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Dynamic properties of isolated acetylcholine receptor proteins: Release of calcium ions caused by acetylcholine binding

(calcium binding/murexide/multiple binding analysis/conformational changes)

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ABSTRACT **Interaction of Ca and acetylcholine (AcCh) ions with purified acetylcholine receptor (AcChR) from *Torpedo californica* and *Electrophorus electricus* has been investigated in view of these ions' role proposed in bioelectricity. Spectrophotometric Ca titration using murexide as an indicator and an ultrafiltration method with ^{45}Ca show that AcChR proteins have a high binding capacity for Ca ions. Per macromolecule of 260,000 daltons, up to 60 Ca ions can be bound with at least three Ca dissociation constants. A linear inhibition of AcCh binding to AcChR by Ca was observed in the 0.1–1 mM Ca range, indicating competition of AcCh and Ca for AcChR. The addition of AcCh to a Ca–AcChR solution at 1.2 mM Ca causes release of four to six bound Ca ions from AcChR when a maximum of two AcCh ions are bound per 260,000 dalton macromolecule. The subsequent addition of α-bungarotoxin causes reuptake of up to six Ca ions by AcChR. These results suggest that the neural activator AcCh and the inhibitor α-bungarotoxin induce opposing shifts between different conformational states of isolated AcChR.**

Evidence is accumulating that the acetylcholine receptor (AcChR) protein is involved in controlling rapid changes in ionic conductivity of excitable membranes (see refs. 1 and 2). However, the mechanism by which the AcChR protein translates the binding of acetylcholine (AcCh) into a change in membrane permeability is not known. As early as 1953, Nachmansohn suggested that AcCh induces a conformational change of the AcChR protein and thereby leads to increased membrane permeability to ions (3). This chemically specific proposal is the basis of an integral model of nerve excitability which has recently been proposed (2, 4). The model suggests that the AcCh cycle controls the ion permeability and that Ca ions are involved at the AcChR level. An essential role of Ca in nerve and muscle excitability has been demonstrated in pharmacological and electrophysiological studies (see references in refs. 2 and 5).

Recent progress in the isolation and characterization of the AcChr protein (see references in refs. 1 and 6) made possible a study of the molecular mechanism by which the AcChR is involved in the permeability control system. The present report involves the interaction of Ca and AcCh with the purified AcChR from electric organs of electric fish, *Electrophorus electricus* and *Torpedo californica*. We have developed a spectrophotometric method which allows detection of extremely small changes in free Ca concentration, using murexide (Mu) as a Ca indicator (7). This method is applied to measure the effects of AcCh and α-bungarotoxin (α-Bgt) on Ca ions bound to the AcChR macromolecule. Preliminary results of these studies have been briefly referred to in previous publications (2, 4).

Abbreviations: AcCh, acetylcholine; AcChR, acetylcholine receptor; α-Bgt, α-bungarotoxin; Mu, murexide.

MATERIALS AND METHODS

Purification of AcChR. AcChR proteins were purified from electric organs of either fresh *Electrophorus electricus* (electric eel) or liquid-nitrogen-frozen *Torpedo californica* as described previously (6, 8) and used within a few days. In order to remove excess endogenous Ca ions, buffer solutions containing 1 mM EDTA were used during the salt extraction of soluble proteins (6) from electric tissue while the AcChR was still membrane bound. Purified AcChR was stored as a 1 mg/ml of solution in 0.1% Brij, 15 mM sodium phosphate buffer, 0.1 M NaCl, pH 7.0. Assays of ^{125}I-labeled α-Bgt binding on DEAE-81 filter discs were performed as previously described (6). Protein concentration was estimated by the method of Lowry *et al.* (9). The purified AcChR samples used had a binding capacity of 7.6–10 nM ^{125}I-labeled α-Bgt per mg of protein.

