE-ACTIVITY STUDIES ON SCORPION TOXINS THAT BLOCK POTASSIUM CHANNELS.

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Scorpion venoms contain toxins that block different types of potassium channels. Some of these toxins have affinity for high conductance Ca^{2+}-activated K^+ channels and for dendrotoxin-sensitive voltage-dependent K^+ channels. The structural features that determine the specificity of binding to different channel types are not known. We investigated this using natural and synthetic scorpion toxins. We have tested the effects of charybdotoxin (CbTX), iberiotoxin (IbTX), and kaliotoxin (KTX) from the scorpions *Leiurus quinquestriatus hebreus*, *Buthus tamulus* and *Androctonus mauretanicus mauretanicus*, respectively, and synthetic variants of CbTX, namely $CbTX_{2-37}$, $CbTX_{3-37}$, $CbTX_{4-37}$, and $CbTX_{7-37}$, on a Ca^{2+}-activated K^+ current (I_{K-Ca}) at a mammalian motor nerve terminal, and on the binding of a radiolabelled dendrotoxin, ^{125}I-DpI, to voltage-dependent K^+ channels on rat brain synaptosomal membranes. The native toxins contain 37-38 amino acid residues, they are over 30% identical in sequence (CbTX and IbTX are 68% identical), and they have similar 3-dimensional conformations.

All toxins, except IbTX, displaced ^{125}I-DpI from its synaptosomal binding sites: KTX (K_i=2.1 nM), CbTX (K_i=3.8 nM), $CbTX_{2-37}$ (K_i=30 nM), $CbTX_{3-37}$ (K_i=60 nM), $CbTX_{4-37}$ (K_i=50 nM), $CbTX_{7-37}$ (K_i=105 nM). IbTX had no effect at 3 μM. When variants of CbTX with deletions at the N-terminal portion were tested for their activity on I_{K-Ca} on motor nerve terminals in mouse triangularis sterni nerve-muscle preparations, $CbTX_{2-37}$ was slightly less active than CbTX, which caused maximum block at 100 nM; $CbTX_{3-37}$ and $CbTX_{4-37}$ were ineffective at 100 nM; and $CbTX_{7-37}$ was ineffective at up to 1 μM. IbTX (100 nM) completely blocked I_{K-Ca}, but KTX (100 nM) did not affect the nerve terminal I_{K-Ca}.

Different residues appear to be important for interactions of the toxins with different K^+ channels. IbTX did not displace dendrotoxin binding, but it did block I_{K-Ca}, whereas KTX was as active as CbTX against dendrotoxin binding but it did not affect the I_{K-Ca} of the motor nerve terminals. The N-terminal section of the toxins appears to be particularly involved in block of I_{K-Ca} at the motor nerve terminal: it is truncated in the inactive synthetic CbTX variants; and it is positively charged in KTX (which is inactive), but negatively charged in IbTX and CbTX. Phenylalanine at position 2 seems to be essential: there is a marked loss in activity between $CbTX_{2-37}$ and $CbTX_{3-37}$, and KTX has valine-2. For binding to dendrotoxin sites, it is noticeable that the inactive IbTX lacks the conserved asparagine residues at positions 4 and 30, and contains additional negatively charged residues at positions 4, 6 and 24.

RECOGNITION SITES OF SNAKE CURAREMIMETIC TOXINS AND ACETYLCHOLINE RECEPTORS

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With the view to delineate the sites by which snake curaremimetic toxins interact with the nicotinic acetylcholine receptor from *Torpedo marmorata* (AcChoR) we (i) cloned the cDNA encoding erabutoxin a, a short-chain toxin from the sea snake *Laticauda semifasciata*; (ii) expressed it as a fusion protein either linked to *S. aureus* protein A or inserted into bacterial alkaline phosphatase, (iii) generated *in vitro* the free recombinant erabutoxin (iv) demonstrated that the biological and structural properties of the recombinant toxin are undistinguishable from those of the wild toxin; (v) generated by site-directed mutagenesis a set of 30 mutants and determined their affinities for AcChoR, on the basis of competition experiments. The data obtained show that most residues previously postulated as being functionally conserved within the family of curaremimetic toxins are important, though not essential, for the toxin to bind to AcChoR. These conserved residues all belong to the second and third loops of the toxin. In addition, however, we found that (i) a number of non conserved residues on the first and second loops are also important for the toxin function and (ii) upon appropriate mutations, the affinity of erabutoxin a can substantially increase. Available data led us to propose the first experimentally-based delineation of the site by which a short-chain toxin recognizes AcChoR.

With the view to identify the site by which AcChoR binds to curaremimetic toxins we investigated, using a solid-phase assay, the binding of [^{3}H]-labelled αCobtx, a long-chain toxin and [^{3}H]-labelled toxin α, a short-chain toxin, to the fragments 128-142 (Tα128-142) and 185-199 (Tα185-199) of the α-subunit of AcChoR. The two toxins bind specifically to both fragments with affinities characterized by Kd values ranging from 3 10^{-7}M to 50 10^{-6} M. On the basis of competition experiments with various toxins monoderivatized on functionally conserved residues we show that Lys-53 contributes to the binding of curaremimetic toxins to Tα128-142 whereas Tyr-25, Lys-27, Trp-29, Cys30 and Cys-34 do not. In contrast, none of the monoderivatizations affected the binding affinity of αCobtx for Tα185-199, a fragment which is currently described as being a critical binding region for AcChoR ligands.

A tentative and preliminary model depicting the region to region interactions between curaremimetic toxins and AcChoR will be proposed.

CLONING AND EXPRESSION OF MAMBA TOXINS

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Toxins isolated from the venoms of mamba snakes act on various components (acetylcholine receptor, acetylcholinesterase, or voltage-activated K^+ channels) of the neuromuscular junction involved with neuromuscular transmission. In an attempt to clone, characterize, and express these toxin genes, as well as other toxin genes encoding activities not completely elucidated as yet, a cDNA library was constructed from mRNA isolated from the glands of the black mamba. Numerous cDNAs from this library were characterized by nucleotide sequence analysis. The gene sequence encoding dendrotoxin K (DTX_K), an inhibitor of voltage-dependent K^+ channels, was subsequently expressed as a maltose-binding (MBP) fusion protein in the periplasmic space of *Escherichia coli*. The fusion protein was purified on an amylose affinity resin, and the MBP chaperone was cleaved from the toxin by using Factor X. Purification of the recombinant toxin was achieved by using FPLC Superose-12 and reverse-phase SEP-PAK C-18 column chromatography. Recombinant dendrotoxin K was shown to be identical to native DTX_K in its N-terminal sequence, chromatographic behavior, convulsive-inducing activity, and binding to voltage-activated K^+ channels in bovine synaptic membranes.

Stable heterologous expression of nicotinic acetylcholine receptor in clonal cell lines and the acquisition of α-bungarotoxin binding and channel gating properties. **A.M. Roccamo, E. Aztiria, L. Zanello, M.F. Pediconi, E.L. Politi, B. de los Santos, A. Wolstenholme, I. Werkman and F. J. Barrantes. Instituto de Investigaciones Bioquímicas de Bahía Blanca, 8000 Bahía Blanca, Argentina.**

In order to further our studies on modulation of the functional properties of the nicotinic acetylcholine receptor (AChR) by its lipid microenvironment and the effect of modifications of the plasma membrane lipids on the channel gating properties of the AChR, we have transfected cDNAs encoding the subunits of the adult ($\alpha,\beta,\varepsilon,\delta$) mouse peripheral (muscle) AChR into mutant CHO cells having alterations in their lipid metabolism. Transfected cells were selected for antibiotic resistance and a number of colonies were isolated. Integration and expression of AChR sequences was confirmed by subunit-specific PCR and expression of functional receptor was checked by patch-clamp techniques. The single-channel properties and temperature sensitivity of the AChR heterologously expressed in the mutant clones were compared with those of the embryonic type ($\alpha,\beta,\gamma,\delta$) expressed (homologously) in BC3H-1 cells and (heterologously) in the CHO AR-42 clone. α-Bungarotoxin binding was followed at different times after cell plating. Channel activity has been characterized using the inside-out patch configuration over a range of temperatures (5°C to 40°C). We found that the conductance of the channels increased as temperature augmented, with Q_{10} values of 1.2-1.4. Mean open durations of the channels diminished as the temperature was raised, yielding Q_{10} values of 1.9-2.0. A thermodynamic analysis using Eyring's transition rate theory was carried out for the processes of ion conductance and kinetics. The values obtained for the activation enthalpy and entropy were compared to those reported for other types of channels from animal cells. The relative thermal insensitivity of the channel conductance suggests that ions traverse the pore by diffusion. Comparison of the AChR properties in BC3H-1 muscle cells with those of "wild" CHO cells and lipid-deffective CHO mutants provides further support to the hypothesis that the lipid microenvironment plays a role in the modulation of receptor functional properties.

EFFECTS OF SCORPION VENOM TITYUSTOXIN ON CENTRAL NERVOUS SYSTEM.

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Tityustoxin (TsTX) mimics many effects of electrical stimulation in slices of rat brain cortex, increasing the uptake of Na^+ and Ca^{2+} ions and causing substantial depolarization. These effects of TsTX result in the release of acetylcholine (ACh) which is Na^+ and Ca^{2+} dependent and is inhibited by the sodium channel blocker tetrodotoxin (TTX).As an agent that activates the voltage dependent sodium channel, TsTX also stimulates the turnover of inositol phosphates (InPs) and TTX blocked this effect. Ouabain an inhibitor of Na^+K^+ATPase, causes release of ACh independently of extracellular Ca^{2+} and insensitive to TTX inhibition. Simultaneous incubation of TsTX and ouabain has an additive effect on the release of ACh from brain cortex slices. TsTX also increased the incorporation of ^{32}P in Synapsin I while ouabain did not stimulate the phosphorylation of this protein suggesting dissociation between the processes of ACh release and protein phosphorylation. Based on the above data we have suggested that TsTX and ouabain may mobilize different intracellular pools of ACh. TsTX causes an early mobilization of ACh from vesicular and cytoplasmic pool while ouabain releasing ACh in a non quantal manner only after prolonged stimulation caused mobilization of vesicular ACh. Cholinergic synaptic vesicles contain high levels of ACh and ATP and the exocytotic theory predicts that ACh and ATP would be coreleased as result of a stimulation. In brain synaptosomes TsTX and ouabain increased the release of ATP by different mechanisms. Literature data suggest that La^{3+} pretreatment of the tissue might be usefull to identificate ACh release from different pools. We have found that TsTX, ouabain and K^+ release ACh from the same pool that is inhibited by La^{3+}. The presence of Ca^{2+} ions is a prerequisite for neurotransmitter release and for the InPs production in brain preparation. Neuronal Ca^{2+} channel serve as routes for Ca^{2+} entry through the nerve terminals. These calcium channels have been typified as N, L and T. The calcium channel blockers verapamil, nifedipine and diltiazen inhibited the release of ACh induced by TsTX but had no effect in the release of ACh induced by ouabain. The peptide ω-Conotoxin a blocker of N and L types inhibited the the release of ACh by TsTX. Neurotransmitter release from different neurons appears to be regulated by different types os calcium channel and TsTX might be a tool in studies of calcium channel and neurotransmitter release.

