BINDING STUDIES OF Mn^{+2} TO ISOLATED ACETYLCHOLINE RECEPTOR AS A PROBE FOR CATION-RECEPTOR INTERACTION[1]

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SUMMARY: The paramagnetic cation Mn^{+2} binds to Torpedo californica acetylcholine receptor (AcChR) at sites with at least two different affinity constants. For each α-Bungarotoxin (α-Bgt) binding site AcChR has between 3 to 4 Mn^{+2} sites with Kd values of 1.74 ± 1.0 x 10^{-4} M. An additional 10-12 sites/α-Bgt site have a weaker affinity for Mn^{+2} (Kd ≅ 1 mM). The α-Bgt does not displace bound Mn^{+2}, however Ca^{+2} displaces all bound Mn^{+2} in a competitive fashion with Kd of 0.90 x 10^{-3} M and Mg^{+2} is as effective as Ca^{+2} in the displacement. Decamethonium, carbamylcholine and NaCl at high concentrations are also effective in displacing Mn^{+2}. A constant enhancement value (ε_b) for the binary metal·AcChR complexes was obtained when simultaneous EPR measurements and the water proton relaxation rates were made. Similarity of the AcChR environment and/or coordination number for the Mn^{+2} sites in AcChR is inferred. It appears that Mn^{+2} binds to many AcChR sites, different from those responsible for binding cholinergic ligands. The Mn^{+2} site seem to be the same as those responsible for binding the electrophysiologically significant Ca^{+2}.

Postsynaptic depolarization at the neuromuscular junction is mediated by acetylcholine through its interaction with the AcChR[4] and electrophysiological evidence suggests a role for Ca^{+2} in the conductivity changes observed in these membranes (1,2). The purification and characterization of an AcChR from Torpedo californica

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[4]Abbreviations: AcChR, Acetylcholine receptor; α-Bgt, α-Bungarotoxin; EPR, electron paramagnetic resonance; PRR, proton relaxation rate; DAP, bis-(3-amino pyridinium) 1, 10-decane.

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(3) makes possible the study, at a molecular level, of the interaction of the receptor with divalent cations. Our previous findings measuring the binding of DAP to AcChR (4) revealed competition between this ligand and Ca^{+2} with Kd value for Ca^{+2} of 0.2 ± 0.05 mM.

Equilibrium dialysis studies with ^{45}Ca failed to detect significant Ca^{+2} binding to the purified AcChR. Using murexide as an indicator (5) or lanthanide Tb^{+3} (6) as a substitute for Ca^{+2} have encountered mixed success. The murexide·Ca complex is weak (Kd = 1.07 ± 0.05 mM) and the titrations are limited to the alkaline pH range where the indicator is photosensitive (5). On the other hand, the detection of fluorescence changes of Tb^{+3} free or bound to AcChR presents excitation spectra overlapping with the spectra of the detergents needed to maintain the AcChR in solution. The latter studies have also indicated that most of the bound Tb^{+3} cannot be displaced by Ca^{+2} (6). In this communication we report the use of the paramagnetic probe Mn^{+2} as a tool for the study of the interaction between isolated AcChR and divalent cations.

METHODS

Purification and assay of AcChR. AcChR was purified as previously described (3). Before each experiment the AcChR preparation was subjected to filtration on G-25 Sephadex followed by extensive dialysis against 10 mM Tris·Cl, pH 7.4, 0.03% Triton X-100 buffer.

The concentration of α-Bgt binding sites was determined by the DEAE cellulose filter disc assay procedure (7) using I^{125} α-Bgt prepared from Bungarus multicinctus venom (Sigma Chemical Co.) by the procedure of Clark et al. (8).

Binding studies. All binding studies were carried out in Tris 9 mM, pH 7.4, containing 0.027% Triton (Tris/Triton buffer). Under these experimental conditions binding of Mn^{+2} to Triton X-100 and Tris was found to be negligible as judged by the amplitude and the line width of the Mn^{+2} EPR spectrum. The free concentration of Mn^{+2}, in the presence of AcChR was determined by the intensity of the EPR spectrum (9) using a Varian X-band EPR spectrometer (V4500-10A) equipped with 100 KHz modulation. Measurements were

made in quartz capillaries (1 mm internal diameter) with 0.055 ml samples containing varying amounts of metal ion and AcChR.