Binding of AcCh by Equilibrium Dialysis. Two samples of [^3H]AcCh, 49.5 mCi/mmol (New England Nuclear) and 250 mCi/mmol (Amersham/Searle) were used to prepare stock solutions between 1 mM and 0.5 mM. The exact concentration of AcCh in these stock solutions was determined by the Hestrin method (10), using standard solutions of AcCh between 0.1 mM and 1.5 mM. An AcChR solution was incubated for 30 min at 4° with 10 μM Tetram (*O,O*-diethyl *S*-2-diethylaminoethyl phosphorothiolate) to inhibit any trace of acetylcholinesterase. Tetram was synthesized according to Ghosh and Newman (11). Aliquots of 0.13 ml of the resulting solutions (25–40 μg of AcChR) were placed in a 1-cm diameter dialysis bag and dialyzed at 4° against 50 ml of the specified buffer solutions (see Table 3) containing 1 μM Tetram and various concentrations of [^3H]AcCh and Ca. After 8–12 hr of gentle shaking, the dialysis was terminated and 0.1-ml aliquots were transferred from both the inside and the outside of the dialysis bags to 10 ml of Scintisol (Isolab) for scintillation counting.

Calcium Binding by Ultrafiltration. ^{45}Ca (New England Nuclear) binding measurements were performed at 4° and 25° in an ultrafiltration cell (MRA Co., Boston) by the method of Paulus (12). The 0.1-ml samples were placed along with parallel blanks in alternating cells. The sample compartments contained solutions of about 30 μg of AcChR which were incubated in 0.1 M NaCl, 25 mM Tris·HCl, at pH 7.2 with various concentrations of ^{45}Ca. The matching blanks, used to determine nonspecific binding, were identical to the samples except for the absence of AcChR. The solutions were forced through 7 mm diameter PM-10 Diaflo membranes with nitrogen gas at a pressure of 2.7 bar (0.27 MPa). After filtration the undersides of the membranes were washed with ethylene glycol and each membrane was suspended in 10 ml of Scintisol for scintillation counting.

Ca Analysis by Atomic Absorption Spectrophotometry.

The total Ca ion concentration, [Ca°], in AcChR solution either before or after the Ca titration was determined by atomic absorption spectrophotometry on a Pye Unicam SP 90 instrument after hydrolysis of the protein sample in a sealed tube with 5 M HCl at 100° for 12 hr. All solutions in these studies were prepared from Ca-free, double quartz-distilled water.

Spectrophotometric Ca Titrations. Murexide was used as an optical indicator to determine changes in Ca concentrations (7). Since the Ca–Mu reaction has a simple (1:1) stoichiometry and sufficient sensitivity only at alkaline pH, a pH 8.5 (20°) buffer solution of 50 mM Tris·HCl, 0.1 M NaCl (buffer I) was used in all experiments where Mu is involved. Immediately before the titration, the purified AcChR solution was reacted with 10 μM Tetram for 30 min and concentrated by vacuum dialysis in a collodion bag (Schleicher & Schuell) against three changes of buffer I (300 ml each) and centrifuged to remove suspended matter. The titration was carried out in a Cary 118 C spectrophotometer in a semi-micro cell (1 cm) containing 0.8 ml of AcChR solution (2.5–3 mg of protein) and 0.2 ml of Millipore (0.45 μm)-filtered Mu solution (0.4 mM) in buffer I. The thermostated cell holder was equipped with a specially designed magnetic stirring device. A titrant, $CaCl_2$ (50 mM) in buffer I, was supplied through a thin Teflon tubing from an Aglar microsyringe to the cell with stirring. The optical density change at 480 nm was recorded on the strip chart; absorbance recording accuracy was ±0.0003. At the final concentration of 1.2 mM Ca the solution was titrated with freshly prepared AcCh solution (5 mM in buffer I) delivered from a second Aglar microsyringe. Subsequently, portions of α-Bgt (0.1 mM) in buffer I were added by micropipette.

