We have studied the binding of α-bungarotoxin to a particulate fraction of goldfish brain enriched in synaptosomes. The binding is specific and saturable and exhibits the pharmacological properties of a nicotinic cholinergic receptor. Equilibrium binding measurements yield a single dissociation constant (K_d) of 0.92 nM. Kinetic analysis revealed one association rate constant and two dissociation rate constants. Dissociation constants calculated from kinetic measurements were 1.9 nM and 12.5 pm. The toxin-receptor complex is readily solubilized in nonionic detergent. The isoelectric point of the toxin-receptor complex was found to be 5.00 ± 0.01. Sedimentation velocity analysis in sucrose/H_2O and sucrose/D_2O gradients in conjunction with Sepharose 4B chromatography and diffusion experiments yielded a sedimentation constant of 11.45, a partial specific volume of 0.79 cm^3/g for the toxin-receptor-detergent complex, and a molecular weight of approximately 340,000 for the toxin-receptor complex.

The elapid neurotoxin, α-bungarotoxin has been shown to bind specifically and saturably to membrane fragments from muscle cells (1, 2), electroplax (3), sympathetic ganglia (4), and both vertebrate (5–8) and invertebrate (9) brain. The fact that α-Btx inhibits the agonist-induced activation of acetylcholine receptors of muscle and electroplax (10) is well known. Several reports have suggested that the α-Btx binding component of sympathetic ganglia (11, 12) and cultured sympathetic neurons (13–15) may not be an acetylcholine receptor because of the inability of α-Btx to block agonist-induced physiological responses.

Recent studies from our laboratory, however, suggest that the optic tecta of both the marine toad, Bufo marinus (16, 17), and the common goldfish, Carassius auratus (18), contain acetylcholine receptors that are sensitive to α-Btx. In the toad, the intracellularly recorded response of tectal cells to iontophoretically applied acetylcholine and to the native optic nerve transmitter is abolished by α-Btx. In addition, the postsynaptic portion of the visually evoked tectal “off” response, identified by the technique of current source-density analysis (19), is abolished by the topical application of α-Btx. Similarly, in the goldfish, the postsynaptic responses of tectal neurons elicited by all three classes of optic nerve fibers are abolished by the micropipette injection of α-Btx into the tectum (18).

The goal of the present experiments was to characterize the α-Btx binding protein of goldfish brain. Knowledge of the binding parameters and molecular properties of the binding protein are of interest because the molecule is very likely the acetylcholine receptor. Also, Freeman (17) has provided evidence for the role of the acetylcholine receptor in the maintenance of synaptic connections. Thus, the binding protein is of interest not only because of its involvement in ion translocation but also because of its possible role in neuroplasticity.

In this paper, we present evidence that the α-Btx binding protein of goldfish brain has kinetic properties different from those of the α-Btx binding protein of sympathetic ganglia (4), but quite similar to those of the acetylcholine receptors from muscle (1) and electroplax (20). We also present data on the pharmacological specificity, the isoelectric point, and molecular size of the binding protein.

**Experimental Procedures**

**Materials**

α-Btx was purified from lyophilized Bungarus multicinctus venom (Miami Serpentarium) and iodinated with Na^125^I (Amersham/Searle) using lactoperoxidase as described by Papaionannou and Gospodarowicz (21). Iodinated α-Btx was shown by intracellular recording to retain its toxicity. The specific activity ranged from 400 to 700 Ci/mmol. Common goldfish (Carassius auratus) were purchased from Ozark Fisheries and were maintained at room temperature. Sepharose 4B, Sephadex G-100, blue dextran, and ovalbumin were obtained from Sigma. Crystalline bovine serum albumin, ferritin, thyroglobulin, catalase, D_2O (99.8%), nicotine, and Hepes were obtained from Research Products International, and Emulphogene BC-720 [alkoxypoly(ethyleneoxy)ethanol] was obtained from GAF Corp.