The longitudinal nuclear magnetic relaxation rate of water protons (PRR) was measured by the Carr-Purcell sequence using a Seimco pulsed NMR spectrometer operating at 24.3 MHz at 24°. Samples were typically of 0.55 ml. The observed enhancement (ε^*) of the relaxation rate was calculated as follows:

$$\varepsilon^* = \frac{\frac{1}{T_1^*} - \frac{1}{T_{1(0)}}}{\frac{1}{T_1} - \frac{1}{T_{1(0)}}} \qquad (1)$$

Where $1/T_1$ is the observed relaxation rate in the presence, and $1/T_{1(0)}$ is the observed relaxation rate in the absence of Mn^{+2}. The terms with (*) represent the same parameters in the presence of protein. The observed enhancement (ε^*) of PRR is a weighted average of the free and the bound Mn^{+2} and is defined by the following equation:

$$\varepsilon^* = \frac{[Mn_f]}{[Mn_t]}\,\varepsilon_f + \frac{[Mn_b]}{[Mn_t]}\,\varepsilon_b \qquad (2)$$

where Mn_f, and Mn_b and Mn_t refer to the free, bound and total Mn^{+2} concentration; ε_f, the enhancement of free Mn^{+2} is by definition 1, and ε_b is the enhancement of the bound Mn^{+2}, then:

$$\varepsilon_b = \frac{\varepsilon^*\,[Mn_t] - [Mn_f]}{[Mn_b]} \qquad (3)$$

Utilizing equation 3, values of ε_b were obtained by determining ε^* at different ratios of Mn^{+2} to AcChR, and independent measurement of free Mn^{+2} by EPR.

RESULTS

In the insert of Fig. 1 the effect of increasing concentration of AcChR on the observed enhancement shows a linear relationship and an extrapolation to infinite protein concentration gives $\varepsilon_b = 8.7$. Data from the 7 preparations tested showed ε_b values ranging from 8-10.

The stoichiometry and the dissociation constant for the Mn^{+2} binding sites, of AcChR were determined from Scatchard plots of such titrations. Figure 1 demonstrates at least 2 sets of Mn sites in each mole of AcChR with 3.8 ± 0.3 Mn^{+2} sites (Kd = 1.1 x

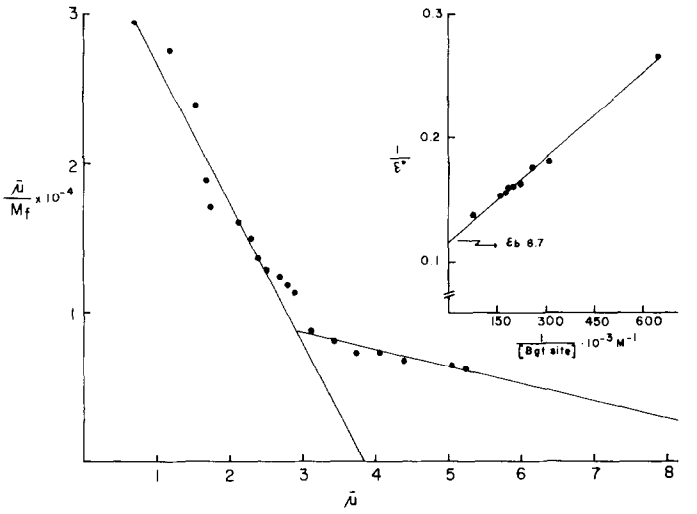

Fig. 1: Scatchard plot of the binding of Mn^{+2} to AcChR obtained by PRR. Moles of Mn^{+2} bound/mol of α-Bgt binding site ($\bar{\mu}$) versus $\bar{\mu}/Mn_f$ are plotted. A solution of AcChR 21 nm in α-Bgt binding sites was titrated with Mn^{+2} (64-800 μM) at 24°. Insert: PRR titration of Mn^{+2} (85 μM) binding to AcChR.

10^{-4} M) and 10.6 ± 1 (Kd = 1 • 10^{-3} M) Mn^{+2} per α-Bgt site. These results are confirmed by EPR titration studies (Fig. 2) in which only the amount of free manganese is detected. The results obtained by both methods with different preparations of AcChR produced Kd values of 0.8 x 10^{-4} to 3.7 x 10^{-4} for the 3 to 4 high affinity sites.

From the simultaneous water relaxation rate and EPR measurements of each samples it is apparent (Fig. 2) that the ε_b values remain constant with increasing Mn^{+2} occupancy.