Titration Data Analysis. In the spectrophotometric Ca titration, the concentration of Ca ions bound to AcChR, $[Ca_b]$, is given by

$$[Ca_b] = [Ca^\circ] - [Ca] - [CaMu] \qquad [1]$$

where [Ca°] is the total Ca concentration; [Ca], the free Ca concentration; [CaMu], the Mu-bound Ca concentration. In the visible range, murexide in Ca-free solution has an absorbance maximum A_λ at 520 nm [$\epsilon_{520} = 1.25\ (\pm 0.05) \times 10^4\ M^{-1}\ cm^{-1}$ at pH 8.5, 20°]. Due to the Tris buffer system, the absorbance of murexide is extremely sensitive to temperature changes. Furthermore, alkaline Mu solutions are photosensitive; the rate of Mu decomposition is practically constant over about 2 hr, and the decomposition rate coefficient was found to be $k = 2.8\ (\pm 0.2) \times 10^{-4}\ min^{-1}$. Thus, at time t, $A_\lambda(t) = A_\lambda(t_0) \times (1 - kt)$. The difference absorbance spectrum of Ca plus Mu versus Mu shows a maximum at 480 nm. The measured absorbance per cm is then $A_{480} = \epsilon_{Mu} \cdot [Mu] + \epsilon_{CaMu} \cdot [CaMu]$. With [Mu] = [Mu°] − [CaMu], where [Mu°] is the total concentration of Mu, it is found that

$$\Delta A_{480} = A_{480} - A'_{480} = [CaMu] \cdot \Delta\epsilon_{480} \qquad [2]$$

where A'_{480} is the absorbance measured in the absence of Ca (corrected for dilution and murexide decomposition) and $\Delta\epsilon_{480}$ is the difference in the molar absorption coefficients. The introduction of [Ca°] and [Mu°] into the equilibrium expression, $K_{Mu} = [Ca] \cdot [Mu]/[CaMu]$ yields:

$$(\Delta A_{480})^{-1} = (\Delta\epsilon_{480} \cdot [Mu^\circ])^{-1}\{1 + K_{Mu}[Ca^\circ]^{-1}\} \qquad [3]$$

valid for $[Ca^\circ] \gg \Delta A_{480}/\Delta\epsilon_{480}$. Both $K_{Mu} = 1.07\ (\pm 0.05)$ mM and $\Delta\epsilon_{480} = 9.0\ (\pm 0.05) \times 10^3\ M^{-1}\ cm^{-1}$ were determined experimentally in buffer I (pH 8.5) at 20° with the use of Eq. 3.

Table 1. Total residual Ca, [Ca°], in solutions of AcChR from *Torpedo californica* (3 mg/ml, ≃10 μM), determined by atomic absorption spectrophotometry after the parallel treatments specified

Treatment of purified AcChR	[Ca°], μM
Vacuum dialysis against Ca-free buffer	180
Incubation (12 hr) in 20 mM EDTA, vacuum dialysis	120
Incubation (12 hr) in 72 mM La^{3+}, vacuum dialysis	60

Defining $\beta = [CaMu]/[Mu^\circ] = \Delta A_{480}/(\Delta\epsilon_{480} \cdot [Mu^\circ])$, Eq. 1 can now be rewritten in terms of β:

$$[Ca_b] = [Ca^\circ] - K_{Mu} \cdot \beta/(1 - \beta) - \beta \cdot [Mu^\circ] \qquad [4]$$

Thus, with [Ca°] obtained by atomic absorption analysis, $[Ca_b]$ can be calculated from spectrophotometric data. The actual calculation, however, has to take into account the fact that AcChR solutions contain Ca ions before the Ca titration. A Ca-free Mu solution has an absorption ratio, $r = A_{520}/A_{480} = 1.63$, whereas an AcChR solution to which Mu was added shows $r < 1.63$. Now, the addition of Ca to a Mu solution leads to a decrease of A_{520} by δA_{520} concomitant with an increase of A_{480} by δA_{480}. It is found that the ratio $r' = \delta A_{480}/\delta A_{520} = -3.4$ is constant, independent of Ca concentration. Using r', A_{520} and A_{480} of an AcChR–Mu solution for the (hypothetical) case of no free Ca ions can be calculated. If A_{520}^m and A_{480}^m are the actually measured absorbances of the AcChR solution containing Ca and murexide, it is found that $\delta A_{480} = -0.52 \cdot (1.63 \cdot A_{480}^m - A_{520}^m)$ and $A'_{480} = A_{480}^m + \delta A_{480}$.

