Equilibrium Properties of Mouse-*Torpedo* Acetylcholine Receptor Hybrids Expressed in *Xenopus* Oocytes

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ABSTRACT This study used messenger RNA encoding each subunit (α, β, γ, and δ) of the nicotinic acetylcholine (ACh) receptor from mouse BC3H-1 cells and from *Torpedo* electric organ. The mRNA was synthesized in vitro by transcription with SP6 polymerase from cDNA clones. All 16 possible combinations that include one mRNA for each of α, β, γ, and δ were injected into oocytes. After allowing 2–3 d for translation and assembly, we assayed each oocyte for (*a*) receptor assembly, measured by the binding of [^{125}I]α-bungarotoxin to the oocyte surface, and (*b*) ACh-induced conductance, measured under voltage clamp at various membrane potentials. All combinations yielded detectable assembly (30-fold range among different combinations) and ACh-induced conductances (>1,000-fold range at 1 μM). On double-logarithmic coordinates, the dose-response relations all had a slope near 2 for low concentrations of ACh. Data were corrected for variations in efficiency of translation among identically injected oocytes by expressing ACh-induced conductance per femtomole of α-bungarotoxin–binding sites. Five combinations were tested for *d*-tubocurarine inhibition by the dose-ratio method; the apparent dissociation constant ranged from 0.08 to 0.27 μM. Matched responses and geometric means are used for describing the effects of changing a particular subunit (mouse vs. *Torpedo*) while maintaining the identity of the other subunits. A dramatic subunit-specific effect is that of the β subunit on voltage sensitivity of the response: $g_{ACh}(-90\ mV)/g_{ACh}(+30\ mV)$ is always at least 1, but this ratio increases by an average of 3.5-fold if β_M replaces β_T. Also, combinations including γ_T or δ_M usually produce greater receptor assembly than combinations including the homologous subunit from the other species. Finally, E_{ACh} is defined as the concentration of ACh inducing 1 μS/fmol at −60 mV; E_{ACh} is consistently lower for α_M. We conclude that receptor assembly, voltage sensitivity, and E_{ACh} are governed by different properties.

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Volume 90 October 1987 553–573

INTRODUCTION

All four subunits of the nicotinic acetylcholine (ACh) receptor have been isolated and sequenced as cDNA clones from muscle and electric organ for several species. This accomplishment has encouraged several theoretical and experimental studies dealing with the relationship between structure and function of this membrane protein (Stroud and Finer-Moore, 1985). Important unanswered questions concern the nature of the coupling between agonist binding and channel activation, structure and selectivity properties of the channel itself, and details of open-channel and closed-channel blockade.

One way to test such theories exploits the fact that the cDNA clones themselves can be combined and mutated in various ways to encode novel receptors. At present, it appears that the most appropriate functional assay for such manipulations consists of in vitro RNA synthesis using a viral RNA polymerase system (Melton et al., 1984; Krieg and Melton, 1984; Mishina et al., 1985; White et al., 1985), followed by injection into *Xenopus* oocytes and by electrophysiological measurements on the newly expressed receptors (Gurdon et al., 1971; Sumikawa et al., 1981; Barnard et al., 1982; Mishina et al., 1984, 1985; White et al., 1985; Sakmann et al., 1985; Methfessel et al., 1986). The more recent work shows an excellent quantitative correspondence between the characteristics of the receptors expressed in oocytes and those in the native tissue; this correspondence extends to functional stoichiometry, desensitization, single-channel conductance and lifetime, and voltage sensitivity (White et al., 1985; Sakmann et al., 1985; Methfessel et al., 1986). The faithful translation and assembly suggest that useful insights will indeed be obtained from the study of modified receptors expressed in *Xenopus* oocytes. Our study therefore extends that of White et al. (1985) and of Sakmann et al. (1985) on interspecies hybrid receptors. We have studied all 16 possible combinations of mouse and *Torpedo* α, β, γ, and δ subunits.

This report is limited to the equilibrium properties of these hybrid receptors: Hill coefficient, steady state activation, voltage sensitivity, and blockade by *d*-tubocurarine. Because we wanted to concentrate on the receptor function rather than on the biosynthesis, assembly, or membrane insertion, α-bungarotoxin binding has been measured on the same oocytes and most of results are expressed on a "per receptor" basis. A preliminary analysis of some of the data has been published (Mayne et al., 1987) and has also appeared in abstract form (Yoshii et al., 1987).

