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MASS-SCREENING OF CURARIMIMETIC NEUROTOXIN ANTAGONISTS

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A screening procedure for inhibitors of alpha-bungarotoxin binding to nicotinic acetylcholine receptors (from Torpedo californica electric tissue) was developed which combines the sample handling capacity of microtiter plate technology with the speed and simplicity of autoradiography; the screening protocol is amenable to further automation by liquid handling robotic equipment. Additional assays are needed to distinguish between blockers of neurotoxin binding that are themselves toxic and those that have therapeutic value; here research focused on identifying a suitable cell line and on adapting the use of microwell plates to isotope flux assays. Good results were obtained with BC3-H1 cells grown and assayed in 24-well plates. (cytotoxicity)

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Introduction - Statement of Objectives

The postsynaptically active proteins found in elapid and hydropheid snake venoms block the acetylcholine receptor (AChR) in the neuromuscular junction and are therefore referred to as curarimimetic neurotoxins. This class of compounds more recently also has been recognized as comprising a number of potent peptide toxins in certain marine snails (e.g. Conus geographus and other Conus species). The action of these proteins differs from that of the alkaloid blockers, e.g. d-tubocurarine itself, in that it is much more selective and essentially irreversible. Curarimimetic proteins such as alpha-bungarotoxin, the major toxic principle of the venom of the Formosan krait Bungarus multicinctus, are toxic because they prevent the agonist acetylcholine from reaching its binding site and thereby from triggering the opening of the receptor ion channel.

A defense against them can be designed in two ways: first, deployment of antitoxin antibodies to intercept incoming toxin molecules before they reach the receptor and second, competition for target sites by antagonists that share some of the neurotoxin's structure and therefore recognition potential without blocking the acetylcholine sites. The objective of the research of the present contract was to develop methods which will eventually lead to the identification of therapeutically useful antagonists of the second type. Such agents can be identified by appropriate competition assays, i.e. a toxin antagonist is recognized by its ability to block or slow down the binding of a suitable radiolabeled derivative of the toxin.

The great majority of neurotoxin antagonists identified in this manner are likely to be receptor agonists and antagonists themselves, and therefore of little preventive or therapeutic value. An additional step is consequently required to select those compounds from among the large number identified in the first round of screening which are physiologically inert. This requires an assay technique that goes beyond binding competition and reveals effects on physiological function.

The first two years of the contract period were dedicated to goal No. 1, i.e. research on simplified techniques of antagonist recognition while during the third year (and extensions thereof) we tackled objective No. 2, i.e. we attempted to develop screening techniques for physiologically inert toxin antidotes. The following is a brief summary of this work.

A Test of the Basic Premise

The assumption on which the contract research rests is that the neurotoxin binding site on the nicotinic acetylcholine receptor differs sufficiently from the acetylcholine binding site that drugs can be found or designed which discriminate between them. More specifically it is implied that compounds exist that interact with portions of the toxin binding site, without blocking the binding of the agonist, and therefore act as physiologically inert toxin antagonists. At the beginning of the contract period little information was available on this question, except for some indirect evidence for the existence of substances (e.g. inorganic cations) that impair the interaction of the receptor with alpha-bungarotoxin without equally affecting the function of the receptor. We undertook to analyze this problem by screening a number of drugs and other compounds for their ability to block the binding of acetylcholine and alpha-bungarotoxin, in the hope of finding substances of the appropriate discriminatory potency, i.e. substances capable of blocking toxin access at concentrations far below those at which they interfere with acetylcholine binding.

That the binding sites for acetylcholine and alpha-bungarotoxin should not be identical is a plausible postulate based on the comparison of the size, structure and affinity of these two ligands. While the agonist is a small molecule of 146 daltons with a binding energy of 7 to 11 kcal/mol, the toxin has a molecular mass of 8000 daltons and binds virtually irereversibly, with an association energy in excess of 20 kcal/mol, implying a larger number of interactions with the receptor molecule.

Using a filtration assay that can be employed with few modifications for both acetylcholine and toxin, we screened a number of cholinergic drugs as well as other compounds for their ability to inhibit the association of the radioactive ligands to Torpedo californica receptor. In the majority of cases excellent correlation of the two pharmacologies was observed; fortunately however it turned out that several small basic proteins (such as lysozyme and cytochrome c) are capable of blocking toxin-receptor association over a wide range of concentrations without any detectable effect on acetylcholine; to our disappointment, protamine, a strongly basic polypeptide which has been in clinical use as a heparin antidote for many years (and therefore would not require costly and time-consuming development and approval procedures) also inhibited acetylcholine binding. Nevertheless the results of this investigation suggest that the toxin site is larger than the agonist site, as could have reasonably been expected, and that it comprises additional

negative subsites for which ligands might be designed that will not interfere with receptor activation. It is conceivable that the design of such toxin antidotes will be based on modifications of small proteins perhaps even on fragments or on chemical derivatives of the curarimimetic neurotoxins themselves.

