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MASS-SCREENING OF CURARIMIMETIC NEUROTOXIN ANTAGONISTS

ANNUAL REPORT

JAKOB SCHMIDT

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The third year of the contract period was devoted to testing several ion flux protocols for the distinction between toxic and therapeutic blockers of curarimimetic neurotoxins. First a cell line suitable for acetylcholine receptor channel analysis was identified; among several rodent and human lines the murine tumor line BC_3-H1 was selected because of its high receptor content. After determining optimal culture conditions for this line attempts were made to miniaturize the flux assay; we were able to perform ^{86}Rb flux measurement in standard 24-well multititer plates.

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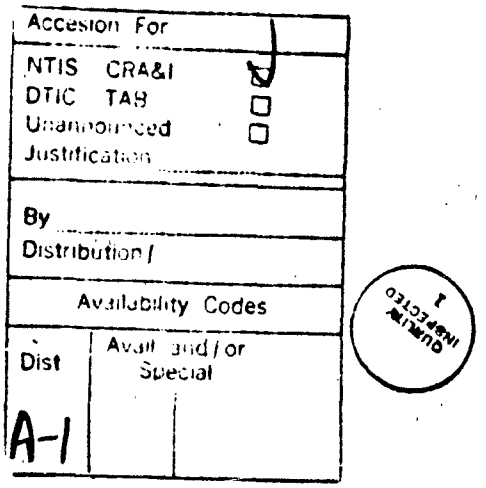

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Statement of Problem

The goal of the contract is to develop methods for the identification of antidotes for the potent curarimimetic neurotoxins which occur in the venoms of elapid and hydropheid snakes as well as certain sea snails (Conus sp.). The great majority of such neurotoxin antagonists, i.e. compounds that in vitro compete with or displace the neurotoxin, are likely to be antagonists themselves and therefore of little therapeutical value. Therefore a major research effort may have to be mounted in order to identify the presumably small number of physiologically inert toxin antagonists. The first 2 years of this contract were devoted to the development of techniques for the identification of neurotoxin competitors, while the third year, including its funded and non-funded extensions have been dedicated to the development of techniques that would allow the distinction between toxic and therapeutic compounds.

Background

Ligands capable of binding to a specific receptor can be readily identified by their ability to compete with radiolabeled compounds known to bind to that same site. During the first 2 years of the contract period we have devised a procedure which permits the identification of compounds capable of blocking the binding of alpha-bungarotoxin, a prominent representative of the curarimimetic protein toxins found in certain animal venoms. This procedure is amenable to automation by means of a robotic workstation. Since most of the competing compounds identified by this binding assay are likely to be physiologically active themselves, either as agonists or antagonists, and thus presumably with little preventive or therapeutic value, an additional screening procedure is required to identify among them the physiologically inert ligands which then could be further investigated as potential antidotes. In vivo the binding of an agonist to the nicotinic acetylcholine receptor, such as carbamoylcholine or acetylcholine itself, results in the opening of a cation-selective ion channel formed by the 5 transmembrane subunits of the receptor (for review of

acetylcholine receptor physiology see Adams, 1981; St. and Finer-Moore, 1985). The ion flux that results fro. action cannot be observed in solubilized receptor preparations but requires intact membranes for membrane potential or ion transfer measurements. The simplest biochemical assay is the determination of flux of radioactive ions through the plasma membrane of intact cells.

Rationale

During the first 2 years of the contract we have developed an autoradiographic procedure for the identification of neurotoxin antagonists which is amenable to further simplification and automation. Among the biochemically identifiable antagonists only relatively few are expected to be potential antidotes, namely those that have no physiological actions of their own. To identify them, a physiological assay is required, i.e. an assay that measures channel activity; we opted for ion flux assays not only because this is a biochemist's way to study channel activity but also because such assays lend themselves to the advanced forms of robotics and automation recently achieved in the field of liquid handling.