Analysis of Multiple Ligand Binding to AcChR. If the equilibrium constants, K, of a system exhibiting multiple binding of a ligand, L, are only different by a factor of about 10, the formal analysis of binding data in terms of independent and identical binding sites requires some comment. Denoting by B_i the amount of ligand bound to sites of type i and by B_i^0 the total amount of i-sites, $K^{(i)} = (B_i^0 - B_i) \cdot [L]/B_i$. The maximum number, $n^{(i)}$, of ligands bound to site i, per macromolecule is calculated from the macromolecular concentration of AcChR. The molecular weight of AcChR is taken as 260,000 (see ref. 1).

If m different types of binding sites are present, the measured amount of bound ligand $B^0 = \Sigma_{i=1}^m B_i$ is given by $B^0/[L] = \Sigma_{i=1}^m (B_i^0 - B_i)/K^{(i)}$. For $m = 2$ and $K^{(1)} \geq 10\,K^{(2)}$, two limiting cases may be differentiated. (*a*) If $[L] \gg K^{(1)}$, so that the approximation $B_2 \simeq B_2^0$ can be used, then one can write, e.g., for the Scatchard form:

$$B^0/[L] = \{(B_1^0 + B_2^0)/K^{(1)}\} - B^0/K^{(1)} \qquad [5a]$$

(*b*) If $[L] \ll K^{(1)}$, so that the approximation $B^0 \simeq B_2$ can be applied, we obtain:

$$B^0/[L] = (B_1^0/K^{(1)}) + (B_2^0/K^{(2)}) - (B^0/K^{(2)}) \qquad [5b]$$

It is noted that Eqs. 5 represent straight lines and the crossing point of which defines the value of B_2^0. If $m > 2$, successive application of Eqs. 5 to pairs of adjacent types of binding sites yields the values of $K^{(i)}$ and B_i^0.

RESULTS

Ca Analysis in AcChR by Atomic Absorption. Atomic absorption spectroscopic analyses of Ca in purified AcChR preparations from *Torpedo californica* are summarized in Table 1. About 18 Ca per AcChR of 260,000 dalton (1) remain

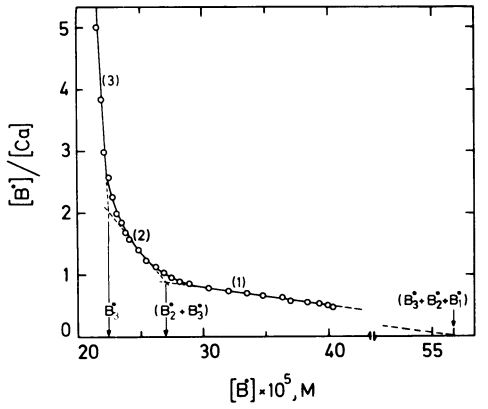

FIG. 1. Ca binding isotherm of purified AcChR protein from *Torpedo californica*: spectrophotometric titration in the presence of Mu, 0.1 M NaCl, 50 mM Tris·HCl, 0.1% Brij, pH 8.5, at 20°. The data are plotted according to Eqs. 5. [B^0], concentration of Ca bound; 9.7 μM AcChR; [Ca], concentration of free Ca.

tightly bound even when the purification procedure is carried out in Ca-free buffer. Furthermore, incubation with either 20 mM EDTA or 72 mM La^{3+} did not result in complete release of Ca from the AcChR. Acid hydrolysis of the protein at 100° was necessary to allow complete Ca exchange with La^{3+} before atomic absorption analysis.