**Tissue Fractionation**

Brains were dissected out under ice anesthesia and placed in a beaker of ice cold 0.7 M sucrose. The brains were quickly blotted on Whatman No. 1 filter paper, weighed, and homogenized (12 passes, 800 rpm) in 0.7 M sucrose at a 10% w/v concentration. A nuclear pellet was obtained by centrifugation at 1000 × g for 10 min. The pellet was washed once by resuspension in 0.7 M sucrose (1000 × g for 10 min),

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1 J. A. Freeman, and J. T. Schmidt, in preparation.
2 J. A. Freeman, in preparation.
3 J. A. Freeman, in preparation.
and the supernatants were combined. The supernatants were centrifuged at 30,000 x g for 30 min to obtain a crude synaptosomal pellet. The pellet was resuspended in 0.7 M sucrose containing 0.02% NaN₃ and 10⁻⁴ M PMSF (225 mg wet weight equivalent/ml) and used directly for binding studies. Electron microscopy confirmed that the crude synaptosomal pellet was highly enriched in synaptosomes.

**Particulate Binding Assay**

To 100 μl of resuspended pellet were added 50 μl of buffer or unlabeled α-Btx. After 10 min, 50 μl of [125I]-α-Btx were added in 50 mM sodium phosphate buffer, pH 7.4, containing 0.02% NaN₃, 10⁻⁴ M PMSF, 1 mM EDTA, and 0.25% albumin. All incubations were carried out at room temperature (20–22°C) in polyethylene tubes. Incubations were terminated by the addition of 8 ml of NaCl/P₃ (50 mM sodium phosphate buffer, pH 7.4, with 0.25 M NaCl) and filtration through EGWP Millipore filters (presoaked in 0.25% albumin and 10⁻⁵ M α-Btx). The filters were washed with five 8-ml aliquots of NaCl/P₃. Radioactivity was determined in a Nuclear Chicago γ scintillation counter. Specific binding was considered to be the difference between samples containing no cold α-Btx and those containing 10⁻⁵ M α-Btx. Nonspecific binding constituted 6 to 30% of the total binding depending on the degree of saturation. At 5 nM, approximately 10% of the binding was nonspecific.

For competition binding experiments, the inhibitor was added in a volume of 50 μl to 100 μl of resuspended synaptosomal pellet. Ten minutes later, 50 μl of [125I]-α-Btx were added giving a final concentration of 5 nM. After 60 min, the incubations were terminated and filtered as described above.

**Solubilization of Toxin-Receptor Complex**

The α-Btx binding component was readily solubilized by treatment of the synaptosomal pellet (10% whole brain equivalent weight/volume) with 1% Emulphogene BC-720 or Triton X-100 in 50 mM sodium phosphate buffer, pH 7.4, containing 10⁻⁴ M PMSF and 0.02% NaN₃. Extraction was allowed to continue for 2 h with vigorous shaking, after which the suspension was centrifuged at 38,000 x g for 1 h. The supernatant contained an α-Btx binding component that is excluded from a Sephadex G-100 column (Fig. 1). For isoelectric focusing, gel filtration, and sedimentation analysis, [125I]-α-Btx (5 nM) was allowed to bind to the synaptosomal fraction before extraction. Free [125I]-α-Btx was separated from bound to synaptosomes by centrifugation at 30,000 x g for 12 min. The pellet was washed twice by resuspension in 3 ml of distilled H₂O followed by the addition of NaCl/P₃ and centrifugation. The final pellet was extracted as described above.

**Isoelectric Focusing**

The solubilized [125I]-α-Btx-receptor complex was desalted on a Sephadex G-100 column (15 x 60 cm) equilibrated in 10 mM sodium phosphate buffer containing 1% Emulphogene BC-720, 10⁻⁴ M PMSF and 0.02% NaN₃. Isoelectric focusing was performed in a 110-ml LKB ampholine preparative isoelectric focusing column. The anode solution was 0.16 M H₂PO₄ containing 60% sucrose, and the cathode solution was 0.25 M NaOH. The isoelectric focusing solution was a 5 to 50% sucrose gradient containing 0.25% Emulphogene BC-720 and 1% LKB ampholine (either pH 4 to 6 or pH 3 to 10). The column was prefocused for 12 h at 800 V before the addition of [125I]-α-Btx-receptor complex. The position at which the complex was added was different in each run to avoid artifacts resulting from loading at any particular pH or sucrose density. Focusing continued at constant voltage and temperature (6°C) until an increase in the voltage had negligible effect on the current drawn, indicating that equilibrium had been reached (approximately 12 h).

The column was drained from the bottom, and 40 drop fractions were collected. The radioactivity was monitored and pH determined for each tube.