Methods and Materials

Cell culture: Culture plates were obtained from Falcon; microtiter plates (6, 12, 24, and 96 wells) were products of Costar. Plates (single and multi-well) were coated with a 1% solution of collagen prior to use.
Media: Dulbecco's modified Eagle's medium (DME) was purchased from GIBCO, and supplemented with 10-20% heat-inactivated fetal calf serum (FCS, also from GIBCO), depending on cell line. Cell lines: BC_3-H1 cells (a murine brain tumor derived non-fusing muscle cell line that expresses the nicotinic acetylcholine receptor at high levels) were obtained from the laboratory of Dr. Paul Adams, Howard Hughes Med. Inst., S.U.N.Y. Stony Brook, and grown in 80% DME, 20% FCS; they were transferred to 1% FCS (in DME) to induce differentiation. L6 (a rat skeletal muscle line), CRL (a mouse muscle line) and RD, A673, A204, and TE671 (human rhabdomyosarcoma lines; with regard to TE671 see Stratton et al., 1989) were all obtained from the American Type Culture Collection and grown in 90% DME, 10% FCS; C2C12 cells (a murine myoblast line) were obtained from either ATCC or Dr. Bruce Paterson, NIH. Primary chick myogenic cells were prepared from leg muscles of 11-day embryos by established procedures (Pezzementi and Schmidt, 1981) and

grown in 10% horse serum, 2% embryo extract in DME. Al cultures were kept at 37 in a 5% CO2, water-saturated atmosphere.

Assays: Acetylcholine receptor densities were determined by binding of ^{125}I-alpha-bungarotoxin as described previously; cell extracts weres assayed by the DEAE-cellulose disk procedure (Schmidt and Raftery, 1973); intact cells were labeled by adding the radiotoxin to the medium, followed by a 1-hour incubation at 37^{o}C, aspiration of medium and repeated washes with phosphate-buffered saline to remove unboud toxin (Pezzementi and Schmidt, 1981). The ion flux protocol was adapted from the procedure of Sine and Taylor (1979), except for substituting the less hazardous $^{86}Rb^{+}$ for $^{22}Na^{+}$. Single-channel recordings were performed by Dr. J. Dilger in the Dept of Anesthesiology at S.U.N.Y. Stony Brook Health Sciences Center.

Ligands and other chemicals: Several batches of ^{125}I-alpha-bungarotoxin with specific activities ranging from 200 to 1200 cpm per femtomole were prepared as described previously (Schmidt, 1984). Drugs including d-tubocurarine chloride and carbamoylcholine were from Sigma (St. Louis, MO). ^{86}Rb was purchased from Amersham (specific activity: 37 to 300 MBq/mg).

Results and Discussion

Identification of a suitable cell line

The binding assays were all performed with the acetylcholine receptor from Torpedo californica which had been originally selected because it is available in bulk; receptor structure has been sufficiently conserved throughout vertebrate evolution to obviate work on the far less plentiful receptors from higher vertebrates. Unfortunately channel activity measurements are not as easily performed with this preparation; receptor-containing vesicles can be prepared, but the ion flux signals are small because of the small volume controled by each receptor molecule. Torpedo electrocytes can be grown in culture but require a constant supply of embryonic rays - a virtual impossibility, even at suitable marine biology stations, in view of the reproductive behavior of these animals. We therefore explored a number of skeletal muscle cell lines (both rodent and human) for their feasibility for this investigation. Results are presented in Table I. As is obvious from this survey, there is considerable variability in the receptor content of these cells. The human

rhabdomyosarcoma lines RD and TE671 are rich in recep but only a small fraction appears to be accessible on surface. BC_3-H1 cells exhibits a large density of su[illegible] receptors, and since they have been used successfully in the past for biochemical receptor analysis (Merlie, 1984) as well as channel studies (Sine and Taylor, 1979; 1980) we chose them for subsequent ion flux analysis. The major drawback in working with these cells is that they differentiate rather slowly.

