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Title: A Comparative Investigation of the Pharmacology of Fish and Mammalian Neuromuscular Systems

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Lavern J. Weber

Neuromuscular pharmacology has been extensively studied in mammals but there have been few investigations examining the neuromuscular systems of fish. In situ experiments have shown that the basic cholinergic characteristics of fish neuromuscular junctions are different from those of mammals. In order to further understand the nature of these differences, the nicotinic acetylcholine receptors (AChR) of rat and buffalo sculpin (Enophrys bison) neuromuscular junctions and the AChR of electric ray (Torpedo california) electroplax, were investigated using receptor binding analysis. A rapid filtration assay was utilized to measure [^{125}I]-α-BGT binding to tissue membranes.

Scatchard analysis of [^{125}I]-α-BGT binding was performed on sculpin pectoral muscle, rat gastrocnemius,

rat denervated gastrocnemius, and Torpedo electroplax. The affinity constant was similar for all tissues studied (K_D = 1-5 nM). The maximal number of binding sites for each tissue (pmole/g tissue) is : Torpedo electroplax = 190; sculpin pectoral = 2.07; rat innervated gastrocnemius = 0.04; rat denervated gastrocnemius = 0.50.

Kinetic analysis produced association rate constants for sculpin pectoral muscle: 3.96×10^{-3} min^{-1} $nmole^{-1}$; rat dennervated gastrocnemius: 6.48×10^{-3} min^{-1} $nmole^{-1}$, and Torpedo electroplax: 4.78×10^{8} min^{-1} $mole^{-1}$. Dissociation was biphasic in sculpin pectoral with fast and slow dissociating components, whereas only a very slow dissociating component was detected in Torpedo electroplax and denervated rat gastrocnemius.

In competition studies, d-tubocurarine had the highest affinity for the [^{125}I]-α-BGT binding site in all tissues, illustrating the nicotinic nature of the binding sites. Acetylcholine had high affinity for the rat gastrocnemius binding site and low affinity for the sculpin pectoral muscle and Torpedo electroplax binding site.

Atropine had high affinity for the sculpin pectoral muscle binding site when compared to the rat gastrocnemius and Torpedo electroplax binding site, indicating that the sculpin pectoral site may have some

mixed muscarinic-nicitinic characteristics.

These results indicate that there are definite qualitative as well as quantitative differences between the fish skeletal muscle nicotinic receptor and the nicotinic receptor of fish electroplax and rat skeletal muscle.

A Comparative Investigation of the Pharmacology of Fish and Mammalian Neuromuscular Systems

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A COMPARATIVE INVESTIGATION OF THE PHARMACOLOGY OF FISH AND MAMMAMIAN NEUROMUSCULAR SYSTEMS

Introduction

The classical concept of neuromuscular transmission involves the propagation of an action potential into the nerve terminal resulting in membrane depolarization. Calcium ions in the extracellular medium enter the terminal. Acetycholine (ACh) is released and diffuses across the junctional cleft and binds to nicotinic receptors (AChR) on the endplate of the muscle. ACh is also rapidly hydrolyzed by acetylcholinesterase (AChE). The reaction of ACh with the AChR initiates a series of conformational changes resulting in the activation of an ionic channel, which is an integral part of the AChR molecule. Channel activation causes a transient conductance increase to sodium and potassium ions resulting in a local potential charge across the muscle endplate. This leads to the production of a propagating action potential and a muscle twitch (Steinbach and Stevens, 1980).

The state of knowledge of the AChR is the most advanced for any type of receptor. The highly enriched AChR content of fish electric organs has attributed to much of the progress on AChR (Dolly and Bernard, 1984).

These receptors are related in both their function and evolution to those found in mammalian neuromuscular systems (Stroud, 1983). The AChR is a membrane protein with a molecular weight of approximately 250,000. It is composed of five subunits, two of which appear to be identical in the stoichiometry $\alpha_2\beta\gamma\delta$ (Dolly and Bernard, 1984). Noda et al. (1983) has deduced the amino acid composition for all four subunits using complementary DNA sequences. The ACh binding sites are on the α chains (Karlin, 1983).

The use of snake α-neurotoxins, particularlly α-bungarotoxin (α-BGT), as highly selective probes that bind to the AChR has also attributed to the progress on AChR (Dolly and Bernard, 1984). α-BGT is purified from the venom of the elapsid snake Bungaris multicinctus (many banded krait). α-BGT binds irreversably with the mammalian AChR and produces an antagonistic action similar to curare (Lee, 1972). The primary structure of α-BGT consists of 74 amino acids in a single chain, crosslinked by five dilsulfide bridges with a molecular weight of 8000 (Mebs et al., 1971). Audioradiographs of mouse diaphragm muscle labeled with [^{3}H]-α-BGT have revealed that the toxin is associated with the postjunctional fold, while labeling elsewhere is negligible (Bernard et al., 1973).

Although there have been extensive investigations

on the AChR of fish electric tissue and mammalian skeletal muscle (Dolly and Bernard, 1984), no studies on fish skeletal muscle AChR have been reported. The results of several investigations of the pharmacological aspects of fish neuromuscular systems indicate that the basic characteristics of their AChR may vary from those of mammels. Schneider and Weber (1975) and Gant et al (1984) examined the significance of AChE activity on neuromuscular transmission in the pectoral muscle of the largemouth bass (Micropterus salmoides) and the buffalo sculpin (Enophrys bison), respectively. Schneider and Weber (1975) reported that inhibition of muscle AChE in excess of 97% was always accompained by a well maintained tetanus which is not typical of mammalian neuromuscular systems. Bone (1978) reported that there are indications in some fish motoneurons of transmitter substances other than ACh, and of dual innervation of the muscle fibers.

In the present study, the AChR of fish and rat skeletal muscle, and fish electric tissues were characterized using receptor binding procedures. In receptor labeling studies a radioactive form of the transmitter, hormone or biologically active agent is bound to membrane or cell preparations of the target tissue. The binding of radioactive ligands to high affinity binding sites in these preparations usually

follows the kinetics and mathematical models similar to those of enzyme-substrate interactions. Saturation, kinetic, and pharmacological criteria are used to support the hypothesis that a binding site represents a particular receptor. From these tests, parameters such as number, distribution and identification of binding sites can be obtained. In this study the binding properties of [^{125}I-α-BGT to fish and rat skeletal muscle AChR and fish electric organ AChR are examined. The development of an assay using [^{3}H]ACh of high specific activity to characterize the AChR from these tissues is also attempted. This information provides a comparative characterization of the AChR of these tissues and may provide insight into the differences between fish and mammalian neuromuscular systems.

[^{125}I]-α-Bungarotoxin Binding to Fish and Rat Skeletal Muscle and Fish Electroplax Nicotinic Acetycholine Receptors

INTRODUCTION

Nicotinic acetylcholine receptors (AChR) mediate chemical communication at the neuromuscular junction by an interaction of neuronally-released acetylcholine with its recognition site on the AChR of the post junctional membrane. Much of the progress on the study of AChR can be attributed to the high concentration of AChR in fish electric organ and to the use of snake neurotoxins, particularly α-bungarotoxin, which binds specifically and with high affinity to the AChR at the neuromuscular junction (Albuquerque et al. 1979). The study of AChR of vertebrate skeletal muscle has been hindered by the low number of receptors and by the presence of high levels of proteolytic enzymes (Dolly 1979). Although the electroplax model system has yielded useful information, it is not always directly applicable to the AChR of muscle (Dolly and Bernard 1984).

The results of several investigations of the pharmacological aspects of fish neuromuscular systems indicate that the basic characteristics of the AChR at the fish neuromuscular junction may vary from those in mammals. Schneider and Weber (1974, 1975) examined the

significance of acetylcholinesterase to neuromuscular transmission in the pectoral muscle of the largemouth bass (Micropterus salmoides). They evaluated the effects of diisopropylflurophosphate (DFP) on acetylcholinesterase activity in the muscle, and correlated the attendant acetylcholinesterase inhibition with response to nerve stimulation. Bass do have true acetylcholinesterase (AChE) and doses of DFP producing inhibition in excess of 97% were always accompanied by a well maintained tetanic response, which is not typical of mammalian cholinergic systems. Similar results were found with the buffalo sculpin (Enophrys bison) after the administration of high doses of acetylcholinesterase inhibitors ,AChE-I (Gant et al., 1984). In mammals, the adminstration of AChE-I results in two dose dependent responses in indirectly stimulated muscles; (i) potentiation of twitch and (ii) depression in twitch and a brief tetanic tension followed immediately by partial or complete relaxation (Hobbinger, 1976).

In the present study, the binding properties of [^{125}I]-α-bungarotoxin to the AChR of fish pectoral and myotomal muscle, electroplax, and denervated rat skeletal muscle were examined to compare the pharmacological properties of the receptors. Attempts to correlate the differences evident from the in situ studies (Schenider and Weber, 1975; Gant et al, 1984) to

differences noted in the receptor binding analysis are also made. The results indicate that there are definite qualitative as well as quantitative differences between fish skeletal muscle AChR and the ACHR of fish electroplax and rat skeletal muscle.

MATERIALS AND METHODS

Materials

[^{125}I]-α-bungarotoxin (BGT) (15.5-18 uCi/ug) was purchased from the New England Nuclear Corp., Boston, MA. All drugs and reagents were purchased from the Sigma Chemical Co., St. Louis, MO. Buffalo sculpin (Enophrys bison) and red Irish lord (Hemilepidotus hemilepidotus) weighing between 400 and 700 grams were collected from Yaquina Bay, Newport, Oregon, by otter trawl and held in tanks with aerated seawater at 12°C. Coho salmon (Oncorhynchus kitsutch) were obtained from the Department of Fisheries and Wildlife, Oregon State University. Pacific electric ray (Torpedo california) was obtained from Marinus Inc., West Chester, CA.

Preparation of Tissues

Pectoral, slow myotomal, and fast myotomal muscle sections from freshly killed buffalo sculpin, red Irish lord, and coho salmon were dissected over ice and used immediately. Torpedo electroplax organs, slow myotomal muscle, and fast myotomal muscle from a freshly killed

animal, were dissected over ice and connective tissue was removed. The organs and skeletal muscle were cut into small pieces, frozen in liquid nitrogen and stored at -70°C until use.

The gastrocnemous of male Sprague-Dawley rats (200-350 g) were unilaterally denervated by section of the sciatic nerve in the mid-thigh region. Ketamine hydrochloride (100 mg/kg, i.p.) was used to produce anesthesia. The animals were killed 14-30 days after surgery and the gastrocnemius from both legs was dissected over ice and stored at -70°C.

Skeletal muscle and electroplax homogenates were prepared by a procedure modified from Potter (1980). Tissue was weighed, minced and homogenized with a Brinkman Polytron in ice cold 20mM, .1M NaCl, pH 7.5, phosphate buffer containing protease inhibitors (5mM EGTA, .1mM phenylmethylsulfonyl fluoride (PMSF)). The homogenate was filtered through cheesecloth to remove large fragments of connective tissue, and then centrifuged at 35,000xg (5°C) for 30 minutes. The resulting pellet was resuspended in 20mM pH 7.2 phosphate buffer (.4M NaCl, .1mM EGTA, .1mM PMSF) and then centrifuged at 10,000xg (5°C) for 10 minutes. The supernatant was decanted and then centrifuged at 35,000xg for 30 minutes. The resulting pellet was resuspended in 20mM, pH 7.2, phosphate buffer (.1M NaCl,

.1mM EGTA, .1mM PMSF) with a Potter - Elvehjem glass homogenizer. Sodium azide (.01%) was added to the final buffer if the homogenate was to be stored at - 70°C.

Binding Assay

Binding of [^{125}I]-α-BGT to muscle and electroplax membranes was measured by a rapid filtration assay modified from Potter (1980). Aliquots of muscle membranes (100 µl, 50-200 µg protein) were incubated in triplicate for 60 minutes at 20 C with 25 µl of [^{125}I]-α-BGT and 25 µl of buffer or competing drug as indicated. In electroplax binding experiments, aliquots of membranes (900 µl, 5 µg protein) were incubated in triplicate for 90 minutes at 20 C with 50 µl of [^{125}I]-α-BGT and 50 µl of buffer or competing drug. After incubation, all tissue samples were diluted with 4 ml of ice cold 20 mM, phosphate buffer (pH 7.2) containing .1 mM NaCl, .1 mM EGTA, .1 mM PMSF, .01% BSA. The samples were immediately filtered under reduced pressure (15 psi) through GF/B glass fiber filters (Whatman). The filters were then washed two times with 4 ml aliquots of buffer The radioactivity bound to the filters was measured with either a gamma counter with an efficiency of 75% , or the filters were placed in vials containing 5 ml of liquid scintillation cocktail (Beckman Ready-Solv EP) and counted on a liquid scintillation spectrophotometer with an efficiency of 68%. The glass

fiber filters employed in the assay were presoaked in ice cold, 0.03% polyethylenimine for at least two hours prior to use to eliminate displaceable binding of [^{125}I]-α-BGT to the filters in the absence of tissue (Bruns et al., 1983). Specific binding of [^{125}I]-α-BGT to membranes was defined as the difference between the amount of [^{125}I]-α-BGT bound in the absence and presence of 2.5 μM nonradiolabeled α-BGT.

Protein content was determined by the method of Lowry, et al. (1951) using BSA as the standard. In experiments of ACh inhibition of [^{125}I]-α-BGT binding, homogenates were preincubated with DFP (sculpin and rat: 0.1 mM; electroplax: 1.0mM) for 1 hour at 20°C. These concentrations were found to inhibit AChE by greater than 99.9% using the procedure of Ellman et al. (1961).

RESULTS

Total binding of [^{125}I]-α-BGT to muscle membrane preparations was linear up to 800ug protein/sample, as was binding to electroplax membranes up to 40ug protein/sample. There was no specific binding when the homogenates from all tissues were heat-denatured in a 60 C water bath for 1 hour and cooled before being assayed. Pretreatment of the glass fiber filters with polyethylenimine resulted in the filters becoming cationically charged which increased total [^{125}I]-α-BGT

binding and decreased nonspecific binding in all tissues studied.

The equilibrium dissociation constant, K_D, and the maximal number of binding sites, Bmax, were determined for all tissues studied (Table 1.). The K_D values were similar for all tissues with a range of 1.5 to 4.8 nM. Torpedo electroplax had the highest Bmax value at 190 pmoles/mg protein, while innervated gastrocnemius had the lowest at 0.04 pmoles/mg protein. Denervation of the rat gastrocnemius increased the Bmax approximately 12 fold to 0.50 pmoles/mg protein. Sculpin pectoral muscle had the highest Bmax of the muscles studied with a value of 2.07 pmoles/mg protein.

Specific binding of [^{125}I]-α-BGT to sculpin pectoral muscle saturated at concentrations above 20nM (Figure 1A). Scatchard analysis of this data (Figure 1B) is linear, suggesting a single class of binding site. Hill plots of the saturation data yielded a Hill coefficient of 0.99 ± 0.02 (mean ± s.e., n=3) (Figure 1C), indicating lack of cooperativity and that the labeled BGT is binding to a single non-interacting site. Specific binding of [^{125}I]-α-BGT to Torpedo slow and fast myotomal muscle also saturated at 20 nM (Figure 2A,B) producing linear Scatchard plots (Figure 2C). The Hill coefficient of these binding sites calulated to be 0.95 for Torpedo slow myotomal muscle (n=1) and 1.12 for fast

TABLE 1

[^{125}I]-α-Bungarotoxin dissociation constant (K_D) and maximum binding site concentration (Bmax) values of various tissues.

Species	Tissue	K_D (nM) (mean ± s.e.)	Bmax (pmoles/mg protein) (mean ± s.e.)
Sculpin			
	Pectoral Muscle	4.83 ± 0 .32	2.07 ± 0.06
Torpedo			
	Electroplax	2.90	190.51
	Fast Myotomal Muscle	2.60	0.15
	Slow Myotomal Muscle	1.81	0.58
Rat			
	Innervated Gastrocnemius	2.21 ± 0.07	0.04 ± 0.002
	Denervated Gastrocnemius	1.53 ± 0.11	0.50 ± 0.11

[a] The K_D and Bmax values for sculpin and rat muscle were determined by analysis of Scatchard plots of three separate experiments involving six to eight concentrations of [125I]-α-BGT. Torpedo electroplax and muscle values were determined from single experiments.

Figure 1. Saturation analysis of [^{125}I]-α-BGT binding to sculpin pectoral muscle

(A.) Saturation isotherm of [^{125}I]-α-BGT binding using incubation conditions described in methods section. Aliquots of pectoral muscle homogenate were incubated withvarying concentrations of [^{125}I]-α-BGT (1 - 30 nM). Specific binding was defined as the difference between the amount of [^{125}I]-α-BGT bound in the presence and absence of 2.5 μM nonlabeled α-BGT.

(B.) Scatchard analysis of the specific [^{125}I]-α-BGT binding. The values from this representative experiment were: K_D = 4.52 nM and Bmax = 2.0 pmoles/mg protein.

(C.) Hill plot of specific [^{125}I]-α-BGT binding. The Hill coefficient from this experiment was 0.97.