METHODS

Plasmids

A cDNA clone for the mouse ACh receptor α subunit precursor was generously provided by Dr. J. P. Merlie (Washington University, St. Louis, MO) (Isenberg et al., 1986) and was transferred to the pGEM1 vector (Promega Biotec, Madison, WI) containing the SP6 promoter. Two sequenced cDNA clones covering the 5′ and 3′ portions of the mouse ACh receptor β subunit were also provided by Dr. Merlie in the vector M13mp18. A composite cDNA sequence coding for the entire β subunit precursor was constructed from restriction fragments. Both plasmids were digested with SacII, which has a unique recognition site in the β sequence, as well as with PvuI, which cuts once in the vector but not in the β sequence. The desired DNA fragments were isolated by agarose gel electro-

phoresis and treated with T4 DNA ligase. The sequence was confirmed by the dideoxy nucleotide technique (Sanger et al., 1977). The complete protein-coding cDNA sequence was then recloned into the SP6 vector pGEM2 (Promega Biotec).

The cDNA clones for the mouse ACh receptor γ and δ subunits were isolated at the California Institute of Technology (LaPolla et al., 1985; Yu et al., 1986) and recloned into the vectors pSP65 (Melton et al., 1984) and pSP64T (Krieg and Melton, 1984), respectively. The clones for the *Torpedo* ACh receptor subunits were as described by White et al. (1985).

In Vitro Transcription

The protocol of White et al. (1985) was used for in vitro transcription of ACh receptor mRNAs. The linearized DNA templates were present at a concentration of 30 μg/ml and the SP6 RNA polymerase at 300 U/ml. The reaction was carried out for 2 h at 37°C, followed by 10 min incubation with 2 U/ml ribonuclease-free DNAase. Unincorporated nucleotide precursors were removed by spun column (Penefsky, 1977). The RNA was extracted once with phenol-chloroform and twice with chloroform, precipitated twice with ethanol, and redissolved in distilled water (1 mg/ml) for microinjection into oocytes.

Preparation of Oocytes and RNA Injection

Mature female *Xenopus* were obtained from commercial sources. They were anesthetized by immersion in water containing 0.17% tricaine (3-aminobenzoic acid ethyl ester). An incision was made in the abdomen and a portion of the ovary was removed and placed in 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES-NaOH, pH 7.5. Follicle cells were removed by incubating the tissue in this solution containing collagenase (type IA, Sigma Chemical Co., St. Louis, MO), 2 mg/ml, for 3 h at room temperature.

50 nl of the mRNA solution was injected into the ooplasm of stage V and VI oocytes (Dumont, 1972) with a microdispenser (Drummond Scientific Co., Broomall, PA) through a needle of tip diameter ~20 μm. The oocytes were then transferred to Barth's medium supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Oocytes were incubated at room temperature for 48–72 h.

Electrophysiology

Individual oocytes were transferred to a recording chamber (volume, 0.3 ml) continually perfused by a system of valves and stopcocks, at a rate of 3.5 ml/min. The Ringer solution contained 96 mM NaCl, 2 mM KCl, 0.3 mM $CaCl_2$, 1 mM $MgCl_2$, 0.3 μM atropine sulfate, and 5 mM HEPES-NaOH, pH 7.5, plus ACh as indicated.

We employed a two-microelectrode voltage-clamp circuit (Axoclamp-2A, Axon Instruments, Burlingame, CA). Electrodes were filled with 3 M KCl and had tip resistances of 0.5–1 MΩ. Oocytes were continually clamped to a membrane potential of −60 mV and 100-ms steps were generated to various test potentials using standard instrumentation (Sheridan and Lester, 1977; Kegel et al., 1985). Oocytes were typically exposed to each test solution for ~30 s. For the conditions of these experiments, holding currents reached a plateau with essentially the time course of the fluid change (<10 s) and showed little or no desensitization. Marked desensitization occurs in the presence of higher ACh concentrations or with ACh receptors containing chick α subunits (Yoshii, K., and K. M. Mayne, unpublished data). ACh-induced currents were measured by subtracting voltage-clamp currents in the absence and presence of ACh. All experiments were conducted at room temperature.

