MOLECULAR PHARMACOLOGY, 18, 413–420

Ion Exchange between Agonists and Inorganic Ions at the Acetylcholine Receptor of *Torpedo californica*

CHARLES E. SPIVAK[1] AND DERMOT B. TAYLOR

Department of Pharmacology and Brain Research Institute, U.C.L.A., School of Medicine, Los Angeles, California 90024

Received November 8, 1979; Accepted June 23, 1980

SUMMARY

SPIVAK, C. E., AND D. B. TAYLOR. Ion exchange between agonists and inorganic ions at the acetylcholine receptor of *Torpedo californica*. *Mol. Pharmacol.* 18: 413–420 (1980).

Our objective was to investigate the nature of the reactions by which agonists trigger the nicotinic acetylcholine receptor. A series of experiments on the binding of tritiated decamethonium and muscarone to the particulate-bound nicotinic acetylcholine receptor from *Torpedo californica* was carried out using a filtration assay. Specific binding was assessed by subtracting the nonspecific binding, found when the receptors were saturated with α-bungarotoxin, from the total binding. The binding curves obtained by varying the concentration of the physiological ions can be explained by stoichiometric ion-exchange equations, in accordance with the principle of electroneutrality, without invoking positive and negative cooperativity or complicated ad hoc assumptions. In all of our experiments, saturation of the receptors was produced by equimolar quantities of decamethonium, muscarone, and α-bungarotoxin. These results indicated that an additional anchoring or acceptor site is required to bind decamethonium and that decamethonium is monovalent at the receptor site. The receptor recognition site can change its electrovalence and exchange divalent or monovalent counter ions for either decamethonium or muscarone, depending on the agonist and the ionic environment. In all our experiments the receptor behaved with an electrovalence of either one or two and never with an intermediate value. Using decamethonium and low calcium concentrations (5 mM) the receptor exchanged one decamethonium for one magnesium ion, whereas at higher calcium concentrations (15 mM and above) it exchanged one decamethonium for two monovalent cations. Calcium ions do this by acting on sites other than the agonist recognition site. Similar effects of calcium ions were seen with muscarone. In the divalent state the receptor's recognition site binds Mg^{2+} but not Ca^{2+}, and in the monovalent state it binds K^+ but not Na^+. Attempts to analyze the data in terms of other ionic models were unsuccessful.

INTRODUCTION

In earlier papers evidence, from depolarizer uptake and muscle depolarization, was presented that the anionic receptor groups in voluntary muscle occurred in pairs less than 4 Å apart bridged by a magnesium ion and that agonists and antagonists united with the nicotinic receptor by stoichiometric ion exchange (1, 2).

This research was supported by grants from the Muscular Dystrophy Association, the UCLA School of Medicine General Research Support Grant RR05354, USPHS Grant 11708, and USPHS Graduate Training in Pharmacological Sciences Grant GM 02040. We are also indebted to our wives, Ruth Spivak and Dr. Charlotte Taylor, for reading the manuscript and for many useful suggestions.

[1] In partial fulfillment of the requirements for the Ph.D. degree in Pharmacology. Present address: Department of Pharmacology, School of Medicine, University of Maryland, Baltimore, Maryland 21201.

The general ion-exchange reaction may be written (3)

$$D^+ + R^-X^+ \rightleftharpoons R^-D^+ + X^+ \qquad [1]$$

without implying any value for the electrovalence of D^+ or X^+, where X^+ is some naturally occurring ion which can function as a counter ion and where the line represents a solid phase not in true solution. The quantitative equation to represent the equilibrium is (3)

$$K_D^X = [D_s^+]^m[X_r^+]^n/[X_s^+]^n[D_r^+]^m, \qquad [2]$$

where m is the electrovalence of X^+, n is the electrovalence of D^+, and K_D^X is the equilibrium selectivity constant. The electrovalence of each competing ion enters the equation as the exponent of the other ion. The subscripts r and s refer to the receptor and solution, respectively, and K_D^X reflects the relative affinities of the drug D and the ion X for the receptor.

0026-895X/80/060413-08$02.00/0

Fitting experimental data to Eq. [2] yields the electrovalence m of the counter ion, and the effective electrovalence of the receptor site will also be m. The fact that the electrovalence of ClO^{2+} is 2 does not mean, however, that the electrovalence of the receptor site is 2 when it is in combination with ClO^{2+}, because both ends of each ClO^{2+} ion may not be bound to the receptor recognition site. The view that only one end of a long bifunctional ion like ClO^{2+} was bound to a receptor and that the other end was bound to a nonreceptor or acceptor anchoring site about 12 Å away was previously proposed (4). This idea was adopted and modified (2, 5) as an extension of the ion-exchange receptor pair model. In this modification the ends of two ClO^{2+} ions are bound by the receptor pair and the other ends by the anchoring or acceptor pair. The individual groups of the receptor pair will be monovalent when holding the quaternary ends of two ClO^{2+} ions, and when the ends of two ClO^{2+} ions displace a Mg^{2+} ion from the receptor pair, the electrovalence of the receptor pair will change from 2 to 1. This will not show in the ion-exchange equation representing the event because the acceptor pair also holds a Mg^{2+} ion, and two ClO^{2+} ions will therefore displace two Mg^{2+} ions.

Our objectives were to investigate the nature of the reactions by which agonists trigger the nicotinic acetylcholine receptor and to test whether the ion-exchange model for agonist binding is valid.