FIG. 1

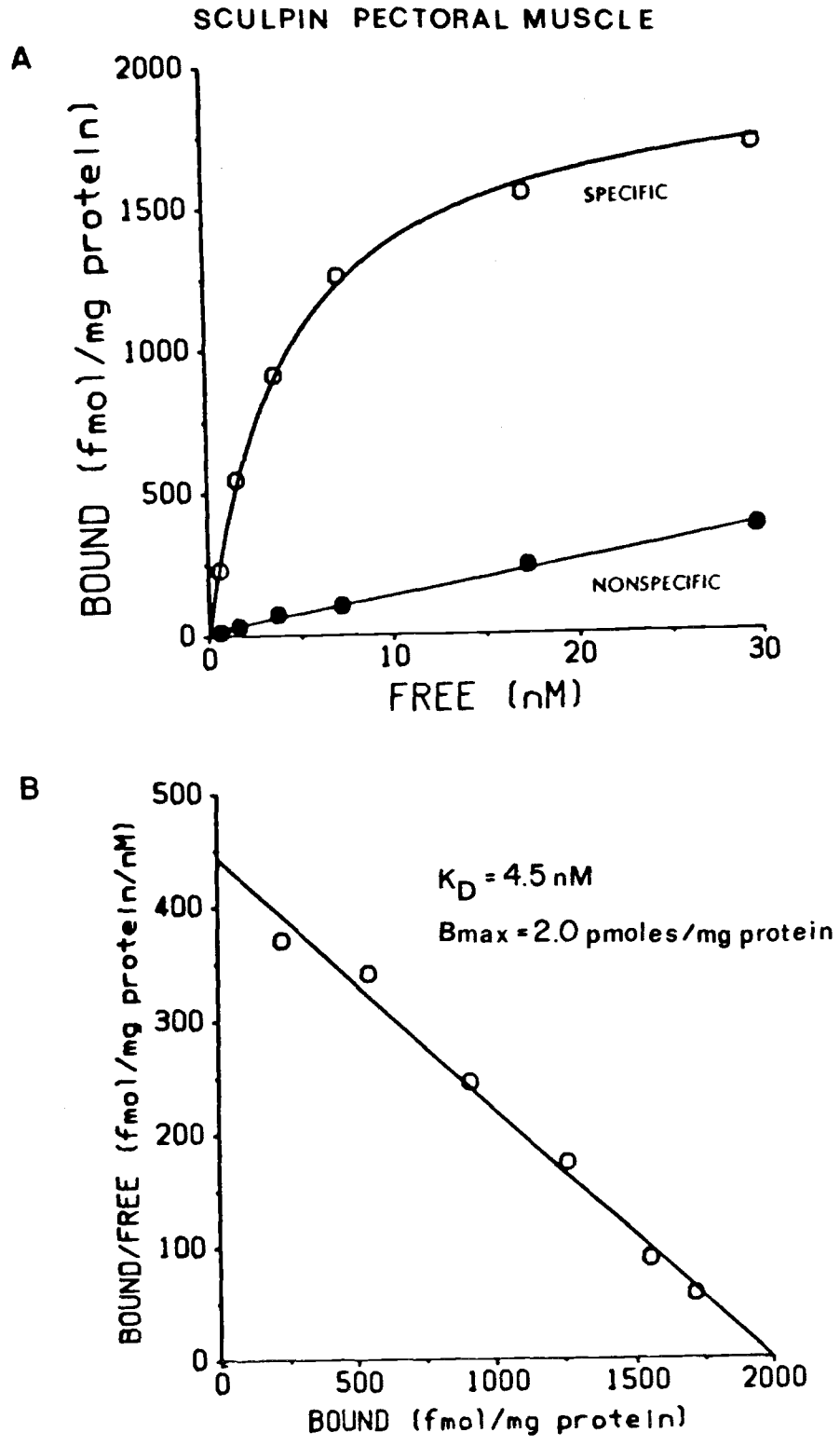

SCULPIN PECTORAL MUSCLE
A
BOUND (fmol/mg protein)
2000
1500
1000
500
0
SPECIFIC
NONSPECIFIC
0
10
20
30
FREE (nM)
B
BOUND/FREE (fmol/mg protein/nM)
500
400
300
200
100
0
K_D = 4.5 nM
Bmax = 2.0 pmoles/mg protein
0
500
1000
1500
2000
BOUND (fmol/mg protein)

FIG. 1 Continued

C

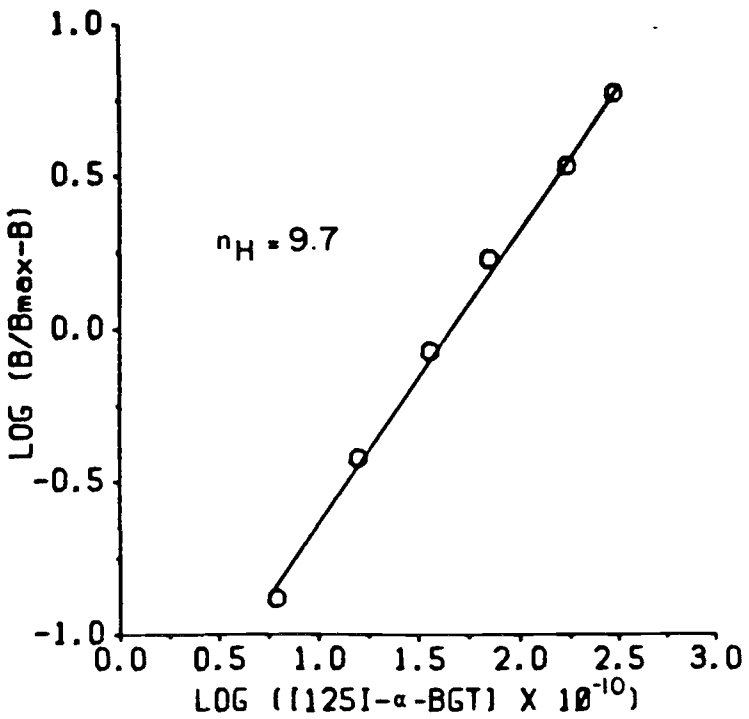


Figure 2. Saturation analysis of [^{125}I]-α-BGT binding to Torpedo slow and fast myotomal muscle.

Saturation isotherm of [^{125}I]-α-BGT binding to Torpedo (A) slow myotomal muscle and (B) fast myotomal muscle. Aliquots of muscle homogenates were incubated with 0.5 - 32 nM [^{125}I]-α-BGT under incubation conditions described in methods section. Specific binding was defined as the difference between the amount of [^{125}I]-α-BGT bound in the presence and abscence of 2.5 μM nonlabeled α-BGT. (C.) Scatchard analysis of specific [[^{125}I]-α-BGT to slow (0) and fast (0) myotomal muscle. The values from these single experiments are K_D = 1.81 and Bmax = 0.58 pmoles/mg protein for slow myotomal muscle and K_D = 2.6 and Bmax = 0.15 pmoles /mg protein for fast myotomal muscle.

D. Hill plots of saturation data. The Hill coefficients were determined to be 0.95 for slow myotomal muscle and 1.12 for fast myotomal muscle.

FIG. 2 TORPEDO

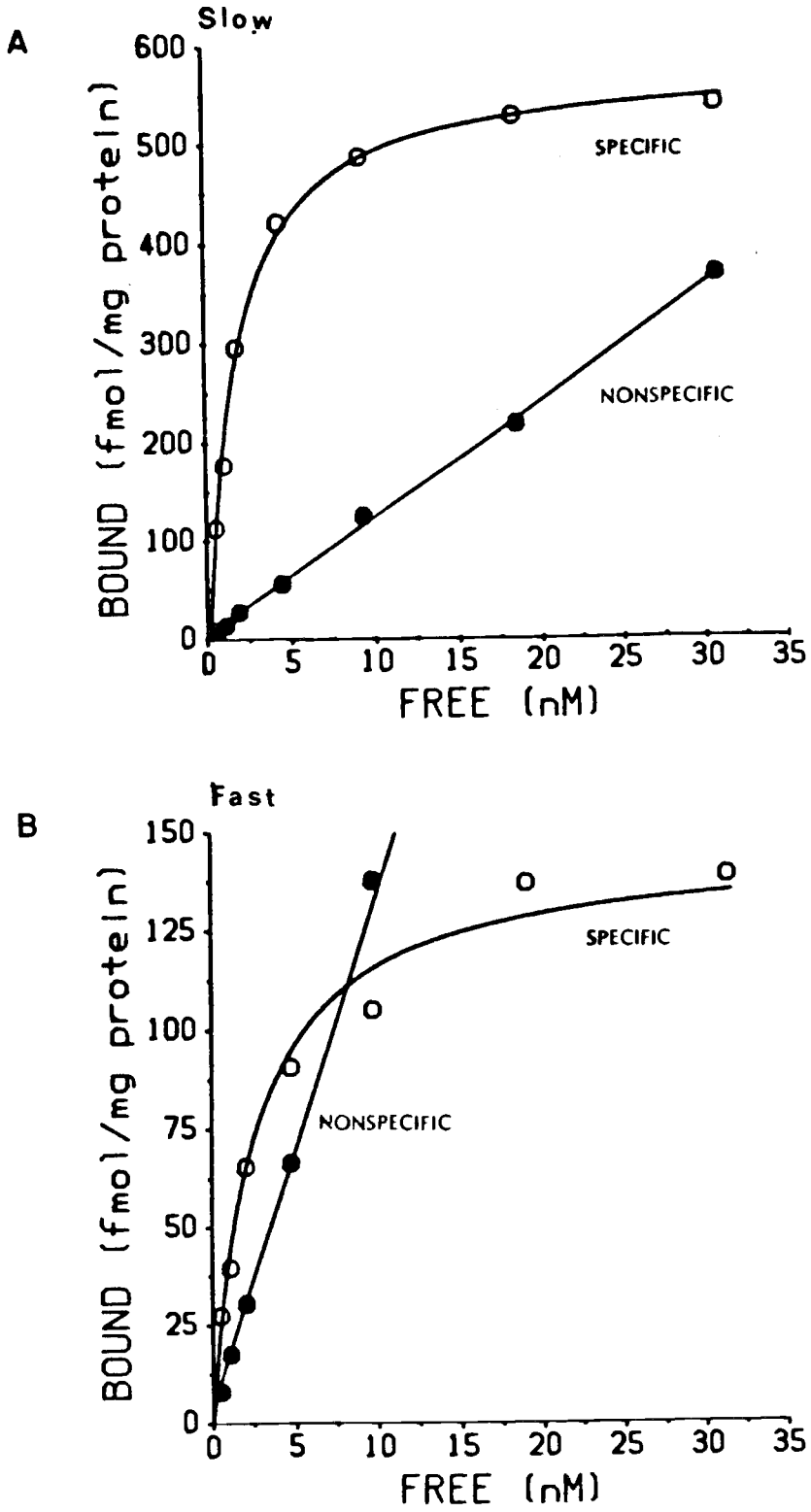
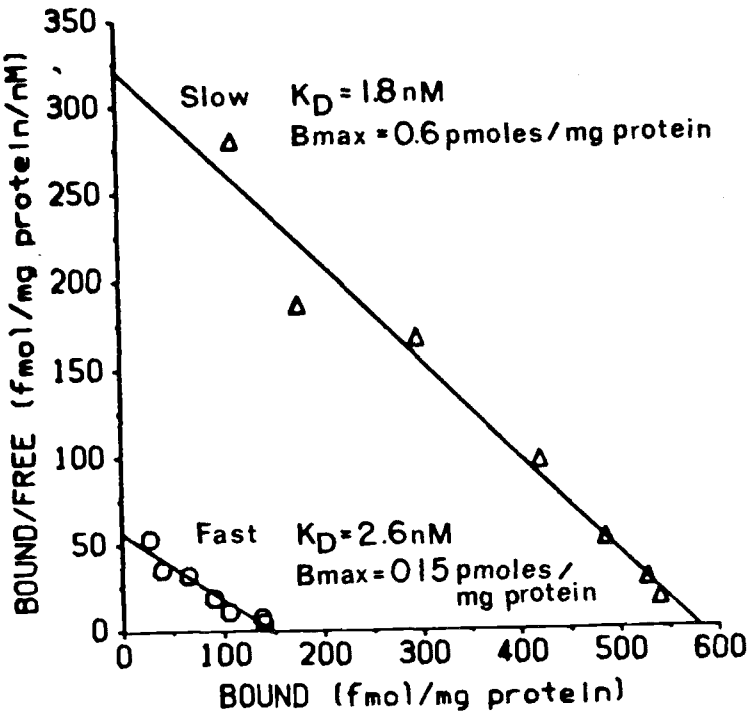
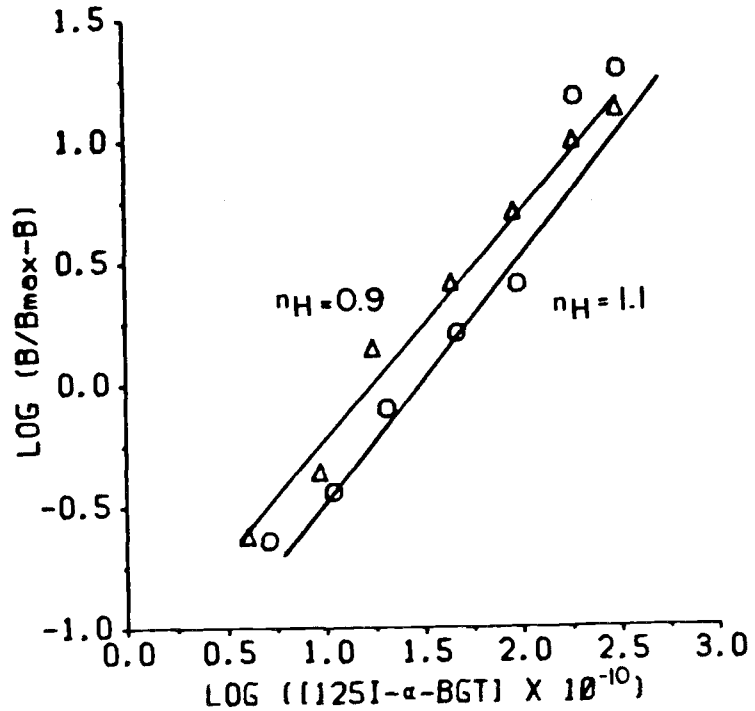


FIG. 2 Continued

C



D



muscle (n=1) (Figure 2D).

Saturation analysis of electroplax membranes was performed according to the method of Akera and Cheng (1977) due to the high percentage of the added [^{125}I]-α-BGT concentration bound to electroplax. In this method, increasing concentrations of unlabeled α-BGT were added to a constant amount of .07 nM [^{125}I]-α-BGT. The concentration of nonlabeled α-BGT which caused a 50 % inhibition of [^{125}I]-α-BGT binding (IC_{50}) was determined from a logit-log plot of the inhibition data. The K_D value was calculated by subtracting the concentration of [^{125}I]-α-BGT from the IC_{50}. A Hill coefficient of 1.16 was calculated from the inhibition data (Figure 3). $Bmax = B_a \times (IC_{50}/a)$, where B_a is the [^{125}I]-α-BGT concentration bound in the abscence of unlabeled α-BGT and "a" is the concentration of [^{125}I]-α-BGT. Saturation analysis of both innervated and denervated rat gastrocnemius indicated that specific binding reached a plateau at 10 nM (Figure 4A,B) and yield linear Scatchard plots (Figure 5A,B). The Hill coefficients calculated for innervated and denervated rat gastrocnemius were 0.96 ± 0.05 and 0.92 ± 0.02 (mean ± s.e., n=3), respectively (Figure 6A,B). All other experiments on rat muscle were performed on denervated gastrocnemius.

Kinetic analysis in which binding is determined as

Figure 3. Hill plot of [^{125}I]-α-BGT binding to Torpedo electroplax. Increasing concentrations were obtained by adding nonlabeled α-BGT (0.1 -5 nM) to 0.7 nM [^{125}I]-α-BGT and incubating under conditions described in the methods section. A Hill coefficient of 1.16 was determined from this data.

FIG. 3

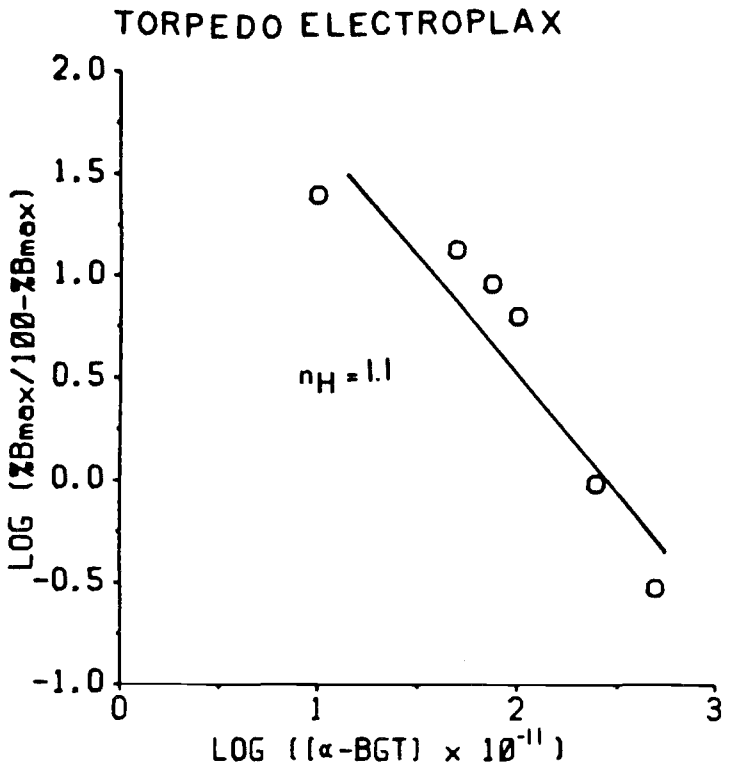

TORPEDO ELECTROPLAX
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
0
1
2
3
LOG (%Bmax/100-%Bmax)
LOG ([α-BGT] x 10^-11)
nH = 1.1

Figure 4. Saturation isotherms of innervated (A) and denervated (B) rat gastrocnemius muscle. Aliquots of both innervated and denervated gastrocnemius were incubated with varying concentrations of [^{125}I]-α-BGT (0.45 - 34 nM, innervated ;0.3 - 18.8nM,denervated) under conditions described in the Methods section. Specific binding was defined as the difference between [^{125}I]-α-BGT binding in the presence and absence of 2.5uM nonlabeled α-BGT. This experiment in which the gastrocnemius was denervated for 26 days is representative of three separate experiments.