Equilibrium Binding of Ca. The result of a continuous spectrophotometric Ca titration of AcChR protein from *Torpedo californica* in the Ca concentration range 10 μM–1 mM is plotted in Fig. 1. If Ca binding is assumed to be independent, at least three classes of binding sites may be differentiated. There are a total of about 60 Ca binding sites per AcChR molecule of 260,000 dalton. Equilibrium binding of ^{45}Ca was measured over a narrower range of Ca concentrations and is presented in Fig. 2. The thermodynamic parameters obtained in both these studies are summarized in Table 2. Good agreement is seen between the K_{Ca} values obtained by the two methods. A value similar to the lowest affinity we observed (site $i = 1$ in Table 2) has been reported previously (13). It is noteworthy that the dissociation constant in this class of low affinity sites at 26° is lower than that at 4°.

AcCh Binding. The result of equilibrium binding studies of [^{3}H]AcCh to both crude and purified AcChR from electric eel and *Torpedo* are presented in Table 3 and Fig. 3. The extent of hydrolysis of AcCh bromide at pH 8.5 (buffer I) at 4° for 15 hr was less than 2.5% determined by the Hestrin method (10). Little variation in the observed K_A values and the relative AcCh/α-Bgt binding stoichiometries is seen between crude and

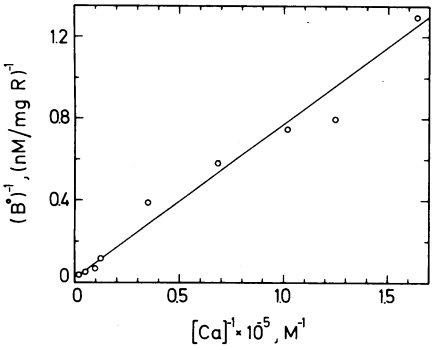

FIG. 2. Ca binding isotherm at 26° (low affinity region) of AcChR from *Torpedo californica* in 0.1 M NaCl, 25 mM Tris·HCl, 0.1% Brij, pH 7.2, obtained from ^{45}Ca ultrafiltration data. Symbols are as in Fig. 1. R = AcChR.

Table 2. Apparent dissociation equilibrium constants, K_{Ca}, for the binding of Ca ions to AcChR from *Torpedo californica* in buffer I

i	$K_{Ca}^{(i)}$, μM	$n^{(i)}$*	T, °C	pH	Tris mM
	Data from spectrophotometric titrations (Fig. 1)				
1	330 (±30)	30 (±3)	20	8.5	50
2	40 (±20)	4 (±2)			
3	2.5 (±0.5)	23 (±3)			
	Data from ^{45}Ca ultrafiltration (Fig. 2)				
1	300 (±200)		26	7.2	25
2	10 (±20)				
1	500 (±200)		4	7.2	25

Uncertainties are expressed as SEM.
* $n^{(i)}$ is maximum number of Ca ions bound to site type i, per 260,000 daltons; $n^{(3)}$ includes contributions from all other higher affinity site(s).

purified eel AcChR preparations (see also ref. 14). More striking variations are observed among *Torpedo* AcChR preparations; low affinity (higher K_A) sites appear only after purification, and relative AcCh/α-Bgt binding stoichiometries fluctuate. The Triton X-100 extract of *Torpedo californica* tissue exhibits only high affinity sites ($K_A = 25 \pm 5$ nM), as has also been observed in other laboratories (15, 16). Despite such variations, the major purified fraction of *Torpedo* AcChR used in the previous Ca studies is associated with low affinity sites to which a maximum of about two AcCh ions can be bound per 260,000 daltons of AcChR. It should be noted that observed concentrations of stock [^{3}H]AcCh were only 30–80% of those reported by Amersham for higher specific activity [^{3}H]AcCh, but the reported New England Nuclear value for lower specific activity [^{3}H]AcCh was close to that observed.