MATERIALS AND METHODS

Electroplax pellet. All our experiments were done with either of two methods of pellet preparation, both similar to that of Ref. 6. The original preparation contained traces of Na^+ (flame test) and other ions from the electroplax tissue. The second well-washed preparations had been washed six times with calcium–Mops (7) buffer.

For the original preparation 15 to 20 g of tissue was trimmed of skin, diced, and homogenized with a Sorvall Omnimixer for three 1-min runs at 0 to 5°C. Three milliliters of 0.02 M phosphate buffer, pH 7.4, was used per gram of tissue. The suspension was filtered through a fine nylon mesh (100 threads/in.) to remove strands of connective tissue and centrifuged at 34,000g for 1 h. It was resuspended in buffer, centrifuged again, and stored as the pellet at −10°C. To make the suspension of bound receptors, approximately 4 mg of stored pellet was added to each milliliter of the appropriate buffer. This, overall, represented an 8×10^3 times dilution of the original tissue fluid but still gave a positive flame test for sodium. The sodium and other ions may have been trapped at an early stage by vesicle formation.

The well-washed preparation, used in most of the [^{3}H]-muscarone binding experiments, was made by first slicing (0.5 mm thick or less) the frozen electroplax perpendicular to the plane of cells to facilitate removal of soluble cellular substances. A buffer containing 10 mM Mops and 5 mM calcium ($Ca(OH)_2$) was adjusted to pH 7.4 and was used throughout. It contained no Na^+, K^+, or Mg^{2+}. The slices (1:9, tissue to buffer) were alternately agitated gently for an hour and centrifuged (12,000g, 30 min) six times. The sodium flame test of the suspension was negative. The tissue was then homogenized in the Omnimixer as before, centrifuged, and stored as the pellet at −10°C. The tissue was well washed first because possible vesicle formation by the homogenization would interfere with removal of traces of ions.

Reagents and chemicals. The NaCl used was Merck's reagent grade for biological work. The KCl, $MgCl_2 \cdot 6H_2O$, and $Ca(OH)_2$ were reagent grade and the $CaCl_2 \cdot 6H_2O$ was Alfa ultrapure grade. The elasmobranch saline contained Ca^{2+} (5 mM), Mg^{2+} (5 mM), K^+ (6 mM), Na^+ (250 mM), and 3-(*N*-morpholino)propanesulfonic acid (Mops) buffer (10 mM) (7).

Tritium-labeled decamethonium was purchased from Amersham Searle. The radiochemical purity, checked by paper chromatography, agreed with the data provided by the manufacturer and was about 98%. The concentration was found by gravimetric analysis using saturated ammonium reineckate. The precipitate was collected on a 0.2-μm-pore 25-mm Nuclepore filter, washed three times with ice-cold distilled water, air-dried, and weighed on a microbalance. Unlabeled ClO^{2+} was used to check recovery. The radioactivity was determined separately. None of the activity was volatile.

Tritium-labeled muscarone was made from normuscarine (8, 9). The normuscarine was oxidized to normuscarone and crystallized as the oxalate (10). It was recrystallized four times from isobutanol to constant mp 125–126°C [lit., 121°C (11)]. The normuscarone, as the free base, was labeled with tritiated methyl iodide in dry benzene (12) at the Biochemical and Nuclear Corp., Burbank, California. It was crystallized, collected, recrystallized from acetone ether, and stored refrigerated. It had a specific activity of 270 mCi/mmol. Radiochromatograms run by the manufacturer in *N*-butanol, pyridine, water (1:1:1) and in *n*-butanol, acetic acid, water (4:1:2) showed no extraneous peaks.

The α-bungarotoxin (Miami Serpentarium) was labeled by the iodine chloride method to give the diiodo derivative (13, 14). Specific activity was obtained by two independent methods. In the first the radioactive and protein concentrations of the purified toxin were determined; the second estimate was based on the nonradioactive toxin displacement method (9). The results of the two assays agreed with the specific activity of the iodine chloride. The radiochemical purity of the labeled toxin was determined by the pellet saturation method and was never higher than 94%.

Ligand binding assays. The standard binding assay was similar to that used earlier (15). A suspension of 4 mg/ml of electroplax pellet in buffer was divided into two equal parts, and 4 μg/ml of α-bungarotoxin was added to one. This was a 10-fold supersaturating excess of toxin, and it blocked all the receptors in 1 h. After 1 h 3.2-ml aliquots were placed in six pairs of clean polyethylene vials, and 4.2-ml aliquots in a seventh pair. This latter pair was for the labeled α-bungarotoxin assay of receptor capacity. Both of these last tubes received 200 pmol of ^{125}I-labeled α-bungarotoxin, a fourfold excess, and were swirled gently in the dark at room temperature for 3 h before filtering. The labeled ligand was added to each of the six pairs of vials and the contents were mixed gently for 20 min at room temperature before filtering.

On milliliter of the particulate suspension was applied

to the cup-shaped filter (15) at a suction of 50 cm Hg. After the last trace of visible solution had gone, the suction was continued for a further 30 s. The membrane was then lifted off, air-dried, and weighed. The process was repeated for an identical sample to which a saturating concentration of α-bungarotoxin had been added. The difference was taken as the specific binding. Each filtration was run in triplicate, and the air-dried filters were weighed and counted in Beckman Filter Solv scintillation fluid. The results were expressed as ligand bound per milligram of dry particulates.