Optimal conditions for growing these cells to high receptor densities were worked out. As a rule, they were seeded at 10^5 per 35mm plate; after 4 days in 20% FCS in DME they were switched to 1% FCS; this was, fairly reproducibly, followed by a rise in receptor content that within another 5 to 6 days could reach 200 femtomoles and more. Levels of 500 femtomoles could be reached when culture time was extended to 2 weeks and beyond.

Flux assays in 35-mm plates

Ion flux assay conditions were worked out using 35mm plates as described by Sine and Taylor (1979). Initially, agonist and radioisotope were added in small volumes to the culture medium at the start of the incubation; this however resulted in considerable data scatter. To improve the signal/noise ratio, ion flux was initiated by replacing the

medium with medium containing 0.1 mM carbamoyl choline a 50,000 cpm of $^{86}Rb^{+}$. Flux experiments were carried out after slowly cooling cultures to 4°C to inhibit other ion transport processes. After the desired interval, uptake was stopped by aspiration of the supernatant followed by repeated washes with ice-cold phosphate-buffered saline. The cells were then dissolved in a small volume of 1 N NaOH, 1% SDS, and the extract transferred to a counting vial; the plate was then washed once more with NaOH/SDS and extract, and washes combined and counted in an LKB 1214 Rackbeta liquid scintillation spectrometer (Cerenkov radiation). Control cultures were pre-treated for 60 min at 37°C with 10^{-7} M non-radioactive alpha-bungarotoxin to completely block all receptors. An example of such an experiment is given in Fig. 1. Despite repeated attempts we have been unable to reduce the nonspecific background in a reliable way such as reported by Sine and Taylor (1980); note that the signal-noise ratio reaches a maximum after 1 min.

Flux assays in multiwell titer plates

Once a reproducible flux assay had been established with single 35mm plates, attempts were made to adapt the procedure to the smaller multi-well titer plates. Surface receptor levels appeared adequate; in a preliminary experiment receptor densities of ca 200, 150, and 70 femtomoles were reached in single 35mm plates and in

individual wells of 6- and 12-well plates, respective. Encouraged by these results we proceeded to explore tl possibility of utilizing 24-well plates also. Not unexpectedly, success depended very much on receptor levels achieved, and consequently receptor densities were carefully monitored during cell culture. In general, there was good agreement between the healthiness of a culture as assessed by morphological criteria and receptor levels. Attainment of sufficient receptor densities usually required extended culture periods, to 10 days or beyond. The protocol for the flux assay was a modification of the single-plate procedure described above. To reduce background noise, incubation volumes were kept at a minimum (ca 0.2 ml), and each well was subjected to 4 washes (4 times 0.3 ml ice-cold DME) before counting. Even at fairly low receptor concentrations (<20 femtomoles per well) evidence for agonist-triggered activity was quite obvious; however, data scatter was very pronounced. At higher receptor levels (50 to 100 femtomoles per well) which we could routinely obtain in older preparations, specific uptake could be readily measured. As a rule specific uptake was nearly complete in 1 - 2 min at which time the signal/noise ratio also reached a maximum. An experiment in which receptor densities reached high levels (ca 150 femtomoles) is shown in Fig. 2. Under favorable conditions, i.e. high cell and receptor densities and short incubation times, this assay can be employed to assess inhibitor potency; such an ion flux inhibition

experiment is shown in Fig. 3. It is noteworthy that the inhibition constant for d-tubocurarine determined by this assay is about 2 orders of magnitude higher than would have been expected on the basis of toxin competition experiments with Torpedo californica receptor preparations.

This success encouraged us to attempt the use of 96-well titer plates. However, although toxin binding activity i.e. acetylcholine receptor levels) could be fairly well quantitated in individual wells, no convincing evidence for carbamoylcholine-induced ion flux was obtained. Numerous attempts to utilize 96-well plates failed, primarily, we feel, because receptor number did not exceed 5 femtomoles per well. In addition, cells had a tendency to detach from the substratum either upon the addition of the 'flux mix' (i.e. agonist and ^{86}Rb) or subsequently during the wash procedure.