The effect of Ca on the AcCh dissociation constants is shown in Fig. 4. The dependence appears to be linear and thus con-

Table 3. AcCh binding parameters of AcChR

Preparation	Buffer*	K_A^i, nM	B_A^i/B_T °
	Electrophorus electricus		
Fresh tissue, 1% Triton extract	III	46 (±3)	0.3
		500 (±50)	0.2
Purified AcChR, absence of Ca	II	30 (±2)	0.37
		130 (±20)	0.27
Purified AcChR	II	45 (±5)	0.4
	1 mM Ca	440 (±50)	0.26
	Torpedo californica		
Fresh tissue, 1% Triton extract	III	25 (±5)	0.3†
Purified AcChR, preparation 1	III	32 (±4)	0.04
		880 (±80)	0.42
		5000 (±400)	0.64
	I	260 (±50)	0.21
		2400 (±300)	0.98
Purified AcChR, preparation 2	III	12 (±10)	0.02
		500 (±100)	0.66

K_A^is are the equilibrium dissociation constants for AcCh at 4°; for each preparation, multiple values represent different binding sites, i. B_A^i/B_T^0 is the ratio of AcCh bound to site i and the total α-Bgt bound.
* All dialysis buffer solutions contain 0.1% Brij, 0.1 M NaCl; I: 50 mM Tris·HCl, pH 8.5; II: 25 mM Tris·HCl, pH 7.2; III: 20 mM Na-phosphate, pH 7.0.
† 0.96 nmol AcCh/g of tissue.

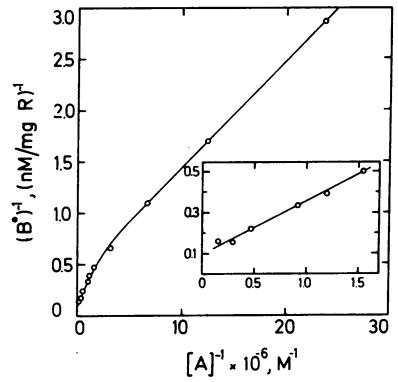

FIG. 3. [^{3}H]AcCh binding isotherm at 4° of purified AcChR from *Torpedo californica* in 0.1 M NaCl, 50 mM Tris–HCl, 0.1% Brij, pH 8.5. B^0, nM [^{3}H]AcCh bound per mg of protein; [A], concentration of free AcCh. Inset: an enlarged replot of the low affinity region.

sistent with competitive inhibition between Ca and AcCh as expressed by Eq. 6,

$$K_A = K_A^\circ(1 + [Ca]/K_{Ca}) \qquad [6]$$

where K_A^0 is the (extrapolated) value in the absence of Ca and K_{Ca} is the apparent Ca dissociation constant, associated with the reduction of the apparent AcCh affinity by Ca. The example given in Fig. 4 shows that $K_A^{(1)}$ is associated with K_{Ca} = 0.11 (±0.01) mM and that the low affinity constant $K_A^{(2)}$ is correlated with K_{Ca} = 3.1 (±0.2) mM.

Release of Ca from AcChR by AcCh. The competition of Ca and AcChR indicated by Fig. 4 predicts a release of bound Ca from AcChR upon binding of AcCh and *vice versa*. The change in the concentration of free Ca caused by AcCh binding to electric eel or *Torpedo* AcChR was measured spectrophotometrically and was analyzed according to Eqs. 1–4. A typical experiment involving AcChR from *Torpedo* is presented in Fig. 5. Increasing amounts of added AcCh release increasing amounts of Ca ions from AcChR until saturation is reached at about 20 μM AcCh. There are slight variations observed among different preparations, but about four to six Ca are released from *Torpedo* AcChR (260,000 daltons) per two AcCh molecules bound at 1.2 mM Ca (see Fig. 5), whereas electric eel

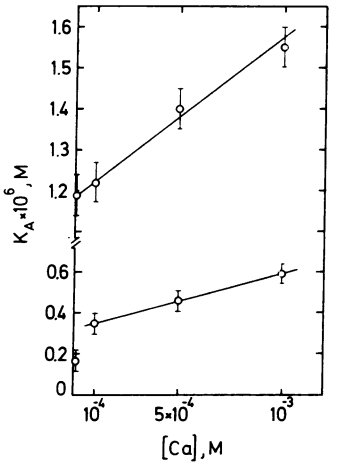

FIG. 4. Apparent equilibrium dissociation constants, K_A, of the [^{3}H]AcCh binding to AcChR from *Torpedo californica*, as a function of Ca concentration. The equilibrium dialysis was carried out in the medium 0.1% Brij, 50 mM Tris·HCl, 0.1 M NaCl, pH 8.5, and 1 μM Tetram at 4°. Upper line, K_A of the lower-affinity site; lower line, K_A of the higher-affinity site.