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CHARACTERIZATION OF CALCIUM CHANNELS INVOLVED IN TRANSMITTER RELEASE AT THE MAMMALIAN NEUROMUSCULAR JUNCTION

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Transmitter release at the nerve terminals is mediated by the influx of Ca^{2+} through voltage-dependent calcium channels (VDCCs). At least four distinct types of VDCCs are known to exist in nerve cells, namely L, N, T and P types, which have been characterized by their electrical and pharmacological properties. The high-threshold N type channel is very sensitive to the blocking effect of W-conotoxin (W-CgTx) and the L type channel is blocked by the Ca^{2+} channel antagonist of the 1-4,dihydropyridine (DHP) class. The P-type channel was found to be insensitive to DHP and W-CgTx, but very sensitive to a low molecular weight fraction (FTX) and to a peptide (W-AGA-IVA) both purified from the venom of the funnel web spider *Agelenopsis aperta.* (1)

In order to determine whether a particular type of VDCC is more commonly involved in synaptic transmission we studied the effect of the Ca^{2+} channel blockers on transmitter release at the neuromuscular junction of mouse phrenic diaphragm and human intercostal muscle . Intracellular microelectrodes were used to record endplate potentials at 0.5 Hz and 40 Hz frequency stimulation .We confirmed that dihydropyridines and W-CgTx produced no effect on quantal content of release. In contrast FTX, as well as W-AGA-IVA, strongly inhibited transmitter release at submicromolar concentrations.

In muscles treated with FTX or W-AGA-VIA high frequency stimulation induces an early facilitation of the endplate potential amplitude, instead of the normal rundown, as expected from nerve terminals with diminished influx of Ca^{2+} (2)

To further investigate the inhibitory mechanisms of FTX and W-AGA-IVA on synaptic transmission , the effect of the toxin on the presynaptic Ca^{2+} currents was studied.

Presynaptic currents were recorded with microelectrodes placed inside the perineural sheath of small nerve branches near the endplate areas of the *levator auris* muscle of the mouse. The muscles where treated with curare and with K channel blockers. In these conditions the records showed a slow , long lasting Ca^{2+} dependent component sensitive to Cd^{2+} which was blocked by FTX an W-AGA IVA but was not affected by W-CgTx.

These results confirmed that the predominat VDCC in the motor nerve terminals is the P type channel.

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BOTULINUM AND TETANUS TOXINS : MULTI-FUNCTIONAL PROBES FOR INVESTIGATING PROTEIN TRAFFICKING AND NEUROTRANSMITTER RELEASE

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Botulinum neurotoxin (BoNT), a family (serotypes A to G) of proteins produced by Clostridium botulinum and consisting of a disulphide-linked heavy (HC) and light (LC) chain, inhibit selectively and irreversibly the release of acetylcholine at peripheral synapses (reviewed by Dolly, 1992). This specific action has been shown for BoNT types A and B to entail (i) targetting to motor nerve endings by binding to distinct ecto-acceptors, via their HC in its 'native' conformation, that are located exclusively on cholinergic terminals; (ii) temperature- and energy-dependent internalisation requiring the toxin's inter-chain disulphide or half cystines (de Paiva et al 1993) and involving the N-terminal moiety of HC (Poulain et al 1989) and (iii) a blockade of acetylcholine release mediated by LC alone (de Paiva and Dolly 1990). Paralysis of hyperactive muscles in humans induced by injecting type A BoNT persists for several months until 'new' synapses, formed by nerve sprouting, restores neurotransmission. Several movement disorders (blepharospasm, torticolis and other dystonias) and dysphonia can be treated successfully by 3-4 monthly toxin administration without any serious side effects (Brin, 1990).

On the other hand, tetanus toxin (TeTX), a structurally similar protein from Clostridium tetani, is transported to the spinal cord and lower brain where it blocks preferentially the release of inhibitory transmitters such as glycine and GABA. These contrasting neuron specificities arise from different targetting of the two toxins; thus, each prevents the release of all transmitters tested when administered intracellularly. Although TeTX or BoNT type B, but not A, proteolytically cleave VAMP/synaptobrevin II (Schiavo et al 1992) - a synaptic vesicle protein thus considered intimately concerned with exocytosis - LC of BoNT A also possesses the conserved and essential Zn^{2+} binding motif characteristic of Zn^{2+} neutral endoproteases. Accordingly, liposomal delivery inside motor nerve terminals of inhibitors of the latter (phosphoramidon, captopril) antagonised the blockade of neuromuscular transmission induced by BoNT A or B; likewise, bath application of chelators of divalent metals or excess Zn^{2+} diminished their neuroparalytic action. Thus, type A probably acts via selective proteolysis of a substrate likely to be another 'intermediate' of the exocytotic pathway. The intriguing subtle differences in the effects of the various BoNT serotypes and TeTX highlight that they provide an invaluable array of research tools. For example, ongoing experiments on permeabilised adrenochromaffin cells are pinpointing distinct stages in the exocytotic pathway that are susceptible to BoNT A and TeTX; this information, in turn, will help establish the precise roles of their targets in the fundamental process of exocytosis.

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Organization of the botulinum neurotoxin complex genes in *Clostridium botulinum* C, and genetic transfer in *C. botulinum*

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The botulinum neurotoxins (BoNT) are associated with other non toxic proteins (ANTP). The BoNT complexes vary in molecular weight (230 to 900 kDa) depending on the toxin type. In *C. botulinum* C, the BoNT/C1 locus consists of 6 genes organized in 3 clusters. The cluster 1 encompasses the BoNT/C1 and ANTP139 genes. ANTP139 could be an important component of the BoNT/C1 complex responsible of the resistance to acidic pH and protease degradation. The cluster 2 consists of 3 genes (ANTP33, 17 and 70) which encode hemagglutinin components. The one gene of the third cluster encodes a DNA binding protein (Orf22) which is probably involved in the regulation of the BoNT/C1 complex genes.

The tetanus and botulinum neurotoxins display a similar structure. Their identity at the amino acid level ranges from 34 to 98%, indicating that the clostridial neurotoxin genes derive probably from a common ancestor. The fact that other *Clostridium* than *C. botulinum* such as *C. butyricum* and *C. baratii* can produce BoNT suggests that the BoNT genes can be transfered between clostridial strains.The BoNT/E genes from *C. botulinum* E and toxigenic *C. butyricum* strains are closely related (98%). DNA fragments from BoNT/E gene and its flanking regions do not hybridize with DNA from non toxigenic *C. butyricum* strains suggesting that the gene transfer between *C. botulinum* E and *C. butyricum* involves a larger DNA fragment than the BoNT/E gene. A study of the C3 gene in *C. botulinum* C and D, showed that this gene is localized on a mobile transposon like element of 21.5 kb. Transposons could be involved in BoNT gene transfer in *C. botulinum*.

αLATROTOXIN AND EXOCYTOSIS

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Development of a Molecularly Engineered Vaccine for Botulinum Toxin

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Botulinum toxin is a highly potent and deadly toxin produced by the bacterium *Clostridium botulinum*. The toxin is responsible for the neuromuscular paralysis and death resulting from food poisoning by this organism. There is a protective toxoid available which is produced by treating the toxin with formaldehyde until it is inactive. There is good reason to believe that using the native form of the antigen would provide better immunity, if, of course, the antigen was nontoxic. We attempted to develop a vaccine based on the 50 kD carboxy-terminal (H_c) part of the molecule. Initially, H_c was produced by expression in *E. coli* of a maltose-binding protein fusion product using a fragment of the natural gene. In crude form, this product was used to vaccinate mice with a resultant protection from challenge with the toxin. The fusion protein product was expressed in low quantities, was insoluble and was difficult to purify. This problem could be due in part to codon usage and an unfavorable A-T content of the natural gene. Therefore, we made an entirely synthetic genes for the H_c fragments of both serotypes A and B. These genes were designed with *E. coli*-preferred codons and have a much lower A-T content than the natural genes. Using nucleotide oligomers of 60-114 bases in length, large seqments of the genes were produced and then assembled in total. Several deletion errors found after sequencing were corrected by *in vitro* mutagenesis. In addition, a second gene was produced which codes for the H_c fragment of serotype B of botulinum toxin. Expression of the serotype A-H_c has been accomplished in *E. coli* and vaccination of mice with a crude extract protected the animals from up to 10^6 LD_{50} of the toxin.

ANTAGONISM OF BOTULINUM TOXIN-INDUCED MUSCLE WEAKNESS BY 3,4-DIAMINOPYRIDINE IN RAT DIAPHRAGM PREPARATIONS

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The actions of 3,4-diaminopyridine (3,4-DAP) and its quaternary analog 3,4-diamino-1-methyl pyridinium iodide (3,4 DAP $^+$) were evaluated for their ability to antagonize the depression of nerve-evoked isometric twitch and tetanic tensions in rat diaphragm muscles *in vitro*. Twitch tensions were elicited with supramaximal rectangular current pulses of 0.1 msec at a frequency of 0.1 Hz. Muscles were exposed to 1 nM botulinum neurotoxin A (BoNT A) for 1 hr at 15 °C, washed and warmed to 32 °C until twitch tensions were reduced by 90-100% (141.5 ± 28.3 min). Addition of 100 μM 3,4-DAP resulted in a prompt and nearly complete antagonism of the BoNT A induced muscle paralysis. The onset of 3,4-DAP was apparent within 30 sec, and peak values were attained within 5 min of drug addition. The beneficial effect of 3,4-DAP was persistent, and twitch tensions remained within 88% of control values even after 4 hr of treatment with the K^+ channel blocker. When subjected to a stimulation pattern that resembles the *in vivo* respiratory rhythm, muscles exposed to BoNT A and 3,4-DAP were able to generate sustained tensions with 50 Hz, 200 msec tetanic pulses.

Since the aminopyridines have been found to produce seizures in patients, especially after high doses or prolonged exposures, we examined the actions of the quaternary analog 3,4-DAP $^+$, a compound not expected to penetrate the blood-brain barrier. In contrast to the marked efficacy of the parent compound, 3,4-DAP $^+$ was unable to antagonize BoNT A-induced muscle paralysis at any concentration tested (up to 1 mM). Consistent with this finding, 3,4-DAP $^+$ also failed to prolong the action potential decay phase. Thus, the quaternary analog has lost its ability to block K^+ channels, making it ineffective in antagonizing the inhibition of transmitter release by BoNT A. These results suggest that addition of a methyl group on the pyridine nitrogen leads to a loss in activity. Perhaps other substitutions will generate aminopyridine compounds that retain their efficacy but will be free of undesirable central side effects.