**Density Gradient Centrifugation**

Six 13-ml linear sucrose-H₂O or sucrose/D₂O gradients, 10 to 34% sucrose containing 10 mM Hapes, 0.15 M NaCl, and 2 mg/ml of Triton X-100 at pH 7.5, were prepared. A 2.0-μl aliquot containing 150 μl of solubilized [125I]-α-Btx-receptor and 50 μl of catalase (50 mg/ml) was carefully layered onto each gradient. The tubes were centrifuged at 33,900 rpm (100,000 x g) for 20 h at approximately 15°C in a Spinco SW41 rotor. The run was terminated by deceleration with the brake off. The tubes were drained using a Buchler Auto-Densi-Flow conductivity drainer. Sucrose concentrations were determined in alternate tubes by measuring the refractive index. The toxin-receptor complex was assayed by precipitation of the complex in 37% saturated ammonium sulfate and collection on Whatmann GF/B glass fiber filters (8). The filters were washed with 40 ml of 30% saturated ammonium sulfate before counting.

**Fig. 1. Sephadex G-100 chromatography of the solubilized [125I]-α-Btx complex.** The column was equilibrated in 10 mM sodium phosphate buffer containing 1% Triton X-100, 10⁻⁴ M PMSF, and 0.02% NaN₃ and was developed with descending chromatography, as described under "Experimental Procedures," at a rate of 0.7 ml/min. Three-milliliter fractions were collected, and the activity in each fraction was counted. The toxin-receptor complex eluted with the void volume (Fractions 7 to 10), and the free toxin eluted in Fractions 30 to 38.
Data Analysis

**Receptor-Toxin Interactions**—Equilibrium binding data were fit directly to the equation: 

\[ \frac{RT_t}{RT_0} = \frac{a_2e^{-k_1t} + a_3e^{-k_3t}}{RT + \frac{RT_0}{RT_t}} \]

where \( k_1 \) and \( k_3 \) are the first order dissociation constants, \( a_2 \) and \( a_3 \) are the relative proportions of molecules having dissociation constants \( k_1 \) and \( k_3 \), respectively, \( RT_i \) is the concentration of the receptor-toxin complex at time, \( t_i \), and \( RT_0 \) is concentration of the receptor-toxin complex at the beginning of the experiment.

The second order association rate constant was determined using the integrated form of the rate equation given by Maelicke et al. (20):

\[ k_i = \frac{RT_0}{t_i RT_0^2 - RT_0 T} \left( \ln \frac{RT_0 - RT_i}{RT_0 - RT} + \ln \frac{RT_0}{RT_i} \right) \]

where \( R_0 \) is the initial receptor concentration and \( T_0 \) is the initial toxin concentration. When \( k_i \) was calculated at nonsaturating levels of toxin, \( R_0 \) was calculated from the following expression:

\[ R_0 = \frac{RT_0 (K_D + T - RT_0)}{T_0 - RT_0} \]

The value of \( k_i \) was determined by an unweighted linear least squares regression through the origin with time as the independent variable and \( \ln \left( \frac{RT_0 - RT_i}{RT_0 - RT} + \ln \frac{RT_0}{RT_i} \right) \) as the dependent variable.

**Competition Binding Experiments**—The binding of small unla- beled cholinergic ligands was assessed by observing the inhibition of \(^{125}\text{I}-\alpha\)-Btx binding due to the presence of the unlaabeled ligand. As described by Maelicke et al. (20), the equation:

\[ \frac{RI}{RT + R} = \frac{1}{1 + T/K_i} \]

where \( R_0 \) is the initial receptor concentration and \( T \) is the initial toxin concentration, when \( k_i \) was calculated at nonsaturating levels of toxin, \( R_0 \) was calculated from the following expression:

\[ R_0 = \frac{RT_0 (K_D + T - RT_0)}{T_0 - RT_0} \]

The value of \( k_i \) was determined by an unweighted linear least squares regression through the origin with time as the independent variable and \( \ln \left( \frac{RT_0 - RT_i}{RT_0 - RT} + \ln \frac{RT_0}{RT_i} \right) \) as the dependent variable.