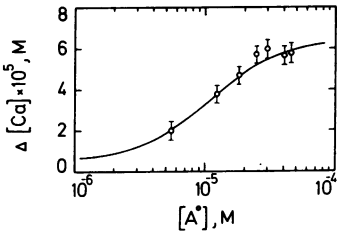

FIG. 5. The change in the concentration of Ca ions reflecting the release of bound Ca, $-\Delta[Ca_b] = \Delta[Ca]$, from AcChR of *Torpedo californica* in 0.1 M NaCl, 50 mM Tris·HCl, 0.1% Brij, 1.2 mM Ca, pH 8.5 at 20°. Δ[Ca] is plotted as a function of total AcCh concentration, [A^0], at 10 μM (2.6 mg/ml) AcChR concentration [R^0].

AcChR binds about one AcCh per 260,000 dalton macromolecules and releases two to three Ca. Furthermore, it was found that subsequent addition of α-Bgt led to reuptake of four to six Ca for *Torpedo* AcChR and of two to three Ca for electric eel AcChR.

DISCUSSION

Ca Binding of AcChR. The data listed in Table 1 and 2 demonstrate a remarkably high capacity of isolated AcChR for Ca. Another result indicating an extremely high binding capacity of AcChR for Ca has been reported recently (17). It appears that AcChR contains strongly bound Ca ions that are not displaced by a chelating agent and a lanthanide. Long exposure of AcChR to EDTA or to La^{3+} results in protein precipitation. It is therefore likely that the tightly bound Ca ions are responsible for the structural stability of the AcChR protein. Table 2 and Figs. 1 and 2 demonstrate that the Ca binding of AcChR protein involves multiple sites and the linear ranges of the binding isotherms support an assumption of independent binding.

The present study demonstrates that Ca has a higher affinity for AcChR at higher temperatures, and the low-affinity Ca binding, $K_{Ca}^{(1)}$, is associated with a dissociation enthalpy ΔH° = −1.3 (±0.3) kcal/mol (−5.4 ± 1.2 kJ/mol). This negative value of ΔH° suggests that the dominant contribution to the Ca–AcChR interaction may be ionic-electrostatic (18) rather than by chelating interaction.

AcCh Binding of AcChR. AcCh binding parameters are frequently used to characterize AcChR. As seen in Table 3, the dissociation constants, K_A, for AcChR from electric eel are about the same for the crude 1% Triton X-100 extract of fresh tissue and the purified preparation. However, both our values and previously reported values of K_A for AcChR from *Torpedo marmorata* and *Torpedo californica* (15, 16, 19–22) show large variations. While our current studies indicate that such variations are due to alterations in the state of the AcChR protein, it is noteworthy that the actual AcCh concentrations of commercial [^{3}H]AcCh are often significantly lower than indicated by the label; this technical problem may have contributed to previously reported AcCh binding variations. Without doubt, the affinity of AcCh to AcChR decreases during purification under present conditions. The chemical change responsible for this decrease is not clear, but may involve oxidation of sulfhydryl groups in AcChR (16).

Competition Between AcCh and Ca for AcChR. The linear dependence of K_A on [Ca] suggests a competition between AcCh and Ca ions for the AcChR macromolecule. Since the temperature dependence of K_{Ca} is small (see Table 2), we may compare K_{Ca} = 3.1 mM from the AcCh competition experiments at 4° with $K_{Ca}^{(1)}$ = 0.33 mM, the largest Ca equilibrium constant directly observed (Fig. 1) in the absence of AcCh. The

two values differ by an order of magnitude. The competition reaction appears to involve receptor sites which are not seen in the Ca titration experiments. Perhaps the numerous Ca binding sites associated with $K_{Ca}^{(1)}$, $n_{Ca}^{(1)} = 30$ (±3) mask less numerous sites with $K_{Ca} = 3.1$ mM. Because solutions of concentrated AcChR become turbid at [Ca] ≥ 3 mM, because of protein aggregation, no reliable spectrophotometric data can be obtained in this [Ca] range.