Development of a Toxin Competition Screening Procedure

The original plan was to devise a screening procedure based on the chemistry of the so-called DEAE-cellulose disk procedure. This procedure which has been the basis of the most widely used receptor assay over the past 20 years has many features suitable for adaptation to mass screening. It is simple, economic, fast, and exquisitely sensitive and specific; in particular, it is adaptable to multiwell titer plate technology which has figured so prominently in hybridoma research and development. To further reduce the time required for an individual data point which normally is determined sequentially, one at a time, in a gamma spectrometer we showed that analysis is possible by autoradiography. In such a protocol many data points (at least several hundred) are obtained at a time.

The ion-exchange protocol involves a number of pipetting steps and final transfer of the incubation mix onto the DEAE-cellulose sheet whose handling (clamping into blotting device; removal therefrom; repeated washes; drying; and autoradiography) requires considerable care and labor. In addition there are drawbacks specific for the chosen membrane (fragility of DEAE-cellulose paper; high cost of Nylon derivatives). Therefore and because of the explicitly stated goals 2b and c of the contract, much of the effort in the second year was devoted to the development of a solid-phase binding assay.

Since electroplaque membranes as well as Triton X-100 solubilized receptor stick poorly to plastic a procedure had to be developed for the immobilization of a sufficient amount of receptor onto the container wall, i.e. the individual wells of the 96-well microtiter plate. Several proteinaceous anchoring agents were tested, of which only monospecific anti-receptor antibodies proved adequate; pre-treatment of wells with anti-receptor antisera, anti-receptor hybridoma supernates, or receptor-binding lectins (concanavalin A) did not detectably improve receptor attachment, presumably because of strong competition of (a) nonspecific proteins for protein binding sites on the plastic surface and of (b) non-receptor glycoproteins for the lectin. We then tested <u>Torpedo</u> receptor dissolved in a variety of detergents, and discovered that cholate-solubilized receptor binds significantly to the PVC plates employed. Consequently, for all subsequent experiments receptor was

prepared by extraction from Torpedo californica electric organs using 1% cholate.

Further Improvements

Further experiments were carried out to optimize the test procedure. Parameters studied included: choice of titer plate; optimal receptor concentration; increases in receptor adsorption by means of special coating agents; and improvements in the autoradiographic procedure. We found that flexible PVC plates are preferable because of their relatively high protein-sorptive capacity when compared to the more commonly used rigid polystyrene plates. That these plates are readily cut with scissors proved to be an additional advantage: it allows quick removal of the rim of the plate so that the bottom of the plate can be brought into direct contact with the film for autoradiography; in addition autoradiographic results can be confirmed by counting individually cut wells in a gamma spectrometer. Millipore's 'Millititer plates' were abandoned, despite their excellent protein binding properties, because of their price and the need for additional steps in the analysis. We found that the amount of receptor adsorbed to the wells not only reaches a limit, presumably reflecting the saturation of protein binding sites on the plastic; it actually decreases at high protein concentrations presumably because the receptor, which after all represents only a relatively small fraction of the total protein, is competed off by more strongly binding proteins. Eventually a protocol was adopted calling for the use of dilute receptor preparations (ca .5 picomole of toxin binding site per well). Since only a rather small fraction (ca 7%) of applied receptor binds to PVC wells even under the optimal conditions described above several nonspecific treatments of the plates prior to the sample adsorption step were explored. Polyethyleneimine was found to increase receptor binding by 3- to 4fold and to reduce nonspecific binding of radioligand to PVC even if plates are not quenched (with protein solutions such as 3% casein, to block nonspecific protein binding sites) prior to receptor addition. To improve the autoradiographic images we swiched from V-bottom to flat bottom wells; to further reduce the autoradiographic halo around each well the incubation volumes were kept to a minimum (.025 ml). Standard autoradiographic casettes cannot hold objects as thick as a microtiter plate; to securely hold the titer plates against film for the duration of the exposure a casette of adequate dimensions was constructed from plexiglass and lined with photographer's felt. Although this prototype only holds 4 plates, the manufacture of larger versions should not be difficult. At this point the autoradiographic version of the multiwell titer plate assay is ready for further automation such as employment of a robotic workstation and computer-assisted analysis of densitometric data.