TOPOGRAPHIC MODELS FOR THE TRANSMEMBRANE CHANNELS FORMED BY BOTULINUM TOXIN

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The translocation of the toxic, light (L) chain of botulinum neurotoxin (BoNT) into the cytoplasm of the presynaptic terminal of the neuromuscular junction may involve the ability of the toxin's heavy (H) chain to form transmembrane (TM) channels within endocytotic vesicular membranes. By knowing which amino acid residues are involved in the channel structure, it is our long-term goal to predict and test potentially useful channel blockers that could inhibit this translocation process.

The approach we used in predicting the residues is based upon the known primary, secondary and tertiary structure of another channel former, diphtheria toxin (DiTX). From this information, we calibrated several computer programs to estimate the free energies of transfer of residues from an aqueous to a lipid environment. As a result, and based on the primary toxin structures of six BoNT serotypes and tetanus toxin, we identified four adjacent amphipathic regions (including about 80 residues) that could be involved in channel formation.

From our thermodynamic calculations, we formulated ten different topological models that have either two or four TM regions in the H-chains of these toxins. We derived an estimate for the optimal pH for toxin-channel formation in a given model by using the pKa values of the residues with chargeable groups within TM and appropriate inter-TM regions. The resulting estimates were then compared to the published values of the optimal pH (ca., 4.5 for BoNT-A, B and TeTX; ca. 6 for serotype-C1). By using the estimated values for the optimal pH and other criteria, we eliminated several of the topological models from consideration.

We hypothesize that these predicted channel-forming regions are involved in a process that is common to all the BoNT serotypes. If correct, these regions may be important when developing single chemical pharmaceuticals or medical products that could counteract the paralytic and lethal effects of all BoNT serotypes.

INTERACTIONS BETWEEN Zn-CHELATORS AND BOTULINUM NEUROTOXINS AT THE NEUROMUSCULAR JUNCTION.

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Research into the structure of tetanus and botulinum neurotoxins suggests a role for zinc ions as enzymatic cofactors in their intracellular action. Recent work by Schiavo et al. (1992) and Wright et al. (1992) suggests that native tetanus toxin contains Zn in approximately a 1:1 molar ratio to toxin light chain peptide and that removal of this Zn with a heavy metal chelator interferes with the ability of the toxin to block synaptic transmission in a central synapse of *Aplysia californica*. Similar studies at the neuromuscular junctions on skeletal muscle (Simpson, personal communication) have shown that membrane permeant heavy metal chelators such as TPEN (N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine; Arslan et al., 1985) interfere with the neuromuscular blockade produced by botulinum neurotoxins. In the results reported here, we examine the interactions of zinc chelation with the development of botulinum intoxication.

Isolated mouse hemidiaphragms were incubated at 36°C and stimulated via the phrenic nerve. Exposure to 0.1 nM BoNT-A or BoNT-B blocked neuromuscular transmission such that a 50% loss of peak isometric tension was observed at 45 min (BoNT-A) to 70 min (BoNT-B) after application. Either coincubation in TPEN and BoNT, preincubation with TPEN followed by BoNT, or application of TPEN after BoNT but before neuromuscular block, delayed the onset of muscle failure in a dose-dependent manner by up to five-fold over controls with BoNT alone. TPEN doses between 2 and 10 μM were required to significantly antagonize muscle block produced by BoNT and the delay in onset was maximal between 10 and 50 μM TPEN. Treatment of muscles with a Zn·TPEN coordination complex, rather than TPEN alone, eliminated any beneficial effects of TPEN on BoNT intoxication, indicating that these effects were mediated by chelation of Zn.

Given the requirements for micromolar concentrations of TPEN to antagonize nanomolar concentrations of BoNT, it seems likely that TPEN acts by removing readily exchanged Zn from the presynaptic terminal and prevents its binding to BoNT rather than stripping Zn already bound to BoNT. Similar conclusions were reached by Schiavo et al. (1992) for *in vitro* manipulations of purified tetanus toxin with TPEN. This putative mechanism for TPEN is also supported by the observations that Zn binding to tetanus toxin is markedly reduced at pH < 6.5 (Wright et al., 1992) and that the amount of intracellular Zn available for TPEN chelation is in the micromolar range (Arslan et al., 1985). Thus it appears that native BoNT light chain loses its Zn during the acidification stage in endocytotic vesicle processing and must then reacquire intracellular Zn before exerting its neuromuscular block.

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CONOTOXINS: PEPTIDE LIGANDS FOR RECEPTORS AND ION CHANNELS

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The venoms of the predatory marine gastropods belonging to the genus *Conus* ("cone snails") are a rich source of small peptide neurotoxins ("conotoxins"), each targeting specifically to a receptor or ion channel. There are ca. 500 species of cone snails; each venom has 50-200 different peptides. Because of hypermutation during speciation, there is no sequence identity between venoms. Thus, many thousands of neuroactive peptides remain to be characterized.

Conus peptides which target nicotinic acetylcholine receptors, voltage-sensitive Na channels and vasopressin receptors have been characterized. Among the most notable neurotoxins are two peptide families which affect Ca channels. These include the ω-conotoxins, which inhibit voltage-sensitive Ca channels, and the conantokins, which inhibit NMDA receptors, a major family of ligand-gated Ca channels. Multiple homologous forms have been characterized for both peptide families.

The ω-conotoxins have been widely used to differentiate between voltage-sensitive Ca channel subtypes. Some ω–conotoxins (i.e., ω–GVIA, ω–MVIIA) are highly specific for the N-type Ca channel; other peptides of this family (i.e., ω–MVIIC, ω–MVIID) will also target other Ca channel subtypes including P-type and a novel class, different from all previously characterized channel subtypes. These peptides have proven to be potent inhibitors of neurotransmitter release in a variety of systems.

Conotoxins are generally between 10-30 amino acids in length; despite their small size, those which have been analyzed by NMR are all conformationally rigid. Most conotoxin structures are stabilized by multiple disulfide cross-links, which greatly restrict conformational mobility. However, conantokin structure is stabilized by the unusual post-translational carboxylation of glutamate residues to γ–carboxyglutamate (Gla); in the presence of Ca^{++}, these Gla residues greatly stabilize an α-helical conformation for the conantokins.

The conantokins are specifically targeted to NMDA receptors and do not affect other glutamate receptors. These peptides may have differential effects on different NMDA receptor subtypes; one member of the family, conantokin G, completely blocked the major rat brain NMDA receptor expressed in oocytes; however, the NMDA receptor in cerebellar cells in culture was only partially blocked, and with lower affinity. Using radiolabeled conantokins, an NMDA receptor complex has been biochemically purified. The major protein subunit in the purified complex radiolabeled by chemical cross-linking to ^{125}I-conantokin G derivatives has an MW of ca. 170 Kd.

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SMALL NEUROTOXINS: GLUTAMATE ANALOGUES AND NITRIC OXIDE. Keith Tipton, Gethin McBean and Harald Kollegger, Department of Biochemistry, Trinity College, Dublin 2, Ireland.

Coronal slices of rat brain were used to assess the neurotoxicity of glutamate receptor antagonists and the interactions between neurons and glial cells in toxicity. Neuronal toxicity was assessed microscopically after thionin staining as well as by immunochemical estimation of $\gamma\gamma$-enolase. Measurement of glutamine synthase activity was used as a measure of glial cell function. The transient gilal cell-toxin DL-α-aminoadipate protects against the neurotoxicity of kainate but not of N-methyl-D-aspartate. These results may indicate that the toxicity of kainate involves release of glutamate from stores in intact and functioning glia. Treatment of the coronal slices with methionine sulphoxide, which is an inhibitor of glial cell glutamine synthase, resulted in some neurotoxicity, perhaps as a result of glutamate release from these cells. Such pretreatment considerably potentiates the neurotoxicity of both kainate and N-methyl-D-aspartate, perhaps as a result of the reinforcing effects of decreased glial cell glutamate uptake. Pretreatment with the inhibitor of nitric oxide synthase N^G-nitroarginine reduces the toxicity of N-methyl-D-aspartate but that of kainate. These results suggest that the action of Nitric oxide synthase is not important to the neurotoxicity of kainate, whose actions may involve osmotic stress as a result of K^+ movements, whereas the stimulation of this enzyme by N-methyl-D-aspartate may play an important role in its neurotoxicity, perhaps as a result of radical-induced tissue damage. A number of compounds that have been reported to be "neuroprotective" were found to be ineffective in protecting against the neurotoxicity of these receptor agonists.

NEW NATURAL PRODUCTS AND THEIR POTENT SYNTHETIC ANALOGUES: POTENTIATORS AND ANTAGONISTS OF RECEPTOR OPERATED ION CHANNELS

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Polyamine amides comprise a new class of bioactive agents [1] and their antagonism of glutamate and acetylcholine receptors affords opportunities to target hitherto unexploited systems for the control of calcium influx. The potential of these compounds and their many synthetic analogues as pharmaceutical agents is currently under intensive development for the regulation of intracellular calcium levels with respect to stroke and neurodegenerative diseases [2].

A number of low molecular weight polyamine amide toxins which non-competitively antagonize ionotropic receptors gated by either glutamate or acetylcholine, in vertebrates and in invertebrates, have been isolated from the venoms of several predatory arthropods. In the search for new toxins, preferably with new sites of action, polyamine amides constitute a new class of compounds with potential as insecticides and as pharmaceutical agents due to their antagonism of these ligand-gated cation channels. The discoveries of philanthotoxins, argiotoxins, agatoxins, and hettoxins have aroused intense interest. Our on-going synthetic studies include the design and production of hybrid analogues of spider and wasp toxins, designed neuroprotectants, and targeted at the selective block of ligand-gated ion channels.

Whereas acylated amino acids and smaller, less-charged polyamine amides (e.g. 2,4-dihydroxyphenylacetyl-L-asparagine and PhTX-3.3 respectively) are not potent antagonists of glutamate receptors, polyamines and especially polyamine amides carrying three or four positive charges, at physiological pH, are potent antagonists. The biological effects of these compounds are further complicated by the potentiation of ionotropic receptor function which can be demonstrated with polyamines or polyamine amides. These highly polar compounds are challenging targets in synthetic medicinal chemistry.

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Sites and modes of action of polyamine amides toxins - a 10-year checkup

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Abstract

Almost 10 years have past since the original discoveries of polyamine amide toxins in venoms of Araneid spiders and their antagonism of ionotropic glutamate receptors (GluR) of arthropod skeletal muscle (1-5). These discoveries overtook and somewhat overshadowed previous work undertaken by Piek and colleagues on toxins in the venom of a wasp, *Philanthus triangulum*, the mode of action of which had already been established qualitatively and which were also subsequently identified as polyamine amides (6-7). There has been considerable interest in polyamine amide amides as pharmacological tools and as lead structures for the chemical industry (8), but have they fulfilled their early promises?