Toxin-binding Assay

Oocytes were prewashed for 5 min in 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6, and 1 mg/ml bovine serum albumin. They were then transferred to the

same solution containing 5 nM [^{125}I]α-bungarotoxin (New England Nuclear, Boston, MA) and incubated for 1 h. Oocytes were then washed four times and counted individually in a gamma counter. All incubations were done at room temperature.

Graphical Presentation of the Data

Figs. 2, 3, and 5–8 represent our solution to the problem of identifying data from the various subunit hybrid combinations. We have devised a system using circular symbols divided into sectors of equal area. Each subunit is represented by a unique sector that appears in a constant position for all combinations and for all figures. For this study, there are four subunits; thus, each sector is a quadrant. The α subunit is represented by the upper right quadrant, with the other subunits following in clockwise order according to their Greek letter names.

The shading of a quadrant denotes the source of the subunit. For this study, there are only two sources: mouse is denoted by an empty quadrant (mnemonic: eMpty for Mouse) and *Torpedo* by a filled quadrant.

The symbols are shown superimposed on the appropriate error bars. The system may be generally useful for the following two reasons: (*a*) it can be used with proteins composed of any (small) number of subunits; (*b*) shading patterns can be complex to denote several sources. Because of the wide range in the data, we have elected to display most of the plots on logarithmic coordinates.

RESULTS

Conductance per α-Bungarotoxin–binding Site: a Measure That Minimizes Variations

Among oocytes from the same ovary receiving identical injections, there was a >10-fold range in the conductance induced by a given concentration of ACh. This variation among oocytes is a general phenomenon in our laboratory and in many others that study channels and receptors induced by foreign RNA; it is seen, for instance, with Ca and Na channels from rat RNA (Dascal et al., 1986; Goldin et al., 1986; Leonard et al., 1987). In the present study, we attempted to draw functional conclusions despite the presence of these large variations. For this purpose, we subjected individual oocytes to two experimental manipulations: we measured both ACh-induced conductance and surface α-bungarotoxin binding. The large variations in ACh-induced conductance were indeed accompanied by large variations in the α-bungarotoxin binding, and there was a good correlation between the two parameters with each of the combinations studied. An example is given in Fig. 1 for the case of all-*Torpedo* receptors. We therefore express most of the conductance data with a normalization to the number of α-bungarotoxin–binding sites. This ratio (microsiemens per femtomole) has a coefficient of variation of ~30% for all cells injected with a given combination of subunits.

We also found that the average microsiemens per femtomole values varied little among oocytes from different frogs, typically by only 30%. Nonetheless, the data presented in this article were all gathered from a single frog's oocytes, tested over a period of 1 wk.

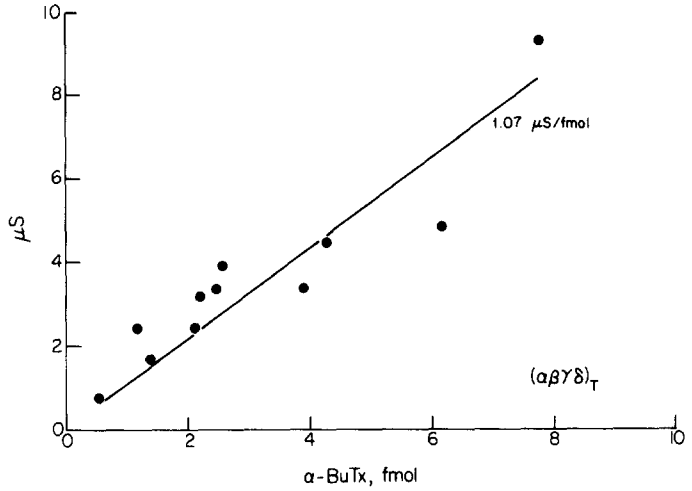

FIGURE 1. Binding and conductance compared for 11 individual oocytes from the same ovary that received identical injections with $(\alpha\beta\gamma\delta)_T$ RNA. ACh-induced conductance was measured at −60 mV. α-Butx, α-bungarotoxin. The line is a least-squares fit to the data, constrained to pass through the origin, and has a slope of 1.07 μS/fmol.