FIG. 4

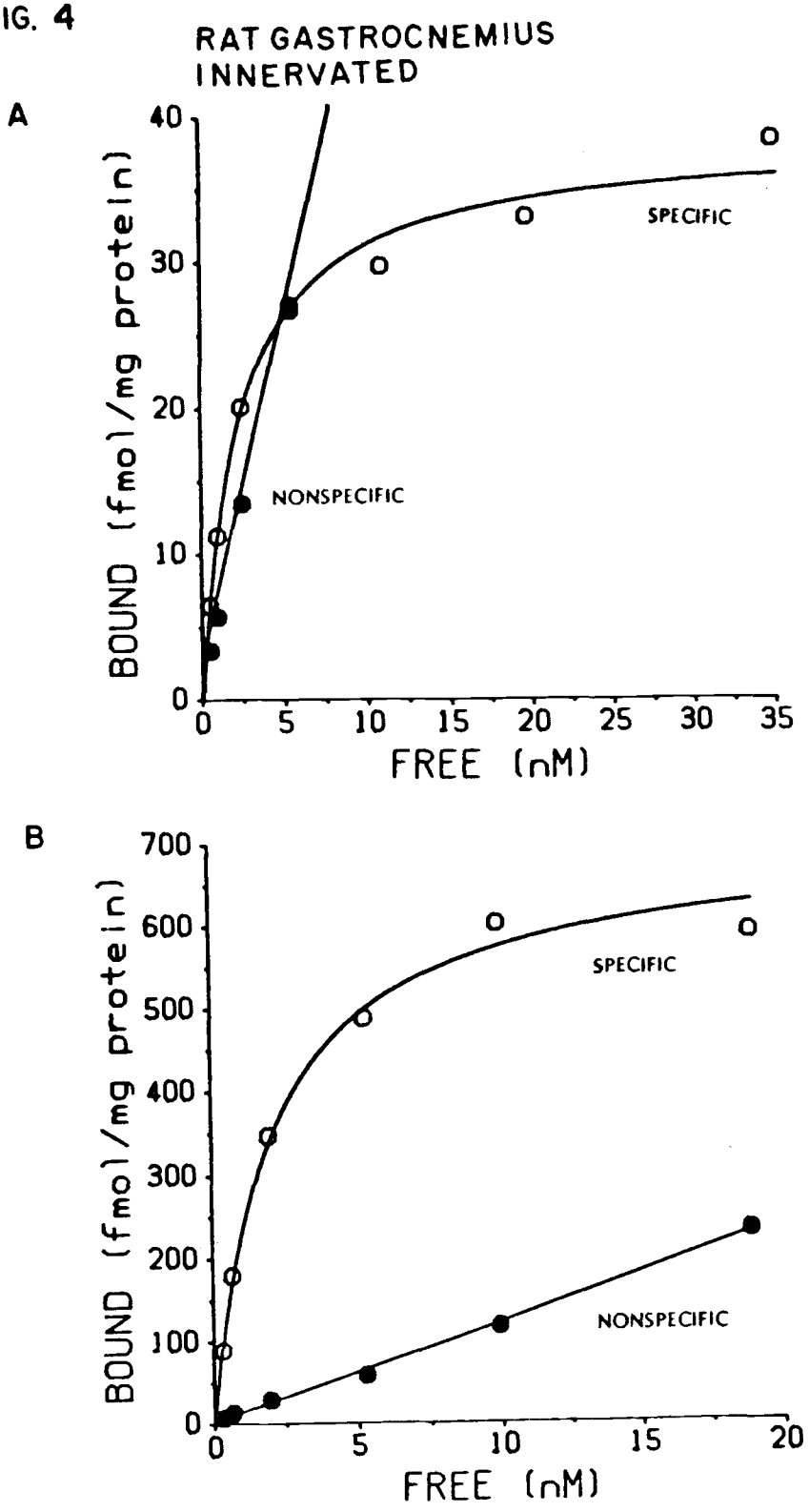

RAT GASTROCNEMIUS
INNERVATED
A
BOUND (fmol)/mg protein)
FREE (nM)
SPECIFIC
NONSPECIFIC
0
10
20
30
40
0
5
10
15
20
25
30
35
B
BOUND (fmol)/mg protein)
FREE (nM)
SPECIFIC
NONSPECIFIC
0
100
200
300
400
500
600
700
0
5
10
15
20

Figure 5. Scatchard analysis of [^{125}I]-α-BGT binding to innervated (A) and denervated (B) rat gastrocnemius. Figure 5 (B) also displays the Scatchard plot for innervated gastrocnemius ( ). The values from these representative experiments were K_D = 2.32 and Bmax = 0.04 pmoles/mg protein for innervated gastrocnemius and K_D = 2.11 pmoles/mg protein and Bmax = 0.70 pmoles/mg protein for denervated gastrocnemius.

FIG. 5

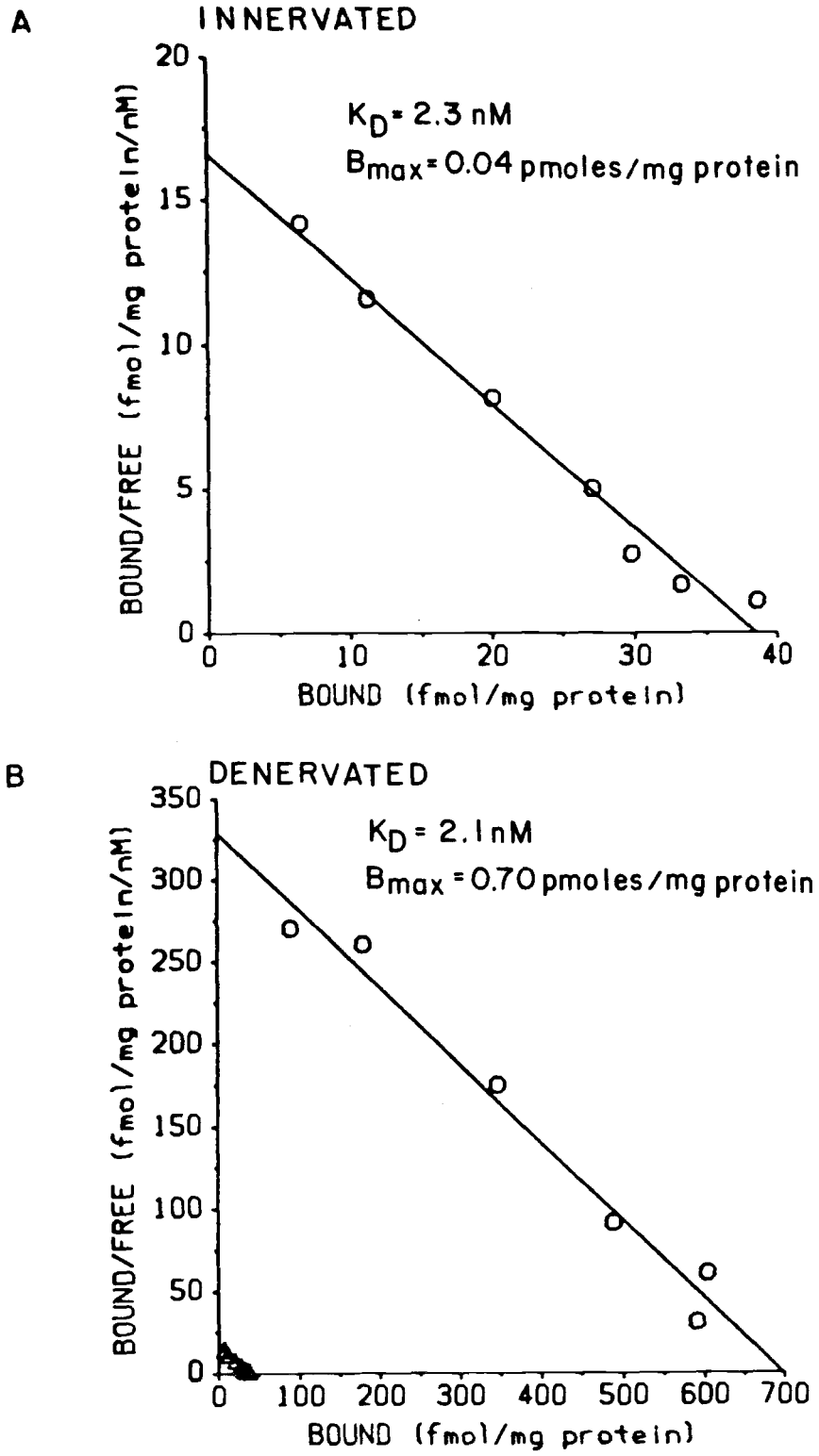
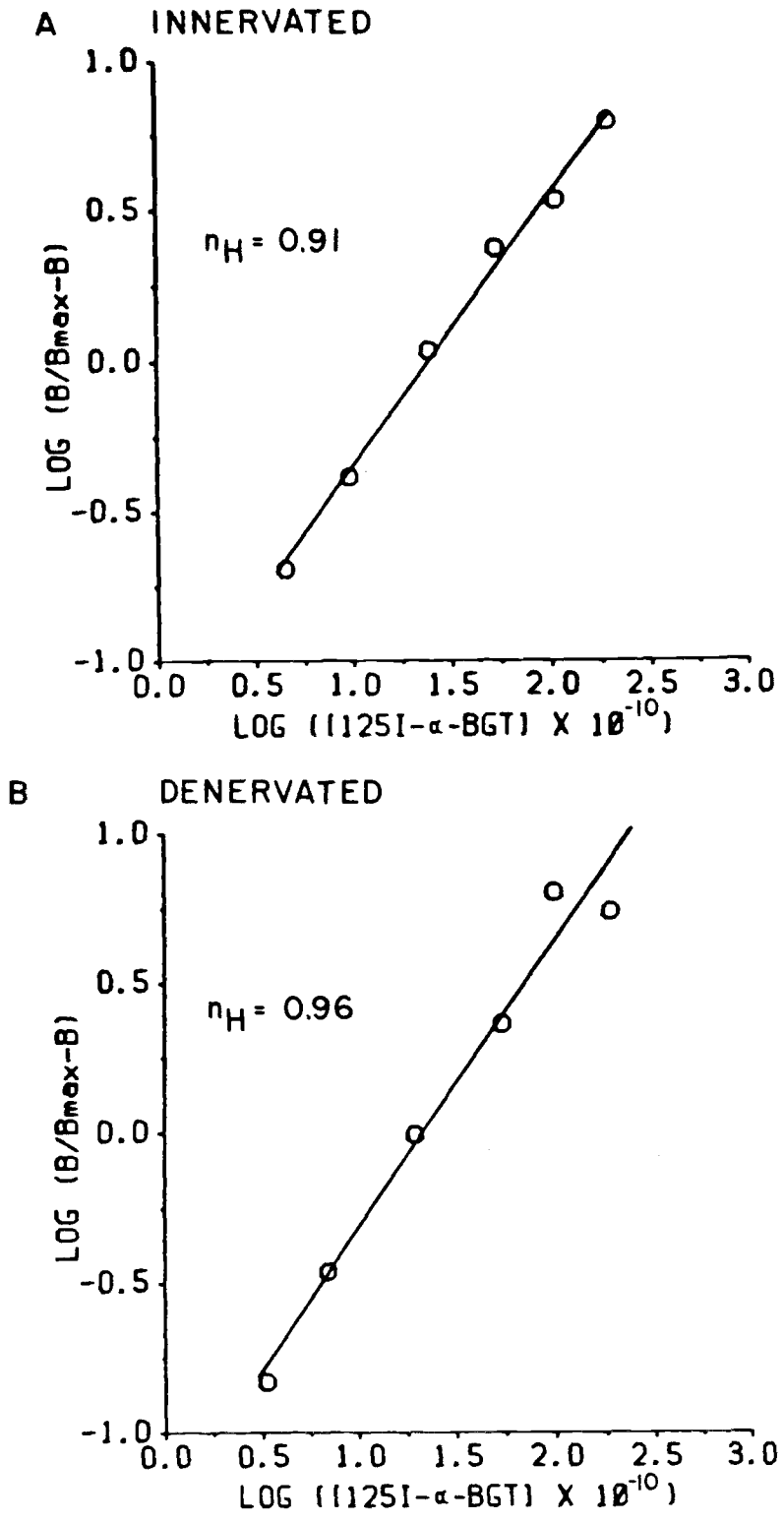

A
INNERVATED
K_D = 2.3 nM
B_{max} = 0.04 pmoles/mg protein
BOUND/FREE (fmol/mg protein/nM)
BOUND (fmol/mg protein)
0
5
10
15
20
0
10
20
30
40
B
DENERVATED
K_D = 2.1 nM
B_{max} = 0.70 pmoles/mg protein
BOUND/FREE (fmol/mg protein/nM)
BOUND (fmol/mg protein)
0
50
100
150
200
250
300
350
0
100
200
300
400
500
600
700

Figure 6. Hill plots of saturation data from innervated (A) and denervated (B) rat gastrocnemius muscle. The Hill coefficients from these representative experiments were: 0.91 for innervated and 0.96 for denervated muscle.

FIG.6


A INNERVATED
1.0
0.5
0.0
-0.5
-1.0
n_H = 0.91
LOG (B/Bmax-B)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
LOG ([125I-α-BGT] X 10^{-10})
B DENERVATED
1.0
0.5
0.0
-0.5
-1.0
n_H = 0.96
LOG (B/Bmax-B)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
LOG ([125I-α-BGT] X 10^{-10})

a function of time was completed for all tissues studied. The time course of binding of [^{125}I]-α-BGT was determined for sculpin pectoral muscle and denervated rat gastrocnemius. The specific binding of [^{125}I]-α-BGT to sculpin pectoral muscle required 30 minutes to reach equilibrium at 20 C, while equilibrium was reached after approximately 60 minutes in the rat (Figure 7). The association rate constant (k_{+1}) for both sculpin pectoral and rat gastrocnemius was determined by performing the association binding assay at several concentrations of [^{125}I]-α-BGT. When this data is plotted as ln(Be/Be-B) vs time, where Be is the amount of specific [^{125}I]-α-BGT bound at equilibrium and B_t is the amount specifically bound at time t, the slope is equal to the observed rate constant of association (K_{obs}). A plot of K_{obs} vs concentration of (^{125}I)-α-BGT has a slope of K_{+1} ($k_{obs} = k_{+1} L_T + k_{-1}$) (Figure 8). The K_{+1} determined for sculpin pectoral muscle is 3.96 x 10^{-3} min^{-1} $nmole^{-1}$ and the K_{+1} for denervated rat gastrocnemius is 6.48 x 10^{-3} min^{-1} $nmole^{-1}$.

Binding of [^{125}I]-α-BGT to electroplax membranes reached equilibrium by 60 minutes (Figure 9A). A k_{obs}= 0.026 was calculated from this plot (Figure 9B.) and the

Figure 7. Time course of [^{125}I]-α-BGT binding to sculpin pectoral (A) and rat denervated gastrocnemius muscle (B). Aliquots of both sculpin and rat muscle were removed at various times for rapid filtration from a batch incubation (0°C) with 4.0 nM [^{125}I]-α-BGT. Specific binding was determined as the difference between batches incubated in the presence and absence of 2.5 uM nonlabeled α-BGT. The data are representative of three different experiments.

FIG.7

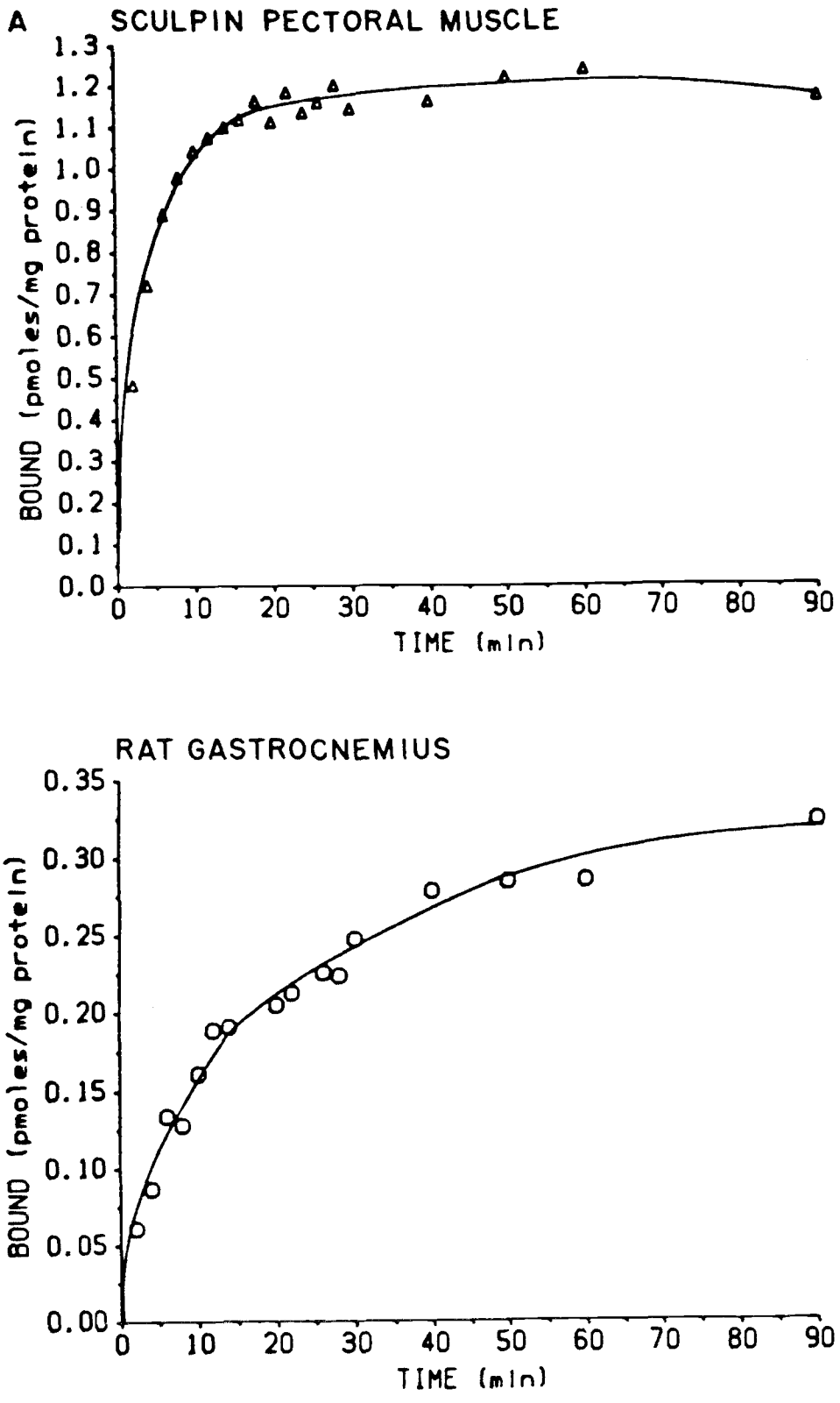

A
SCULPIN PECTORAL MUSCLE
BOUND (pmoles/mg protein)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
90
TIME (min)
B
RAT GASTROCNEMIUS
BOUND (pmoles/mg protein)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
80
90
TIME (min)

Figure 8. Rate of association of [^{125}I]-α-BGT binding to sculpin pectoral (A) and rat denervated gastrocnemius (B) muscle. The association rate was determined by performing the association binding assay at several concentrations of [^{125}I]-α-BGT (0.1 - 16 nM,sculpin; 0.1 - 16 nM,rat) and the observed association rate constant (K_{obs}) was determined for each tissue as described in the results section. The K_{+1} is equal to the slope of the K_{obs} vs [^{125}I]-α-BGT plot.