**Dissociation of \(^{125}\text{I}-\alpha\)-Btx-Receptor Complexes**—The dissociation of labeled cholinergic toxin complexes was measured by observing the inhibition of \(^{125}\text{I}-\alpha\)-Btx binding due to the presence of unlabeled ligand. As described by Maelicke et al. (20), the equation:

\[ \log \left( \frac{RT_i}{RT_0} \right) = n \log I - n \log K_i \]

In the equations above, \( R_i \) refers to the concentration of receptor-inhibitor complex at equilibrium, \( I \) refers to the free inhibitor concentration at equilibrium, \( K_i \) to the inhibition constant, and \( n \) to the ratio of inhibitor to toxin binding sites. All inhibitors were present at concentrations that allowed the approximation \( I = IO \) to be used, where \( IO \) is the total inhibitor concentration. \( K_i \) can be determined from the log \( I \) axis intercept, and \( n \) from the slope of the line. Linear least squares were used to fit the data to Equation 5.

**Sedimentation Velocity Experiments**—The sedimentation con- stant \( (s_{20,w}) \) and effective partial specific volume \( (\rho*) \) of the toxin-receptor-detergent complex were determined using the procedure described by Smigel and Fleischer (23). In this procedure, measurements of refractive index are used to determine the density and viscosity gradients present in the tube. These gradients are used to integrate numerically the sedimentation equation using Simpson's rule. This yields a series of \( \rho* \) and \( s_{20,w} \) values for each detergent concentration. The actual values of \( s_{20,w} \) and \( \rho* \) are those values at the intersection of the \( D_20 \) and \( H_20 \) curves. Using the Stokes radius \( (R_s) \) computed from Sepharose 4B chromatography, the molecular weight of the toxin-receptor-detergent complex is given by the equation:

\[ M^* = \frac{s_{20,w} + 0.6\pi s_{20,w} N R_s}{1 - \phi^* f_{20,w}} \]

Using a partial specific volume for Triton X-100 of 0.908 cm³/g (28) and assuming a partial specific volume \( (\pi) \) for the toxin-receptor complex of 0.735 cm³/g (see “Discussion”), the weight fraction of protein \( (X_p) \) and detergent \( (X_D) \) can be determined using the relationship:

\[ \phi^* = \frac{\pi}{\phi^* f_{20,w}} \]

The molecular weight of the toxin-receptor complex is \( X_p M^* \).

**Free Diffusion Experiments**—The distribution profile was fit directly to the equation:
where \( C(x, t) \) is the concentration \( x \) cm from the initial boundary \( t \) s after the formation of the boundary; \( C_0 \) is the initial concentration, and \( D \) is the diffusion coefficient. Equation 6 is derived by the integration of Fick's second law as described by Crank (29). A nonlinear least squares fit was obtained with the Levenberg-Marquardt (26, 27) algorithm described above using Squire's (30) numerical integration routine, SIMPER, to calculate the value of the integral by Simpson's rule.

RESULTS

Toxin-Receptor Interaction—The amount of \(^{125}\text{I}-\alpha\)-Btx bound to particulate fractions of goldfish brain is a saturable function of the amount of toxin added. Fig. 2 shows the results of a typical experiment. The \( K_D \) calculated from experiments using 5 mg to 25 mg wet weight equivalent per assay tube was 0.917 \( \pm \) 0.020 nM. In contrast to the report of Speth et al. (31), no dependence of \( K_D \) on the amount of tissue present was observed.

The dissociation of toxin-receptor complexes was studied by the addition of a 1000-fold excess of cold \( \alpha \)-Btx after a saturating dose of \(^{125}\text{I}-\alpha\)-Btx had equilibrated with the receptor. Aliquots were removed at various times following the addition of cold \( \alpha \)-Btx and the amount of bound \(^{125}\text{I}-\alpha\)-Btx was measured. To control for the effects of proteolysis and denaturation a parallel set of measurements in which buffer replaced cold \( \alpha \)-Btx was made. The results, shown in Fig. 3, are expressed as normalized binding, i.e. binding observed when excess cold \( \alpha \)-Btx was added following equilibration divided by binding when no cold \( \alpha \)-Btx was added. The semilog plot of Fig. 3 demonstrates clearly that two rates of dissociation are present. The fast dissociation site has a first order dissociation constant of \( 6.57 \times 10^{-4} \) s\(^{-1}\), yielding a half-time of 17.6 min. It comprises approximately 20% of the toxin-receptor complexes. The slow dissociation site has a dissociation constant of \( 4.31 \times 10^{-6} \) s\(^{-1}\) (\( t_{1/2} \) = 44.7 h) and comprises about 80% of the receptor sites.