Exploration of Non-radioactive Technologies

As a rule non-radioactive assays are far less sensitive than assays involving radioactive compounds, but they offer advantages with respect to environmental safety and reagent stability. We therefore spent some time to explore such non-radioactive alternatives. In particular we worked on an ELISA assay for the detection of receptor-bound alpha- bungarotoxin. A major problem, in view of the limited sensitivity of such assays, is the amount of receptor that can be bound to the well. Non-specific anchoring methods (e.g. the coating with polyethyleneimine employed so successfully in the radiotoxin assay) increase adsorbed receptor levels toward the threshold of detectability by ELISA methods, but not significantly beyond it. A further increase in receptor density on the well wall cannot be achieved without either the isolation of the receptor itself or the purification a receptor-specific anchoring agent. In view of the limited supply of electric tissue and the difficulties and low yield of receptor purification protocols, and further considering the problems of isolating monospecific anti-receptor antibodies (which were found to be excellent anchoring agents), we settled on the preparation and isolation of mAb35, a monoclonal antibody which was originally raised against receptor from another species but meanwhile has been found to crossreact with virtually all vertebrate muscle receptors. Since the antibody constitutes only a small fraction of the total protein in the hybridoma supernatant, it must be purified or at least considerably enriched prior to use. Conventional procedures failed, but affinity chromatography on either immobilized receptor or anti-rat IgG antibodies showed some promise. The second technical problem in ELISA type assays is how to detect unblocked toxin binding sites. We investigated the use of cold (non-radioactive) toxin followed by anti-toxin antibodies, secondary antibody-enzyme conjugate, and enzyme substrate (we used alkaline phosphatase as the reporter enzyme). Although the feasibility of this protocol was established and although such a colorimetric assay could be automated, the number of manipulations (additions, transfers, washes) appears excessive. A simplification of the above protocol, involving competition of ligand with a toxin-enzyme conjugate, was tested. Unfortunately the conjugates lacked high-affinity binding.

Finding toxin antidotes among the toxin antagonists

The antagonists that can be identified using the procedures whose development was described above still need to be sorted into physiologically active substances that are presumably of little therapeutic value and real toxin antidotes. To identify

the latter a physiological assay is required, i.e. an assay that measures the activity of the channel associated with the receptor. This can be accomplished in a variety of ways. Most obvious would be an electrophysiological method; however we opted for ion flux assays, not only because this is a biochemist's way of analyzing channel activity but also because such assays lend themselves to the advanced forms of robotics ansd automation recently achieved in the field of liquid handling.

In our initial proposal we had suggested carrying out channel studies on the mouse muscle cell line BC3-H1; nevertheless in the interest of improved signals we investigated a number of different cell lines, including human rhabdomyosarcomes as they express the acetylcholine receptor of the species of interest. Unfortunately they express far lower levels of receptor, and a large fraction does not even reach the cells urface so that we returned to the murine line whose advantages include, in addition to a high copy number, their extensive previous use both for biochemiocal analysis as well as channel studies. We first duplicated published procedures using individual 35mm plates and substituting $^{86}Rb^{+}$ for $^{22}Na^{+}$, to reduce the radiation hazard. Once a reproducible flux assay had been established, we switched to multi-well plates. Reduction in well size was tolerated down to, and including, 24-well plates, especially when care was taken to optimize culture conditions so as to reach the highest possible receptor densities. We were however unable to carry the protocol to the 96-well plate level. Much of this failure was due to low receptor levels (ca 5 femtomoles per well), but cell detachment was also partly responsible. An attempt to circumvent this problem by using cell suspensions for the flux assay failed likewise.

Summary

I would like to summarize by comparing the accomplishments with the original contract goals.

Objective (a): "Isolate and purify nicotinic acetylcholine receptor and receptor-enriched membrane preparations from electric organ of Torpedo californica by standard methods. Prepare radioiodinated purified alpha-bungarotoxin for use in binding assays. Prepare anti-receptor antibodies by conventional methods in rabbits." - All of these preparations were made; with the exception of the last one they were actually necessary prerequisites for subsequent work.

Objective (b): "Develop and adapt two assay procedures for detecting competition of putative neurotoxin antagonists for toxin binding sites on the acetylcholine receptor. 1. Modify the DEAE-cellulose binding assay to permit processing of larger

numbers of samples by adapting this standard assay to microtiter plates with evaluation of results by autoradiography. 2. Develop and test a solid-phase binding assay where purified receptor is the immobilized target for binding." - Both of these targets were reached. It was shown that the DEAE-cellulose binding assay can be combined with autoradiographic analysis; the feasibility of a solid-phase assay was established using cholate extracts of Torpedo electric tissue, without any additional receptor purification step being required. The solid-phase assay is amenable to automation.

Objective (c): "Develop and assess the sensitivity of an ELISA assay using anti-toxin antibody conjugated with enzyme rather than radioiodinated toxin." - The feasibility of an ELISA assay was explored, but although such an assay was found possible in principle, in practice it turned out to be too insensitive.

Objective (d): "A cultured cell line, BC3-H1, shall be used in a radioactive sodium flux assay to assess whether the inhibitory effects of curarimimetic toxins can be prevented or reversed by administration of putative toxin antagonists identified using the screening procedures developed during years 1 and 2 (objectives a - c)." - A slightly modified version ($^{86}Rb^{+}$ instead of $^{22}Na^{+}$) of the proposed assay was found to be adequate for the proposed purpose; however efforts to miniaturize the assay for use with multi-well titer plates proved only partly successful.

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