To answer this question it is necessary to review the formative stages in the grooming of these compounds for centre stage, to address the interpretive mistakes that were made, and to underline extant misconceptions, concerning their pharmacological properties.

In attempting such a review I will address the following questions:

(i) How do polyamine amides antagonise ionotropic GluR?
(ii) Do they differentiate between the different types of ionotropic GluR present in vertebrates and invertebrates?
(iii) Are they specific antagonists of ionotropic GluR?
(iv) Are their pharmacological actions on ionotropic GluR qualitatively different from those of polyamines such as spermine?
(v) What is the current status of their role as lead structures for the pharmaceutical and pesticide industry?

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Poster Abstracts

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NOVEL NICOTINIC ANTAGONISTS BASED UPON METHYLLYCACONITINE

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The norditerpenoid alkaloid methyllycaconitine (MLA) is the principal toxic component of *Delphinium brownii*; this complex hexacycle is also present in other species of *Delphinium* and *Consolida* (Larkspur). MLA is a substituted anthranilate ester of lycoctonine which is an *N*-ethyl piperidine norditerpenoid incorporating a homocholine moiety. MLA is a novel competitive nicotinic acetylcholine receptor (nAChR) antagonist [1,2,3] and is selective for neuronal nAChR identified by α-bungarotoxin binding. As a low molecular weight ligand able to discriminate between different nAChR sub-types, MLA displays considerable potential as a selective probe for nAChR.[3] We are investigating the significance of the acyl moiety in the supposed pharmacophore of MLA, as part of our MLA structure-activity studies.

In our initial studies, we have optimised the extraction of crude alkaloids from the seeds of Garden Hybrid *Delphinium*. Preparative TLC, crystallisation, and vacuum liquid chromatography have been assessed in the isolation and purification of MLA. Also, a series of small molecule MLA analogues has been designed and acylated cholines and homocholines have been prepared from isatoic anhydride. The succinimide ring was synthesized by fusion of the anthranilate ester with methylsuccinic anhydride. These esters of substituted anthranilic acid and the norditerpenoid alkaloid natural products have been assayed for nicotinic potency in competition binding assays to rat brain membranes, using [^{125}I]α-bungarotoxin and [^{3}H]nicotine as nAChR ligands. The synthetic anthranilate esters, lacking the norditerpenoid skeleton, were not active. In contrast, two alkaloid fractions from *Delphinium* were potent at [^{125}I]α-bungarotoxin sites (with IC_{50} values of 3-200 nM).

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AN HPLC ASSAY FOR THE NORDITERPENOID ALKALOID, METHYLLYCACONITINE

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Methyllycaconitine (MLA), which occurs in many species of *Delphinium* and *Consolida* (Ranunculaceae), is an extremely potent and selective neuronal nicotinic acetylcholine receptor antagonist having an IC_{50} of 2 x 10^{-9}M in an *in vitro* competitive binding assay against [^{125}I]α-bungarotoxin. We are engaged in structure-activity studies of MLA and related alkaloids. It is essential that the alkaloids used in this work are not contaminated with traces of MLA which would seriously distort the biological assay results. Accordingly, we have developed a reverse phase HPLC assay for MLA for routine monitoring of alkaloid samples isolated from MLA-containing plant sources. The system uses an octadecylsilane column, a mobile phase mixture of 0.2M ammonium acetate adjusted to pH 5 with formic acid and acetonitrile (70:30^{v}/$_{v}$), UV detection at 270nm and lappaconitine as internal standard. We are able to detect 0.07 nmoles of MLA on column.

A sample of the alkaloid delpheline, isolated from seeds of garden *Delphinium* hybrids by vacuum liquid chromatography and recrystallisation, was shown by HPLC to contain 0.18 moles % of MLA. This sample had an IC_{50} of 3 x 10^{-7}M in the binding assay. Less than 30% of this activity can be accounted for by the MLA content and thus delpheline also binds at α-bungarotoxin sensitive brain receptors with considerable potency.

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ANATOXIN STRUCTURE ACTIVITY STUDIES: (I) SYNTHETIC CHEMISTRY

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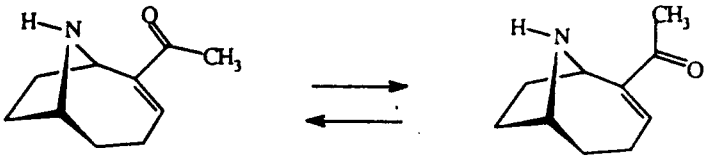
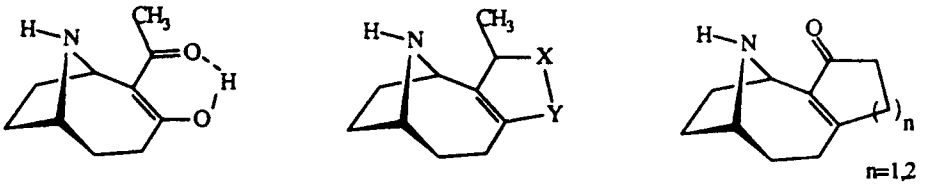
Anatoxin-a (1) is a naturally occuring neurotoxin which is the most potent known agonist for the nicotinic acetylcholine receptor.



(1) *s-trans* (1) *s-cis*

There is some controversy over which of the two possible enone configurations (*s-cis* or *s-trans*) is the bioactive conformation. Our approach is to synthesise anatoxin-a variants with further conformational constraint. Hydroxyanatoxin (2) is an *s-cis* variant of anatoxin and also a useful precursor to a wide range of heterocycles (3).



(2) (3) (4)

Our target for an *s-trans* variant is a tricyclic compound such as (4) and several synthetic approaches from N-Boc anatoxin have been studied, including Diels-Alder and thermal rearrangement methodology. The synthesis of side chain (C 10) analogues can be achieved by the alkylation of key dithioacetal intermediate (5) followed by synthetic manipulation to give the compounds homo, propyl and isopropyl-anatoxin (figure 1).

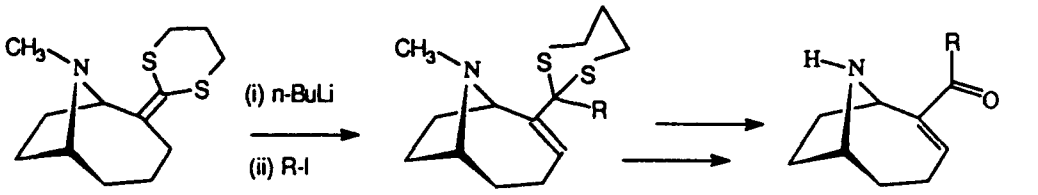


Figure 1

The biological effects of these modifications have been assessed relative to the parent anatoxin (see companion abstract "Anatoxin Structure-Activity Studies: (II) Biological and Computational Evaluation", Thomas *et al*).

ANATOXIN STRUCTURE-ACTIVITY STUDIES: (II) BIOLOGICAL AND COMPUTATIONAL EVALUTION.

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The natural alkaloid of the fresh water cyanobacterium *Anabaena flos aqua*, (+)anatoxin-a (AnTx), poses a significant environmental hazard as a result of its extreme neurotoxicity. AnTx is a potent and stereoselective agonist at the vertebrate muscle end plate nicotinic acetylcholine receptor (nAChR) and is the most potent agonist at neuronal nAChRs[1]. The semi-rigid structure of this bicyclic alkaloid and the presence of a flexible acetyl group ammenable to modification make this molecule an attractive candidate for structure-activity studies, focusing on the pharmacophore and agonist recognition site of nAChR. Previously an extensive series of analogues with alterations to the acetyl side chain, or N-methylation, all had diminished biological activity relative to the parent compound[2,3]. This study highlighted the secondary amine and carbonyl function as crucial for activity. These features have been retained in the current series of analogues. However the conformation of the carbonyl moiety in the nicotinic pharmacophore is controversial, as both *s-cis* and *s-trans* low energy forms exist in solution. Synthesis of a conformationally locked *s-cis* analogue of AnTx, hydroxyanatoxin, has low activity and suggests that the *s-trans* conformation may be most relevant to biological activity[4].

Further attempts to constrain the geometry of the enone moiety were introduced by the synthesis of an epoxide (epoxyanatoxin) and through an anatoxin-heterocycle hybrid, yet neither have activity. Subsequently the addition of methyl and ethyl groups to C10 of the parent AnTx extended the side chain length, creating the structures homo-, propyl-, and isopropyl-anatoxin respectively - (Refer to companion abstract "Anatoxin structure-activity studies: (I) Synthetic Chemistry" Brough *et al.*). The biological effects of these modifications were then assessed relative to the parent AnTx. Activity was measured in competition binding assays on two rat brain nAChR subtypes labelled by [^{3}H]-(-)-nicotine and [^{125}I]αbungarotoxin (αBgt). Simultaneously a functional study was carried out using whole cell voltage clamp of the neuronal α7 nAChR subtype reconstituted in *Xenopus* oocytes. The IC_{50} values for (±)AnTx binding to [^{3}H]-(-)-nicotine and [^{125}I]αBgt are $4x10^{-6}$M and $5x10^{-7}$M respectively, and all the C10 methylated derivatives retained the affinity of the parent compound for these sites (~10-50nM range at [^{3}H]-(-)-nicotine sites and ~100-500nM at [^{125}I]αBgt sites). Similarly in the functional electrophysiological system the C10 derivatives were able to activate the α7 receptor rapidly and to the same extent as AnTx with no significant alteration in EC_{50} values, which were approximately $6x10^{-7}$. EC_{50} values were typically lower than IC_{50}, a trait of nicotinic agonists, reflecting the propensity of the nAChR to adopt a desensitised, high affinity state in binding assays. The rectification properties of the receptor were unchanged for all of the conformers.

Computational chemistry studies define the enone moiety of AnTx as adopting a preferred *s-trans* conformation and similarly the C10 methylated derivatives favour a more *trans* than *cis* minimum energy conformation due to the bulky methyls around the carbonyl. The ability of C10 methylated AnTx derivatives to retain affinity and functionality at neuronal nAChRs has significant implications for the electrostatic properties, conformation and volume associated with the carbonyl moiety of the pharmacophore model for the receptor.

We acknowledge the financial support of RJR Tobacco Co. and SERC.

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RECOMBINANT FUSION PROTEINS FOR IMMUNODIAGNOSIS: Application to the detection of curaremimetic compounds

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ELISAs are based on the use of conjugates that consist of an enzyme coupled to either an antigen or an antibody. Classically, conjugates are made by chemical means, a tedious task which usually leads to heterogeneous coupling. We overcome these difficulties by producing, in *E. coli*, both types of active conjugates, using appropriate expression vectors. The system will be described in the frame of diagnosis of curaremimetic compounds.The enzyme which used in our system is alkaline phosphatase (Phoa), a well-known enzyme which is active as an homodimer.