The association rate was linear for a wide range of toxin concentrations and had a second order association rate constant of \( 3.456 \pm 0.391 \times 10^{-5} \) M\(^{-1}\) s\(^{-1}\). Using this association rate constant with the dissociation rate constants calculated above, two equilibrium dissociation constants can be calculated. The “low affinity site” has a \( K_D \) of 1.90 nM and the “high affinity site” has a \( K_D \) of 12.47 pm. The equilibrium binding is dominated by the “low affinity site” because the assay is not sufficiently sensitive to determine accurately an equilibrium dissociation constant of that low magnitude. The equilibrium dissociation constant in the range of toxin concentrations studied should, however, be a composite of the dissociation constants for both sites. It can be shown that:

\[
K_D = \frac{kK_H + K_L}{k + 1}
\]

which relates the \( K_D \) obtained from Scatchard analysis or direct fit to \( RT = R_0T/(T + K_D) \) to the two known dissociation constants obtained from kinetic analysis. \( K_H \) refers to the dissociation constant for the high affinity site, \( K_L \) to the low affinity constant, and \( k \) to the ratio of the number of high to low affinity sites. Using this expression with the dissociation constants calculated above from the kinetic experiments yields a \( K_D \) of 0.39 nM, in reasonable agreement with the value calculated from equilibrium binding.

Competition Binding Experiments—The pharmacological specificity of the toxin binding sites was investigated using the ligands nicotine, d-tubocurarine, atropine, and carbamylcholine. Table I shows the values of \( n \) and \( K_I \) for each inhibitor. The \( K_I \) for each ligand is the intersection of the log I axis (see “Experimental Procedures”).

**TABLE I**

| Ligand        | \( n \) | \( K_I \)     |
|---------------|-------|-------------|
| d-Tubocurarine| 0.65  | 1.07 \times 10^{-9} |
| Nicotine      | 0.80  | 3.63 \times 10^{-9} |
| Carbamylcholine| 0.67 | 1.26 \times 10^{-9} |
| Hexamethonium | 0.62  | 1.12 \times 10^{-9} |
| Atropine      | 0.82  | 1.04 \times 10^{-9} |

**FIG. 5.** Isoelectric focusing of the toxin-receptor complex. \(^{125}\text{I}-\alpha\)-Btx was bound to particulate fractions, extracted with 1% Emulphogene BC-720, and loaded into a prefocused pH 4 to 6 gradient. The peak eluting with the anode solution is free \(^{125}\text{I}-\alpha\)-Btx. The isoelectric point of the toxin-receptor complex is 5.00 \( \pm \) 0.01.
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small cholinergic ligands: d-tubocurarine, nicotine, carbamylcholine, hexamethonium, decamethonium, and atropine. Fig. 4 presents the data using the log-log plot described under "Experimental Procedures." The data for decamethonium are not shown because they had no effect on the equilibrium binding of 125I-α-Btx at concentrations lower than 10 mM. Table I summarizes the $K_d$ values determined from the log $I$ axis and the number of ligand binding sites per toxin binding site calculated from the slope of the line.

Isoelectric Focusing—The α-Btx binding protein of goldfish, like that of Torpedo (33) and muscle (34), is an acidic molecule. Six preparative isoelectric focusing experiments yielded a value for the isoelectric point of 5.00 ± 0.01. In each of the six experiments, the toxin-receptor complex was added to a different portion of either a pH 4 to 6 or a pH 3 to 10 ampholine gradient. Fig. 5 shows the results of an experiment using a pH 4 to 6 gradient.

Molecular Characterization—Chromatography of the toxin-receptor complex on Sepharose 4B is shown in Fig. 6 in relationship to the elution of standard proteins. The calculated Stokes radius was 85.3 ± 3.8 Å. The diffusion coefficient ($D$) can be calculated (35) from the Stokes radius ($R_s$) using the equation:

$$D = \frac{kT}{6\pi\eta R_s}.$$  

where $k$ is the Boltzmann constant, $\eta$ is the viscosity of the medium, and $T$ is the absolute temperature. The value of $D_{20,\omega}$ for the toxin-receptor complex from gel filtration is 2.51 × 10$^{-7}$ cm$^2$/s.