Flux assays with cell suspensions:

To overcome the cell detachment problem we attempted to measure ion flux into suspended cell preparations. To this end, cells were intentionally detached by trypsin treatment and after treatment with horse serum (to block trypsin activity) incubated with 'flux mix' for 2 min, followed by centrifugation and repeated washes to remove excess radioisotope. No evidence

for agonist-stimulated, curare-inhibited ion flux was obtained. The reason for this failure is not known.

Cell type	Species	Receptor density (femtomoles/plate)	
		Total	Surface
L6	Rat	.3	N.D.
CRL	Mouse	<5	N.D.
C2C12	Mouse	300	50
BC3-H1	Mouse	500-1000	150
A673	Man	35	N.D.
A204	Man	12	N.D.
RD	Man	300	N.D.
TE671	Man	300	15
primary	Chick	150	50-100

Table I. Receptor densities in various skeletal muscle cell lines.

Cells were grown in 35mm plates under conditions (e.g. confluence, age, morphology) leading to optimal receptor expression. Total receptor content was determined by solubilizing cells in 1% Triton X-100, and surface receptors were measured by labeling cells in situ (see Methods). The human lines are all derived from rhabdomyosarcomas, including TE671 (previously mistaken as a medulloblastoma - see Stratton et al., 1989). Average results are given except for the rat and human lines, which were measured only once. N.D., not determined.