FIG.8

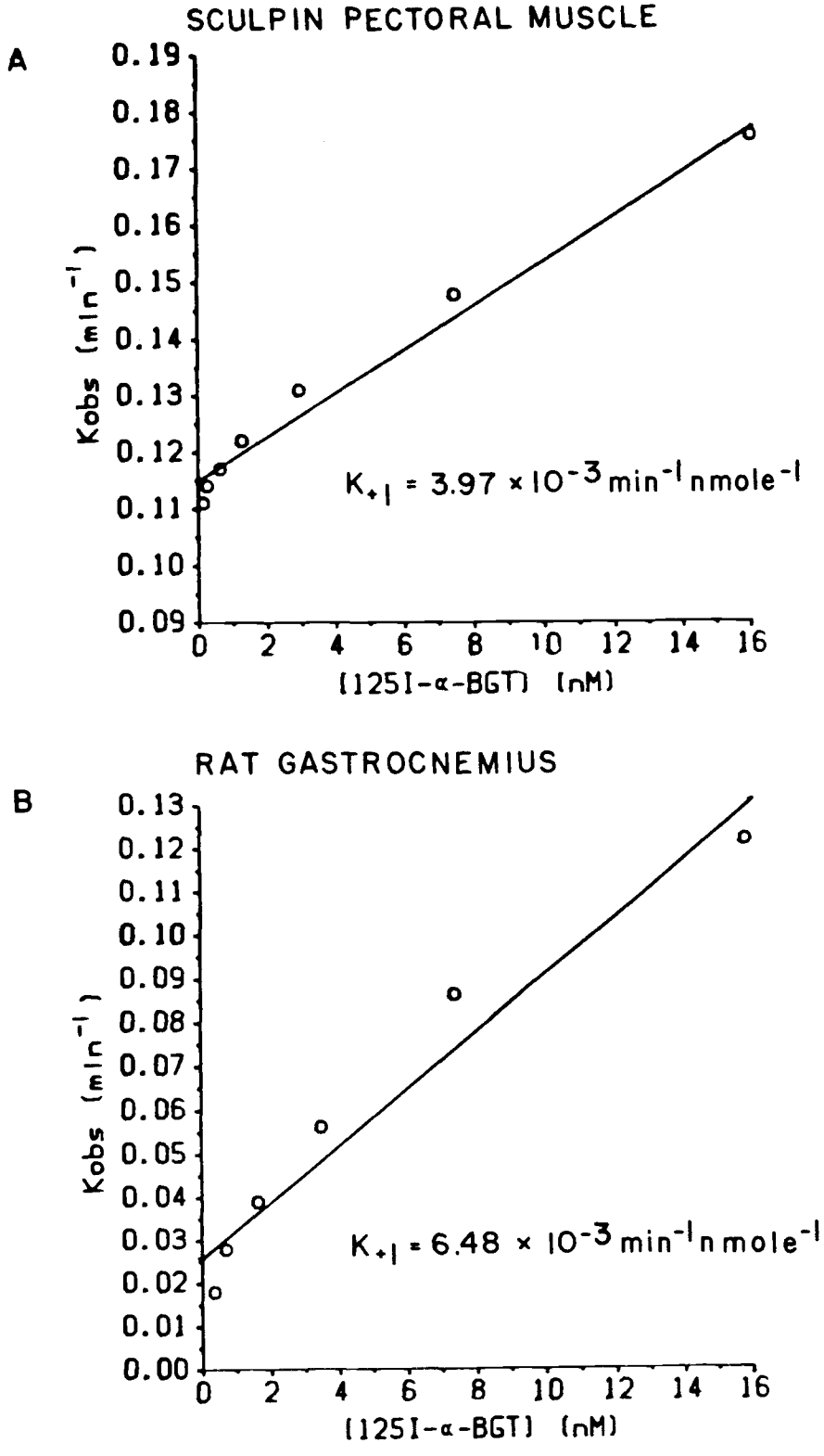

SCULPIN PECTORAL MUSCLE
A
Kobs (min⁻¹)
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
$K_{+1} = 3.97 \times 10^{-3}\ min^{-1}\ nmole^{-1}$
0
2
4
6
8
10
12
14
16
[125I-α-BGT] (nM)
RAT GASTROCNEMIUS
B
Kobs (min⁻¹)
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
$K_{+1} = 6.48 \times 10^{-3}\ min^{-1}\ n\ mole^{-1}$
0
2
4
6
8
10
12
14
16
[125I-α-BGT] (nM)

Figure 9. Time course and rate of binding of [^{125}I]-α-BGT to Torpedo electroplax

(A). Time course of binding of [^{125}I]-α-BGT to Torpedo electroplax homogenates at 20°C. Aliquots of electroplax homogenate were removed at various times for rapid filtration from a batch incubation with 0.07 nM [^{125}I]-α-BG. Specific binding was determined by the difference between batches incubated in the presence and abscence of 2.5 μM nonlabeled α-BGT.

(B). The association rate constant was determined by plotting the time course data as a pseudo-first order plot of specific [^{125}I]-α-BGT binding. The K_{+1} was determined by the equation $K_{+1} = (K_{obs} - K_{-1}/[^{125}I]$-α-BGT).

FIG. 9

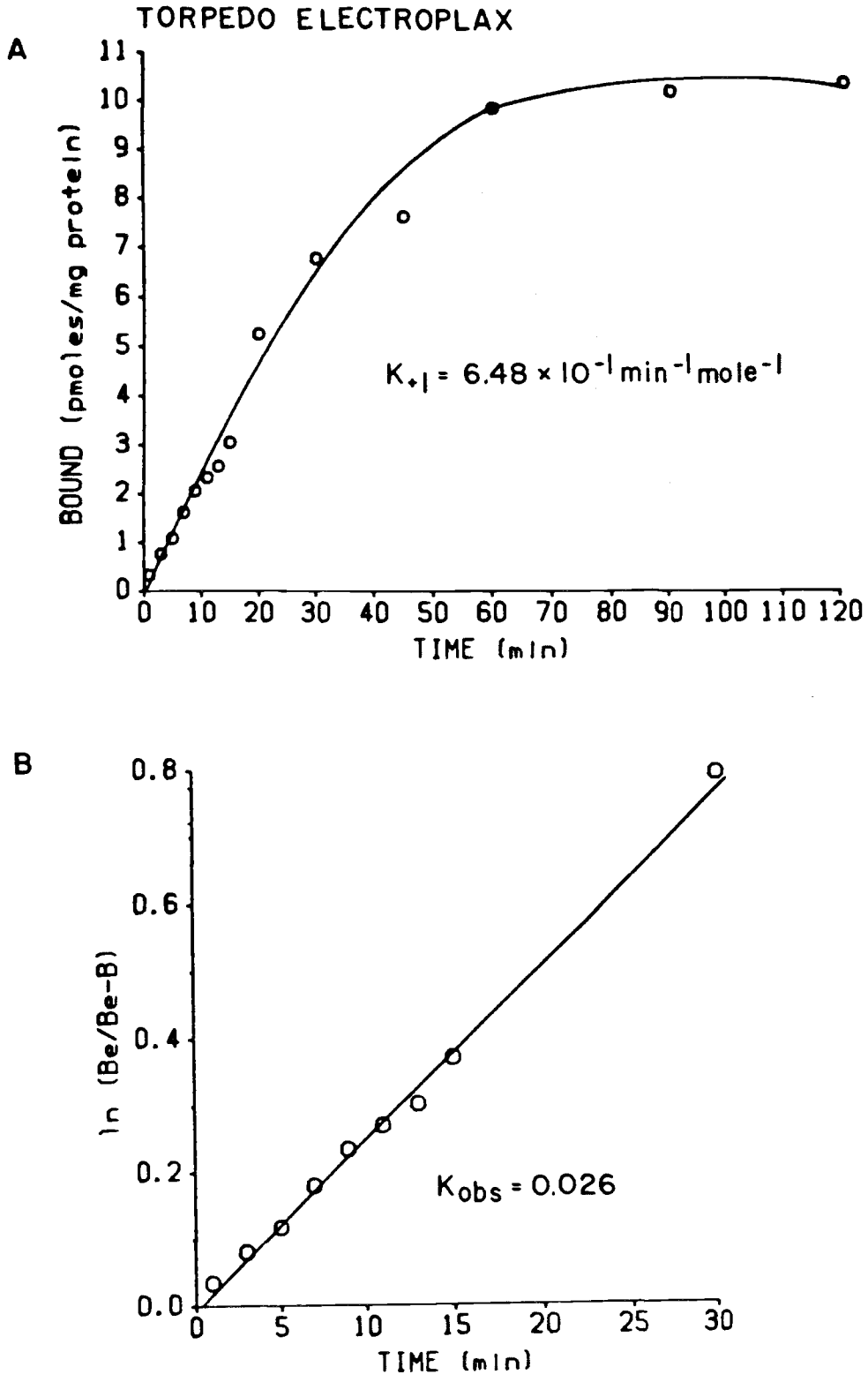

TORPEDO ELECTROPLAX
A
BOUND (pmoles/mg protein)
0
1
2
3
4
5
6
7
8
9
10
11
0 10 20 30 40 50 60 70 80 90 100 110 120
TIME (min)
$K_{+1} = 6.48 \times 10^{-1} min^{-1} mole^{-1}$
B
ln (Be/Be-B)
0.0
0.2
0.4
0.6
0.8
0 5 10 15 20 25 30
TIME (min)
$K_{obs} = 0.026$

Figure 10. Time course of dissociation of [^{125}I]-α-BGT binding to sculpin pectoral and rat denervated gastrocnemius muscle (A) and Torpedo electroplax (B). The [^{125}I]-α-BGT (4nM-muscle, 0.07nM-electroplax) and tissue homogenates were incubated until they reached equilibrium. Dissociation was initiated by adding 2.5uM nonradiolabeled α-BGT to the batch incubation. Aliquots of the incubate were subsequently filtered at the various times indicated. The data for sculpin pectoral and denervated rat gastrocnemius are representative of three separate experiments.

FIG. 10

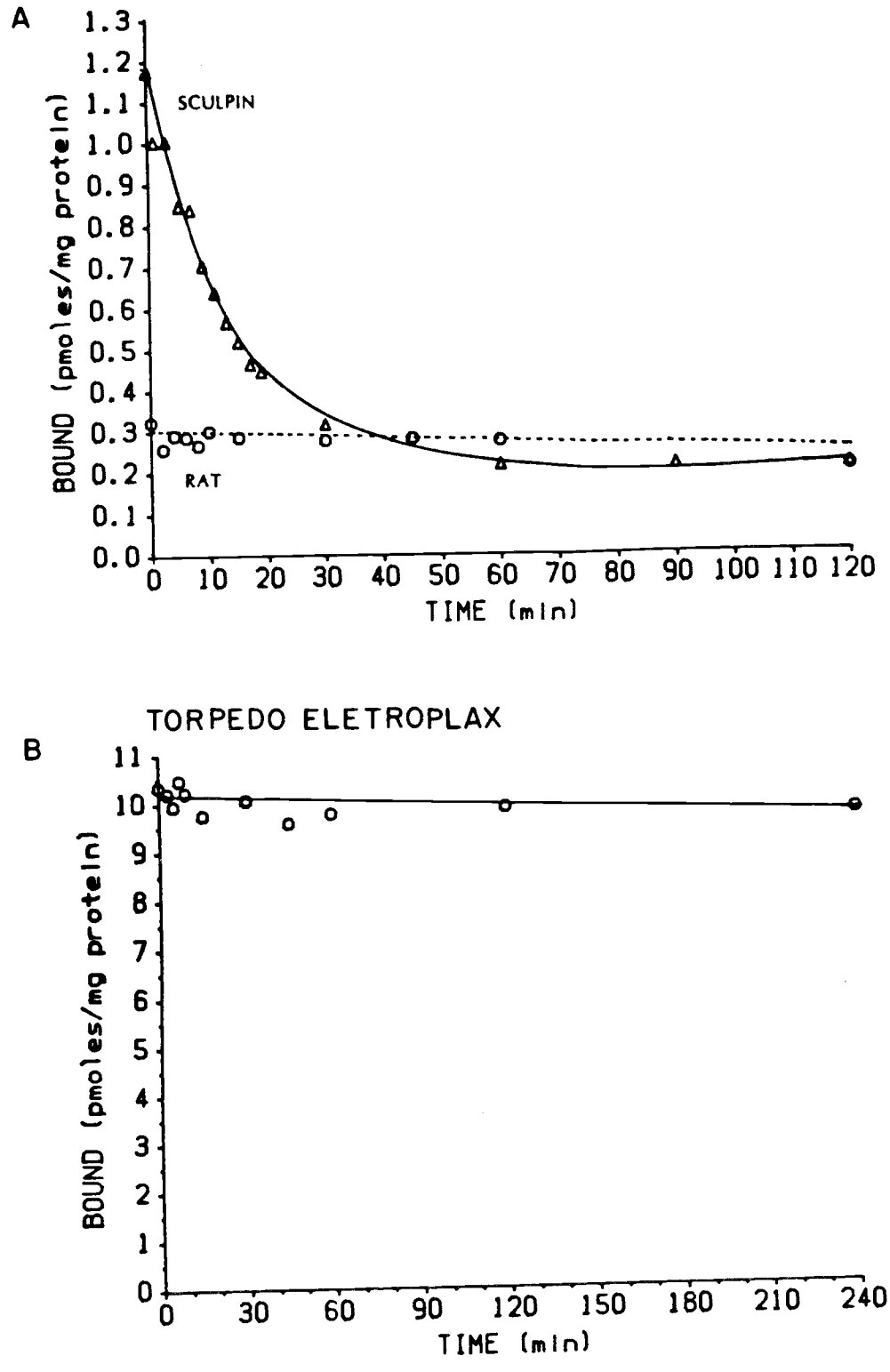
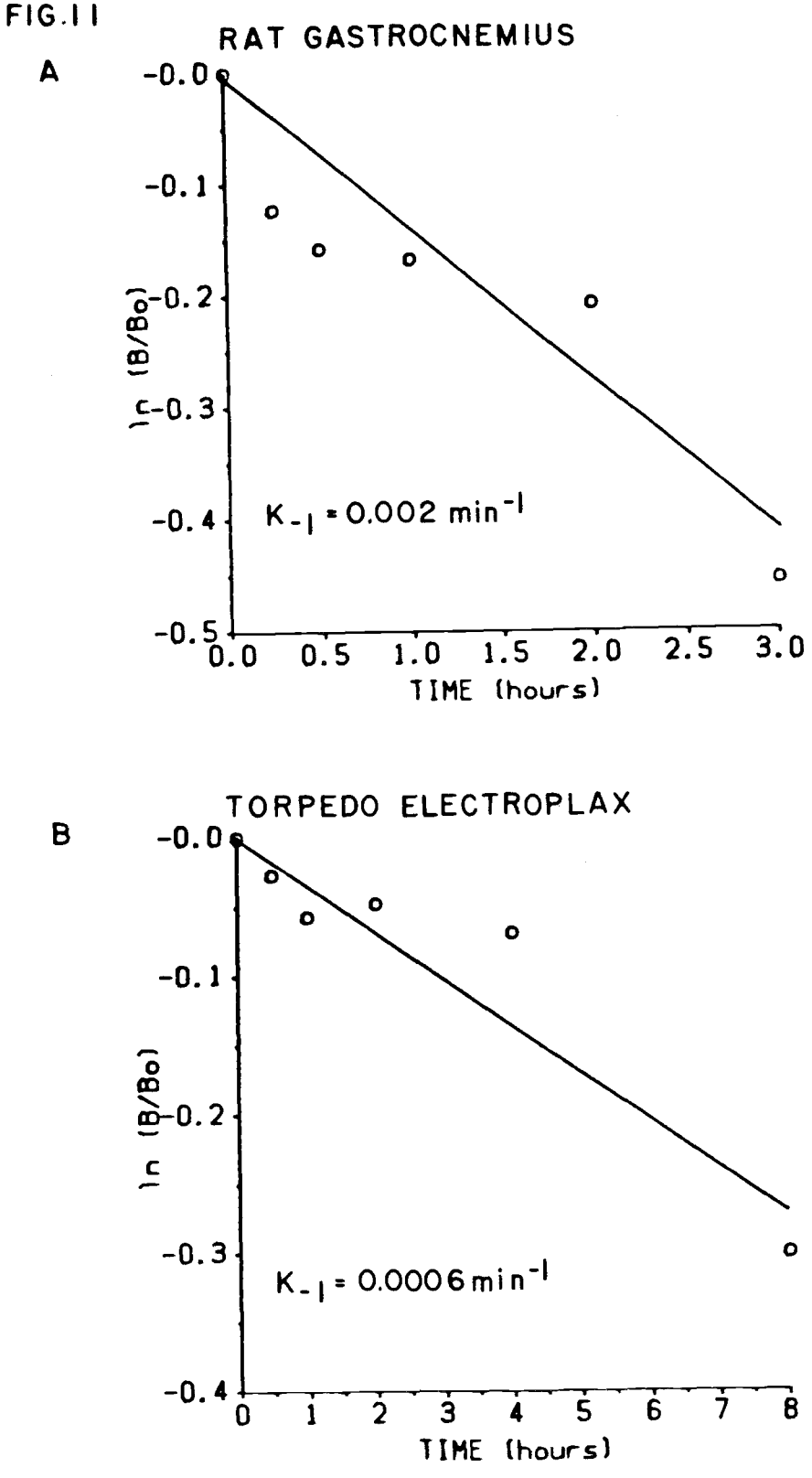

A
SCULPIN
RAT
BOUND (pmoles/mg protein)
TIME (min)
B
TORPEDO ELETROPLAX
BOUND (pmoles/mg protein)
TIME (min)

Figure 11. Rate of dissociation of [^{125}I]-α-BGT from rat denervated gastrocnemius (A) and Torpedo electroplax (B) presented as a first-order kinetic plot of specific binding. The K_{-1} rate constants were determined from the negative slope of the line calculated by linear regression analysis. The K_{-1} determined in these experiments were 0.002 min^{-1} for rat denervated rat gastrocnemius and 0.0006 min^{-1} for Torpedo electroplax.

FIG. 11


A
RAT GASTROCNEMIUS
-0.0
-0.1
-0.2
-0.3
-0.4
-0.5
ln (B/Bo)
K-1 = 0.002 min-1
0.0
0.5
1.0
1.5
2.0
2.5
3.0
TIME (hours)
B
TORPEDO ELECTROPLAX
-0.0
-0.1
-0.2
-0.3
-0.4
ln (B/Bo)
K-1 = 0.0006 min-1
0
1
2
3
4
5
6
7
8
TIME (hours)

$k_{+1} = 4.79 \times 10^{-1}$ min^{-1} $nmole^{-1}$ was determined from the following equation

$$k_{+1} = \frac{k_{obs} - k_{-1}}{[^{125}I]\text{-}\alpha\text{-BGT}}$$

where k_{-1} is the dissociation rate constant. The k_{-1} was determined by allowing binding to reach equilibrium (60 min. incubation for muscle membranes; 90 min. for electroplax), at which time unlabeled α-BGT (2.5 μM) was added to the incubation mixture. Aliquots were filtered at various times and the remaining $[^{125}I]$-α-BGT determined.