The diffusion coefficient of the toxin-receptor-detergent complex was also measured using the free diffusion technique (25). A typical experiment is illustrated in Fig. 7. $D_{20,\omega}$ was calculated from the observed diffusion coefficient of the complex relative to a series of standard proteins (Fig. 8). The $D_{20,\omega}$ for the complex was 2.851 ± 0.541 × 10$^{-7}$ cm$^2$/s. When the standards were plotted in terms of Stokes radius, the Stokes radius of the toxin-receptor complex is 74.3 ± 4.3 Å (95% confidence intervals).

The sedimentation profile in sucrose/H$_2$O of the toxin-receptor complex is shown in Fig. 9. As shown, the major component running near catalase on the gradient specifically binds 125I-α-Btx, as demonstrated by the fact that it is not present when the tissue homogenate is preincubated with unlabeled α-Btx. The sedimentation analysis described under "Experimental Procedures" is shown in Fig. 10. The curves for sucrose/H$_2$O and sucrose/D$_2$O gradients intersect at a value of $s_{20,\omega}$ of 11.45 and $\phi*$ of 0.786 cm$^3$/g. The sedimentation data, in conjunction with the Stokes radius calculated from either the Sepharose 4B column or the diffusion analysis, allow the calculation of the molecular weights of both the protein-detergent complex and the protein portion of the complex. The frictional coefficient ($f/f_0$) of the protein-detergent complex can also be determined. These data are summarized in Table II.

**Discussion**

The α-Btx binding protein of goldfish brain exhibits many properties of the acetylcholine receptor from muscle (1) and electroplax (3). The α-Btx binding protein of sympathetic ganglia (4) can be kinetically distinguished from that of electroplax, muscle, and goldfish brain. Equilibrium binding measurements, however, are similar. For goldfish, toxin-receptor interactions yield a $K_d$ of 0.92 × 10$^{-7}$ M, revealing only a single class of toxin binding sites with Scatchard (not shown) and double reciprocal (Fig. 2) analysis. A similar value for the dissociation constant of 1.1 × 10$^{-5}$ M was observed by Greene (4) in sympathetic ganglia. Association rate constants for the binding of α-Btx are quite similar for Torpedo californica electroplax (1.9 × 10$^7$ M$^{-1}$ s$^{-1}$ (40)), rat muscle (1.1 to 1.5 × 10$^7$ M$^{-1}$ s$^{-1}$ (1)), and goldfish (our value of 3.5 × 10$^7$ M$^{-1}$ s$^{-1}$) but are somewhat slower for sympathetic ganglia (4.3 × 10$^7$ M$^{-1}$ s$^{-1}$ (4)). An additional, very rapid association component was observed for both muscle (4 to 6 × 10$^9$ M$^{-1}$ s$^{-1}$, (1)) and electroplax (1.3 × 10$^9$ M$^{-1}$ s$^{-1}$, (40)).

An important distinction between the α-Btx binding component of sympathetic ganglia, which does not seem to be involved in chemical synaptic transmission, and the α-Btx...
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0 3 6 9 12 15
D 20-W

FIG. 8. The relationship between the observed diffusion coefficients and $D_{20,w}$ of standard proteins. The $D_{20,0}$ values of the standards were thyroglobulin = $2.49 \times 10^{-7}$ (36), catalase = $4.10 \times 10^{-7}$, albumin = $5.94 \times 10^{-7}$ (35), horseradish peroxidase = $7.05 \times 10^{-7}$ (37), and cytochrome c = $13.0 \times 10^{-7}$ (38). The arrow refers to the position of the toxin-receptor complex.

0 10 20 30
FRACTION NUMBER

FIG. 9. Sedimentation profile of the toxin-receptor complex in a 10 to 34% sucrose/H$_2$O gradient containing 5 mM Hepes, 0.15 M NaCl, and 2 mg/ml of Triton X-100. Free toxin runs at the top of the gradients. Also shown is the parallel profile of a solubilized fraction which had been pretreated with cold $\alpha$-Btx before the addition of $^{131}$I-$\alpha$-Btx. It is evident that essentially all of the $^{131}$I-$\alpha$-Btx binding of the component centering about Fraction 21 can be displaced with cold $\alpha$-Btx, indicating that the binding to this molecular species is specific.