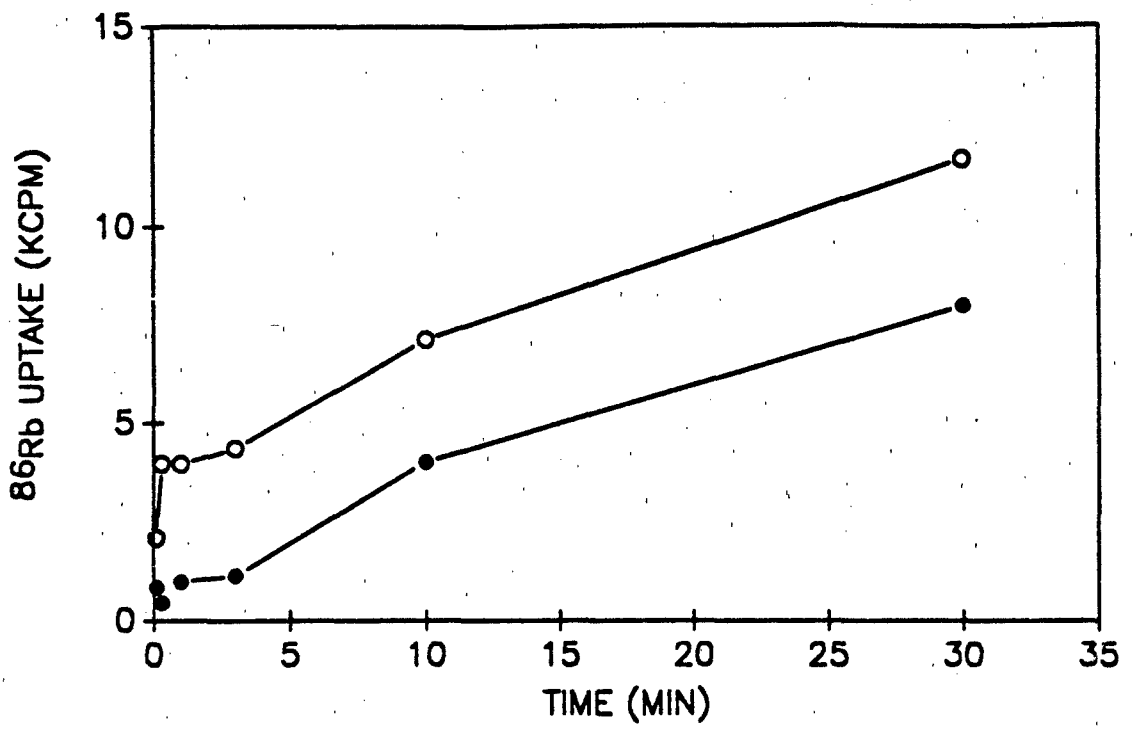

Fig. 1 Time and carbamoylcholine-dependent ^{86}Rb uptake into BC3-H1 cells

Cultures of BC3-H1 cells were grown in 35mm dishes as described in the Methods section. When they had differentiated they were cooled to 4°C, and ion flux started by exchanging the medium with medium containing 50,000 cpm of radioisotope and 0.1 mM carbamoylcholine. Influx of tracer ^{86}Rb was monitored for the durations indicated. O—O, influx in the presence of carbamoyl choline when cells were previously incubated in medium only; ●—●, influx in the presence of carbamoylcholine monitored in cells initially incubated with 10^{-7} M alpha-bungarotoxin for 1 hour at 37°C prior to cooling.

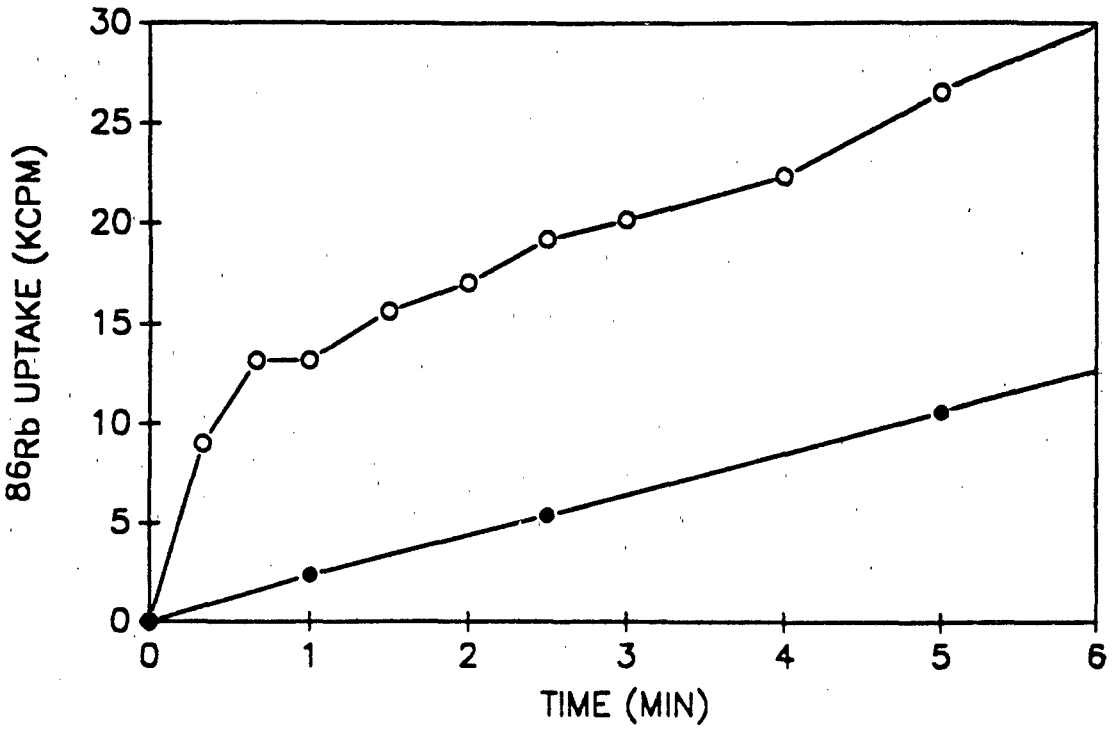

Fig. 2 Use of a 24-well plate to measure ^{86}Rb uptake kinetics

BC3-H1 cells were grown in 24-well microtiter plates as described in Methods. Plates containing differentiated cultures were cooled to 4°C, and the medium replaced with a small volume of ice-cold flux cocktail containing 0.1 mM carbamoylcholine and the radiotracer. After the indicated times the incubation was stopped by aspiration of the medium, followed by thorough washes in ice-cold phosphate-buffered saline. Symbols as in Fig. 1.