Dissociation of $[^{125}I]$-α-BGT from sculpin pectoral muscle was biphasic (Figure 10A) with slow and fast dissociating components, whereas the dissociation from denervated rat gastrocnemius (Figure 10A) and from electroplax (Figure 10B) displayed only a single slowly dissociating component. The first order dissociation constant was determined graphically to be 0.0028 ± 0.001 min^{-1} (n=3) (Figure 11A) for rat gastrocnemius and 0.0006 min^{-1} for electroplax (n=1) (Figure 11B). The two first order dissociation constants were determined for sculpin pectoral muscle using a nonlinear least squares Gauss algorithm (Robinson, 1979) to fit the data to the following equations

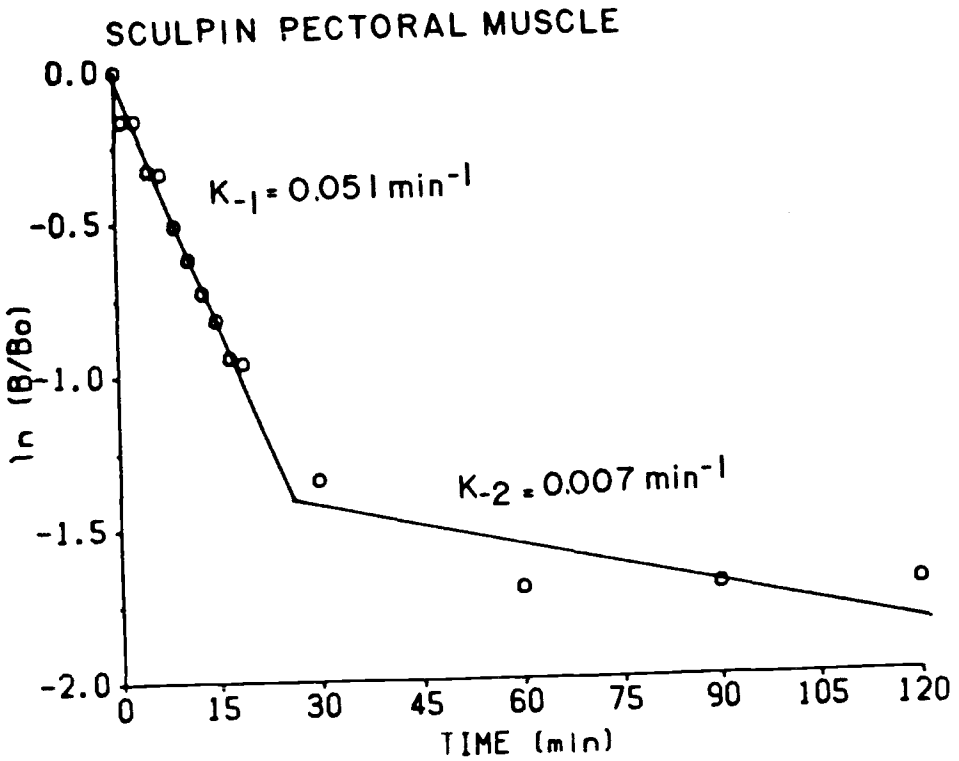
$t_1 < t_s$: $\frac{RT_t}{RT_o} = a * \exp(-k_{-1}t)$

$t_1 > t_s$: $\frac{RT_t}{RT_o} = a * \exp(-k_{-1}t - k_{-2}(t-t_s))$

where a = proportion of molecules bound at time t=0, RT_t is the concentration of receptor-toxin complex at time t, RT_o is the concentration of receptor-toxin complex at the beginning of the experiment, k_{-1} and k_{-2} are the first order dissociation constants for the fast and slow dissociating components, and t_s is the time of separation between the two dissociating components. The k_{-1} and k_{-2} were determined to be 0.0595 ± 0.0054 min^{-1} and 0.0052 ± 0.0008 min^{-1} (mean ± SE, n= 4), respectively (Figure 12). A similar biphasic dissociation was observed in the sculpin pectoral muscle when the incubation mixture was diluted with a 100 fold excess of incubation buffer, indicating that homotropic cooperativity is not part of the binding process. Control experiments in which buffer was added to the incubating tissue instead of nonlabeled α-BGT showed that the fast dissociating component of sculpin pectoral muscle is stable under the incubation conditions. Therefore, the observed decrease in binding cannot be due to denaturation of a specific class of reversable binding site. To test if the biphasic dissociation was due to parallel reactions of [^{125}I]-α-BGT with two different binding sites or to a two stage sequential reaction, the sculpin muscle homogenate was incubated

Figure 12. Rate of dissociation of [^{125}I]-α-BGT from sculpin pectoral muscle presented as a first order kinetic plot. The data was fitted to the equation described in the results section. The dissociation rate constants generated from this representative plot were: K_{-1} = 0.051 min^{-1} and K_{-2} = 0.007 min^{-1}. The 95% confidence intervals calculated from this data were: 0.046 to 0.056 for K_{-1} and 0.002 to 0.015 for K_{-2}.

FIG. 12


SCULPIN PECTORAL MUSCLE
0.0
-0.5
-1.0
-1.5
-2.0
ln (B/Bo)
K-1 = 0.051 min-1
K-2 = 0.007 min-1
0
15
30
45
60
75
90
105
120
TIME (min)

with [^{125}I-α-BGT for 1 or 30 minutes and the the rate of dissociation was measured (Figure 13). In each case the dissociation was biphasic, and a similar percentage of specifically bound [^{125}I]-α-BGT dissociated rapidly whether the incubation was for 1 or 30 minutes, 62% and 69%, respectively. This indicates that the specifically bound [^{125}I]-α-BGT is dissociating from two sites having different rates of dissociation. Dissociation rate experiments were also completed on pectoral and myotomal skeletal muscle from several different species of fish (Figure 14). [^{125}I]-α-BGT appears to dissociate biphasically from sculpin pectoral, slow myotomal, fast myotomal, and red Irish lord pectoral muscle. (^{125}I)-α-BGT has a single slow rate of dissociation from Torpedo fast and slow myotomal muscle, salmon slow and fast myotomal muscle, and salmon pectoral muscle.

The pharmacological characteristics of the [^{125}I]-α-BGT binding sites in sculpin pectoral muscle, rat gastrocnemius muscle, and Torpedo electroplax were studied by drug displacement (Figure 15) and the potencies were determined for d-tubocurarine, nicotine, gallamine, atropine, decamethonium, and acetycholine (Table 2). Hill coefficients calculated from these inhibition curves where less than unity for rat and electroplax membranes while the Hill coefficients for sculpin pectoral muscle homogenates are closer to one

Figure 13. Time course of dissociation of [^{125}I]-α-BGT from sculpin pectoral muscle homogenates after incubations for 1 min (●) or 30 min (○) under conditions as described in the methods section. Nonlabeled α-BGT (8 μM) was then added and the incubation was continued for the indicated times.

FIG.13

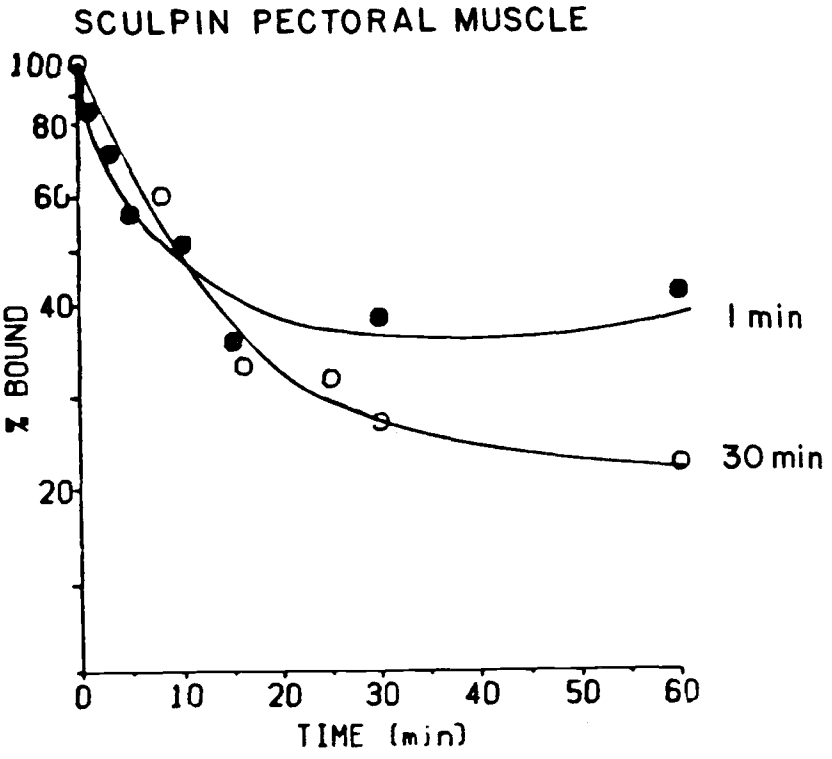

SCULPIN PECTORAL MUSCLE
100
80
60
40
20
% BOUND
0
10
20
30
40
50
60
TIME (min)
1 min
30 min

Figure 14. Time course of dissociation of [^{125}I]-α-BGT from pectoral and myotomal muscle from several species of fish.

(A.) Sculpin slow (●) and fast (0) myotomal muscle were incubated to equilibrium with 2.5 nM [^{125}I]-α-BGT and allowed to reach equilibrium. Dissociation of [^{125}I]-α-BGT was initiated by adding 2.5 μM nonlabeled α-BGT to the batch incubation. Aliquots of the incubate were subsequently removed for filtration at the times indicated.

(B.) Torpedo slow (●) and fast (0) myotomal muscle were incubated wih 4.6nM [^{125}I]-α-BGT and allowed to reach equilibriun . Dissociation of [^{125}I]-α-BGT was initiated by adding 2.5 uM unlabeled α-BGT to the batch incubation.

(C.) Red Irish lord pectoral muscle (□) coho salmon pectoral(△), and coho salmon slow (●) and fast(0) myotomal muscle were incubated to equilibrium with 4.0 nM [^{125}I]-α-BGT. Dissociation was initiated by the addition of 2.5uM nonlabeled α-BGT to the batch incubation homogenate.

FIG.14

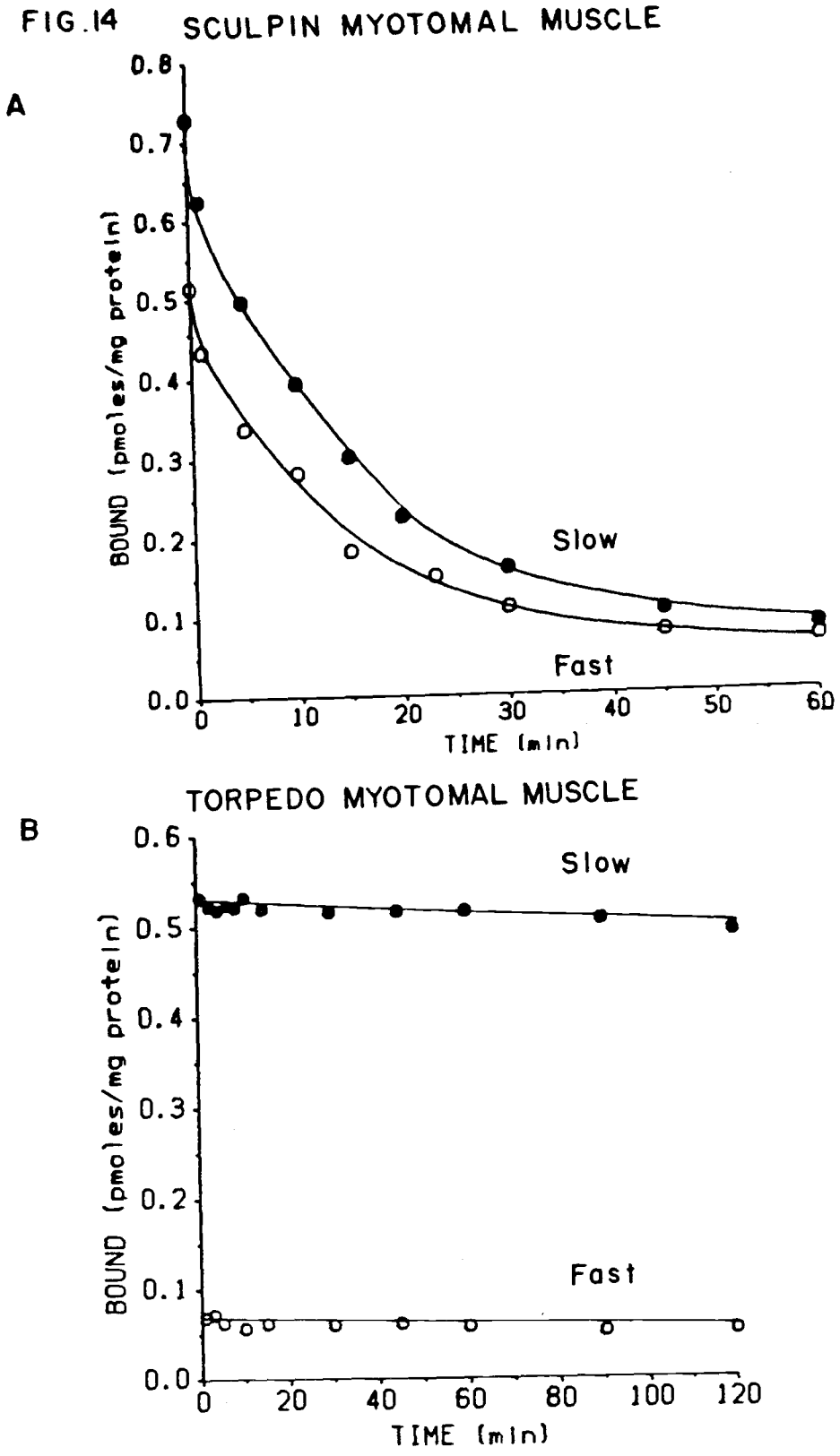

SCULPIN MYOTOMAL MUSCLE
A
BOUND (pmoles/mg protein)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Slow
Fast
0
10
20
30
40
50
60
TIME (min)
TORPEDO MYOTOMAL MUSCLE
B
BOUND (pmoles/mg protein)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Slow
Fast
0
20
40
60
80
100
120
TIME (min)

FIG. 14 Continued

C

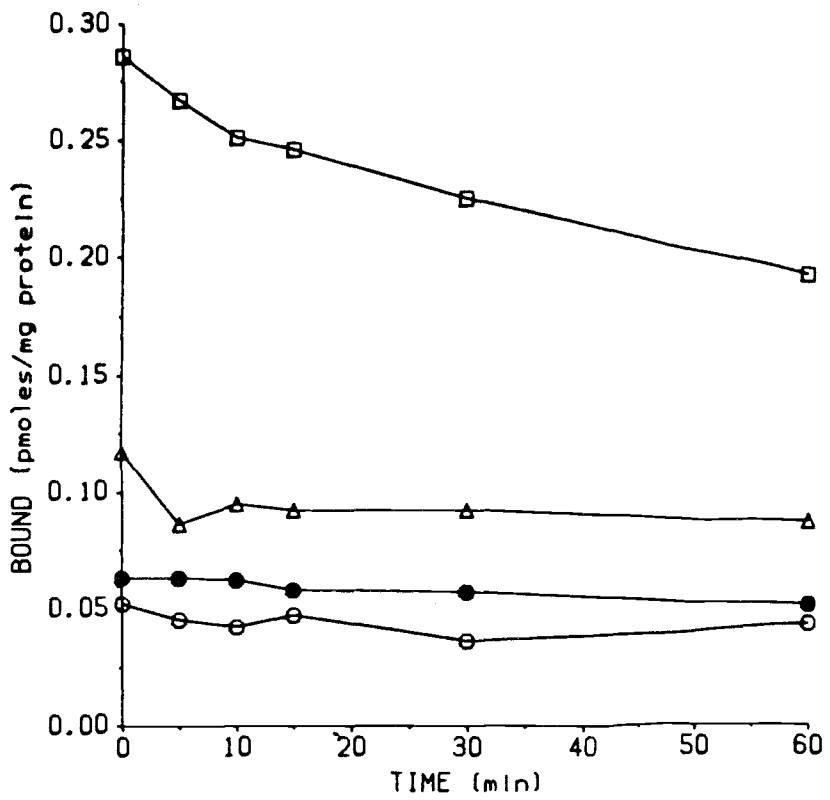


Figure 15. Ligand inhibition of [^{125}I]-α-BGT binding to sculpin pectoral muscle (A), rat denervated gastrocnemius muscle (B), and Torpedo electroplax (C) presented as logit-log plots. Muscle homogenates were incubated with 2.5 nM [^{125}I]-α-BGT and various concentrations of ligands for 60 min prior to filtration. Electroplax homogenates were incubated with 0.06nM [^{125}I]-α-BGT and various concentrations of ligands for 60 min prior to filtration. The plots for inhibition of [^{125}I]-α-BGT to sculpin and rat muscle homogenates are representative of three separate experiments. Each point represents the average of three replicate determinations. The ligands used were: △—△, d-tubocurarine; +--+, nicotine; ▲—▲, gallamine; □—□, atropine; ●—●, decamethonium; O--O, acetylcholine. Acetylcholine inhibition experiments were performed in the presence of 0.1mM DFP in sculpin and rat muscle homogenates, and in the presence of 1mM DFP in electroplax homogenates.