Binding analysis and receptor capacity determination. Ligand binding capacities and other parameters were calculated by the Newton–Gauss method of weighted nonlinear regression analysis, using the Health Sciences Computing Facility and program BMDO7R (16). The weighting factors used were the estimated reciprocal variances. Results were confirmed by linear double reciprocal and Scatchard transforms using the appropriate electrovalencies in the basic ion-exchange equations. More details are available (9).

The way in which the dimensions of the equilibrium constants in ion exchange are expressed is different from that in classical receptor theory. The receptor occupation ratio, $[X_r^+]/[D_r^+]$ of Eq. [2], is first normalized, that is, $[X_r^+]$ and $[D_r^+]$ are calculated as fractions whose sum is unity, each fraction is then raised to the appropriate power, and the overall ratio is calculated. Such a ratio is a dimensionless number and is independent of the units in which receptor occupation is measured. The solution ratio $[D_s^+]^m/[X_s^+]^n$ and the selectivity constant are dimensionless only when m and n are the same, and when they are not the selectivity constant is expressed in terms of the units used in the equation. In cases where we have been able to calculate only a number proportional to a selectivity constant, units have been omitted but the equation is given.

RESULTS

Decamethonium Exchange Experiments

The exchange between decamethonium and magnesium ions in the presence of 5 mM calcium. Figure 1 shows the total binding and the nonspecific binding in the presence of a saturating concentration of α-bungarotoxin; the difference is the specific binding. Using ClO^{2+} as agonist, the specific binding was usually about 30% of the total.

Curves similar to those in Fig. 1 were obtained at 5, 15, 30, 45, 60, and 75 mM Mg^{2+}. Each set of data relating specific binding to ClO^{2+} concentration was analyzed by determining the parameters of the corresponding rectangular hyperbola. This analysis provides an estimate of the asymptote which is the ion-exchange capacity of the receptor for ClO^{2+}. The receptor capacity was also determined by the labeled bungarotoxin method, and the results of these two methods are shown in Table 1. These data were then analyzed in terms of the basic equations of ion exchange.

The exchange between ClO^{2+} and Mg^{2+} ions at the receptor can be written

$$ClO^{2+} + \mathit{Mg}^{2+}R_2^- \rightleftharpoons \mathit{ClO}^{2+}R_2^- + Mg^{2+}, \qquad [3]$$

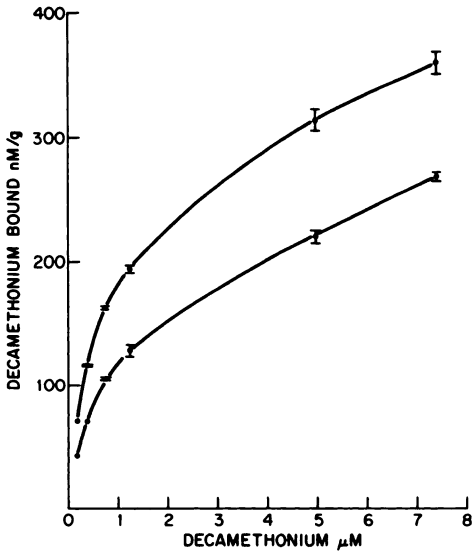

FIG. 1. *Relationship between ClO^{2+} concentration and ClO^{2+} bound in the presence (lower curve) and absence (upper curve) of a saturating concentration of α-bungarotoxin*

The difference between each pair of points is the specific uptake. The magnesium and calcium concentrations were both 5 mM. There was no added Na^+ or K^+. The original method of particulate preparation was used.

where R^- represents an anionic group attached to the receptor macromolecule and the solid line represents a phase not in aqueous solution. The equilibrium ion-exchange equation corresponding to [3] is

$$K_{ClO}^{Mg} = [ClO_s^{2+}]^2\,(1 - \theta_{ClO})^2/[Mg_s^{2+}]^2\,\theta_{ClO}^2, \qquad [4]$$

where θ_{ClO} is the fraction of receptors occupied by ClO^{2+} and $(1 - \theta_{ClO})$ is the fraction occupied by Mg^{2+}.[2]

When the data from the six curves are plotted as Mg_s^{2+} against $[ClO_s^{2+}](1 - \theta_{ClO})/\theta_{ClO}$, a straight line through the origin with a slope of $1/K_{ClO}^{Mg\,1/2}$ should result. The least-squares regression line (Fig. 2, crosses) goes through the origin within the limits of experimental error, showing that the 5 mM calcium and small amounts of other cations do not compete significantly under these conditions. The values of the selectivity constant in Table 1 are dimensionless.

Table 1 shows that there is no evidence of drift in the selectivity constant, and the exchange capacity values indicate that one α-bungarotoxin occupies the same number of sites as each ClO^{2+} ion.

The above experiment indicates that in the resting state the receptor pair of negative groups is occupied by a single Mg^{2+} ion and its electrovalence is 2, but when the individuals of the pair are occupied by the ends of the two ClO^{2+} ions the electrovalency of each member of the pair is 1. Decamethonium converts the receptor from the divalent to the monovalent state. The other ends of the two ClO^{2+} ions attach to the pair of acceptor or anchoring sites also occupied by a Mg^{2+} ion.

The exchange between decamethonium and magne-

[2] This treatment is based on the specificity of the receptor recognition site for Mg^{2+}. Inclusion of terms to account for other possible competing cations has been shown (9) to yield the same results for the data reported here.