binding components of electroplax, muscle, and goldfish brain, which are involved in synaptic transmission, is the dissociation kinetics of $\alpha$-Btx. Both Patrick and Stallcup (14), using a rat sympathetic nerve line, and Greene (4), using chick sympathetic ganglia, have observed a single dissociation rate of $4.9 \times 10^{-5}$ s$^{-1}$ and $4.6 \times 10^{-3}$ s$^{-1}$, respectively. In goldfish, muscle, and electroplax, on the other hand, dissociation of the complex is much slower. In both goldfish brain and rat muscle, $\alpha$-Btx exhibits two distinct dissociation rate constants. The fast component has a rate constant of $4.8 \times 10^{-5}$ for rat muscle (1) and $6.6 \times 10^{-4}$ s$^{-1}$ for goldfish brain. The slow component has a dissociation constant of $1.9 \times 10^{-6}$ for rat muscle (1) and $4.3 \times 10^{-4}$ s$^{-1}$ for goldfish brain. The dissociation of $\alpha$-Btx from electroplax is too slow to be measured (3, 40). Maelicke et al. (20) have thoroughly characterized the kinetic and equilibrium interactions between the $\alpha$-neurotoxin of Naja Naja siamensis venom and the acetylcholine receptor from Electrophorus electricus electroplax. The $\alpha$-neurotoxin of Naja Naja siamensis is homologous with $\alpha$-Btx (41) and has similar biological properties. In a number of elegant experiments, Maelicke et al. (20) found that the dissociation rates arise from the existence of paired toxin binding sites which are interconvertible between high and low affinity states. Our data do not distinguish between the presence of paired interconvertible sites and the presence of two distinct $\alpha$-Btx binding molecules with similar association kinetics but different dissociation kinetics; however, studies are presently underway to do so.

In systems such as electroplax, muscle, and toad and goldfish brain, where $\alpha$-Btx inhibits the activation of the AChR by nicotinic agonists, the dissociation kinetic data include a component with a half-time of dissociation of greater than 40 h; whereas, sympathetic ganglia which bind $\alpha$-Btx but whose AChR activation is insensitive to it exhibits simple dissociation kinetics with a half-time on the order of 4 h. This suggests that measurement of the dissociation kinetics of the $\alpha$-Btx-nAChR complex might provide a useful estimate of its physiological effectiveness in blocking ACh-induced ion flux at other synapses.

The pharmacological properties of the goldfish brain $\alpha$-Btx

0 5 10 15

FIG. 10. Determination of $s_{20,w}$ and $\rho^*$ values from the sedimentation of the toxin-receptor complex in sucrose/H$_2$O and sucrose/D$_2$O gradients. Values of $s_{20,w}$ for a series of $\rho^*$ values were computed using density and viscosity values at each point in the tube calculated from the refractive index. A computer program kindly supplied by Dr. M. Smigel utilizing Squire's (30) numerical integration routine, SIMPER, was used to analyze the data. Error bars represent one standard deviation from the mean of six experiments.
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The symbols used are defined as follows: $S_{0,20}$, sedimentation coefficient at 20°C in water; $\phi^*$, effective partial specific volume of the toxin-receptor-detergent complex; $R_s$, Stokes radius of the complex; $M^*$, molecular weight of the complex; $X_p$, weight fraction of protein in the complex; $X_d$, weight fraction of detergent in the complex; $M_p$, molecular weight of the protein portion of the complex; and $f/f_0$, fractional coefficient of the complex. The degree of hydration ($\delta$) is assumed to be 0.2 g of solvent/g of protein as suggested by Tanford (35). The 95% confidence intervals given for $R_s$ estimates include uncertainty due to the uncertainty in the positioning of the calibration curves as well as variance in the measurements of the unknowns (39).

binding protein are distinctly nicotinic cholinergic. Both $\alpha_2$-tubocurarine and nicotine are more potent inhibitors of $\alpha$-neurotoxin binding ($K_i \sim 10^{-8} \text{M}$) in goldfish brain than has been reported in mammalian brain (8, 31), chick brain (42), sympathetic nerve cells (14), and electrophys (20). The inhibition of $\alpha$-neurotoxin binding by hexamethonium in goldfish brain was similar to that in electrophos (20) but was much more potent than in sympathetic ganglia (43) and mammalian brain (31). The failure of decamethonium to inhibit binding, on the other hand, was more similar to rat brain and sympathetic ganglia than to electrophos.