In order to investigate possible changes in environments for the cationic binding sites, the effect of α-Bgt and other cholinergic ligands upon the binary enhancement value (ε_b) was measured. The presence of α-Bgt at concentrations 10 times higher than those required to saturate the binding sites gives an identical Scatchard plot as in Fig. 1 and does not significantly affect the value for

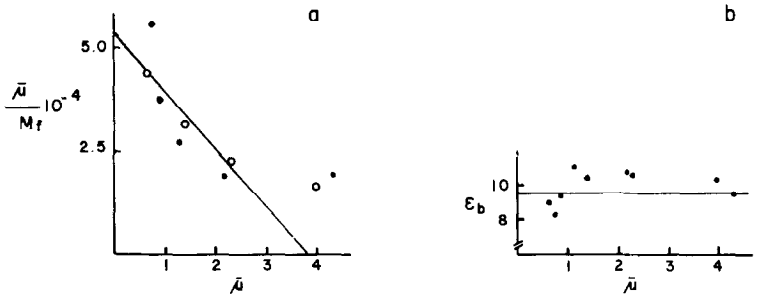

Fig. 2: a) Scatchard plots of the binding of Mn^{+2} to AcChR obtained by measurements of Mn^{+2} in absence ●–● and presence O–O of 70 μM α-Bgt. A solution of AcChR 30 μM in Bgt binding sites was titrated with Mn^{+2} (37-350 μM) at 24°. b) Dependence of the binary enhancement value (ε_b) on the moles of Mn^{+2} bound/mole of α-Bgt binding sites. ε_b was measured using the values of free Mn^{+2} measured by EPR and the observed enhancement (ε^*) of each solution of Fig. 2a, in presence O–O and absence ●–● of α-Bgt.

ε_b. The displacement of Mn^{+2} by cations such as Ca^{+2}, Mg^{+2}, Na^+, decamethonium and carbamylcholine is shown by PRR studies (Fig. 3). The data indicate that the divalent cations are effective in displacing Mn^{+2}. On the other hand, for decamethonium (Kd = 2 x 10^{-6} M) and carbamylcholine (Kd = 5 x 10^{-5} M) concentrations of at least 2 orders of magnitude greater than their dissociation constants are required for a significant displacement to occur. Similar results are obtained using EPR measurements to detect the amount of Mn^{+2} displaced by the cholinergic ligands. Ca^{+2} appears to act as a competitive inhibitor of Mn^{+2} binding. Scatchard plots of Mn^{+2} binding at varying Ca^{+2} concentrations render a set of apparent Mn^{+2} dissociation constants. The simplest fit consistent with this behavior is $Kd^{Mn}_{app} = Kd^{Mn} + \frac{Ca^{+2}}{Kd^{Ca}}$. When the apparent dissociation constant for Mn^{+2} (Kd^{Mn}_{app}) is plotted against the Ca^{+2} concentration (Fig. 4) the extrapolation at the negative abcissa intercept detects the dissociation constant of Ca^{+2}, K_d^{Ca} = 0.9 mM. An excess of Ca^{+2} ions displaces all Mn^{+2} bound to AcChR which can be detected by both the ESR ligand and $\varepsilon^*_{obs} \cong 1$.

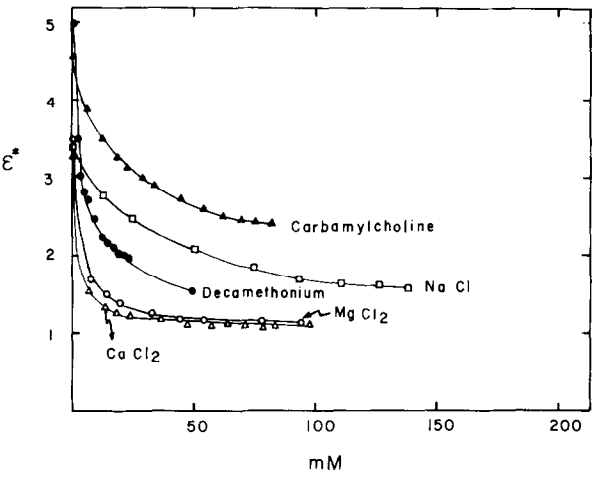

Fig. 3: Displacement of Mn^{+2} from AcChR. ε^* is plotted as function of the total ion or ligand concentration present. Solutions of AcChR 21 μM (O–O) or 24 μM (●–●) in α-Bgt binding sites and 170 μM Mn^{+2} were titrated with solutions containing the same concentrations of α-Bgt binding sites and Mn^{+2} plus the ion or ligand to be tested. Under these conditions between 54-80% of the higher affinity binding sites are saturated.