TABLE 1

Values of the selectivity constant for the exchange between ClO^{2+} and magnesium ions together with the specific receptor binding capacity for ClO^{2+} and α-bungarotoxin

Mg^{2+}	K^{Mg}_{ClO}	Receptor binding capacity (nmol/g ± SE)	
		ClO^{2+}	α-Btx
mM			
5	7.95×10^{-5}	96 ± 3	85 ± 1
15	6.71×10^{-5}	89 ± 6	80 ± 2
30	5.05×10^{-5}	95 ± 4	86 ± 2
45	6.09×10^{-5}	96 ± 3	79 ± 1
60	7.00×10^{-5}	98 ± 5	75 ± 1
75	6.24×10^{-5}	87 ± 4	79 ± 1
Mean	6.51×10^{-5}		

sium ions was repeated in elasmobranch saline (Fig. 2, circles). The regression line has a slightly different slope, corresponding to $K^{Mg\,1/2}_{ClO} = 7.32 \times 10^{-5}$ as opposed to 6.51×10^{-5} for the previous line, and it cuts the vertical axis at −29.5 mM Mg^{2+}. Because of the high sodium concentration the group of data obtained at each magnesium concentration was tested to see whether or not ClO^{2+} was exchanging for one or two inorganic ions by analysis of the individual binding curves corresponding to each point. At no value of Mg^{2+} was there any evidence that 250 mM Na^+ could force a monovalent exchange. Calcium at 75 mM has about the same ionic strength as 250 mM Na^+, but it does not produce the same effect.

The effect of increased calcium concentrations on the exchange behavior of decamethonium at the receptor. Binding curves as in Fig. 1 were done at calcium concentrations of 5, 15, 30, 45, 60, and 75 mM, and all were at a constant Mg^{2+} concentration of 5 mM. The curves were all close together, and none at 15 mM Ca^{+2} or above was a rectangular hyperbola

$$K^{IN}_{ClO} = [ClO^{2+}_s](1 - \theta_{ClO})^2/[IN^+_s]^2\,\theta_{ClO}. \qquad [5]$$

Both curves in Fig. 3 represent the same data (15 mM Ca^{2+}) using Eq. [5] (circles) or Eq. [4] with all squared terms (crosses). From the linearity using Eq. [5], it is evident that each ClO^{2+} ion is exchanging for two monovalent counter cations. The result was the same at the higher calcium contractions. At 5 mM Ca^{2+} the data obeyed Eq. [4], with ClO^{2+} exchanging for a divalent ion (Mg^{2+}) as in the previous experiment.

Between 5 and 15 mM Ca^{2+} the receptor undergoes a profound change, and from 15 to 75 mM Ca^{2+} it exchanges with an electrovalence of 1. This is consistent with the concept that at 5 mM Ca^{2+} the receptor is behaving as an ion exchanger having a higher selectivity for Mg^{2+} ions and that, upon raising the calcium concentration, the receptor changes to a state or states having a higher selectivity for monovalent ions.

Muscarone Exchange Experiments

The data on muscarone binding were obtained by essentially the same method as in the earlier experiments. Figure 4 is a typical binding curve for tritium-labeled muscarone. The upper curve is the binding in the absence of α-bungarotoxin, and the lower curve is the binding in the presence of the toxin, the difference being the specific

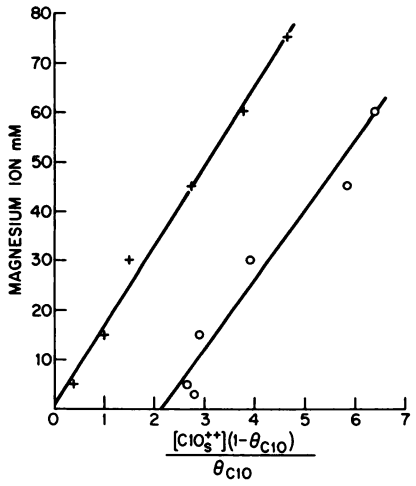

FIG. 2. *Relationship between experimental data and Eq. [4] for exchange between ClO^{2+} and Mg^{2+} at the receptor*

Both graphs are plots of $[Mg^{2+}_s]$ against the square root of the remainder of the right-hand side of Eq. [4] in the presence of 5 mM Ca^{2+} and no added Na^+ or K^+ (+) and in elasmobranch saline (○). Each point is the experimental mean of the term $[ClO^{2+}_s](1 - \theta_{ClO})/\theta_{ClO}$ found at a given Mg^{2+} concentration.

binding. The nonspecific binding was linear in all experiments and was simply the small amount of muscarone deposited on the filter by the evaporation of the fluid residue after as much solution as possible had been sucked through. It appears that the specific uptake measured in this way is as much as 90% of the total uptake and that muscarone, unlike ClO^{2+}, is a specific agent in our experiments. Muscarone binding can be measured without α-bungarotoxin and it confirms the use of the latter.

The exchange behavior of muscarone in the presence of varying concentrations of magnesium ions. The data in these experiments were all obtained from the same original batch of particulates as was used for ClO^{2+}. The solution contained 5 mM Ca^{2+} and concentrations of 5 and 60 mM Mg^{2+}. There was no added Na^+ or K^+, but traces of Na^+ and K^+ were present.