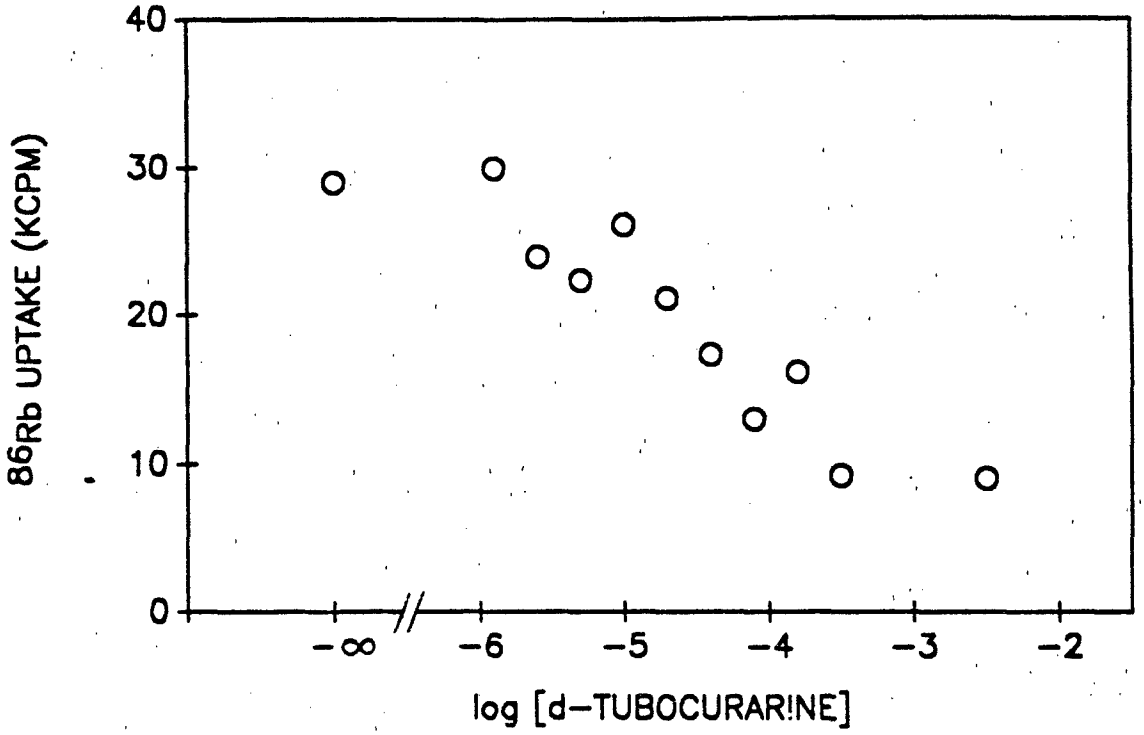

Fig. 3 Inhibition of ^{86}Rb uptake by d-tubocurarine as measured in a multiwell titer plate protocol

Cultures of BC3-H1 cells were grown in individual wells of a 24-well microtiter plate. Prior to the flux assay cells were treated with the indicated concentrations of d-tubocurarine in culture medium, and ion flux was initiated by replacing the pre-incubation medium with the flux cocktail (agonist and radioisotope in medium) containing the same concentration of antagonist. Incubations were stopped after 1 min by removal of the flux mix as described.

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