INTERACTION BETWEEN CALCIUM AND LIGAND-BINDING SITES OF THE PURIFIED ACETYLCHOLINE RECEPTOR STUDIED BY USE OF A FLUORESCENT LANTHANIDE

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Summary: The acetylcholine receptor isolated from Torpedo ocellata binds about 10 moles of a fluorescent lanthanide, terbium, per mole α-bungarotoxin-binding site, a process which is accompanied by a fluorescence enhancement (λexcitation 295 nm, λemission 546 nm) which allows detection of receptor-Tb^{3+} complexes at μM concentrations. In presence of calcium two types of terbium-binding site are revealed, both with terbium dissociation constants of 18 ± 0.5 μM. About 60% of the sites bind calcium with an apparent dissociation constant of 1.1 ± 0.1 mM. Sites which interact with calcium also interact with activators of neural transmission, carbamylcholine and decamethonium, but not with the inhibitors, d-tubocurarine and α-bungarotoxin. Whether the displacement of calcium by chemical mediators is directly responsible for activator-induced changes in ion permeability of neural membranes is an important question raised by our experiments. The results show that fluorescent lanthanides can be an important tool in such studies.

Interaction of activators of neural transmission with the membrane-bound acetylcholine receptor initiates changes in permeability to inorganic ions and thus in the electrical potential across neural membranes (1, 2). Calcium ions have been implicated in regulating membrane permeability (3-6). Although the interaction of chemical mediators with the membrane-bound acetylcholine receptor is being investigated intensively (e.g. 7-16), and a mechanism has recently been proposed (16), very few studies deal with the molecular events by which calcium ions affect flux of inorganic ions across neural membranes. Studies of the binding of Ca^{2+} to the acetylcholine receptor indicate that Ca^{2+} competes with acetylcholine and with a fluorescent cholinergic ligand for a binding site on the receptor protein purified from Torpedo californica (17, 18). In studies using receptor-rich membrane fragments from T. marmorata an increased affinity of the receptor for cholinergic

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ligands in the presence of Ca^{2+} was observed (19). Our report deals with the role of Ca^{2+} in interactions of chemical mediators with acetylcholine receptor which has been purified from T. ocellata and extensively characterized (17, 20-24). We use a fluorescent lanthanide, terbium, which has been employed previously to characterize inorganic ion binding sites of transferrin (25), trypsin (26) and thermolysin (27), to study Ca^{2+}-binding sites on the receptor protein.

RESULTS AND DISCUSSION: The fluorescence excitation spectrum for Tb^{3+}, in presence and absence of acetylcholine receptor, is shown in Figure 1. Since the

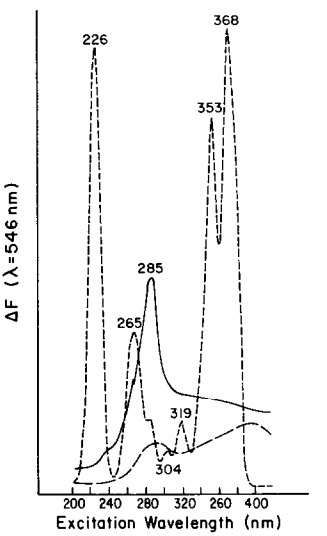

Figure 1: Fluorescence excitation spectra for emission at 546 nm at 20°C, pH 6.5, (- - -), 10^{-2} M $TbCl_3$ in H_2O; (— —), acetylcholine receptor (0.7 μM α-bungarotoxin binding sites); (——), acetylcholine receptor in presence of 1 x 10^{-4} M Tb^{3+}. The acetylcholine receptor was purified as previously described (20) with the addition of 1 mM EDTA to the Tris buffer solutions (17). The electric organs used were from T. ocellata collected in Alexandria, Egypt, and stored at -20°C for less than 6 months. After purification, Triton was added to the receptor to give a concentration of 0.03% and then the receptor was dialyzed extensively at 4°C against 10 mM Pipes buffer (Sigma) pH 6.5 (pH adjusted with Tris), 0.03% Triton X-100 (Sigma), 10 μM $TbCl_3$ (Alfa Ventron), until free of Ca^{2+} as determined by atomic absorption (30). Tb^{3+} was removed by extensive dialysis against 2 mM Pipes/Tris buffer, pH 6.5, 0.03% Triton X-100. Moles α-bungarotoxin binding site/ml solution were determined by reacting the receptor with various concentrations of mono-[^{125}I]-α-bungarotoxin prepared according to a previously published procedure (14). The receptor-toxin complex was then separated from free toxin on a carboxymethylcellulose column (Whatman CM-52). Concentrations of protein were determined by the method of Lowry (31) and of Tb^{3+} in stock solutions by titration with EDTA using arzenazo I (Aldrich) as indicator (32). A Perkin-Elmer MPF-3 spectrofluorimeter and thermostated microcells were used for fluorescence measurements.

emission at 546 nm of free Tb^{3+} is very weak at excitation wavelengths of 285-295 nm, fluorescence in the presence of receptor is due almost entirely to bound Tb^{3+}. The fluorescence excitation maximum is at 285 nm. The experiments were carried out in presence of 0.01%-0.03% Triton X-100 to prevent aggregation of the receptor (21), a concentration which does not interfere with the fluorescence measurements at the excitation wavelength used. (0.03% Triton gives 0.01 OD units at 295 nm.)