Isoelectric focusing of the toxin-receptor complex revealed that like the electrophos (33) and rat muscle (34) nAChR, the goldfish $\alpha$-Btx binding protein is an acidic molecule with an isoelectric point of approximately 5.0 for the nAChR-$\alpha$-Btx complex. Studies are currently under way to determine whether denervation by severing the optic nerve might produce a de novo synthesis of receptor species in the optic tectum. The validity of this assumption cannot be determined without a purified protein sample. The major practical problem is the measurement of the actual temperature during sedimentation. The rotor temperature is assumed to be that which gives catalase (a protein that runs very near the toxin-receptor complex in sucrose/D$_2$O gradients) its correct $S_{0,20}$ and $f/c$ values (23). Using this method, the value of 11.45 S calculated for the goldfish toxin-receptor-detergent complex is smaller than that reported by Meunier et al. (45) for Electrophorus AChR (12.5 S) and larger than that reported by Devreotes et al. (46) for chick muscle nAChR of 10.5 S.

The Stokes radius was determined using both gel filtration and diffusion analysis because of the discrepancies observed with asymmetric particles (47). The two techniques yielded a value of approximately 80 Å for the toxin-receptor-detergent complex. Assuming a value of 0.735 cm$^3$/g for the viscosity of the protein portion of the molecule, approximately 70% of the complex is found to be protein. This yields an average molecular weight for the toxin-receptor complex of 340,000. The frictional coefficients suggest that the molecule behaves as either a prolate ellipsoid with an axial ratio between 5.5 and 8 or an oblate ellipsoid with an axial ratio between 7.5 and 10.5. The value of the frictional coefficient was obtained using the assumption that the degree of hydration ($\delta$) was 0.2 g of solvent/g of protein as suggested by Tanford (35). The accuracy of the axial ratio estimates is thus dependent on the validity of this assumption.

The biochemical characterization of the goldfish brain $\alpha$-Btx binding protein presented in this paper in conjunction with physiological studies demonstrating the ability of $\alpha$-Btx to block synaptic transmission in the goldfish optic tectum provides compelling evidence that $\alpha$-Btx binds to a nicotinic cholinergic receptor protein. Using the regenerating goldfish retinotectal system, studies are currently under way to determine what role the $\alpha$-Btx protein might play in the formation and maintenance of synaptic connections and to determine any dynamic changes in the protein as a function of denervation and reinnervation.

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| Characterization | a Goldfish Brain Cholinergic Receptor |
|------------------|-------------------------------------|
| $S_{0,20}$       | $11.45 \times 10^{-17} \text{S}$   |
| $\phi^*$         | 0.786 cm$^3$/g                     |
| $R_s$ (diffusion)| $74.3 \pm 4.3 \times 10^{-1} \text{cm}$ |
| $R_s$ (chromatography) | $85.3 \pm 8.8 \times 10^{-1} \text{cm}$ |
| $M^*$ (diffusion)| 448,900                             |
| $M^*$ (chromatography) | 515,400                             |
| $X_p$            | 0.705                              |
| $X_d$            | 0.295                              |
| $M_p$ (diffusion)| 316,500                             |
| $M_p$ (chromatography) | 363,400                             |
| $f/f_0$ (diffusion)| 1.33                                |
| $f/f_0$ (chromatography) | 1.45                                |

The method of Smigel and Fleischer (23) used in the present study to characterize the molecular parameters of the goldfish brain $\alpha$-Btx binding protein involves a number of assumptions. The calculation of the Stokes radii requires that the standards that are used do not bind appreciable detergent, in this case Triton X-100. This assumption appears reasonable from the work of Helenius and Simons (44) showing that hydrophobic proteins bind little or no Triton X-100. In addition, the method assumes that the mole fraction of bound detergent ($X_p$) is independent of sucrose concentration and of the use of H$_2$O versus D$_2$O as the solvent. The value of 0.735 cm$^3$/g for a typical, nonglycosylated protein is assumed. This assumption must be considered tentative because at the present time, we have no information concerning whether or not the goldfish $\alpha$-Btx binding protein is glycosylated. The assumption is made that all of the membrane lipids were replaced by detergent. The validity of this assumption cannot be determined without further experiments.

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