In a first expression vector, we inserted the cDNA encoding a snake curaremimetic toxin, earbutoxin a (Ea) from *Laticauda semifasciata*, between codons +6 and +7 of the PhoA gene (1).The resulting hybrid Ea-PhoA that accumulated in the periplasmic space of the bacteria was bifunctionnaly active. It recognized an Ea-specific monoclonal antibody, Mα2-3, like unfused Ea and bound specifically to the nicotinic acetylcholine receptor. It also had the expected PhoA enzymatic activity.

The second vector contained a di-cistronic operon. The cDNA encoding a truncated heavy chain (VH-CH1) of Mα2-3, was inserted between codons +6 and +7 of the Phoa gene while the cDNA encoding the light chain was separately fused to the 3' part of the first six residues of the PhoA gene. The periplasmic space of transformed *E. coli* contained a 200 Kda protein which had the binding properties of Mα2-3 and possessed PhoA enzymatic properties. In a sense, the resulting hybrid resembles an IgG molecule in which the Fc part has been replaced by the dimer of PhoA (2).

The two types of recombinant fusion proteins have been used in immuno- and receptor-assays. They both proved to be at least comparable to more classical tracers.

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STUDIES WITH MONOCLONAL ANTIBODIES AGAINST *Naja naja oxiana* NEUROTOXIN I

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Seven different monoclonal antibodies (mAb) were developed against neurotoxin I (NT 1), a snake postsynaptic neurotoxin isolated from *Naja naja oxiana* venom. All of the mAb strongly recognized another snake long-chain neurotoxin, α-bungarotoxin (α-BT) from *Bungarus multicinctus* venom, but not *N. n. kaouthia* α-cobratoxin (α-CT) in an enzyme linked immunosorbent assay (ELISA). The mAb did not bind to short-chain neurotoxins like *N. n. atra* cobrotoxin, *Laticauda semifasciata* erabutoxin b, or *N. n. oxiana* neurotoxin II. Twenty-two different venoms were tested by ELISA with each mAb and as anticipated, *N. n. oxiana* and *B. multicinctus* venoms were positive. *Dendroaspis polylepsis*, *Acanthophis antarcticus*, and *Pseudechis australis* venoms, which contain known long-chain postsynaptic neurotoxins, were also recognized by each antibody. ELISA results from dithiothreitol-reduced NT1 ranged from 13-27% of the native neurotoxin; reduced α-BT was not immunoreactive. Further work with synthesized NT 1 peptides resulted in recognition by mAb of CAPGQNLCY, PGQNLCYTK, KTWCDAWCG, or DAWCGSRGK. Each mAb bound to two non-contiguous peptides, suggesting that they represent contact sites within a more complex, conformational epitope. Equivalent α-BT peptides were not immunoreactive, further supporting the theory that the mAb optimally recognize a conformational epitope. Based on previously published crystal coordinates, molecular modeling of NT-1 and α-BT revealed a similar, accessible region spanning the immunoreactive peptides but the equivalent region within α-CT is less available for antibody binding. *In vitro* binding of α-BT to *Torpedo* AchR was inhibited 29-69% by the mAb which also prolonged the time to death in mice at a toxin:antibody molar ratio of 2.3:1

BINDING PROTEINS ON SYNAPTIC MEMBRANES FOR CROTOXIN AND TAIPOXIN, TWO PHOSPHOLIPASES A_2 WITH NEUROTOXICITY

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Crotoxin and taipoxin are both neurotoxic phospholipases A_2 capable of affecting the presynaptic activity to bring about ultimate blockade of synaptic transmission. The enzymatic activity has generally been considered to be necessary but not sufficient for the blockade. Since many phospholipases A_2 with comparable or even higher enzymatic activity are not toxic, it has been postulated that the difference lies in strong binding of the neurotoxic ones, and not the nontoxic ones, to the presynaptic membrane. In confirmation of this proposition, we have shown that iodinated crotoxin and taipoxin bind specifically with high affinity to the synaptic membrane fraction from guinea pig brain, whereas specific binding is not detected with the nontoxic pancreatic phospholipase A_2. About 50% of the binding sites for [^{125}I]crotoxin can be blocked by unlabeled taipoxin; in contrast, unlabeled crotoxin enhances the binding of [^{125}I]taipoxin slightly. Experiments using photoaffinity labeling and simple chemical cross-linking techniques have led to the identification of three polypeptides preferentially present in neuronal membranes as (subunits of) the binding protein(s) for crotoxin and/or taipoxin. Some, but not all, other toxic phospholipases A_2 appear to be ligands for the three polypeptides. Partial purification of these polypeptides has been made, using affinity chromatography and other techniques.

In order to learn the normal physiological roles played by the toxin-binding proteins, the phospholipase-independent effects of the toxins on the synaptosomes have been sought. We have found that taipoxin or crotoxin, under Ca^{2+}-free condition, inhibits with IC_{50}'s of 20-1000 nM the Na^+-dependent uptake of norepinephrine, dopamine and serotonin by the synaptosomes. In contrast, choline uptake is not affected. Furthermore, the high-affinity site for [^{3}H]desipramine binding, known to be the norepinephrine transporter, is inhibited with an IC_{50} of 14 nM by taipoxin independent of phospholipase activity. These results are strong indications that some synaptosomal biogenic amine transporters are part of the binding proteins for taipoxin and crotoxin.

Chemical modification at Tyr-21 of the phospholipase subunit of crotoxin greatly reduces the neurotoxicity and the binding affinity with little effect on the enzymatic activity of the toxin. Hence, Tyr-21 may be an important residue for the binding of crotoxin and perhaps other phospholipase A_2 neurotoxins. Even stronger evidence comes from the finding that replacement of the corresponding Phe residue of bovine pancreatic phospholipase A_2 with Tyr by site-directed mutagenesis enables it to compete with an IC_{50} of 1 μM for the binding of [^{125}I]crotoxin, contrasting sharply with the complete lack of such ability with the wild type even at 50 μM. In addition, rat phospholipase A_2, which has a corresponding Tyr residue, can inhibit the binding of [^{125}I]crotoxin.

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PURIFICATION AND CHARACTERIZATION OF A NOVEL PHOSPHOLIPASE A_2 FROM THE VENOM OF *CONUS MAGUS*

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Phospholipase A_2 (PLA_2) has been found in the venoms of diverse organisms; we present for the first time the purification and characterization of a PLA_2 from the venom of a cone snail. Using several steps of reverse phase HPLC the polypeptide exhibiting PLA_2 activity was isolated from an aqueous extract of *Conus magus* venom. Molecular weight was determined by electrospray mass spectrophotometry to be approximately 13.7 kDa. Reduction yielded two sharp peaks, suggesting disulfide bridged chains. Partial sequence of both chains has been obtained; one contains ~77 residues, the other ~42. Surprisingly, neither chain has appreciable homology to previously sequenced PLA_2s.

Conus magus PLA_2 (Cm-PLA_2) was originally identified because of its effects on ion channels; crude venom from several species of cone snails were screened independently for ability to inhibit (i) K^+ depolorization induced ^{45}Ca uptake into synaptosomes and (ii) binding of [^{3}H]isradipine to L-type Ca^{2+} channels. Venom from *Conus magus* showed potent block in both assays. Using these two assays as markers, the single polypeptide Cm-PLA_2 was isolated. PLA_2 is known to release arachidonic acid (AA) and to initiate the AA cascade, which has a regulatory effect on many systems, including L-type Ca^{2+} channels. Further studies are being carried out in an effort to determine if specific AA metabolites mediate Cm-PLA_2's effects on Ca^{2+} channels. *Conus magus* may not be unique among cone snails in possessing PLA_2 in its venom; we have isolated a polypeptide with similar activity from *Conus caracteristicus*.

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AMMODYTOXIN ACCEPTOR IN BOVINE BRAIN SYNAPTIC MEMBRANES
A new approach in the study of the toxic site in ammodytoxins

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Ammodytoxins (ATX) are presynaptically neurotoxic phospholipases A_2 isolated from the venom of long - nosed viper (*Vipera ammodytes ammodytes*). Isotoxins ATXA and ATXC, differ structurally only in two amino acid residues (1) but have considerably different toxicities (ATXC is 17-times less toxic than ATXA). They were radioiodinated and used as a ligands. On bovine brain synaptic membranes specific, high affinity, binding sites were demonstrated. ^{125}I-ATXA showed two binding plateaus. Low affinity binding site was not well characterized while for high affinity binding site Scatchard analysis gave K_d: 3.57 nM and B_{max}: 5 pmol/mg of membrane protein. Results obtained for less toxic ^{125}I-ATXC were K_d: 5.54 nM and B_{max}: 6 pmol/mg of membrane protein, which correlates with their toxicities. Both toxins were covalently crosslinked to their acceptors in synaptic membranes using bifunctional reagent DMS. Subsequent SDS-PAGE analysis in the presence of DTT, followed by autoradiography, revealed a major specific adduct of apparent M_w around 70 kDa. Specific binding was lost when Ca^{++} ions were replaced with Sr^{++} ions in the incubation buffer. Binding of both toxins, ATXA and ATXC, to the acceptor was strongly inhibited by β–neurotoxins: crotoxin, crotoxin B and ammodytoxins. *Vipera berus berus* PLA_2 inhibited binding considerably although it was not reported to be neurotoxic. Ammodytin L, PLA_2 analog with myotoxic action, and ammodytin I_2, a non-toxic PLA_2, both from *Vipera ammodytes ammodytes* venom, inhibited binding only at very high concentrations while α–dendrotoxin, β–bungarotoxin and crotoxin A had no influence on binding what so ever. The treatment of synaptic membranes with proteinase K and *Staphylococcus aureus* proteinase lowered the ^{125}I-ATXA and ^{125}I-ATXC binding, while trypsin treatment had no influence. Heat, acid treatment and incubation of synaptic membranes with mixture of neuraminidase and PNGase F also lowered the binding of ^{125}I-ATXA and ^{125}I-ATXC.

In order to study the so called "toxic site" in ammodytoxin, part of the molecule responsible for neurotoxicity (1,2), the binding characteristics of two ATXA mutants were determined. ATXA (K 98,101 N) behaved similarly as the native ATXA, while ATXA (K 117 E) binding constants were the same as in the case of ATXC. This is a new evidence that amino acid residue 117 is involved in the acceptor binding. The two exposed Lys residues (98 and 101), however, might not be so important for binding as was expected. Four peptides from the ATXA sequence, where the "toxic site" is expected, were synthesised and used in competition experiments. All four peptides had an influence on ATX specific binding, with the strongest shown by L3 peptide, taken from the β–structure of ATXA (amino acid residues 61-69).

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IDENTIFICATION OF A PUTATIVE MEMBRANE RECEPTOR FOR BOTULINUM TOXIN TYPE A AND INTRACELLULAR TARGET FOR TETANUS TOXIN IN CHOLINERGIC NERVE ENDINGS OF THE ELECTRIC ORGAN OF *TORPEDO MARMORATA*.