Binding isotherms of the acetylcholine receptor and Tb^{3+} in presence and absence of 8 mM Ca^{2+}, at 20°C, are presented in Figure 2 as Scatchard plots (28), y versus y/L, where L represents the concentration of free Tb^{3+}, and y is defined

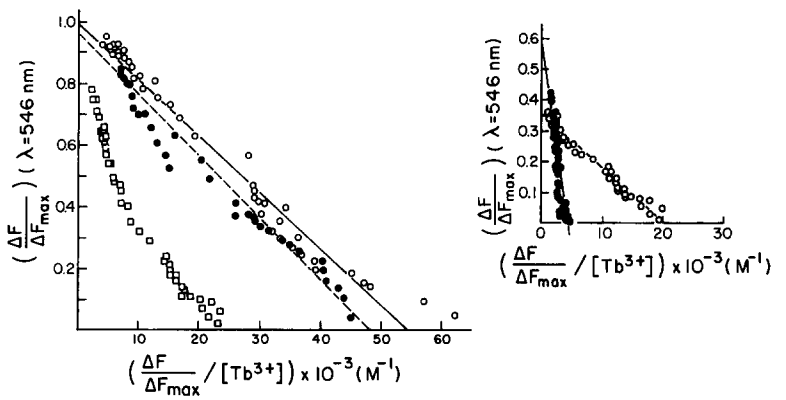

Figure 2: Binding of Tb^{3+} to acetylcholine receptor in presence and absence of 8 mM $CaCl_2$. Acetylcholine receptor (0.54 μM α-bungarotoxin binding sites), 0.01% Triton X-100, 2 mM Pipes buffer, pH 6.5, 20°C. The data are plotted as $\Delta F/\Delta F_{max}$ versus $(\Delta F/\Delta F_{max})$ $([TbCl_3])^{-1}$. ΔF is the observed fluorescence intensity (λEM = 546 nm - 535 nm) in arbitrary units at a given concentration of Tb^{3+} and ΔF_{max} the maximum observed fluorescence intensity when all ion-binding sites are occupied. ○, ●, In absence of $CaCl_2$; ○, μ = 7 mM, ●, μ = 100 mM. □, In presence of 8 mM $CaCl_2$, μ = 55 mM. 0.5 to 2 μl aliquots $TbCl_3$ stock solutions (10^{-2} to 10^{-3} M) were added stepwise to 600 μl acetylcholine receptor. Excitation wavelength was 295 nm. A slit-width of 10 nm for excitation and emission beams and a 430 nm cutoff filter were used. The coordinates of the solid line were computed using a linear least squares computer program. The slope of the line gives a value for K_{Tb} of 18 ± 0.5 μM, μ = 7 mM, and 20 ± 0.7 μM, μ = 100 mM.
Inset: A replot of the Tb^{3+}-binding data obtained in presence of 8 mM $CaCl_2$ (Fig. 2, □) on the basis of two types of binding sites (see text). The coordinates of the solid lines were computed using a linear least squares program. ○, Binding site not affected by 8 mM $CaCl_2$; K_{Tb} = 18 ± 0.05 μM; (1-α) = 0.36 ± 0.01 (see eq (2)). ●, Binding site affected by 8 mM $CaCl_2$; K_{Tb}^{ϕ} = 150 ± 11 μM (see eq (2)); α = 0.57 ± 0.03.