Range of the Data

Assembly. Table I summarizes several aspects of the measurements on each combination. The assembly of receptors is simply expressed as the number of binding sites for α-bungarotoxin per oocyte. There is a >30-fold variation in the

TABLE I

Summary of Some Parameters for Each of the 16 Combinations

RNAs	α-Bungarotoxin per oocyte	E_{ACh} at −60 mV	$\frac{g(-90)}{g(+30)}$	Hill coefficient	K_{dTC}
$\alpha\beta\gamma\delta$	*fmol*	*μM*			*μM*
MMMM	6.49±0.67 (5)	0.20±0.006 (5)	4.3±0.2 (4)	2.0±0.1 (4)	0.27
MMMT	0.49±0.04 (5)	0.61±0.018 (5)	16.0±2.2 (4)	1.8±0.1 (4)	
MMTM	8.06±0.05 (5)	0.16±0.002 (8)	6.6±1.1 (4)	1.9±0.1 (4)	
MTMM	1.01±0.08 (5)	0.09±0.004 (8)	2.8±0.2 (4)	2.0±0.2 (4)	
TMMM	0.91±0.14 (5)	1.67±0.089 (8)	6.1±0.6 (4)	1.5±0.1 (4)	
MMTT	0.76±0.12 (5)	0.45±0.075 (8)	11.0±0.9 (4)	1.5±0.3 (4)	
MTTM	2.02±0.17 (5)	0.16±0.009 (8)	3.7±0.3 (4)	1.9±0.1 (4)	
MTMT	0.67±0.05 (5)	0.29±0.023 (8)	1.3±0.1 (4)	1.6±0.1 (4)	
TMMT	0.39±0.04 (5)	2.75±0.14 (4)	7.1±1.1 (4)	1.8±0.2 (4)	
TMTM	11.4±1.48 (5)	0.76±0.066 (8)	4.1±0.3 (4)	2.1±0.1 (4)	
TTMM	0.53±0.08 (5)	2.20±0.13 (8)	2.8±0.3 (4)	1.8±0.1 (4)	
MTTT	2.53±0.37 (5)	0.20±0.016 (4)	1.7±0.1 (4)	2.3±0.3 (4)	0.08
TMTT	0.65±0.18 (5)	13.5±0.71 (8)	8.9±2.5 (4)	1.5 (1)	
TTMT	1.36±0.21 (5)	1.33±0.099 (8)	1.0±0.1 (4)	1.5±0.1 (4)	0.19
TTTM	5.45±0.64 (5)	0.29±0.02 (8)	3.0±0.1 (4)	1.9±0.1 (4)	0.19
TTTT	3.48±0.27 (11)	0.96±0.044 (11)	1.6±0.2 (4)	1.8±0.2 (4)	0.16

n is given in parentheses.

average number of α-bungarotoxin–binding sites per oocyte for the various combinations, ranging from 0.39 fmol for the $\alpha_T\beta_M\gamma_M\delta_T$ hybrid to 11.4 fmol for the $\alpha_T\beta_M\gamma_T\delta_M$ hybrid.

Functional efficiency. There was an even larger range in the average ACh-induced conductance. At 1 μM ACh and −60 mV, some combinations—for instance, $(\alpha\beta\gamma\delta)_M$—yielded signals too large for reliable clamping (>5–10 μA); others, such as $\alpha_T\beta_M\gamma_T\delta_T$, yielded signals too small for accurate measurement (<5 nA). This range of $>10^3$ is due to three factors. (*a*) There may be differences in single-channel conductance among the combinations. Single-channel measurements are still incomplete, but the data available for some combinations show little or no difference (Yu et al., 1987). (*b*) There are real differences in the fractional receptor activation produced by a given ACh concentration. If these differences arise primarily from the agonist-receptor interaction, they are amplified by the necessity for activation by two bound agonist molecules and the resultant parabolic dose-response relation. (*c*) Finally, there are differences in the assembly for each combination. As explained above, we account for factor *c* by referring to response per femtomole of bound α-bungarotoxin (Fig. 2). We propose to account for point *b* by using a form of "response matching" similar to the principle of the dose-ratio method for studying antagonist dissociation constants. We therefore define E_{ACh} as the equipotent concentration of ACh that induces 1 μS/fmol of α-bungarotoxin–binding sites. Differences in E_{ACh} can eventually be compared with differences in the binding of competitive antagonists and open-channel blockers. E_{ACh} ranged >100-fold among the combinations tested.

Direction of voltage sensitivity. Many combinations showed nonlinear current-voltage relations for the ACh-induced conductance (Fig. 3). Voltage sensitivity is conveniently abstracted as the ratio of two slope conductances: $g(-90\text{ mV})/g(+30\text{ mV})$. This parameter ranged from unity to ~16. The voltage sensitivity does not vary detectably with ACh concentration in the range tested; the constancy shown in Fig. 4 is typical of all the combinations.