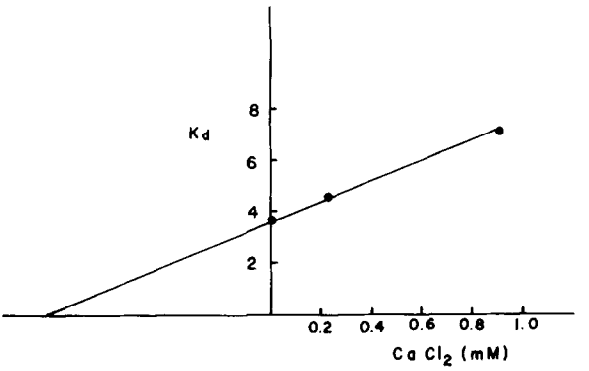

Fig. 4: Secondary plot of the apparent dissociation constants of Mn^{+2} obtained from Scatchard plots of the binding of Mn^{+2} to AcChR by PRR titrations. A solution of AcChR 21 μM in α-Bgt binding sites was titrated with Mn^{+2} (80-800 μM) in absence and in presence of 0.23-0.91 mM Ca^{+2}.

DISCUSSION

The direct titration of isolated AcChR with Mn^{+2} demonstrates the presence of 3-4 high affinity binding sites for each α-Bgt binding site. Extrapolation of the lower affinity binding sites

data indicates that about 11 additional sites may be occupied by Mn^{+2} at high cation concentrations. These results compare with those reported for Tb^{+3} binding (6) and with those obtained (60 Ca^{+2} ions bound/260,000 dalton) by the murexide method (5), if we consider that four α-Bgt molecules may be bound/mole AcChR (10). The dissociation constants obtained are in agreement with those previously reported for the competition of cations with DAP binding (4) and more recently to the competition experiments with Tb^{+3} or the murexide method (5,6).

The relationship of the cation sites to the cholinergic sites is still unresolved. Our experiments indicate that; a) all the Mn^{+2} which binds is displaced by Ca^{+2}; b) α-Bgt does not affect either the stoichiometry or environment (constant ε_b) of Mn^{+2} bound to AcChR; and c) high concentrations of cholinergic ligands, carbamylcholine and decamethonium, are required to displace bound Mn^{+2}. These results contrast with the behavior of Tb^{+3} binding where only half of the bound lanthanide can be displaced by excess Ca^{+2} (6). On the other hand, these results agree with the Tb^{+3} studies in that the cations are not displaced by α-Bgt and that millimolar concentrations of decamethonium or carbamylcholine are required to displace a fraction of AcChR-bound cations. The murexide studies produced some data in disagreement with both the Mn^{+2} and Tb^{+3} data. Contrary to the claim of additional Ca^{+2} binding induced by α-Bgt (5), we do not observe any additional binding of Mn^{+2} in the presence of α-Bgt either with AcChR alone or in the presence of excess carbamylcholine.

Mn^{+2} binding studies are inherently less subject to turbidity problems associated with either fluorescence or spectrophotometric methods since turbidity of detergent solutions of AcChR in the presence of high concentrations of Ca^{+2} and α-Bgt is difficult to

correct. Some of the discrepancies between the interpretations of the experimental observations may be associated with the fact that optical spectroscopic probes were used in the other approaches (5,6). Experiments using ESR and PRR data, which are direct measuments, minimize these ambiguities. It is less likely that these results, dependent on the magnetic properties of bound cation (ε) or free cation concentration (EPR) may be subject to the same source of error.

Both classes of Mn^{+2} sites appear to have equivalent environments based on identical enhancement values. Our results also indicate that cholinergic agents exert little or no specific effect on the amount and mode of binding of Mn^{+} to isolated AcChR. High concentrations of the cholinergic ligands which act as cations displace Mn^{+2} in a non-specific manner. This is indicated by the displacement of Mn^{+2} by Na^{+} or Mg^{+2} which are considered non-specific ligands of AcChR.

In conclusion, it appears that AcChR contains many sites capable of binding cations. The cationic sites can be occupied by Mn^{+2}, Tb^{+3}, or Ca^{+2}. In addition, AcChR may contain sites which bind other cations non-specifically as judged by the presence of additional lanthanide binding sites (6). Most of the cationic sites seem to be unaffected by the binding of α-Bgt or even cholinergic ligands. Since the isolated AcChR appears to exist in different states with dissimilar affinities for ligands from that of the desensitized Torpedo membranes (11,12), it has yet to be demonstrated that relationships similar to those reported in this communication for the isolated AcChR also occur for in situ, desensitized, AcChR.

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