as the fraction of sites occupied at a given Tb^{3+} concentration:

$$y = \Delta F/\Delta F_{max} \quad (1)$$

ΔF is the observed fluorescence intensity in arbitrary units at a given ligand concentration at 546 nm, and ΔF_{max} the maximum observed intensity when all ligand sites are occupied. Experiments in which the occupancy of Tb^{3+}-binding sites was determined directly using neutron activation analysis (29), indicate that the fluorescence intensity is linearly dependent on the amount of Tb^{3+} bound. As can be seen from the upper line (open circles) in Figure 2, the data are consistent with homogeneous Tb^{3+}-binding sites in absence of Ca^{2+}. The apparent dissociation constant of these sites, K_{Tb}, is calculated (from the slope of the line) to be 18 ± 0.5 μM. This value is independent of ionic strength (μ) from 100 mM (solid circles) to 7 mM (open circles). In presence of 8 mM Ca^{2+} (open squares) (μ = 55 mM) two classes of Tb^{3+}-binding sites are revealed, only one of which interacts with Ca^{2+}. y in presence of Ca^{2+} is given by the rational function:

$$y = y_{\alpha} + y_{(1-\alpha)} = \alpha \frac{L}{L + K_{Tb}\Phi} + (1-\alpha) \frac{L}{L + K_{Tb}} \quad (2)$$

$$\Phi = \left(1 + \frac{[Ca^{2+}]}{K_{Ca}}\right).$$

K_{Tb} is the apparent dissociation constant for Tb^{3+}-binding sites in absence of Ca^{2+}, and K_{Ca} the apparent dissociation constant for calcium from receptor sites. L represents the concentration of free Tb^{3+} and α the fraction of Tb^{3+} sites which interact with Ca^{2+}. The parameters α and Φ are evaluated by determining y as a function of L, using equation (2), and inserting the value for K_{Tb} determined in absence of Ca^{2+}. These results were used to calculate y_{α} and $y_{(1-\alpha)}$ at any given concentration of L. The parameters pertaining to each kind of Tb^{3+}-binding site were then evaluated using a linear least squares computer program. The data are shown in the inset to Figure 2. The intercept of the line defined by open

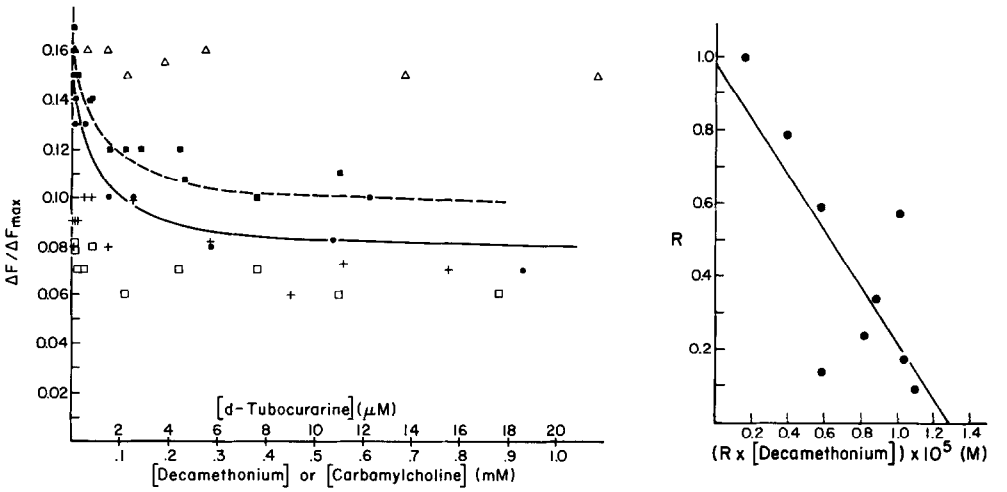

Figure 3: Amount of Tb^{3+} bound to acetylcholine receptor in presence of various concentrations of activators and inhibitors of neural transmission. Acetylcholine receptor (0.48 μM α-bungarotoxin sites), 8.3 μM $TbCl_3$, 0.03% Triton X-100, 2 mM Pipes buffer, pH 6.5, 20°C.
Δ, d-tubocurarine; ●, decamethonium; ■, carbamylcholine; +, decamethonium in presence of 8 mM $CaCl_2$; □, carbamylcholine in presence of 8 mM $CaCl_2$.
Inset: Displacement of terbium from acetylcholine receptor by decamethonium is plotted according to a linear form of the equation given below. Acetylcholine receptor (0.54 μM α-bungarotoxin sites), 3.3 μM $TbCl_3$, 0.01% Triton X-100, 2 mM Pipes buffer, pH 6.5, 20°C. The fraction of the fluorescence signal due to Tb^{3+} binding sites from which decamethonium displaces Tb^{3+} is given by eq (3) and (4). Eq (4) pertains to experiments in which the displacing ligand is present.

$$\frac{\Delta F_\alpha}{\Delta F_{max}} = \frac{L}{L + K_{Tb}} \tag{3}$$

$$\frac{\Delta F'_\alpha}{\Delta F_{max}} = \frac{L'}{L' + K_{Tb}(1 + \frac{[D]}{K_D})} \tag{4}$$

$$R^{-1} = \frac{\Delta F_\alpha}{\Delta F'_\alpha} = \frac{L}{L'} \frac{L' + K_{Tb}(1 + \frac{[D]}{K_D})}{L + K_{Tb}} \tag{5}$$