Analysis of both sets of data indicated that the muscarone ion, unlike ClO^{2+} in the same circumstances, exchanged for a *mono*valent cation. The evidence derives

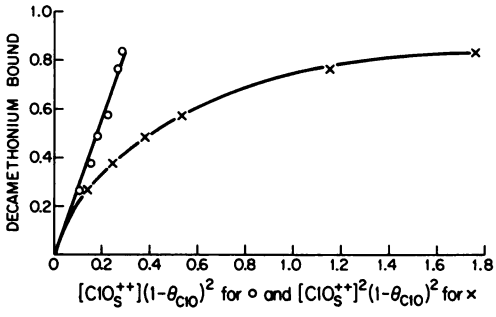

FIG. 3. *Plots of receptor-bound decamethonium from Eq. [5]*

The fraction of receptors occupied against $[ClO^{2+}_s](1 - \theta_{ClO})^2$ is represented by the circles, and that against $[ClO^{2+}_s]^2(1 - \theta_{ClO})^2$ as crosses. The linear plot indicates that each ClO^{2+} is exchanging for two monovalent ions and that the receptor is in the monovalent state. The calcium concentration was 15 mM. The particulate preparation used contained traces of both Na^+ and K^+.

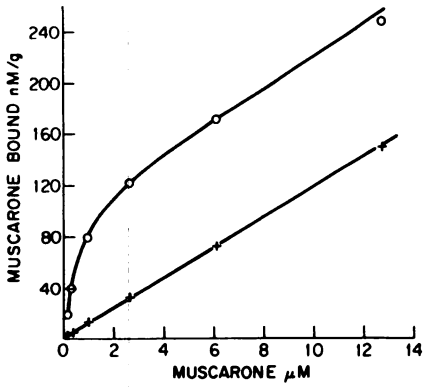

FIG. 4. *Muscarone binding curve in the presence (lower curve) and absence (upper curve) of a saturating concentration of α-bungarotoxin* The difference between the curves is the specific binding.

from the application of the appropriate equation,

$$K^{IN}_{Mu} = [Mu^+_s]^n(1 - \theta_{Mu})/[IN^+_s]\theta^n_{Mu}, \qquad [6]$$

where θ_{Mu} is the fraction of receptors occupied by Mu^+ and $(1 - \theta_{Mu})$ is the fraction occupied by the inorganic counter ion. When muscarone exchanges for a monovalent ion n is 1, and for a divalent ion n is 2.

At both 5 and 60 mM Mg^{2+}, Fig. 5 shows that the data obey Eq. [6] when n is 1, but on going from 5 to 60 mM Mg^{2+}, the decreased value of the slope shows that the selectivity constant increased about 2.7 times. The receptor is in the monovalent state, perhaps because it has been moved into this state by muscarone during the period of equilibration. The receptor displays a very high affinity for the low-concentration monovalent ions relative to the high-concentration Mg^{2+} ions.

The exchange between muscarone and magnesium ions in the presence of very low concentrations of monovalent inorganic ions. Because the previous binding measurements showed muscarone exchanging for monovalent ions, the experiments were continued using well-washed particulate preparations containing minimal amounts of Na^+, K^+, and Mg^{2+} (see Materials and Methods). A series of muscarone binding curves was run at 5, 20, 40, 60, and 80 mM Mg^{2+} and the curves were analyzed individually and as a group in accordance with Eq. [7],

$$K^{Mg}_{Mu} = [Mu^+_s]^2(1 - \theta_{Mu})/[Mg^{2+}_s]\,\theta^2_{Mu}. \qquad [7]$$

A plot of $[Mg^{2+}_s]$ against the remainder of the right-hand side of Eq. [7] gave a linear regression line which did not deviate significantly from the origin (Fig. 6), indicating that now only Mg^{2+} was exchanging for Mu^+ at the receptor. Values of K^{Mg}_{Mu} (Eq. [7], $N = 22$) had a mean value of 2.25 ± 0.15 (SE) $\times 10^{-11}$ μM.

Muscarone receptor binding experiments were also done in the same buffer solution at 2.0, 0.5, and 0.0 mM Mg^{2+}, and detailed analysis indicated that muscarone was exchanging for a monovalent ion at these low magnesium concentrations. The basic equation for this exchange is [6] when n is 1 and may presumably refer to the trace of inorganic monovalent ions not completely removed in the washing procedure. The results indicate that from 0.0 to 2.0 mM Mg^{2+} the receptor is in a monovalent state, occupied by monovalent counter ions, and that from 5 to 80 mM Mg^{2+} the receptor is in the divalent state, occupied by Mg^{2+} ions. Over the range of 0.0 to 80 mM Mg^{2+} the receptor is in the monovalent state when occupied by muscarone ions.

Potassium (5 mM) but not Na^+ restored monovalent behavior and the data gave a value for K^K_{Mu} of $2.5 \pm 0.25 \times 10^{-4}$ (Eq. [6]).

The effect of increased calcium concentrations on the exchange behavior of the receptor in response to muscarone. The salt solutions used for these six muscarone binding curves contained 5, 15, 30, 45, 60, and 75 mM Ca^{2+} and no added Na^+, K^+, and Mg^{2+}. The particulates had been extensively washed with solutions free of Na^+, K^+, and Mg^{2+}. Each binding curve was done at a constant calcium concentration.