FIG. 15

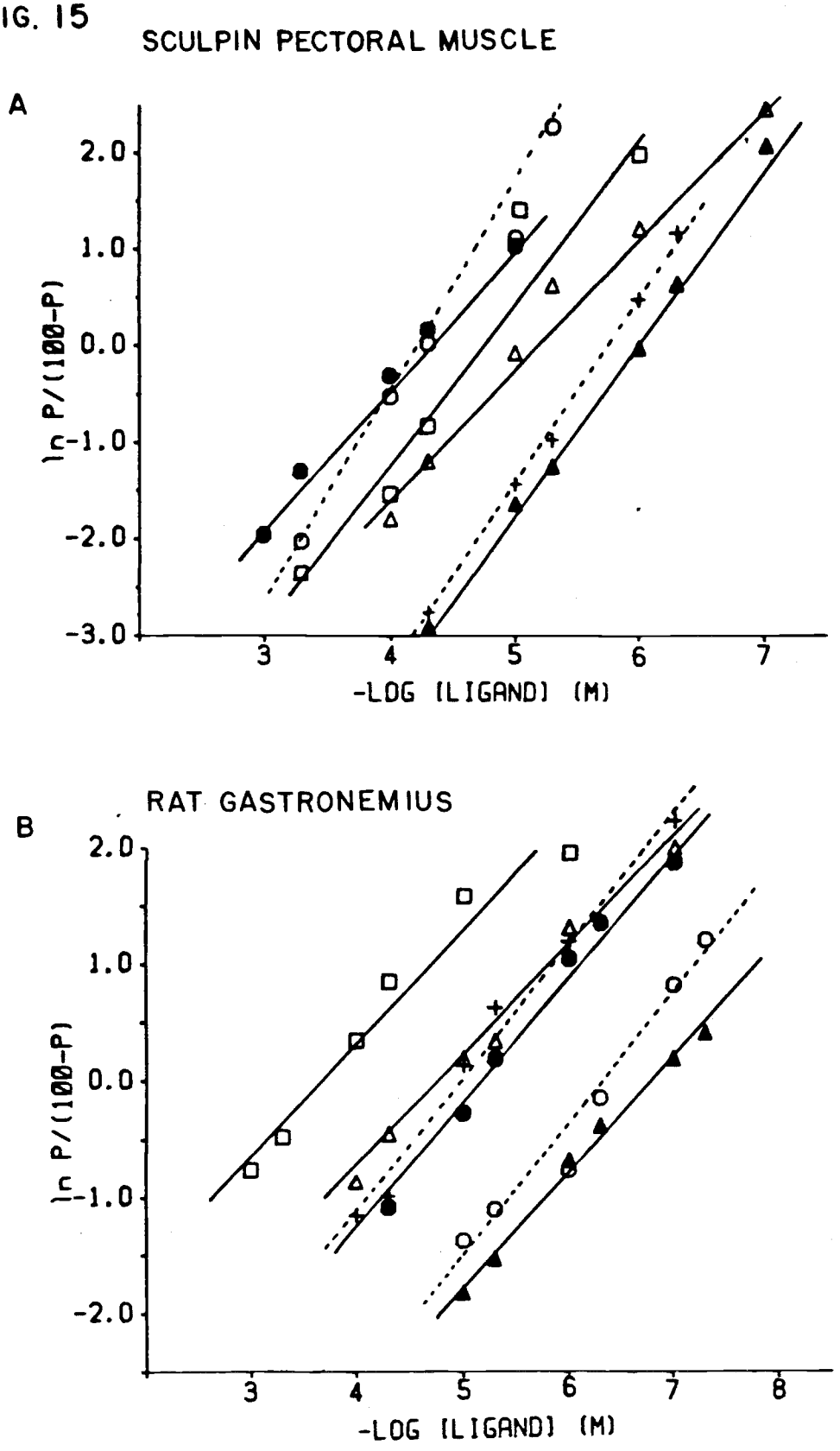
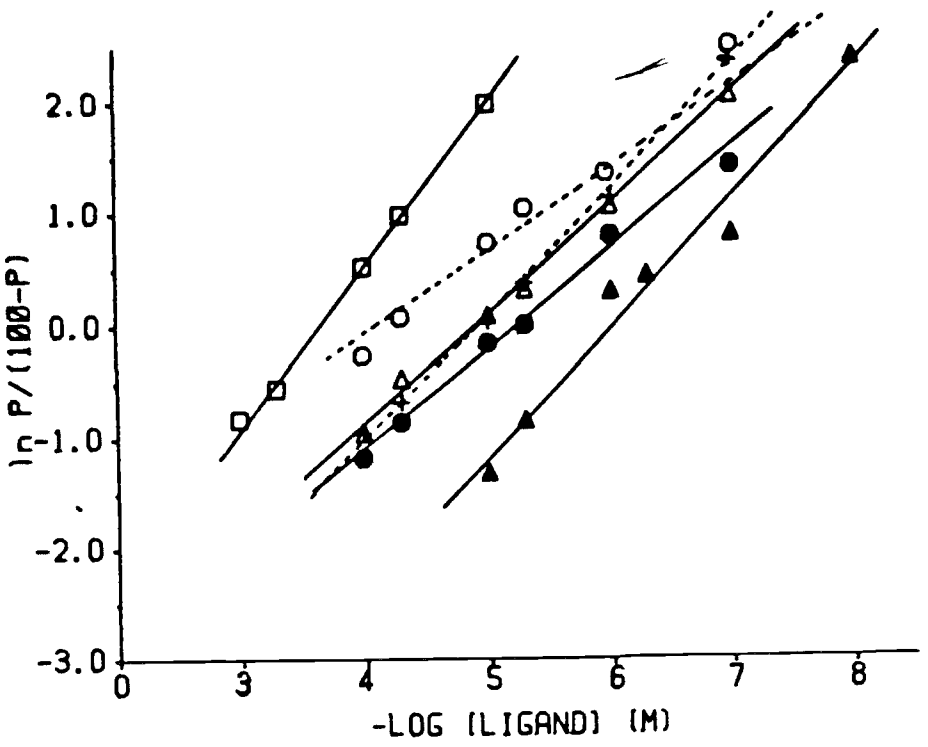

SCULPIN PECTORAL MUSCLE
A
2.0
1.0
0.0
-1.0
-2.0
-3.0
ln P/(100-P)
3
4
5
6
7
-LOG [LIGAND] (M)
RAT GASTRONEMIUS
B
2.0
1.0
0.0
-1.0
-2.0
ln P/(100-P)
3
4
5
6
7
8
-LOG [LIGAND] (M)

FIG. 15 TORPEDO ELECTORPLAX
C



(Table 2). These results suggust that binding to sculpin pectoral muscle occurs at a single class of independent sites whereas binding to rat muscle and Torpedo electroplax occurs at either multiple classes of sites or at a single class of sites exhibiting negatively cooperative site-site interactions. Since the interaction of these drugs with the [^{125}I]-α-BGT binding site in rat muscle and electroplax does not obey the simple mass action law a single equilibrium dissociation constant for the inhibitor (K_I) value cannot be calculated from these data. However, there are some differences in the IC_{50} values between the tissues that should be noted. Tubocurarine, a nicotinic antagonist, was the most potent displacer of specifically bound [^{125}I]]-α-BGT in all three tissues studied. Rat gastrocnemius had the highest affinity for d-tubocurarine with an IC_{50} value approximately one order of magnitude lower than electroplax or sculpin pectoral. Acetylcholine was a potent inhibitor of [^{125}I]-α-BGT binding in rat gastrocnemius with an IC_{50} 62 times lower than sculpin and 225 times lower then the electroplax binding site. The muscarinic receptor antagonist atropine was the weakest inhibitor in electroplax and rat gastrocnemius membranes. However, sculpin pectoral muscle had an IC_{50} value approximately 15 times lower than those of rat gastrocnemius and

TABLE 2

Inhibition constants (IC_{50}) and Hill coefficients (n_H) for ligand inhibition of [^{125}I]-α-bungarotoxin binding to various tissues in order of potency.[a]

Drug	IC_{50} (μM) (mean ± s.e.)	n_H (mean ± s.e.)
Sculpin Pectoral Muscle		
d-Tubocurarine	1.36 ± .45	0.85 ± .07
Nicotine	3.15 ± 1.5	0.82 ± .05
Gallamine	5.77 ± 1.3	0.66 ± .03
Atropine	17.00 ± 4.6	0.85 ± .06
Acetylcholine	45.20 ± 11.0	0.81 ± .04
Decamethonium	45.70 ± 7.0	0.75 ± .14
Rat Denervated Gastrocnemius		
d-Tubocurarine	0.18 ± 0.02	0.39 ± .04
Acetycholine	0.74 ± 0.31	0.43 ± .02
Decamethonium	7.53 ± 0.97	0.44 ± .03
Nicotine	13.80 ± 1.90	0.56 ± .03
Gallamine	28.70 ± 8.20	0.55 ± .17
Atropine	273.00 ± 91.00	0.40 ± .06
Torpedo Electroplax		
d-Tubocurarine	0.99	0.46
Decamethonium	5.65	0.38
Nicotine	12.20	0.48
Gallamine	12.60	0.42
Acetycholine	22.00	0.46
Atropine	238.00	0.63

[a] IC_{50} = Concentration of ligand which inhibits 50% of specific [125I]-α-BGT binding to various tissues. IC_{50} values were determined from logit-log plots of at least three separate experiments for sculpin and rat muscle involving five to seven concentrations of each drug assayed in triplicate. Electroplax IC_{50} values are from single experiments.

electroplax. The order of potency reported here for rat skeletal muscle is consistent with that reported by Colquhoun and Rang (1976) for inhibition of [^{125}I]-α-BGT to denervated rat diaphragm homogenates.

DISCUSSION

When the B_{max} values are expressed as the number of binding sites per gram of tissue (Table 3), there are approximately 25 times more receptors per gram of sculpin pectoral muscle than per gram of innervated rat gastrocnemius. This may be due to differences in the pattern of innervation. In the few species that have been investigated, fish fin muscles have been found to contain both slow and fast fibers with the slow fibers concentrated near the surface (Johnston, 1981). Both fiber types have been found to be multiply innervated with `en grappe' type nerve endings that are distributed evenly along the length of the fiber (Nishihara, 1967; Nakajima, 1969). In a typical mammalian muscle fiber, one axon innervates a single end-plate on a single muscle fiber (Bowden and Duchen, 1976). Torpedo slow myotomal muscle fibers had approximately 9 fold more binding sites per gram of tissue than the fast myotomal fibers, which may also be due to differences in innervation. Torpedo slow myotomal fibers are multiply innervated with `en grappe' type nerve endings distributed along the fibers, whereas the fast fibers

TABLE 3

Concentration of [^{125}I]-α-bungarotoxin binding sites in different tissues.

Tissue	pmoles of α-BGT bound/gram
Electric Organs:	
Torpedo	1100 [a]
	500 - 1000 [b]
	337
Electrophorus	35 [c]
Muscles:	
Sculpin Pectoral	12.21
Torpedo Myotomal-Fast	0.34
Torpedo Myotomal-Slow	3.02
Rat Gastrocnemius	0.47
Rat Gastrocnemius-Denervated	6.14
Rat Diaphragm	3 [a]
Rat Diaphragm-Denervated	60 [a]
Frog Sartorius	1 [a]
Chick Pectoral	0.5 - 0.8 [d]
Chick Pectoral-Denervated	14 - 21 [d]

[a] Albuquerque et al. (1979)
[b] Raftery et al. (1972)
[c] Raftery et al. (1971)
[d] Sumikawa et al. (1982)

are focally innervated with en grappe type endings at only one end of the fiber (Bone,1964).

Denervation of the rat gastrocnemius for 26 to 30 days increased the number of binding sites 13 fold. The magnitude of increase in extrajunctional α-BGT binding sites and the time course of development vary with the species and the muscle type. Chiu et al. (1973) observed a twenty fold increase in rat diaphragm denervated for 20 days. Dolly and Bernard (1977) reported a 44 fold increase in the number of α-BGT binding sites in cat lower leg mixed muscles 28 days after scission of the sciatic nerve. The number of binding sites per gram electroplax determined in the present study was close to those reported by Raferty et al. (1972).

The dissociation constants for innervated and denervated rat gastrocnemius determined from Scatchard plots were very similar. They are in close agreement with the K_D of 1.42 nM reported by Almon et al. (1974) for denervated rat soleus muscle. However, they were greater than values reported by other researchers for mammalian skeletal muscle. Colquhoun and Rang (1976) reported a K_D of 1×10^{-10} M for denervated rat diaphragm homogenates. Brockes and Hall (1975) investigated the binding of [^{125}I]-α-BGT to solubilized extracts from the junctional regions of normal rat

diaphragm muscle and from extrajunctional regions of denervated diaphragm. Scatchard plots derived from their data suggested the existence of two classes of binding sites with K_D's of 3.7×10^{-10} and $< 0.4 \times 10^{-10}$M for the junctional receptor and 1.7×10^{-10} and $< 0.2 \times 10^{-10}$M for the extrajunctional receptor. The K_D for Torpedo electroplax determined in this study was also slightly greater than those reported previously. Quintana and Lobao (1985) report a K_D of 0.9×10^{-9}M for electroplax compared to the value of 2.9×10^{-9}M reported here. The K_D determined from the Scatchard plot for the sculpin is approximately 2 fold greater than that of innervated rat gastrocnemius, indicating slightly less affinity for α-BGT. The Hill coefficients were close to unity for all tissues studied indicating that α-BGT is binding to a single class of non-interacting binding sites.

The rate of association of α-BGT to sculpin pectoral muscle membranes was slightly slower than that of rat gastrocnemius. The rat muscle k_{+1} of 6.48×10^6 min^{-1} M^{-1} from the present experiments is in close agreement with $K_{+1} = 2 - 5 \times 10^6$ min^{-1} M^{-1} for rat diaphragm at 20° C reported by Colquhoun and Rang (1976). The electroplax association constant was approximately two orders of magnitude greater than the constants calculated for the sculpin and rat muscle. Franklin and Potter (1972)

reported a k_{+1} of 2.0×10^{7} M^{-1} min^{-1} for Torpedo electroplax at 20°C.

One possible explanation why the Scatchard plots indicated only one binding site for sculpin pectoral muscle while dissociation kinetics appear to indicate two sites could be the existance of two sites which have equilibrium constants that are so similar that it is difficuly to distinguish them using isotherm experiments. Several investigators have reported two binding sites in mammalian skeletal muscle (Brockes and Hall, 1975; Chiu et al., 1973) but none have reported a binding site that dissociates as rapidly as that seen in the sculpin. The fact that sculpin fast and slow myotomal muscle also appeared to dissociate biphasically while the myotomal muscle of Torpedo and coho salmon is slowly reversable may indicate that the biphasic dissociation is a species specfic characteristic of sculpin skeletal muscle. However, red Irish lord pectoral muscle also dissociated rapidly, but not to the same degree as sculpin pectoral muscle.

Drug inhibition of toxin binding reveals the nicotinic nature of the binding sites in the tissues studied. d-tubocurarine, a nicotinic anatagonist, was the most potent of the displacing drugs studied. However, the high affinity of atropine in inhibiting α-BGT binding to sculpin pectoral muscle in comparison to

rat skeletal muscle and electroplax indicates that the sculpin binding site may have some mixed muscarinic-nicotinic characteristics. The low potency of acetylcholine in displacing α-BGT to sculpin muscle binding sites also shows that there are some pharmacological differences between this site and that of rat skeletal muscle.

Gant et al. (1984) examined the effects of various blocking agents and acetylcholinesterase inhibitors on sculpin pectoral muscle response to nerve stimulation. When the potency of drugs as neuromuscular blocking agents is compared to their inhibition of [^{125}I]-α-BGT, the rank order of potency is similar for both studies. The consistency beteen the *in situ* and the *in vitro* binding experiments suggests a functional relationship between these studies. The high dosage of α-BGT required to block sculpin pectoral muscle response reported by Gant *et al* (1984). when compared to mammalian studies may be related to the fast α-BGT dissociating component or the high dose may be required due to the high density of binding sites seen in sculpin pectoral.

When the [^{125}I]-α-BGT binding sites in sculpin pectoral muscle, rat gastrocnemius, and *Torpedo* electroplax are compared, it is evident that all the receptors are nicotinic but the binding site of sculpin pectoral muscle differs substantially from the other

two. The sculpin pectoral muscle binding site has a K_D that is approximately 2 fold greater than that of rat gastrocnemius and*Torpedo* electroplax. The sculpin [^{125}I]-α-BGT binding site dissociates biphasically with a reversable component and the affinity of the binding site for acetylcholine is lower than that seen in the rat. The data presented along with that of Schneider and Weber (1974,1975) and Gant et al. (1984) indicate that the neuromuscular system of fish differs from that of mammalian systems. There are some indications in the motoneurons of some fish of transmitter substances other than acetylcholine, and of dual innervation of the muscle fibers (Bone,). The data presented here also indicates that the binding characteristics of the nicotinic receptors at neuromuscular junctions are not the same for all muscles and species. This may indicate that there are some structural differences in the AChR of the fish neuromuscular junction, or there are some secondary differences such as association of the AChR with specific membrane lipids or with a cytoskeletal element.

CONCLUSION

Fish neuromuscular systems are able to maintain a tetanus at levels of AChE inhibition in excess of 97% (Schneider and Weber, 1975). The ability to maintain a

tetanus or tetanic fade is lost in the mouse isolated nerve-diaphragm preparation when the level of inhibition of AChE is 80 % (Hobbinger, 1976). Zamis and Head (1976) state that tetanic fade with AChE inhibitors has been explained as due to either a depolarization block at the postjunctioal site or due to desensitization of the AChR. Burns and Patton (1951), using the gracilis muscle of the cat, demonstrated that decamethonium and ACh in the presence of AChE inhibitors cause a persistent depolarization of the end-plate region. They concluded that the inexcitability of the muscle membrane was the principal cause of the neuromuscular block by decamethonium or by tetanization of the motor nerve.

Thesleff (1955) reported that the prolonged neuromuscular block in the frog sartorius muscle caused by decamethonium and ACh in the presence of AChE inhibitors was not due to a persistent depolarization but to a decrease in the sensitivity to ACh or desensitization. He noted that the neuromuscular block persisted in the presence of ACh despite repolarization of the membrane to the normal level. Katz and Thesleff (1957) repeated the same experiments on isolated rat diaphragm and concluded that it was not possible to show any relationship between membrane depolarization and the neuromuscular block and that a major part of the block

was due to desensitization. However, other researchers have been able to demonstrate a well maintained depolarization to ACh and decamethonium using similar mammalian preparations (Zamis and Head, 1976).

Sakman et al. (1980) examined the current through individual frog muscle AChR ion channels with a extracellular patch-clamp and reported that the channel can be found in at least three states: open; closed but activable; and closed and not activable (desensitized). Their results also imply that desensitization is composed of a rapid component with a time range of hundreds of milliseconds and a slow component with a time range of several seconds. Feltz and Trautmann (1982) studied desensitization produced by ionophoretic or bath applied ACh at the frog end-plate. They reported that desensitization occurs in two phases, one of which develops in a few seconds or less, whereas the second one is extended over tens of seconds. The rate of desensitization varies with the concentration of agonist reaching the end-plate. All cholinergic agonists tested are able to desensitize but different agonists can produce different rates of desensitization (Steinbach and Stevens, 1980). For example, desensitization occurs more rapidly in decamethonium than carbachol (Parsons, 1960).

If tetanic fade is a result of a persistent

depolarization of the end-plate, tetanus may be maintained in fish due to the lower affinity of their AChR for ACh and therefore a complete block may not occur. The ability to maintain a tetanus may also be related to the pattern of innervation of the muscle fiber and hence the concentration of AChR per muscle fiber. Chicken multiply innervated fibers are also able to maintain a tetanus after the adminstration of AChE inhibitors (Brown and Harvey, 1938).