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The electric organ of fishes are derived embriologically from the neuromuscular junction of skeletal muscles. They are formed by hundreds of thousands of cholinergic nerve terminals that synaptically contact with transformed myocites. It is possible to obtain subcellular fractions enriched in synaptic vesicles, nerve terminals or presynaptic plasma membrane. This experimental model overcomes the interaction of different neurotransmitters, as in brain synaptosomes, and let us to correlate physiological, morphological, and biochemical changes induced by clostridial toxins. We have previously demonstrated that the electric organ of *Torpedo marmorata* is sensitive to the action either Botulinum toxin (BoNT/A) and Tetanus toxin (TeTx). Both toxins exert a similar action inhibiting the evoked release of acetylcholine and also reducing the spontaneous quantal release, in frequency and shape of miniature end plate currents. However the IC_{50} for BoNT/A is very much lower than for TeTx. This suggest that both toxins share the same extracellular receptor or perhaps they do no act on the same intraterminal target. Using radiolabeled BoNT/A, we have concluded that there is no competitive binding between BoNT/A and TeTx. Our working hypothesis is that the specific BoNT/A receptor has a protein moiety which facilitates either the recognition or the internalization, or both, of BoNT/A in cholinergic terminals. We have detected a specific binding of BoNT/A to the *Torpedo* electric organ presynaptic membranes, and we have identified a presynaptic membrane glycoprotein (P140) which is detected by labelled BoNT/A in overlay experiments. By immunological screening of a cDNA library we have isolated two independent clones that turned out to code for the same cDNA. The longer clone (RBONTX-1, 3 Kb) has been completely sequenced and it displays a single open reading frame coding for a 757 amino acid protein (predicted molecular mass is 86 kDa). The protein is coded by a single gene, and two transcripts of 6 Kb and 3 Kb are detected by northern blotting experiments. Immunoprecipitation experiments of "in vivo" labelled proteins from *Xenopus* oocytes injected with either electric lobe mRNA or RBONTX-1 cRNA suggest that RBONTX-1 contains the complete protein coding sequence. No significant homologies are detected in data banks, except for short boxes (20 to 30 amino acid long) homologous with various membrane proteins. RBONTX-1 protein has a single putative transmembrane domain at the N-terminus, an Asp-rich C-terminus and a Cys-rich central domain. Three putative N-glycosylation sites are also detected. Antibodies against BoNT/A immunoprecipitate BoNT/A-RBONTX-1 complexes after cross-linking experiments. Our results are consistent with the cloning of a putative receptor for BoNT/A from the electric organ of *Torpedo marmorata*, and functional assays will allow to further confirm this function for RBONTX-1 protein.

With respect to the intracellular target of these clostridial toxins, we have incubated subcellular fractions of synaptic vesicles and synaptosomes with BoNT/A and TeTx. After SDS-PAGE and western blot we have tested the immunoreactivity for synaptobrevin. Under reducing conditions the reactivity of the electrophoretic band corresponding to synaptobrevin has a lesser reactivity in the case of TeTx. This result agree with the proposed action of TeTx trough its Zn^{2+}-protease activity . However when the incubation of synaptosomes or synaptic vesicles were made with BoNT/A there was not any change in the reactivity for synaptobrevin band. This suggest that perhaps BoNT/A is acting on another synaptic vesicle protein membrane that blocks equally the exocytosis of acetylcholine.

This work has been supported by grants from DG2ICYT and CIRIT, Spain. We are grateful to Prof. R.Jahn, Yale University, New Haven for providing antisynaptobrevin sera and to Prof B. Bizzini, Institute Pasteur, Paris for the generous gift of TeTx.

IDENTIFICATION AND CHARACTERIZATION OF HYBRIDOMAS SECRETING ANTIBODIES TO BOTULINUM NEUROTOXIN A

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Hybridomas secreting antibodies to botulinum neurotoxin serotype A (BoNT-A) have been identified and are being characterized for neutralizing capacity, chain specificity, pair-wise binding and reagent potential. The goals of this project are to produce a therapeutic antibody product (one or a combination of monoclonal antibodies) and to characterize toxin neutralization at the molecular level.

Mice were hyperimmunized with pentavalent (serotypes A, B, C, D, E) botulinum toxoid. They received four immunizations. Mice surviving a 10^5 mouse LD_{50} challenge with BONT-A were identified for fusion. These mice were boosted two more times with 10^5 mouse LD_{50} (1.0 µg neurotoxin) each time. Seventy-two hours after the last boost, four mice were bled and splenectomized. Pooled sera from the mice had an ELISA inverse serum dilution titer greater than 10^6. Half of the pooled B cells were fused to SP2 myeloma cells by standard procedures.

The fused cells were plated into 2,400 wells. More than half the wells had growth and 98 cultures were identified as positive for BoNT-A by ELISA. Six supernatants showed some protection *in vivo* (4 x LD_{50} challenge) in two independent experiments. Subcloning and further characterization are underway.

Our approach differs from previous efforts in that we developed protective immunity using toxoid and then repeatedly boosted mice with native toxin. The boosts (1.0 µg neurotoxin/mouse) were intended to establish or reinforce toxin-specific immunity while toxoid-unique (not toxin cross-reactive) epitopes were not boosted.

The experiments conducted herein were performed according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

PHARMACOLOGICAL MANIPULATION OF CELLULAR PROCESSING OF BOTULINUM TOXINS

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Paralysis produced by botulinum neurotoxins type A and B (BoNT A and B) at the neuromuscular junction involves three steps - binding of the toxin to the extracellular surface of the nerve terminal plasma membrane, its internalization by the process of endocytosis and finally the intracellular toxic step leading to the inhibition of transmitter release (Simpson, 1986).

We have attempted to intervene in these steps with potential pretreatment compounds by examining their ability to delay the onset or to prolong the time to 50 and 90 % block (T_{50} and T_{90}) of nerve -elicited muscle twitch tension in the mouse phrenic-neve diaphragm (in vitro at 36°C) after BoNT A and B. Addition of BoNT A or BoNT B (10^{-10} M) produced 50 and 90% block of the twitch response at 86 $\pm$ 7 and 145 $\pm$ 15 min for BoNT A and 62 $\pm$ 3 and 93 $\pm$ 5 min for BoNT B respectively. Preincubation (1 hr) of muscles with triticum vulgaris lectin (TVL, 2 X 10^{-5} M) which has been shown to inhibit toxin binding (Bakry et al., 1992) prolonged T_{50} and T_{90} values to 97 $\pm$ 13 and 173 $\pm$ 14 min respectively with BoNT A ($p > 0.05$ with respect to BoNT A alone). Ammonium chloride (8 mM) which is known to block endocytosis produced a 2 to 3- fold prolongation of BoNT A-induced paralysis times. Combination of TVL and ammonium chloride did not have a potentiating or additive effect. The clinically used drug amantadine, is a lysosomotropic agent. Preincubation of muscles with amantadine at concentration $< 10^{-5}$ M for up to 1 hr failed to alter the time-course of paralysis induced by BoNT A or B while higher concentrations of the drug blocked post-synaptic receptors precluding its use. Two ionophores, nigericin and monensin are known to increase membrane permeability to H^+. Of the two drugs, nigericin was least toxic when applied in concentrations < 0.05 μM. When nigericin (0.04 μM) or monensin (0.05 μM) were present during development of block with BoNT A or BoNT B (10^{-11} to 10^{-9}), the onset of neuromuscular block was delayed 2 to 3 - fold in comparison to untreated muscles. The delay in onset was approximately equivalent to a 10-fold reduction in effective concentration of BoNT. Preincubation of diaphragms with captopril (1 to 10 μM), an inhibitor of metalloendopeptidase for up to 1 hr, failed to antagonize BoNT A- and BoNT B- induced neuromuscular block. The results of this study indicate that searches for drugs capable of blocking endocytosis, neutralizing endosomal acidification and preventing selective proteolysis of BoNT substrates should prove useful in counteracting BoNT toxicity.

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THE ENTEROTOXIN OF *CLOSTRIDIUM PERFRINGENS* TYPE A INHIBITS NEUROMUSCULAR TRANSMISSION IN MOUSE NERVE-DIAPHRAGM

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The enterotoxin of *Clostridium perfringens* type A is a simple protein with a molecular weight of about 35,000 and forms ion-permeable channels in planar lipid bilayers. We found that the toxin inhibited neuromuscular transmission under conditions of low calcium in the medium. Isolated phrenic nerve-diaphragm preparations were exposed to the purified enterotoxin and the twitch tension elicited by electrical stimulations to the phrenic nerve was recorded. The enterotoxin (20 μg per ml) reduced in the presence of 0.5 mM calcium within 10 min the amplitude of the twitch tension to 34% of that before toxin treatment. The effects of the toxin on the twitch tension were irreversible and proceeded independently of stimulation. The reduction of the twitch tension by the toxin was detected at calcium concentrations below 0.6 mM and at temperatures above 20°C. The degree of reduction was inversely related to the concentration of calcium and was dependent on the concentration of the toxin, reaching a plateau of 65% at 6.5 μg/ml of the toxin. The effects of the toxin were antagonized by physostigmine, an inhibitor of acetylcholinesterase, but pretreatment with the toxin did not antagonize the neuromuscular block by decamethonium, suggesting that the enterotoxin did not act like curare which occupies acetylcholine receptors in the postjunctional membrane and competitively inhibits the effects of decamethonium. Guanidine, which antagonizes the prejunctional block of neuromuscular transmission by botulinum toxin, also antagonized the reduction of twitch tension by the enterotoxin. The toxin reduced the frequency of miniature endplate potentials from 0.91 to 0.71/sec, but did not influence its mean amplitude and distribution. The enterotoxin did not affect the tension of the muscular twitch elicited by direct electrical stimulation to the muscle. Thus the prejunctional nerve terminal is the most likely site of toxin action. The enterotoxin of *C. perfringens* type A, with known mechanism of its action on the cell membrane will provide a new tool for the studies on the mechanism of the neuromuscular transmission.