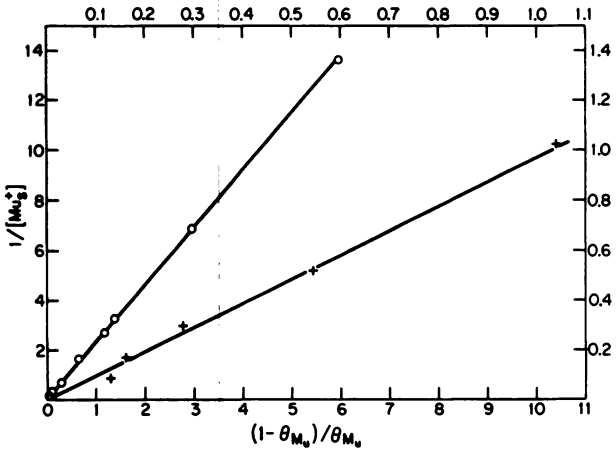

FIG. 5. *Relationship between experimental data and Eq. [6] for the exchange between muscarone and a monovalent ion*

Both graphs are plots of $1/[Mu^+_s]$ against $(1 - \theta_{Mu})/\theta_{Mu}$). The left and bottom axes are for data at 5 mM Mg^{2+} (O), and the top and right axes are for 60 mM Mg^{2+} (+). The data are in agreement with each muscarone ion exchanging for one monovalent ion at the receptor. The particulates were prepared by the original method and contained traces of both Na^+ and K^+.

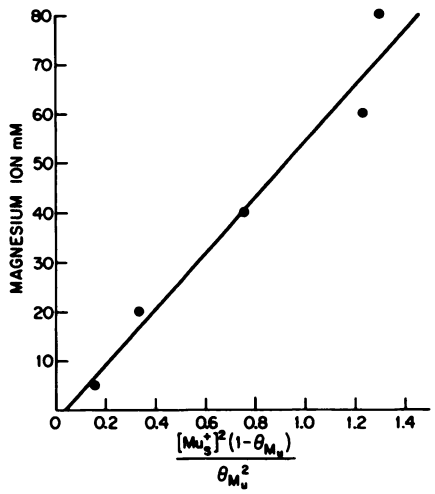

FIG. 6. *Plot of magnesium ion concentration against the mean of the remainder of the right-hand side of Eq. [7] for combined specific muscarone binding data at 5, 20, 40, 60, and 80 mM magnesium*

It is in agreement with the exchange of two muscarone ions for each magnesium ion. The particulate preparation had been well washed with buffer containing no Na^+, K^+, or Mg^{2+}.

TABLE 2

Values proportional to the selectivity constant showing the constancy of selectivity constants at each calcium concentration

Each muscarone is exchanging for one monovalent ion, and the receptor is acting with an electrovalence of 1.

Increasing muscarone concentration	Values of $[Mu_s^+](1-\theta_{Mu})/\theta_{Mu}$ at $[Ca^{2+}]$						Mean
	5	15	30	45	60	75	
↓	0.30	0.42	0.56	0.74	0.65	0.64	0.55
	0.28	0.41	0.56	0.75	0.66	0.65	0.55
	0.30	0.40	0.54	0.73	0.65	0.64	0.54
	0.33	0.38	0.61	0.73	0.74	0.65	0.57
	0.33	0.48	0.73	0.79	0.55	0.44	0.55
↓	0.37	—	—	0.99	—	0.78	0.71
Mean	0.32	0.42	0.60	0.79	0.65	0.64	

At all calcium concentrations muscarone exchanged as a single monovalent counter ion rather than two muscarone ions for one divalent counter ion. That is, Eq. [6], where n was 1, was obeyed rather than Eq. [7], in which Ca^{2+} was substituted for Mg^{2+}. From Eq. [6], since $[IN_s^+]$ was constant, $[Mu_s^+](1-\theta_{Mu})/\theta_{Mu})$ should be constant for each calcium concentration. Table 2 shows these values. The three gaps in Table 2 are all at high receptor occupancies where experimental errors are high and are all due to the measured values of muscarone binding being above the asymptote to the binding curve.

In this group of experiments, where the inorganic monovalent ion concentration has been reduced by as many as seven washings, each with monovalent and magnesium ion-free solutions, the conditions have been optimized for calcium ions to exchange for two muscarone ions at the receptor. No such exchanges involving calcium have been found under any conditions, so it seems that the receptor discriminates completely against Ca^{2+} at its specific ligand binding site.

The effect of calcium (60 mM) and magnesium (40 mM) on the exchange behavior of the receptor in response to muscarone. Using preparations of well-washed particulates containing 5 mM Ca^{2+} and concentrations of Mg^{2+} from 5 to 80 mM, two muscarone ions exchanged for each magnesium ion and the receptor exchanged from the initial divalent state. The present experiment again used well-washed particulates and a magnesium concentration of 40 mM which would ensure exchange of two Mu^+ ions for one Mg^{2+} ion. The experiment was conducted at 60 mM Ca^{2+}, and this again caused a profound change in the behavior of the receptor. Analysis of the data clearly indicated that the receptor now exchanged one monovalent ion for each muscarone ion and was monovalent. The evidence derives from Eq. [6] where n is 1. Figure 7 is a plot of the data. Exchange with a monovalent counter ion requires a straight line passing through the origin, and Fig. 7 shows this. Although there was 60 mM Ca^{2+} and 40 mM Mg^{2+} present, the receptor exhibited a marked preference for trace amounts of monovalent ions, presumably K^+, whereas in the absence of 60 mM Ca^{2+} the receptor showed a preference for Mg^{2+}.