In rats, the depolarizing drugs such as decamethonium produce a "dual" type of blockade which combines the features of both a depolarization block of the end-plate, followed by a competitive block which can be antagonized by AChE inhibitors (Zamis, 1953). The competitive block is associated with a gradual repolarization of the membrane resembling receptor desensitization (Elmquist and Thesleff,1962). The "dual" type of blockade does not occur in sculpin pectoral muscle in which both the end-plate and the muscle fibers remain depolarized (Gant et al., 1984), this may indicate that desensitization is occuring at the fish end-plate at very slow rate or not at all. Zamis and Head (1976) have stated that there is a marked species variation in the intensity and time course of desensitization. The lower affinity of the sculpin AChR for ACh determined in the binding study may indicate

that a higher concentration of ACh is required to result in desensitization. When ACh receptors are desensitized, they exhibit "bursts" of active states at irregular intervals (Sakmann et al., 1980). The high concentration of AChR found on the sculpin pectoral may result in a larger number of receptors in the active state allowing tetanus to occur. Electrophysiological techniques are required to further investigate the fish neuromuscular junction and the effects of AChE-I on this system.

[^{3}H]Acetylcholine Binding to Fish and Rat Skeletal Muscle and Fish Electroplax Nicotinic Acetycholine Receptors

INTRODUCTION

[^{125}I]-α-bungarotoxin ([^{125}I]-α-BGT) has been used by many investigators to characterize the nicotinic acetylcholine receptors (AChR) in electric organs and skeletal muscle (Dolly and Barnard, 1984). However, there have been few attempts to label and measure nicotinic cholinergic receptors with the natural transmitter acetylcholine. Receptor binding studies using [^{3}H]-acetycholine ([^{3}H]-ACh) are difficult due to the rapid hydrolysis of ACh by acetylcholinesterase (AChE) which is present in high concentrations in tissues rich in AChR, and because [^{3}H]ACh has only been available at relatively low specific radioactivity.

The use of [^{3}H]ACh to study the nicotinic receptors in Torpedo electric tissue was first reported by Eldefrawi et al. (1971) using an irreversable ACHE inhibitor to prevent the hydrolysis of [^{3}H]ACh . Binding was measured by equilibrium dialysis and they reported the presence of two high affinity binding sites which were blocked by nicotinic drugs. Boyd and Cohen (1980) also studied the binding of [^{3}H]ACh to Torpedo membranes using a rapid-mixing ultrafiltration assay.

They observed that [^{3}H]ACh binds to a single population of receptors that exists in two interconvertable conformations in the presence of agonist, one with low affinity (K_D = 800 nM) and and the other binding with high affinity (K_D = 2 nM).

Schwartz et al. (1982) synthesized [^{3}H]ACh of high specific activity to measure nicotinic cholinergic binding sites in the brain. The pharmacological characteristics of the binding site suggest that the brain receptor may differ from the two types of peripheral nicotinic receptors found in the ganglia and skeletal muscle.

In the present study, an attempt is made to develop an assay using [^{3}H]ACh of high specifc activity to characterize the nicotinic receptors in Torpedo electric organs, fish skeletal muscle, and rat skeletal muscle. The investigation of [^{125}I]-α-BGT binding to rat and sculpin skeletal muscle indicated differences between their acetylcholine receptors. Acetycholine had a much lower affinity for the toxin binding site in fish skeletal muscle when than that of rat skeletal muscle. An investigation of [^{3}H]ACh binding to electroplax, rat skeletal muscle, and fish skeletal muscle would allow further characterization of their AChR and may provide insight into the differences between the neuromuscular systems of fish and mammels.

MATERIALS AND METHODS

Materials

[Methyl-^{3}H]choline chloride (80 Ci/mmole) was purchased from New England Nuclear Corp., Boston, MA. All drugs and reagents were purchased from the Sigma Chemical Co., St. Louis, MO. Buffalo sculpin (Enophrys bison) were collected from Yaquina Bay, Newport, Oregon by otter trawl and held in tanks with aerated seawater at 12°C. Pacific electric ray was obtained from Marinus Inc., West Chester, CA.

Synthesis and Purity Determination of [^{3}H]ACh

[^{3}H]ACh was synthesized according to the method of Schwartz et al. (1982). For each synthesis, 250 µCi of [methyl-^{3}H] choline chloride in ethanol was evaporated to dryness under N_2 at room temperature. To the dried residue, 50 µl of ethyl acetate, 5 µl triethylamine, and 5 µl of acetic anhydride were added. The mixture was then incubated at room temperature for 1 hour, followed by the addition of 500 µl of 95% ethanol to destroy any remaining acetic anhydride. The mixture was then evaporated to dryness and redissolved in 1 ml of 95% ethanol. [^{3}H]ACh was stored at -20°C under N_2.

The percent conversion of [^{3}H] choline to [^{3}H]ACh was determined according to the method of McGee et al. (1978) under which choline is phosphorylated and

separated from ACh using a liquid/liquid cation exchange process. In the assay, 10 µl of stock [^{3}H]ACh was evaporated to dryness under N_2 and redissolved in 100 µl of Hepes buffered salt solution (pH 7.4) with choline kinase (10 mU/ml), $MgCl_2$ (10 mM) and in the presence or abscence of ATP (10 mM). The mixtures were incubated for 30 min at 37°C during which any choline was phosphorylated while the acetylcholine remained unchanged. The reaction was terminated by the addition of 2 ml of 3-heptanone containing 10 mg/ml tetraphenylboron. The mixtures were vortexed and let stand until the layers had separated. A 1 ml aliquot of the upper organic phase was removed, placed in a scintillation vial, and was evaporated at 60°C. The dried residue was redissolved with liquid scintillation solution and the radioactivity was measured with a liquid scintillation counter. In this assay, [^{3}H]phosphorycholine remains in the aqueous phase while [^{3}H]ACh is extracted into organic phase. Control samples with [^{3}H]choline were run to test the authenticity of the assay. The [^{3}H]ACh was > 97% pure for each batch synthesized. [^{3}H]ACh was reacetylated approximately every two weeks.

Preparation of Tissues and Binding Assays

Torpedo electric organ, buffalo sculpin pectoral muscle, rat diaphragm, rat denervated gastrocnemius, and

rat cerebral cortex were dissected over ice from freshly killed animals. The tissues were either used immediately or frozen in liquid N_2 and stored at -70°C until use.

In initial experiments, rat cerebral cortex and diaphram muscle were prepared according to the method of Schwartz et al. (1982). Tissues were weighed, minced and homogenized with a Brinkman Polytron in 50 mM Tris-HCl buffer containing 1 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, and 2mM $CaCl_2$ (pH 7.4 at 0°C). The buffer for rat cerebral cortex also contained 1.5 µM atropine sulfate to block muscurinic ACh binding sites. The tissue was centrifuged at 49,000xg for 10 minutes and the resulting pellet was resuspended in fresh buffer and centrifuged at 49,000xg for an additional 10 minutes. The final pellet was resuspended in buffer containing 100 µM diisopropylflurophosphate (DFP).

In the binding assay for this tissue preparation, aliquots of tissue, prepared at various protein concentrations ranging from 70-1000 µg of protein, were incubated in tripilicate with [^{3}H]ACh (80 Ci/mmole). Tubes containing either 100 µM carbachol or 100 µM d-tubocurarine were assayed to determine nonspecific binding. The reaction mixtures were incubated for 40 minutes at 0°C and then filtered under reduced pressure through Whatman GF/C filters which were presoaked with

0.05% polyethylenimine (PEI). PEI pretreatment eliminated displaceable binding of [^{3}H]ACh to the filters.

Assays of [^{3}H]ACh to Torpedo electroplax, sculpin pectoral muscle, and denervated rat gastrocnemius were completed by procedures modified from Potter (1980) for [^{125}I]-α-BGT. Tissues were weighed minced and homogenized with a Brinkman Polytron in ice cold 20 mM, pH 7.0, phosphate buffer containing protease inhibitors (0.1 mM NaCl, 5mM EGTA, 0.1 mM phenymethylsulfonyl fluoride (PMSF)). The homogenates were filtered through cheesecloth to remove fragments of connective tissue, and then centrifuged at 35,000xg (5 C) for 30 minutes. The pellet was resuspended in 20 mM, pH 7.0, phosphate buffer (0.4 mM NaCl, 1.0 mM EGTA, 0.1 mM PMSF) and then centrifuged at 10,000xg for 10 minutes. The supernatant was centrifuged at 35,000xg for 30 minutes, and the pellet was resuspended in 20 mM, pH 7.0, phosphate buffer (0.1 mM NaCl, 1.0 mM EGTA, 0.1 mM PMSF, 1 mM DFP). The membranes were incubated at 18 C for 1 hour and then diluted to 0.1 mM DFP with buffer.

The binding of [^{3}H]ACh to muscle and electroplax membranes was measured by a rapid filtration assay. Aliquots of membranes (200 µl; electroplax: 50 µg protein, muscle: 100-1000 µg protein) were incubated in triplicate for 10 minutes with 200 µl of[^{3}H]ACh (electroplax: 10

Ci/mmole, muscle: 80 Ci/mmole) and 100 µl of buffer or competing drug. Tissues and competing drugs were preincubated for at least 30 minutes prior to the addition of [^{3}H]ACh. After incubation at 0 C, each sample was diluted with 4 ml of ice cold 20 mM, pH 7.0, phosphate buffer (0.1 mM NaCl, 1 mM EGTA, 0.1 mM PMSF) and filtered under reduced pressure through Whatman GF/B glass fiber filters. The filters for electroplax binding were presoaked in ice cold 0.3% PEI, pH 10.0 for at least 2 hours prior to use to eliminate displaceable binding to the filter (Bruns et al., 1983). Each filter was then placed in a vial, liquid scintillation solution was added, and the radioactivity was measured by liquid scintillation spectrometry. Specific binding of [^{3}H]ACh to membranes was defined as the difference between the amount of [^{3}H]ACh bound in the absence and presence of 1 uM α-bungarotoxin.

Protein content was determined by the method of Lowry, et al. (1951) using BSA as the standard.

RESULTS

Specific binding of 10 nM [^{3}H]ACh to rat cerebral cortex (200 µg protein) represents approimately 50 % of total binding with 1.1 pmole bound/gram of tissue, which is similar to the values reported by Schwartz et al. (1982). However, under these assay conditions there was

no detectable specific binding of [^{3}H]ACh to rat diaphragm over the range of protein concentrations studied with either carbachol or d-tubocurarine used to determine nonspecific binding. This may indicate that either the number of binding sites in innervated rat skeletal muscle is too low to be measured by this assay procedure or that nonspecific binding is too high to detect any specific binding. Since high levels of proteolytic enzymes have been reported in mammalian skeletal muscle (Dolly and Bernard, 1984), the assay was performed on rat diaphragm homogenized in buffer containing protease inhibitors: 5 mM EGTA, 0.1 mM PMSF in initial buffer and 1 mM EGTA, 0.1 mM PMSF in subsequent resuspensions. However, no [^{3}H]ACh specific binding to rat diaphragm was detected.

Electroplax membrane preparations were then used in an attempt to develop an optimal assay for [^{3}H]ACh binding to AChR that could also be used to characterize skeletal muscle AChR. Binding of [^{3}H]ACh to electroplax membranes linear for protein concentrations ranging from 50 to 250 µg of protein. In an experiment to optimize the number of filter washings in the [^{3}H]ACh binding assay, it was found that a single 4 ml wash of the incubation mixture gave the highest number of specific counts, with specific binding representing 95% of total binding. Heat denaturation of the tissue by placing the

homogenate in 60°C water for 60 minutes, followed by cooling, resulted in the elimination of all specific binding. Incubation of the tissue with 0.1 mM DFP inhibited 99.9% of the AChE in the electroplax tissue preparation, as determined by the spectrophotometric method of Ellman et al. (1961). Anaylsis of the [^{3}H]ACh after incubation with DFP treated tissue was performed by extracting the [^{3}H]ACh from the tissues with ethanol after various times of incubation. Aliquots of ethanol from these samples were evaporated under N_2 and the purity of the [^{3}H]ACh was determined according to the assay described in the Methods section. The lowest rate of [^{3}H]ACh hydrolysis was seen in tissues which were incubated with 1 mM DFP for 1 hour at room temperature and then diluted to 0.1 mM DFP with ice cold buffer prior to the addition of [^{3}H]ACh. In this experiment, there was no hydrolysis of [^{3}H]ACh over the first 10 minutes and 5 % hydrolysis over the first 30 minutes.

When the time course of [^{3}H]ACh binding to electroplax membranes was studied using 0.5 nM [^{3}H]ACh with a specific activity of 80 Ci/mmole, the binding reached equilibrium within 25 seconds. To decrease the observed rate of association, the specific activity of the [^{3}H]ACh was lowered to 10 Ci/mmole by dilution with nonradiolabeled ACh. The binding of [^{3}H]ACh at this lower specific activity reached equilibrium within

approximately four minutes at 0°C (Figure 16). To examine the dissociation of [^{3}H]ACh, electroplax membranes were incubated with [^{3}H]ACh for 10 minutes at 0°C to reach equilibrium, at which time 100 μM carbachol was added and the decrease in binding was measured with time. [^{3}H]ACh completely dissociated from the electroplax membranes within 5 seconds. A similar dissociation was observed in the electroplax when the incubation mixture was diluted with a 100 fold excess of incubation buffer.

A saturation analysis of [^{3}H]ACh binding to electroplax homogenates indicated a dissociation constant (K_D) of 89.0 nM and a binding capacity (Bmax) of 85.4 pmoles/mg protein (44.2 pmoles/gram tissue) (Figure 17 A,B). Although complete saturation was not achieved at a concentration of 100 nM, Scatchard analysis was linear over the range studied. A Hill plot of the saturation data revealed a Hill coefficient of 0.99, indicating a lack of cooperativity (Figure 17 C).

The same tissue preparation procedure and [^{3}H]ACh binding assay used for electroplax was also applied to sculpin pectoral muscle and denervated rat gastrocnemius. [^{3}H]ACh with a specific activity of 80 Ci/mmole was used due to the lower number of AChR found in these tissues. No specific binding was seen in sculpin pectoral muscle or denervated rat gastrocnemius

Figure 16. Time course of binding of [^{3}H]ACh to Torpedo electroplax. Aliquots of electroplax were removed at various times for rapid filtration from a batch incubation (0 C) with 5 nM [^{3}H]ACh. Specific binding was determined as the differences between batches incubated in the presence or abscence of 1.0 uM α-BGT.

FIG. 16

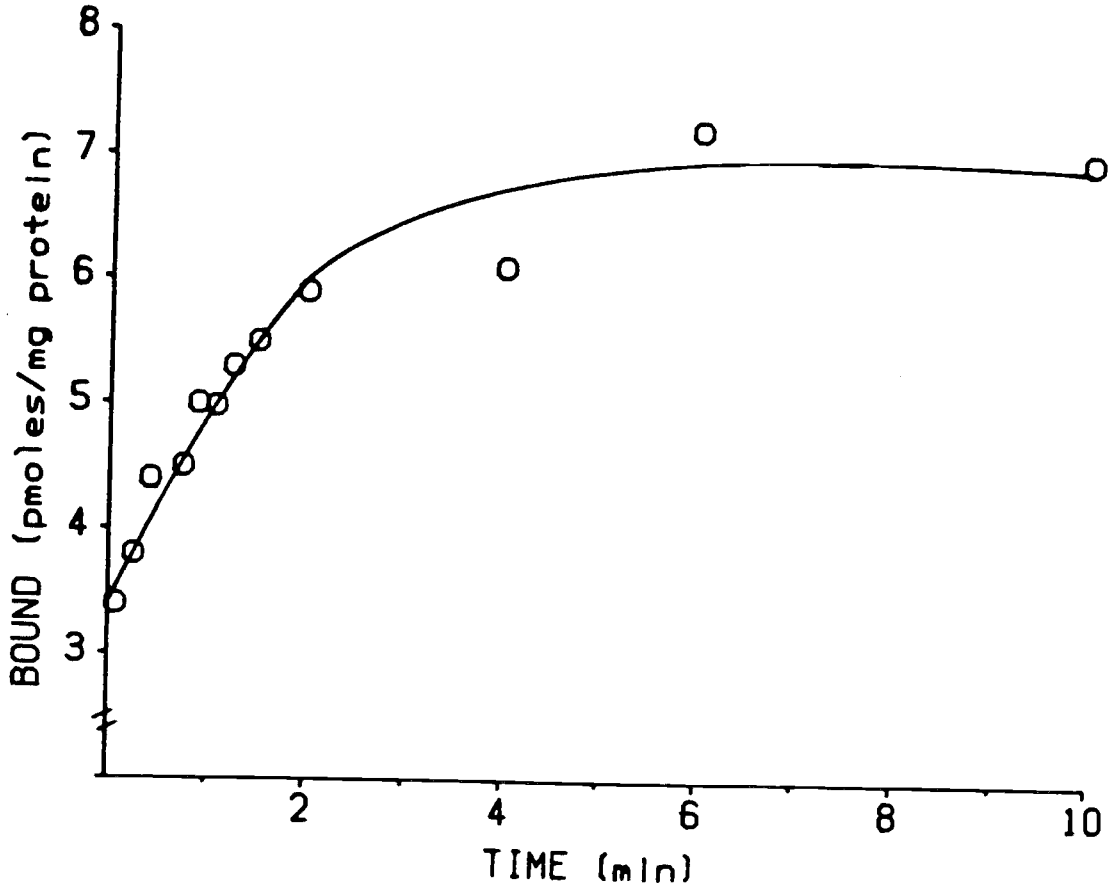


Figure 17. Saturation analysis of [^{3}H]ACh binding to Torpedo electroplax.

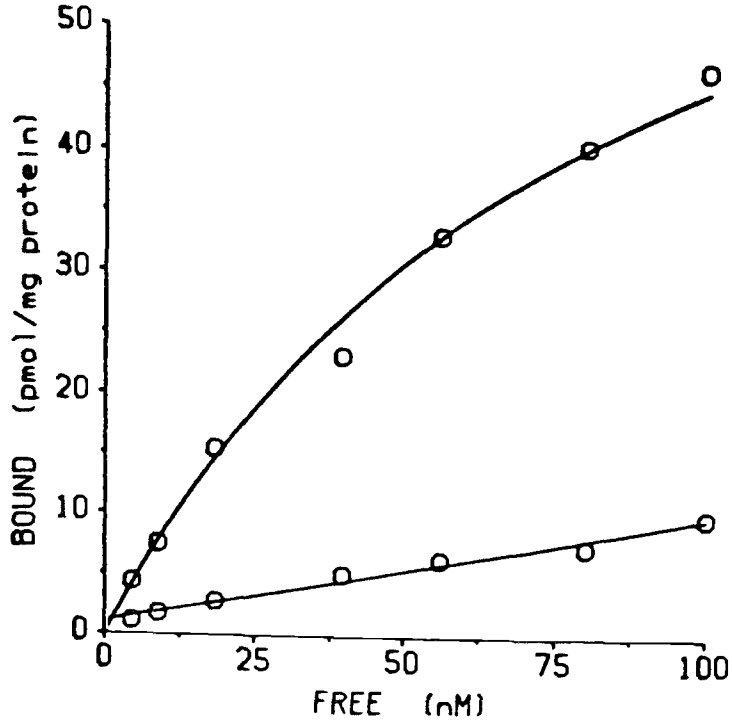
(A.) Saturation isotherm of [^{3}H]ACh binding using incubation conditions described in methods section. Aliquots of electroplax homogenate were incubated with varying concentrations of [^{3}H]ACh (4.5 - 100nM). Specific binding was defined as the difference between the amount of [^{3}H]ACh bound in the presence and absence of 1 μM α-BGT.