ACTION OF KAINIC ACID ON D-[^{3}H]ASPARTATE RELEASE FROM RAT CORTICAL SYNAPTOSOMES AND MICROSLICES

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Kainic acid's (KA) action on the D-[^{3}H]aspartate (^{3}H-Asp) release from rat brain cortical synaptosomes and slices has been investigated. Synaptosomes were loaded with ^{3}H-Asp during different time that varied from 2 till 30 minutes. Depending on ^{3}H-Asp accumulation time into synaptosomes the speed of spontaneous release of the radioactive amino acid changed from 0.84 till 0.14 nmol/min per 1 mg protein . In the presence of 0.5 mM KA the spontaneous release was markedly inhibited and constituted 0.11±0.01 nmol/min per 1 mg protein for each of the accumulation times. The spontaneous release of ^{3}H-Asp from cortical slices which were loaded previously with the labeled amino acid was not affected by addition of 1 mM KA under superfusion speeds 0.25-1.6 ml/min. K^+-stimulated release of ^{3}H-Asp from the slices elevated from 0.09 till 1.89 nmol per 1 mg protein with increasing of superfusion speed. In the presence of 1 mM KA the K^+-evoked release was markedly inhibited and constituted only 43.5% of control for maximal superfusion speed. So it was found that KA (0.5-1.0 mM) did not affect the spontaneous release of exogenous Asp from rat cortical slices and inhibited the release from synaptosomes. The different action of KA may be explained by the difference in the objects of investigations and by the fact that glia in the brain tissues can accumulate ^{3}H-Asp too. K^+-stimulated release of the radioactive amino acid from cortical slices is inhibited by 1 mM KA as well as the release of glutamate from synaptosomes - the fact that was found in previous experiments [1].

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THE EFFECT OF LESIONS INDUCED BY KAINIC ACID ON THE CONTENT AND STATE OF PHOSPHORYLATION OF THE ASTROCYTE MARKER, GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP), IN THE RAT CAUDATE.

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Kainic acid is a neurotoxin that appears to damage neurones by opening voltage sensitive calcium channels via cell surface receptors. Kainic acid receptors controlling calcium entry also occur on astrocytes, but these cells are known to be more resistant to the toxic effects of the drug and astrocyte proliferation and hypertrophy (gliosis) are well known consequences of kainic acid lesions. Recent work (e.g. Matsuoka et al. 1992) has shown that the disassembly of the cytoskeleton that occurs during mitosis in dividing astrocytes is regulated by the phosphorylation of the intermediate filament protein glial fibrillary acidic protein (GFAP). Since GFAP only occurs in astrocytes, investigation of its phosphorylation state is possible in intact brain tissue. In the present study kainic acid (10 nmoles) was injected into the striatum of adult rats and the immunocontent and phosphorylation state of GFAP were followed during survival periods of up to 90 days. After 7 days immuno-detectable GFAP was decreased by 50%; however after 15 days this fall in content was reversed and immuno-reactive GFAP was increased 6-fold. At all intervals after lesioning the phosphorylation state of GFAP was decreased by 50-70%, suggesting that GFAP of the fibrous astrocytes that predominate in gliotic lesions, is less phosphorylated than the GFAP of the protoplasmic astrocytes of normal tissue. The fall in immuno-detectable GFAP after 7 days was surprising and contrasts with the situation following ischaemic lesions of the hippocampus where GFAP content is massively increased 7 days after lesion.

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ALKALOIDS FROM A TROPICAL MARINE SPONGE INTERACT WITH THE SODIUM VOLTAGE GATED CHANNELS IN CHICK EMBRYO NEURONS.

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We have used the whole cell configuration of the patch clamp technique to examine the neurotoxic effects of dibromosceptrin (DBS) and clathrodin (CLA), two alkaloid compounds purified from the tropical sponge Agelas conifera. Membrane ionic currents were activated applying depolarizing pulses to cells isolated from sympathetic ganglia of ten days old chick embryos kept in culture for 48 hrs. N-methylglucamine was used as the intrapipette ion to block outward potassium currents. Cobalt chloride was added to the extracellular medium to abolish inward calcium currents keeping sodium currents intact. Adding DBS 10^{-6} M to the medium decreased by nearly 40% the average maximum current but CLA 10^{-5} M decreased it only 27%. Current-voltage data, fitted using Boltzman's equation, did not show any effect of this alkaloid on the voltage dependence of activation. However, CLA shifted the voltage dependence of the activation toward more positive potentials and the voltage for 50% activation changed by 6 mV. Voltage dependence of inactivation was shifted toward more negative potentials by DBS and the voltage for 50% inactivation changed by an average of 20mV. CLA shifted the voltage dependence of the inactivation toward more positive potentials and changed the voltage for 50% inactivation by 14 mV. Recovery from channel inactivation was complete in 15 to 20 ms in both normal and alkaloid containing medium. DBS delayed the time for 50% recovery by 1.8 msec, while CLA shortened the time for 50% recovery by 0.5 msec. We conclude, thus, that DBS and CLA are new marine neurotoxins that act on voltage activated sodium channels in different ways. DBS by altering specifically their inactivation process and CLA by altering both the activation and inactivation processes. (Supported by NIH grants GM 08102, MH 48190 and, in part, by MH 19547.)

SYNTHETIC STUDIES OF POLYAMINE AMIDES: POTENT ION CHANNEL BLOCKERS

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In recent years, considerable interest has been shown in isolating and identifying the constituents of arthropod venoms, in particular the non-proteinaceous toxins from spiders and wasps. These toxins are important due to their potency, selectivity, and novel modes of action as potentiators and, at higher concentrations, as non-competitive antagonists of certain ligand-gated or voltage sensitive ion channels.

Polyamine amides generally contain a hydroxy-aromatic chromophore, an amino acid spacer residue, and an unsymmetrical polyamine terminating in a primary amino or guanidino functional group. The toxin PhTX-4.3.3, isolated from the solitary, parasitic digger wasp *Philanthus triangulum*, has been synthesized and we also report a two-step synthesis of the analogous PhTX-3.4.3 from L-tyrosine and the symmetrical polyamine spermine (3.4.3). *N*-Acylation of tyrosine (butanoyl chloride followed by silica gel separation from *O*-mono- and *N*,*O*-di-acylated material) and then controlled (25°C) mono-acylation of spermine using only two equivalents of the polyamine afforded the desired polyamine amide. Synthesis of PhTX-4.3.3 requires selective protection of thermospermine (4.3.3), and we have shown that BOC is a suitable protecting group. The unsymmetrical polyamine was generated by cyanoethylation of mono-BOC-putrescine [69% yield from putrescine (1,4-diaminobutane) and BOC-anhydride] and then reduction of the nitrile to the primary amine with LAH in anhydrous ether, at 0°C, with no detectable concomitant reduction of the *tert*-butyl carbamate protecting group. A second Michael addition and LAH reduction gave the desired product.

In the synthesis of 5.3.3-Arg, found in argiotoxins-636 and 673, we have prepared the spider toxin sFTX-3.3 (Arg-3.3) which is an analogue of FTX from *Agelenopsis aperta*. The proposed FTX structure is remarkable, containing an arginine derived peptide bond which has been reduced to the corresponding secondary amine. Therefore, we prepared this structure by acylation of differentially protected L-ornithine with 3,3'-imino-bis-propylamine and then LAH reduction of the amide to the secondary amine.

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SYNTHESIS OF ACRIDINE-LINKED SPERMINES AS BIFUNCTIONAL LIGANDS

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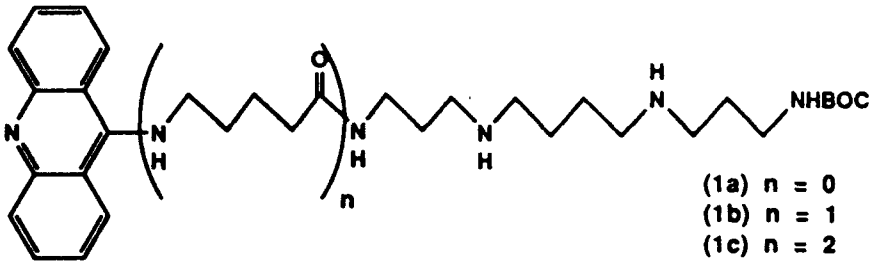

(1a) n = 0
(1b) n = 1
(1c) n = 2

The importance of molecular recognition for ligand-protein and ligand-DNA interactions is significant in the design of selective low molecular weight ligands. We decided to prepare polyamine conjugates of 9-aminoacridine (**1a, b, c**) as bifunctional ligands with utility as DNA receptor probes dependent upon the nature of both the aromatic and polyamine moieties. The importance of DNA interchelators (substituted acridines) and groove binders (polyamines) as anti-tumour agents and anti-viral antibiotics has been well documented [1]. The synthesis of acridine linked spermine derivatives, as novel bifunctional ligands capable of both interchelation and groove-binding modes, is described.

Compounds (**1a, b, c**) comprise three distinct functional fragments, namely, the acridine interchelator, the 5-aminovaleric acid linker, and the polyamine as a groove binder. To synthesize these target molecules, 9(10*H*)-acridone was treated with thionyl chloride to give 9-chloroacridine which was immediately reacted with phenol to give 9-phenoxyacridine as a more stable activated aromatic heterocycle. The phenol was displaced with methyl 5-aminovalerate to give the corresponding 9-monoalkylaminoacridine. Thus, a spacer has been introduced which contains a carboxylic acid for the covalent attachment of groove binding agents. Alternatively, a longer spacer can be incorporated, as in (**1c**), and this was prepared by activation of CBZ-5-aminovaleric acid with HOBt and DCC in dichloromethane and coupling with methyl 5-aminovalerate. Removal of the protecting group (hydrogenolysis) and coupling to the phenol activated acridine gave the precursor to (**1c**). Spermine was selectively protected as a mono-BOC carbamate. The free primary amine was then selectively acylated with activated acridine (**1a**) or acridine-5-aminovaleric acid p-nitrophenyl ester for (**1b**) or (**1c**). Interchelators and groove-binders have been covalently attached and therefore the possibility of selective DNA interaction as a function of these moieties exists.

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FUNCTIONAL DOMAIN STUDY OF BLACK WIDOW SPIDER NEUROTOXINS

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Vertebrate specific neurotoxin (α-LTX) from the black widow spider venom binds with high affinity to the presynaptic membrane, causes an increase of plasma membrane calcium permeability and massive neurotransmitter release. Highly purified α-LTX consists of two polypeptides - 130kDa (LTX) and 8kDa (LMWP). Homogeneous LMWP was isolated by reverse phase HPLC from low molecular weight fraction of the venom . To discover functional activities of each polypeptide in α-LTX complex study of the induced calcium influx and release of transmitter were undertaken. 10^{-9}M of α-LTX (LTX:LMWP ratio is 1:1)as well as 10^{-7}M of the fraction containing LMWP and LTX in ratio 100:1 activates the calcium influx into rat brain synaptosomes and PC12 cells and [^{14}C]-GABA release from the synaptosomes. However, homogeneous LMWP was inactive in these experiments. At the same time preincubation of each preparation with polyclonal antibodies against C-terminal part of LMWP (57-70a.a.residues) decreases substantially induced calcium influx and increases secretagogue action. In case of 100-times excess of LMWP antibodies caused 60% inhibition of calcium influx and twice rise of neurosecretion. At the same time both effects of antibodies were slightly expressed in experiments with α-LTX. Present results may confirm functional interaction between LTX and LMWP, which results in LMWP participation in biological effects of α-LTX complex.