The ion exchange and α-bungarotoxin binding capacities of the particulate preparations. The results, from those experiments where decamethonium was used as a ligand, were obtained from 17 binding curves. The ClO^{2+} and α-bungarotoxin binding capacities were determined for each of the curves to give 17 pairs of measurements. The ratios of the computer-determined ClO^{2+} receptor capacities to the corresponding α-bungarotoxin saturation values had a mean value of 1.06 ± 0.144 (SD). When muscarone was the ligand there were 21 pairs of values and the ratio of the mean value of the Mu^+ saturation values to the corresponding α-bungarotoxin saturations was 0.996 ± 0.106 (SD). It seems evident that each mole of decamethonium occupies the same number of receptors as does a mole of muscarone and not twice that number as would be required if the ends of ClO^{2+} were bound to two different receptor groups.

DISCUSSION

The unifying concept of almost all the experiments in this paper is that the data can be represented quantitatively by the general thermodynamically valid Eq. [2] where the appropriate electrovalencies have been inserted. Moreover, it is the high selectivity, especially for the counter ions, that has enabled us to represent the data with binary equations rather than having to contend with more than two competing ions at the same time.

Sodium

We have not been able to find evidence that Na^+ can compete for the receptor's active site under any conditions.

Potassium

Extensive washing with Na^+- and K^+-free buffer enabled the receptor to exchange one Mg^{2+} ion for two muscarone ions. The receptor could then be changed back to monovalent behavior by simply adding 5 mM K^+, but not by 5 mM Na^+. In the monovalent state the receptor's active site seems specific for K^+.

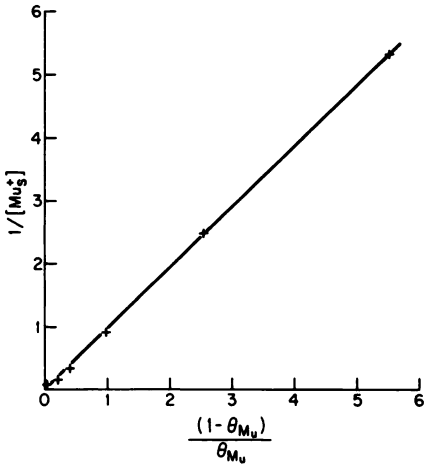

FIG. 7. *Plot of $1/[Mu_s^+]$ against $(1-\theta_{Mu})/\theta_{Mu}$ (Eq. [6], n = 1) at 40 mM Mg^{2+} and 60 mM Ca^{2+} showing exchange of one monovalent ion for each muscarone ion*

The receptor is behaving monovalently. At 5 mM Ca^{2+} two muscarone ions exchanged for one bivalent magnesium ion and the receptor was bivalent. The high calcium concentration caused the receptor to change its electrovalence from 2 to 1.

Calcium

Using the original preparation of particulates containing 5 mM concentrations of both Mg^{2+} and Ca^{2+}, each ClO^{2+} ion exchanges for one Mg^{2+} ion. As the calcium ion concentration is raised, the receptor changes from divalent counter ion exchange to monovalent exchange between 5 and 15 mM Ca^{2+} and maintains this up to 75 mM Ca^{2+}.

Using muscarone as an agonist, the receptors in the original particulate preparation exchanged monovalent ions for the ligand. They could, however, be caused to exchange divalent counter ions by extensive washing with monovalent cation-free solutions, provided Mg^{2+} was present at 5 mM or higher. An effort was made to induce Ca^{2+} to act as a counter ion for Mu^{+}. Using the well-washed particulates, with no added Na^{+}, K^{+}, or Mg^{2+}, the Ca^{2+} ion was unable to act as a divalent counter ion. So there is no evidence using either ClO^{2+} or Mu^{+} that calcium can compete for the receptor recognition site.

To further determine the effects of Ca^{2+}, the well-washed particulates were used at a Mg^{2+} concentration of 40 mM, where the receptor is known to behave divalently and exchange Mg^{2+}. The addition of 60 mM Ca^{2+} caused the receptor to behave monovalently, and Ca^{2+} had an effect similar to its action on ClO^{2+}. In all our experiments, irrespective of the agonist, high Ca^{2+} concentrations seem incompatible with divalent behavior, by the receptor.

Magnesium

Experiments on living muscle operating under physiological conditions provided evidence (2) that the receptors in the resting state occurred in pairs occupied by either a magnesium or a calcium ion. In the present experiments no evidence for Ca^{2+} bound at the recognition site was found. When ClO^{2+} was used as an agonist the receptors behaved as if the ion normally occupying the receptors was Mg^{2+}, and when Mu^{+} was used the receptors behaved as if K^{+} was the normal occupant. The monovalent state of the receptors in the muscarone experiments may have been induced by the ligand, but why ClO^{2+} does not do this is not clear at present.

That the physiological ions have markedly different and almost no overlapping specificities and functions rules out any explanations of their effects based on Gouy–Chapman double layer-type effects or ionic strength or dielectric constant change and makes possible the treatment of our data by simple binary exchange equations.

Positive and negative cooperativity, the Hill equation, multiple binding sites, and various allosteric models have been examined individually and in various combinations. The only approach that we have been able to apply generally and consistently is derived at least in part from the physical chemistry of ion exchange, and this has proved to be basic to any other approaches we have made.