If competition between AcCh, α-Bgt, and Ca simply involved binding at a single active site on AcChR, it would be anticipated that α-Bgt, which blocks the binding AcCh, would also block the binding of Ca. This is not observed; instead, α-Bgt induces reuptake of the Ca displaced by AcCh. These opposing actions of the neural activator AcCh and the neural inhibitor α-Bgt suggest a more complex interaction between AcChR, AcCh, Ca, and α-Bgt than that suggested by direct competitive inhibition. The interactions presumably involve AcChR conformations stabilized alternatively by activators and inhibitors. Such an equilibrium is represented in the overall reaction

$$A + RCa_x = AR' + xCa. \quad [7]$$

given in terms of AcCh binding sites. In this scheme the AcChR conformation R is preferred by α-Bgt, whereas the conformation R' is favored when AcCh is bound.

This reaction scheme may be used to analyze the dependence of Ca release on the total AcCh concentration [A^0] (Fig. 5). The data suggest that changes in bound Ca, $\Delta[Ca_b]$, accompany changes in bound AcCh, $\Delta[A_b]$. If the fractional uptake of AcCh, α, equals the fractional Ca release, i.e.: $\alpha = \Delta[A_b]/\Delta[A_b^0] = \Delta[Ca_b]/\Delta[Ca_b^0]$, the experimental data of Fig. 5 follow the relationship

$$\alpha[R_A^0] + K_A \cdot \alpha/(1-\alpha) = [A^0] \quad [8]$$

when $K_A = 1.0$ (±0.1) μM, and $[R_A^0]$ is the total concentration of AcCh binding sites. The values of K_A and $[R_A^0]$ quantitatively describing the release curve in Fig. 5 are the same as derived from independent equilibrium dialysis data. Since the saturation level of AcCh uptake corresponds to the saturation level of Ca release, it is found that $x = 3$ (±1) at 1.2 mM Ca.

Purified AcChR protein from electric eel exhibits similar behavior. The eel AcChR macromolecule (260,000 daltons) binds one AcCh associated with two K_A values of ~100 and ~10 nM. At [Ca] = 1 mM, two to three Ca ions are released per bound AcCh; subsequent addition of α-Bgt causes reuptake of these Ca ions.

Thus the binding of AcCh and α-Bgt to isolated AcChR can quantitatively account for Ca release. Whether Ca release and uptake, however, is a general indicator for the immediate effects on AcChR of neural activators and inhibitors remains to be established.

In a recent study, Eldefrawi *et al.* (17) report that AcCh binding to AcChR from *Torpedo marmorata* is inhibited by Ca ions; the inhibition is associated with an inhibition constant (K_{Ca}) of 7 mM. Rübsamen *et al.* (23) have found that the activators carbamylcholine and decamethonium displaced Tb ions from AcChR sites which are associated with a Ca dissociation constant (K_{Ca}) of about 1 mM. The K_{Ca} values obtained by these authors are of the same order of magnitude as our data derived from direct AcCh and Ca binding experiments.

Recalling Eq. 8, it is seen that $[AR']/[RCa_x] = (K_{Ca}/K_A) \cdot [A]/[Ca]^x$. Thus, the degree of AcCh binding to AcChR is controlled by the ratio of the AcCh and Ca concentrations. In this way Ca ions exert a regulatory effect on the conformation by limiting the extent of AcCh binding. The association of AcCh with AcChR is believed to control the permeability of excitable membranes to certain ions. In an integral model of nerve excitability, developed recently on the basis of biochemical and pharmaco-electrophysiological data, a competitive interaction of AcCh and Ca ions with AcChR is proposed to be involved in the rapid control of transient ion flows during nerve and muscle excitation (2, 4). As demonstrated by the quantitative results of the present study, the isolated AcChR proteins (from electric fish) exhibit dynamic properties consistent with the molecular interpretations of the integral model.

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