Lack of correlation among function, assembly, and voltage sensitivity. Figs. 5–7 present scatter plots comparing these parameters for the 16 combinations. It is evident that these three parameters have little or no correlation with each other.

Features Common to All Combinations

Functional stoichiometry of the response to ACh is near 2. We have abstracted the functional stoichiometry as the slope of the dose-response relation at low ACh concentration on double-logarithmic coordinates. This slope is near 2 for all of the hybrids (Table I), which suggests that, as usually found for ACh receptors, the open state of the receptor channel is more likely to be associated

FIGURE 2. *(opposite)* Dose-response relations for representative oocytes injected with each of the 16 combinations. In this and subsequent figures, the source of each subunit RNA is represented by the pattern of the symbol. The form of the symbols bears no intended relation to the molecular structure of the receptor. (*A*) Combinations containing mostly or all mouse subunits. (*B*) Combinations containing two mouse and two *Torpedo* subunits. (*C*) Combinations containing mostly or all *Torpedo* subunits.

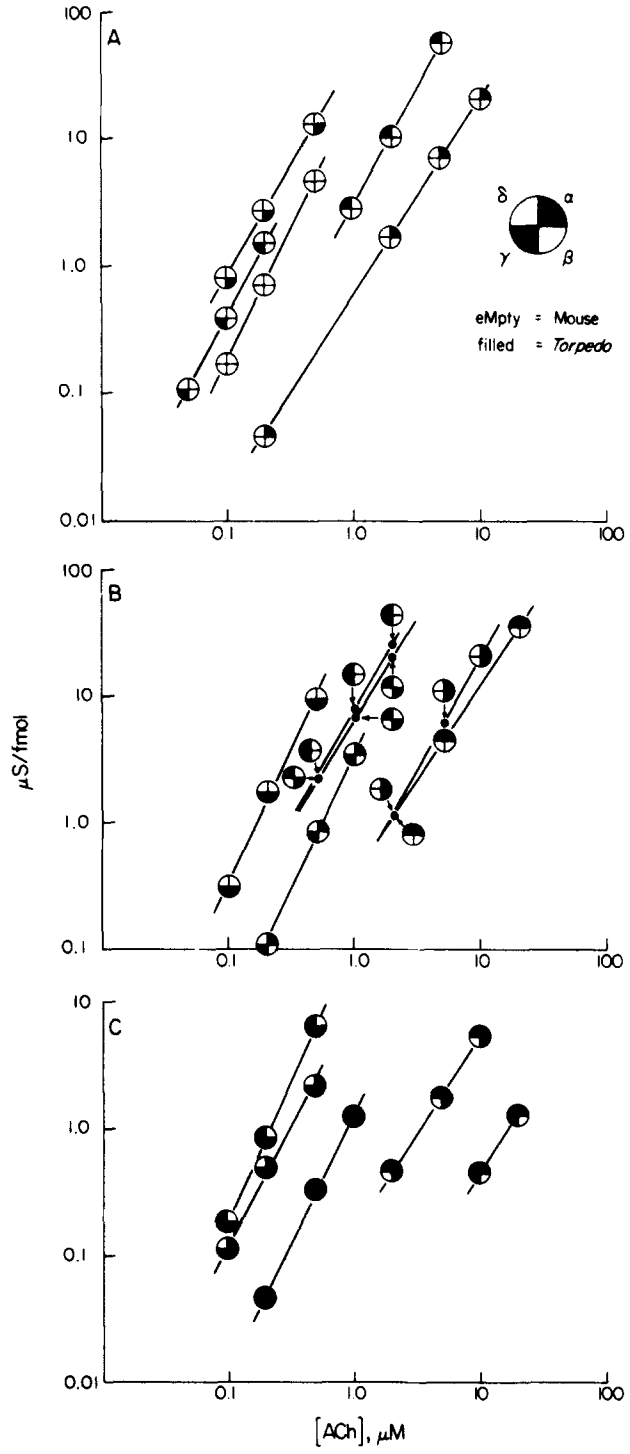
A
B
C
100
10
1.0
0.1
0.01
0.1
1.0
10
100
δ
α
γ
β
eMpty = Mouse
filled = Torpedo
μS/fmol
[ACh], μM

FIGURE 2.