AMINO ACID SEQUENCES OF SPIDER NEUROTOXINS

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Intracerebral ventricular (i c v), subcutaneous (s c) or intraperitoneal (i p) injection of the whole venom of the South American "armed" spider Phoneutria nigriventer (Keyserling) induces neurotoxic signs and symptoms in mice. By the combination of chromatographic methods and observation of the behaviour of the injected mice it was possible to separate three distinct classes of toxins named $PhTx_1$, $PhTx_2$ and $PhTx_3$. The neurotoxic symptoms induced by i.c.v injection of $PhTx_1$ were tail elevation, excitation and spastic paralysis of posterior extremities. LD_{50} was 0.05mg/Kg mouse (whole venom 0.04mg/Kg). The neurotoxin Tx_1 consists of a single chain of 77 amino acids residues and a high content of cysteine. $PhTx_2$ is a mixture of four neurotoxic polypeptides and exhibit obvious homologies with one another (73-84% sequence identities). In mice (i.c.v. and s.c.) $PhTx_2$ produces excitatory symptoms as salivation, lacrymation, priapism, convulsion and spastic paralysis of anterior and posterior extremities. LD_{50} (i.c.v.) 0.24 - 1.62Hg mouse. $PhTx_3$ is a mixture of six polypeptides that caused flacid paralysis in mice and death (LD_{50} 7.2Hg/Kg mouse)

Rezende Jr., L.; Cordeiro,M.N., Oliveira, E B. and Diniz, C.R (1991) Toxicon, 29, 1225-1233

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NOVEL TOXIN FROM *ECTATOMMA TUBERCULATUM* ANT VENOM

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Novel toxin from the *Ectatomma tuberculatum* ant venom responsible for lethal activity of the crude venom to mice and cockroaches was isolated. Its complete amino acid sequence was determined by protein chemistry methods. It was shown that molecular weight of this small protein is 7925Da and pI - 8,2, and its molecular structure is exraodinary as compared with other well known low molecular weight toxins. Toxin contains two polypeptide chains (α and β), which are linked to each other by disulfide bond. Chains α and β accordingly consist from 37 and 34 amino acid residues and, moreover, there is an internal disulfide bridge in each polypeptide chain. This toxin at concentration 10^{-7}-10^{-6}M forms a potential-dependent nonselective cation channel in the artificial and cell membranes.

RABBITS' MODEL OF MPTP-RELATED PARKINSONISM.

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces parkinsonism-like neurological disorder in human and in some laboratory animals, the main criterion of which is irreversible reduction of dopamine (DA) concentration in striata. MPTP - neurotoxicity in susceptible animals is considered the most satisfactory model of Parkinson's disease. The rabbit is one of the most useful species for a neurological research, but until now there have been no publications about MPTP action on this species. The neurotoxic action of MPTP and its 4-(o-tolyl) analog (2'Me-MPTP) on two breeds of rabbits was investigated. Acute toxicity of MPTP was about 12-13 mg/kg for the "little silver-black", and about 32 mg/kg for the "chinchilla" rabbits. Acute toxicity of 2'Me-MPTP was 5-6 mg/kg for both breeds of rabbits. No significant changes in DA content were perceived in the groups "chinchilla" given MPTP idependently from dosing regimens and method of injectios. MPTP causes marked reduction (about 40%) of striatal dopamine content given at a dosage of 6 mg/kg for two days in rabbits of the "little silver-black" breed. 2'Me-MPTP did not produce a reproducible change in the dopamine content in rabbit striata. It can be assumed that the more effective biotransformation of 2'Me-MPTP in rabbits' periferal tissues results in their unsusceptibility to its neurotoxic action.

NEURAL CELL ADHESION MOLECULE (N-CAM) DISTRIBUTION MAY PREDICT THE EFFECT OF NEUROTOXINS ON BRAIN

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Neurotoxicity is one of the most serious toxicological events since damaging even a small number of neurons can cause profound consequences for the overall performance of the organism. Due to the some unique features of the nervous system (spatial separation of the cell body and axon; interdependence of the nerve and glia; myelination; synaptogenesis; neurotransmission) nervous cells are specifically targeted by neurotoxicants. The using of various cultured cells and tissues models give more chance to analyse the effects of neurotoxins that using of laboratory animals. Immunohistochemical investigation of neurospecific proteins extend the possibility to study the neurotoxins effects. The risk or occurrence of toxicity during drug therapy often influences the choice of drugs or limits the success of drug therapy. This is true with valproate (antiepileptic drug). We were revealed the effects of valproate on the NCAM distribution on the plasma membrane of hippocampal neurons and glial cells during development. Monolayer cultures of dissociated hippocampal cells of the mouse foetuses were treated by IgG purified from monospecific rabbit anti-rat-NCAM sera (7 mg/ml) after washing and prefixation with 0.1% glutaraldehyde. Control cultures were incubated with preimmune serum. Protein A conjugated with 17 nm colloid gold particles was used for visualization of specifically bounded IgG. Then cultures were washed and postfixed with 1.5% glutaraldehyde followed by dehydration and embeded in Epon. In controls (5th day *in vitro* culture) NCAM-labels were distributed throughout the neuron plasma whereas glial cell surfaces possessed only single labels. When hippocampal cells were taken from the foetuses of females injected with valproate (200 mg/kg, 40 min before sacrifice) on the 5th day *in vitro* culture multiple pores were visible on the neuronal cell bodies and NCAM-labels seem to be internalized. Elevated amount of NCAM labels in the places of neurities sprouting was clearly demonstrated. On the 12th day *in vitro* the NCAM distributions picture were close to the control. NCAM possess a potential histogenetical plastic functions during CNS development (cell-cell adhesion, migration, synapse formation)[1] and under the treatment with several neurotoxins. It was suggested that experimental approach applied can be useful for prediction of neurotoxins effects on developing nervous system.

1. Rutishauser, U., et al. Science, 240 (1988) 53-57.

PURIFICATION OF THREE IMMUNOLOGICALLY AND STRUCTURALLY SIMILAR NEUROTOXINS FROM THE AUSTRALIAN PARALYSIS TICK, *IXODES HOLOCYCLUS*.

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Envenomation by the Australian paralysis tick, *I. holocyclus*, is a considerable veterinary problem affecting tens of thousands of domestic animals and wildlife each year. Envenomation is characterised by a progressive ascending flaccid paralysis and is due to a neurotoxin present in the salivary secretions of the tick. Purification of the neurotoxic component has been largely unsuccessful mainly due to the insensitivity of the biological assay, the minute quantities available and labile nature of the neurotoxin. Recently, the neurotoxin has been identified as having a molecular weight of 5 kD by SDS-PAGE using an innovative technique involving adsorption to synaptosomes (Thurn *et al.*, 1992). The identification has subsequently enabled the final purification of three closely related neurotoxins.

The neurotoxins were purified from extracts of engorged adult female *I. holocyclus* by a series of conventional chromatography techniques (heparin Sepharose, DEAE Affi-gel blue, Alkyl Sepharose) followed by C4 and C8 reverse phase HPLC. Purity was demonstrated by SDS-PAGE and biological activity confirmed by injection into neonatal mice. The neurotoxins were all inactivated by reverse phase HPLC, however activity was regained in the presence of BSA. Total amino acid analysis of the three neurotoxins revealed that the neurotoxins were essentially identical. Amino acid sequence was restricted to peptides generated by proteolytic digest with trypsin. Direct N-terminal sequencing was unsuccessful due to an amino-terminal blockage. The tryptic maps produced for the three neurotoxins share several peptides in common, however distinct diffences are apparent. Amino acid sequence of several of the peptides has also been obtained. No apparent homology to any other neurotoxins or proteins has been observed. An accurate molecular weight of 5,640 and 5,460 daltons has been determined by Mass Spectrometry for two of the neurotoxins. In addition, immunological similarity has been demonstrated using a non-neutralising monoclonal antibody produced against a partially purified preparation of neurotoxin. The monoclonal recognises a common epitope shared by all three neurotoxins.

The amino acid sequence determined to date will be used to construct oligonucleotide primers for PCR. The amplified DNA will then be used to probe a cDNA library prepared from unengorged adult female *I. holocyclus*. The long term aim is to produce a veterinary vaccine that will be capable of providing protection against the three neurotoxins. The shared structural characteristics and immunological similarity identified above provide the basic evidence for the feasibility of such an approach.

Thurn,M.J, Gooley,A. and Broady,K.W. (1992) Recent advances in Toxinology Research, Vol.2, National University of Singapore, p243.

A EXTRACTION PROCEDURE MODIFICATION CHANGES THE TOXICITY, CHROMATOGRAPHIC PROFILE AND PHARMACOLOGIC ACTION OF *OSTREOPSIS LENTICULARIS* EXTRACTS

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Ciguatera is a human intoxication caused by the ingestion of tropical fish associated with the coral reef. It has been suggested that ***Ostreopsis lenticularis*** is one of several benthic dinoflagellates implicated as producers or primary vectors of ciguatera related toxins. We have studied cells from five different laboratory cultures of these benthic dinoflagellate that were extracted with methanol. Methanolic extracts were also prepared from two other cultures after pre-extraction of cell pellets with acetone. The latter treatment increased by a factor of 10 the mouse toxicity of these extracts as assayed by i.p. injection. Applied to frog sartorius muscles the acetone pre-treated extracts stimulated fast contractions or twitches. In contrast, the pure methanolic extracts inhibited, with approximately equal potency, the response of these muscles to nerve released or bath applied acetylcholine (ACh). The mean membrane resting potential in presence of 0.01 mg/mL of acetone pre-treated extracts changes from -81.521 ± 8.29 (n=70 fibers, 3 muscles) to -26.68 ± 10.13 (n=70 fibers, 3 muscles). Perfusion of the muscles with Tetrodotoxin (10 μM) was effective in antagonizing (75%) of the induced membrane depolarization. In contrast, mean membrane resting potentials in control and pure methanolic extracts treated muscles were no significantly different. Chromatographic analyses done by reverse phase HPLC showed a major component in the excitatory extracts that had a column retention time (R_t) of 5.25 min and represented 65 to 85% of the total area of the chromatograms. This component was termed ostreotoxin-3 (OTX-3). The major component in chromatograms of the ACh inhibitory extracts had a R_t of 2.1 min and represented 53 to 70% of their total area. This component was termed ostreotoxin-1 (OTX-1). In all the chromatograms only the relative concentration of OTX-3 correlated positively ($r = 0.9748$, $p = 0.005$) with the respective mouse toxicities of the extracts. Binding of these extracts to sites in the voltage gated sodium channel was studied using rat brain synaptosomal membranes and the radiolabeled ligands ^{3}H-saxitoxin (for site 1) and ^{3}H-brevetoxin type 3 (for site 5). None of the extracts displaced the specific binding of these ligands indicating that neither of these sites were targets for OTX-1 or OTX-3. (Supported by NIH grants GM 08102 and MH 48190.)

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