The Constancy of the Selectivity Constants

In ligand binding curves where all inorganic ions were constant, the selectivity constants did not change. In experiments at increasing Mg^{2+} concentrations, with either ClO^{2+} or Mu^{+} as ligand, and where Mg^{2+} was the exchanging counter ion, the selectivity constants also held constant. It would seem that the Mg^{2+} ion binds to the recognition site but does not have any other marked effect on the receptor macromolecule. Increasing the sodium ion concentration to 250 mM did not affect either the magnitude or the constancy of the ClO^{2+}, Mg^{2+} selectivity constant appreciably.

Using the original preparation of receptors, the receptor exchanged monovalent ions, presumably K^{+} for Mu^{+}, but increasing the Mg^{2+} concentration from 5 to 60 mM caused close to a threefold increase in the selectivity constant. Where Ca^{2+} was increased, using either ClO^{2+} or Mu^{+}, the selectivity constant was increased by Ca^{2+} acting at a nonagonist recognition site.

In all our experiments the receptor population behaved as a homogeneous group. There was no evidence for receptor desensitization even in experiments on the effect of time on ligand binding (9).

As pointed out earlier (2) the concept of a single bivalent inorganic ion being displaced by two monovalent quaternaries, as the initiation of depolarization, suggests a model of the process. In the resting state the Mg^{2+} ion is in contact with and electrostatically bound to two receptor oxyanions close together. As the Mg^{2+} ion is displaced by two ACh^{+} ions, the binding energy of the Mg^{2+} ion is replaced by a repulsion between the newly formed receptor ACh^{+} dipoles and a large contribution from the rehydration of the Mg^{2+} ion is added. The energy made available by the ion exchange now causes the receptor groups to move apart and the previously divalent receptor site is replaced by a site with two monovalent groups. That is, the electrovalence of the receptor site changes from 2 to 1.

REFERENCES

1. Taylor, D. B., J. Steinborn and T. C. Lu. Ion exchange processes at the neuromuscular junction of voluntary muscle. *J. Pharmacol. Exp. Ther.* **175**: 213–227 (1970).
2. Taylor, D. B. The role of inorganic ions in ion exchange processes at the cholinergic receptor of voluntary muscle. *J. Pharmacol. Exp. Ther.* **186**: 537–551 (1973).
3. Helfferich, F. *Ion Exchange*. McGraw-Hill, New York (1962).
4. Gill, E. W., and H. R. Ing. The problem of hexamethonium. *Il Farmaco Ed. Sci.* **13**: 244–256 (1958).
5. Taylor, D. B., K. P. Callahan and I. Shaikh. Synthesis of a bifunctional coordination complex of osmium with curariform activity. *J. Med. Chem.* **18**: 1088–1094 (1975).
6. Franklim, G. I., and L. T. Potter. Studies of the binding of α-bungarotoxin to membrane bound and detergent-dispersed acetylcholine receptors from Torpedo electric tissue. *FEBS Lett.* **28**: 101–106 (1972).
7. Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izawa and R. M. M. Singh. Hydrogen ion buffers for biological research. *Biochemistry* **5**: 467–477 (1966).
8. Mudryi, F. V., V. V. Simirnov and S. B. Zotov. Synthesis of a mixture of racemates of muscarine and its stereoisomers. *Khim. Geterotsikl Soedin* **5**: 579–581 (1970).
9. Spivak, C. E. *Cation Exchange Equilibria at a Particulate-Bound Nicotinic Acetylcholine Receptor*, Ph.D. thesis. Department of Pharmacology, UCLA School of Medicine; University Microfilms International, Ann Arbor, Mich. 48106 (1978).
10. Bollinger, H., and C. H. Eugster. Konfigurative Zusammenhänge in der Muscarinreike; Chiralität des *epi-*, *allo-* und *epiallo*-Muscarins, des Muscarons und *allo*-muscarons. Zur Biogenes des Muscarins. *Helv. Chim. Acta* **54**: 2704–2730 (1971).
11. Hardegger, E., H. Corrode and N. Chariatte, Über Muscarin. Synthese von Salzen des DL-Muscarons und DL-Allomuscarons uber die Norbasen. *Helv. Chim. Acta* **44**: 1193–1198 (1961).
12. O'Brien, R. D., and L. P. Gilmore. A muscarone-binding material in electro-

plax and its relation to the acetylcholine receptor. I. Centrifugal assay. *Proc. Nat. Acad. Sci. USA* **63**: 496–503 (1969).
13. Vogel, Z., A. J. Sytkowski and M. W. Nirenberg. Acetylcholine receptors of muscle grown *in vitro*. *Proc. Nat. Acad. Sci. USA* **69**: 3180–3184 (1972).
14. Colquhoun, D., and H. P. Rang. Effect of inhibitors on the binding of iodinate α-bungarotoxin to acetylcholine receptors in rat muscle. *Mol. Pharmacol.* **12**: 519–535 (1976).
15. Spivak, C. E., and D. B. Taylor. A filter assay for binding of labeled ligands to membrane-bound receptors. *Anal. Biochem.* **77**: 274–279 (1977).
16. Dixon, W. J. (Ed.). *BMS Biomedical Computer Program*. University of California Press, Los Angeles (1973).

Send reprint requests to: Dermot B. Taylor, Department of Pharmacology, UCLA School of Medicine, Los Angeles, Calif. 90024.