$\Delta F'_\alpha$ is the fluorescence signal obtained at a given concentration of displacing ligand. L and L' represent the concentration of free Tb^{3+} in absence and presence of displacing ligand and D the concentration of decamethonium. K_D is the dissociation constant of the receptor-decamethonium complex. Subtracting the value for $\Delta F/\Delta F_{max}$ obtained at saturating concentrations of decamethonium (Fig. 3) from the observed values obtained in absence and presence of decamethonium gives the values of $\Delta F_\alpha/\Delta F_{max}$ and $\Delta F'_\alpha/\Delta F_{max}$ respectively. Since experimental conditions were chosen such that $K_{Tb} >> L$, L' and only some of the ligand is displaced by decamethonium ($L \approx L'$), the data can be plotted according to a simplified form of eq (5):

$$R = 1 - R[D]/K_D \tag{6}$$

circles gives the fraction of Tb^{3+}-binding sites which are not affected by Ca^{2+}. The intercept of the line defined by solid circles indicates that 60% of the Tb^{3+}-binding sites are affected by Ca^{2+}, and the slope of the line gives a value for $K_{Tb}\Phi$ of 1.5×10^{-4}M. Assuming that Ca^{2+} interacts competitively with the Tb^{3+}-binding sites one can evaluate Φ and therefore obtain an apparent dissociation constant for Ca^{2+} from these sites of 1.1 ± 0.1 mM (eq 2).

The data in Figure 3 indicate that the activators of neural transmission, decamethonium (solid circles) and carbamylcholine (solid squares), displace bound Tb^{3+} from its receptor sites. In these experiments the concentration of receptor and of Tb^{3+} was kept constant and fluorescence of the solution was recorded as a function of ligand concentration. The reason $\Delta F/\Delta F_{max}$ has a value of only 0.16 in absence of decamethonium or carbamylcholine is that an initial Tb^{3+} concentration less than the value of K_{Tb} was chosen in order to maximize the signal change associated with displacement of Tb^{3+} by ligands. When the Tb^{3+} which interacts with Ca^{2+}-binding sites is first displaced from the receptor by 8 mM Ca^{2+} (Fig. 3) addition of decamethonium (crosses) or carbamylcholine (open squares) does not cause displacement of any more Tb^{3+}. This indicates that the Tb^{3+} which is displaced by activators of neural transmission is displaced from the sites which interact with Ca^{2+}. In contrast to activators, two inhibitors of neural transmission, d-tubocurarine and α-bungarotoxin, do not compete with the Tb^{3+}-binding sites. Displacement of Tb^{3+} from the receptor is not observed, even when the concentration of d-tubocurarine (Fig. 3, open triangles) exceeds 100 times the value of its apparent dissociation constant of 0.24 μM (36). In another series of experiments 3.3 μM α-bungarotoxin was added to the receptor (0.3 μM α-bungarotoxin-binding sites) at pH 6.5 and 20°C in presence of 5.8 μM Tb^{3+}. The fluorescence intensity was observed for 1.5 hours and did not decrease. Control experiments indicate that under these conditions all the toxin sites have reacted. These results are in agreement with previous studies which indicated that activators and inhibitors of neural transmission occupy separate binding sites on the membrane bound receptor (14-16, 33).

For a simple mechanism involving competitive inhibition between Tb^{3+} and decamethonium the dissociation constant of the receptor-decamethonium complex can be obtained from the data in Figure 3 as shown in the inset. The slope of the line (inset), computed by a least squares method, reflects the dissociation constant of the receptor-decamethonium complex. The value obtained, 13 ± 4 μM, is in good agreement with that determined in equilibrium dialysis experiments with decamethonium and the same receptor preparation.

In order to correlate the fluorescence changes which accompany binding of Tb^{3+} to the receptor with the moles of Tb^{3+} bound, we determined the amount of Tb^{3+} bound to the receptor, using neutron activation analysis (29). The value estimated is about 10 moles of Tb^{3+} per α-bungarotoxin-binding site. The data in Figures 2 and 3 indicate that about 6 moles of Tb^{3+} per α-bungarotoxin-binding site are displaced from the receptor by either Ca^{2+} or activators of neural transmission. Whether the receptor forms channels through neural membranes which are closed by Ca^{2+}, and whether changes in the permeability of the membranes to sodium and potassium ions are initiated by activator-induced displacement of Ca^{2+} from the channels, as has been suggested by Nachmansohn and Neumann (34 and 35), are interesting problems for future work. Our experiments show that fluorescent lanthanides can be an important tool in such studies.

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