(B.) Scatchard analysis of the specific [^{3}H]ACh binding. The values determined were: K_D = 89.0 nN and Bmax = 85.4 pmoles/mg protein.

(C.) Hill plot of specific [^{3}H]ACh binding. The Hill coefficient was 0.99.

FIG. 17

A


50
40
30
20
10
0
BOUND (pmol/mg protein)
0
25
50
75
100
FREE (nM)

B

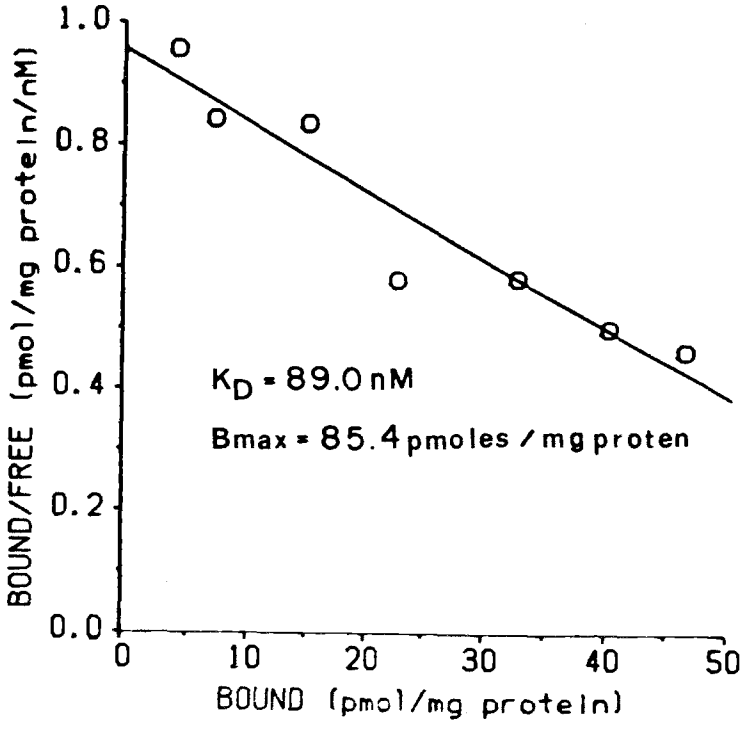
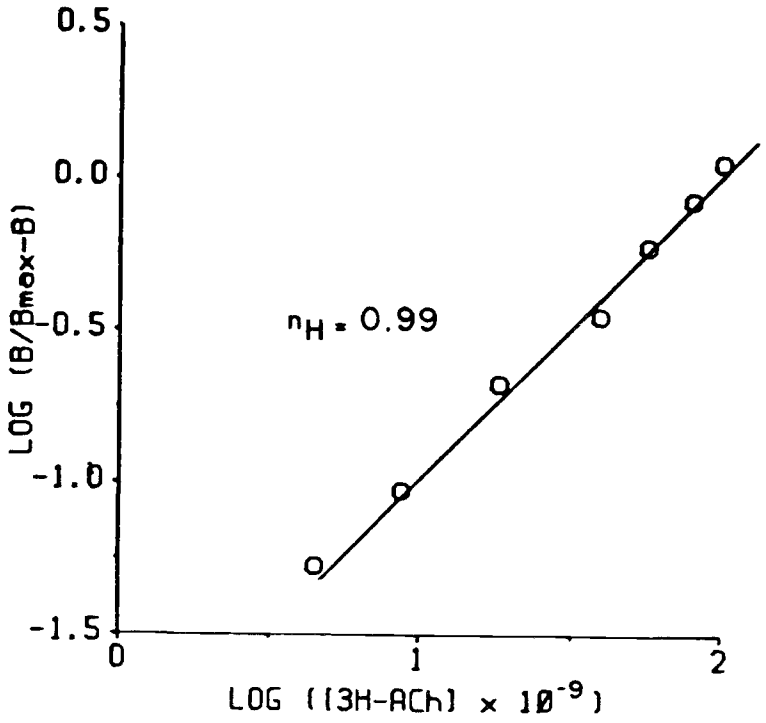

1.0
0.8
0.6
0.4
0.2
0.0
BOUND/FREE (pmol/mg protein/nM)
K_D = 89.0 nM
Bmax = 85.4 pmoles / mg proten
0
10
20
30
40
50
BOUND (pmol/mg protein)

FIG. 17 Continued

C



homogenates over a protein range of approximately 200 to 1000 µg and an incubation time of up to 30 minutes. [^{3}H]ACh concentrations used ranged from 10 to 50 nM. In one experiment, denervated rat gastrocnemius homogenate was incubated in 20 mM, pH 8.0 phosphate buffer containing 2 M KCl for 15 minutes to cause dissolution of the contractile proteins (Pardee and Spudich, 1982) and attempt to decrease nonspecific binding. After this incubation, the mixture was centrifuged at 48,000xg for 10 minutes and the resulting pellet was resuspended in buffer containing 1 mM DFP and incubated for 1 hour. The mixture was then diluted to 0.1 mM DFP prior to incubation with 50 nM [^{3}H]ACh. There was no specific binding of [^{3}H]ACh to the KCl treated homogenate. However when the same homogenate was incubated with 10 nM [^{125}I]-α-BGT for 1 hour at 18 C, there were 0.79 pmoles bound/mg protein with 95% of the [^{125}I]BGT specifically bound.

DISCUSSION

The K_D of 89.0 nM for [^{3}H]ACh binding to electroplax membranes determined in this study is greater than the value of 8 nM determined from Scatchard plots by Boyd and Cohen (1980). Kinetic analysis of [^{3}H]ACh binding to electroplax by Boyd and Cohen (1980) indicated a single population of receptors that exists

in two interconvertable conformations, one with low agonist affinity (K_D = 800 nM) and the other binding with high affinity (K_D = 2 nM). Eldefrawi et al. (1971) reported a nonlinear Scatchard plot for [^{3}H]ACh equilibrium binding to electroplax. Their data indicated a high affinity binding site with a K_D of 8 nM and a low affinity site with a K_D of 68 nM. The absence of the high affinity binding site in the present study may be due to washing since Boyd and Cohen (1980) determined binding without washing the filters. When the assay used in the present study was performed without washing a K_D of 21 nM was determined by Scatchard analysis of a saturation isotherm.

The absence of specific binding of [^{3}H]ACh to muscle homogenates indicates that the assay used is not sensitive enough to detect binding to the low number of AChR present in these tissues. Boyd and Cohen (1980) report a 1.1 : 1 ratio between the number of [^{3}H]ACh and α-neurotoxin binding sites in electroplax. If this ratio is also correct for skeletal muscle there should be 11.1 pmoles of [^{3}H]ACh binding sites per gram of sculpin pectoral muscle and 5.6 pmoles/gram in dennervated rat gastrocnemius. Since the assay works in rat cerebral cortex preparations with a Bmax of 4.6 pmoles/gram of tissue, the abscence of specific binding may be due to higher amounts of nonspecific binding in the muscle

preparations. Further purification of the membranes by sucrose-density gradient centrifugation followed by solubilization (Elliott et al., 1980) or affinity-chromatographic procedures (Gotti et al.,1982) may be useful in decreasing nonspecific binding, but they also result in a lower yield of AChR. In order for a [^{3}H]ACh binding assay to work on rat skeletal muscle one may have to use some other denervated muscles such as the diaphragm which has been reported to have 60 pmoles of [^{125}I]-α-BGT binding sites per gram of muscle (Albuquerque et al., 1979). Denervation of the sculpin pectoral muscle may also increase the number of AChR to a concentration where the [^{3}H]ACh assay may detect specific binding. [^{125}I]]-α-BGT binding sites increased 3 fold in sculpin pectoral muscle denervated for 22 days.

Bibliography

Albuquerque, E. X. , A. T. Eldefrawi and M. E. Eldefrawi. 1979. The use of snake venoms for the study of the acetylcholine receptor and its ion conductance modulator. In: Snake Venoms. C. Y. Lee, ed. Hndbch. d Exper. Pharmakol., Vol. 52. Springer-Verlag, Berlin. pp. 377-402.

Akera, T. and V. K. Cheng. 1977. A simple method for determination of affinity and binding site concentration in receptor binding studies. Biochem. Biophys. Acta. 470:412-423.

Almon, R. R., C. G. Andrew, and S. H. Appel. 1974. Acetylcholine receptor in normal and denervated slow and fast muscle. Biochem. 13(27):5522-5528.

Bernard, E. A., T. H. Chiu, J. Jedrzejcyzk, C. W. Porter and J. Wieckowski. 1973. Acetylcholine receptor and cholinesterase molecules of vertebrate skeletal muscles and their nerve junctions. In: Drug Receptors. H. P. Rang ed. pp. 211-224.

Bone, Q. 1964. Patterns of muscle innervation in lower chordates. Int. Rev. Neurobiol. 6:99-147.

Bone, Q. 1978. Locomotor muscle. In: Fish Physiology. W. S. Hoar and B. J. Randall, eds. Academic Press, New York. Vol. VII. pp. 361-424.

Bowden, R. E. and L. W. Duchen. 1976. The anatomy and pathology of the neuromuscular junction. In: Neuromuscular Junction. E. Zaimis, ed. Hndbch. d Exper. Pharmakol., Vol. 42. Springer-Verlag, New York. pp. 23-97.

Boyd, N. D. and J. B. Cohen. 1980. Kinetics of binding of [^{3}H] Acetylcholine and [^{3}H] Carbamoylcholine to Torpedo postsynaptic membranes: Slow conformational transitions of the cholinergic receptor Biochem. 19:5344-5353.

Brockes, J. P. and Z. W. Hall. 1975. Acetylcholine receptors in normal and denervated rat diaphragm muscle. I. Purification and innervation with [125I]-α-bungarotoxin. Biochem. 14(10):2092-2099.

Brown, G. L. and A. M. Harvey. 1938. Reactions of avian muscle to acetylcholine and eserine. J. Physiol. 94:101-117.

Bruns, R. F., K. Lawsen-Wendling, and T. A. Pugsley. 1983. A rapid filtration assay for soluble receptors using Polyethylenimine-treated filters. Anal. Chem. 132:74-81.

Burns, B. D. and W. D. M. Patton. 1951. depolarization of the motor end-plate by decamenthonium and acetylcholine. J. Physiol. 151:41-73.

Chiu, T. H., J. O. Dolly, and E. A. Barnard. 1973. Solubilization from skeletal muscle of two components that specifically bind α-bungarotoxin. Biochem. Biophys. Res. Commun. 51(1):205-213.

Colquhoun, D. and H. P. Rang. 1976. Effects of inhibitors on the binding of iodinated α-bungarotoxin to acetylcholine receptors in rat muscle. Mol. Pharmacol. 12:519-535.

Dolly, J. O.. 1979. Biochemistry of acetylcholine receptors from skeletal muscle. In: Neurochemistry and Biochemical Pharmacology. K. F. Tipton, ed. International Review of Biochemistry, University Park Press, Baltimore. Vol. 26. pp. 257-309.

Dolly, J. O. and E. A. Barnard. 1977. Purification and characterization of an acetylcholine receptor from mammalian skeletal muscle. Biochem. 16(23): 5053-5060.

Dolly, J. O. and E. A. Barnard. 1984. Nicotinic acetylcholine receptors: An overview. Biochem. Pharmacol. 33(6): 841-858.

Eldefrawi, M. E. , A. G. Britten, and A. T. Eldefrawi. electroplax: Relationship to acetylcholine receptors. Science 173:338-340.

Elliott, J. E., S. G. Blanchard, W. Wu, J. Miller, C. D. Strader, P. Hartig, H. Moore, J. Racs, and M. A. Raftery. 1980. Purification of Torpedo californica post-synaptic membranes and fractionation of the constituent proteins. Biochem. J. 185:667-677.

Ellman, G. L., K. D. Courtney, V. Andrea, Jr., and R. M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharm. 7(2):88-95.

Elmquist, D. and S. Thesleff. 1962. Ideas regarding receptor desensitization at the motor end-plate. Rev. Canad. Biol. 21:229-234.

Feltz, A. and A. Trautmann. 1982. Desensitization at frog neuromuscular junction. J. Physiol. 322:257-272.

Franklin, G. I. and L. T. Potter. 1972. Studies of the binding of α-bungarotoxin to a membrane bound and detergent - dispersed acetylcholine receptors from Torpedo electric tissue. FEBS Letters. 28:101-106.

Gant, D. B., L. J. Weber, and J. R. Smith. 1984. Effects of neuromuscular blocking agents and acetycholinesterase inhibitors on the sculpin (Enophrys bison) pectral fin response to indirect stimulation. Proc. Soc. Exp. Biol. Med. 177:151-155.

Gotti, C., B. M. Conti-Tronconi, and M.A. Raftery. 1982. Mammalian muscle acetycholine receptor purification and characterization. Biochemistry 21:3148-3194.

Hobbinger, F. 1976. Pharmacology of anticholinesterase drugs. In: Neuromuscular Junction. E. Zamis, ed. Hndbch. d. Exper. Pharmakol., Vol. 42 Springer-Verlag, Berlin. pp. 487-581.

Johnston, I. A. 1981. Structure and function of fish muscles. Symp. Zool. Soc. Lond. 48:71-113.

Karlin, A. 1983. The anatomy of a receptor. Neurosci. Comment. 1(3):111-123.

Katz, B. and S. Thesleff. 1957. A study of the 'desensitization' produced by acetylcholine at the motor end-plate. J. Physiol. 138:63-80.

Lee, C. Y. 1972. Chemistry and pharmacology of polypeptide toxins in snake venoms. Ann. Rev. Pharmacol. 12:265-286.

Lowery, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265-267.

McGee, R., P. Simpson, C. Christian, M. Mata, P. Nelson, and M. Nirenberg. 1978. Regulation of acetylcholine release from neuroblastoma X glioma hybrid cells. Proc. Natl. Acad. Sci. U.S.A. 75:1314-1318.

Mebs, D., K. Narita, S. Iwanga, Y. Samejima and C. Y. Lee. 1971. Amino acid sequence of α-bungarotoxin from the venom of Bungaris multicinctus. Biochem. Biophys. Res. Comm. 44:711-716.

Nakajima, J. 1969. Fine structure of red and white muscle fibers and their neuromuscular junctions in the Snake fish (Ophiocephalus argus). Tissue and Cell. 1(2):229-246.

Nishihara, H. 1967. Studies on the fine structure of red and white fin muscles of the fish (Carassius auratus). Arch. Histol. Jpn. 28:425-447.

Noda, M., H. Takahashi, T. Tanabe, M. Toyosato, S. Kikyotani, T. Hirose, M. Asai, H. Takashima, S. Inayama, T. Miyata, and S. Numa. Nature 301:251-255.

Pardee, J. D. and J. A. Spudich. 1982. Purification of muscle actin. Methods Enzymol. 85:164-181.

Parsons, R. L. 1960. Changes in post-junctional receptors with decamethonium and carbamylcholine. Amer. J. Physiol. 217:96-74.

Potter, L. T. 1980. Methods for nicotine and muscarine receptors. In: Receptor Binding Techniques. Society for Neuroscience, Short Course Syllabus. pp. 123-139.

Quintana, E. G. and H. N. Lobao. 1985. Influence of denervation on the cholinergic membrane of the electric tissue of Electrophorus electricus. Comp. Biochem. Physiol. 81C(2):461-467.

Raferty, M. A., J. Schmidt, and D. G. Clark. 1972. Specificity of α-bungarotoxin binding to Torpedo californica electroplax. Arch. Biochem. Biophys. 152:882-886.

Raferty, M. A., J. Schmidt, D. G. Clark, and R. G. Wolcott. 1971. Demonstration of a specific α-bungarotoxin binding component in Electrophorus electricus electroplax membranes. Biochem. Biophys. Res. Commun. 45:1622-1629.

Robinson, B. 1979. SPSS Subprogram Nonlinear - Regression. In: Univ. Wisconsin Nonlinear Regression Package. Document No 328. Evanston, Il.

Sakmann, B., J. Patlak, and E. Neher. 1980. Single acetylcholine-activated channels show burst kinetics in presence of desensitizing concentrations of agonist. Nature 286:71-73.

Schneider, P. W., Jr. and L. J. Weber. 1974. Identification and characterization of acetylcholinesterase from fish (Micropterus salmoides) pectoral muscles. Proc. Soc. Exp, Biol. Med. 146:1071-1075.

Schneider, P. W., Jr. and L. J. Weber. 1975. Neuromuscular function and acetylcholinesterase in pectoral abductor muscle of the black bass (Micropterus salmoides) as evidenced by the effects of diisopropylflurophosphate. J. Fish. Rev. Board Can. 32(11):2153-2161.

Schwartz, R. D., R. McGee, Jr., and K. J. Keller. 1982. Nicotinic cholinergic receptors labeled by [3H]acetylcholine in rat brain. Mol. Pharmacol. 22:56-62.

Steinbach, J.H. and C. F. Stevens. 1980. Neuromuscular Transmission. In: Neurobiology of the Frog. R. Llinas ed. pp. 33-92.

Stroud, R. M. 1983. Acetycholine receptor structure. Neurosci. Comment. 1(3):124-138.

Sumikawa, K., F. Mehraban, J. O. Dolly, and E. A. Barnard. 1982. Similarity of acetylcholine receptors of denervated, innervated, and embryonic chicken muscles. I. Molecular species and their purification. Eur. J. Biochem. 126:465-472.

Thesleff, S. 1955. Neuromuscular block produced by acetylcholine. Nature 175:594-595.

Weber, M. and J. P. Changeux. 1974. Binding of Naja nigricallis [3H]-toxin to membrane fragments from Electrophorus and Torpedo electric organs: II Effect of cholinergic agonists and antagonists on the binding of tritiated α-neurotoxin. Mol. Pharmacol. 10:15-34.

Zamis, E. 1953. Motor end-plate differences as a determining factor in the mode of action of neuromuscular blocking substances. J. Physiol. (London) 122:238-251.

Zamis. E. and S. Head. 1976. Depolarizating neuromuscular blocking drugs. In: Neuromuscular Junction. E. Zamis ed. Hndbch. d Exp. Pharmakol., Vol. 42. Springer-Verlag, Berlin. pp. 365-486.