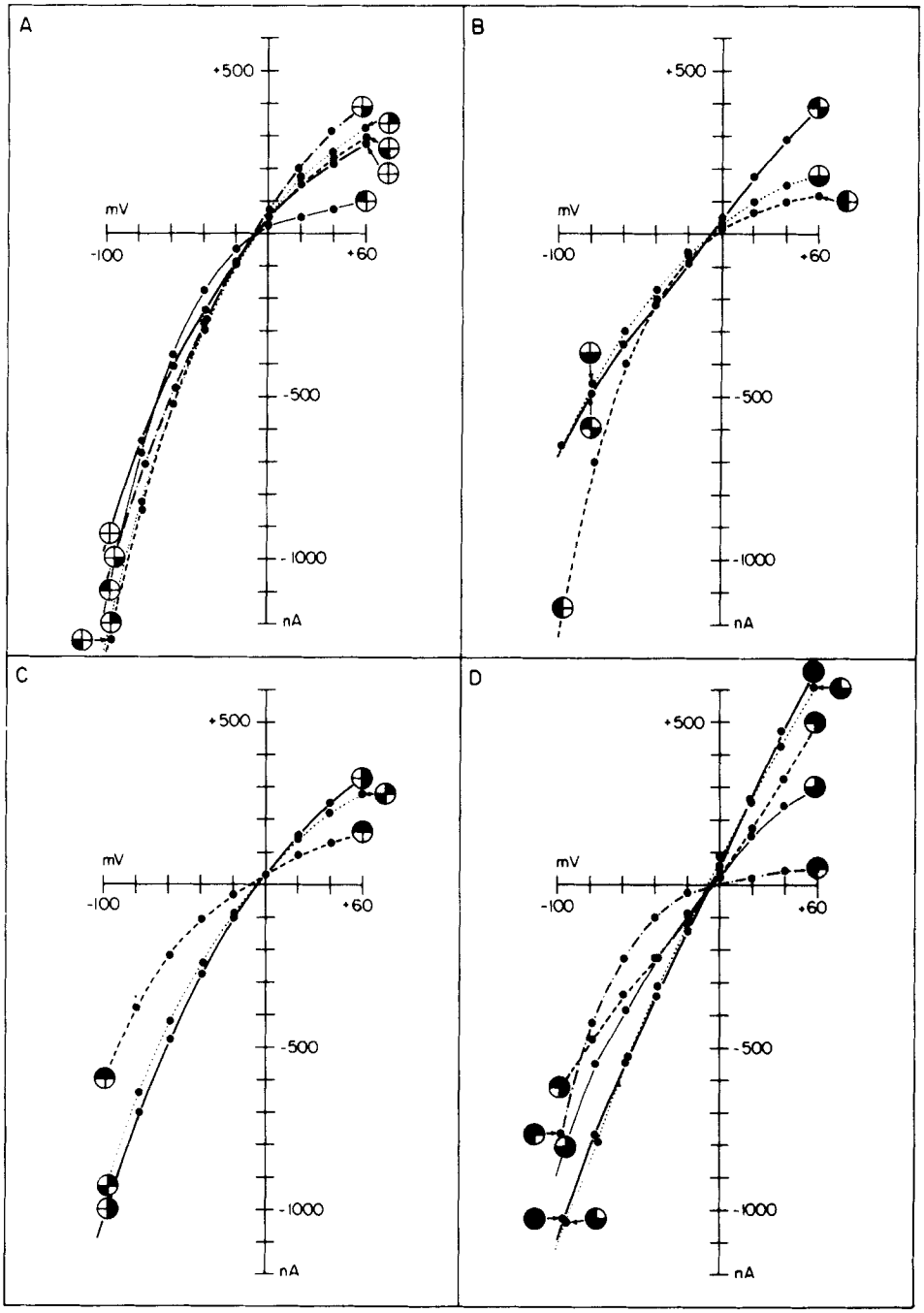

FIGURE 3. Current-voltage relation for representative responses from all 16 combinations. For clarity, each combination is identified only at the extrema of the plot; for the key to the combinations, see Fig. 2. (*A*) Combinations containing mostly or all mouse subunits. (*B* and *C*) Combinations containing two mouse and two *Torpedo* subunits. (*D*) Combinations containing mostly or all *Torpedo* subunits.

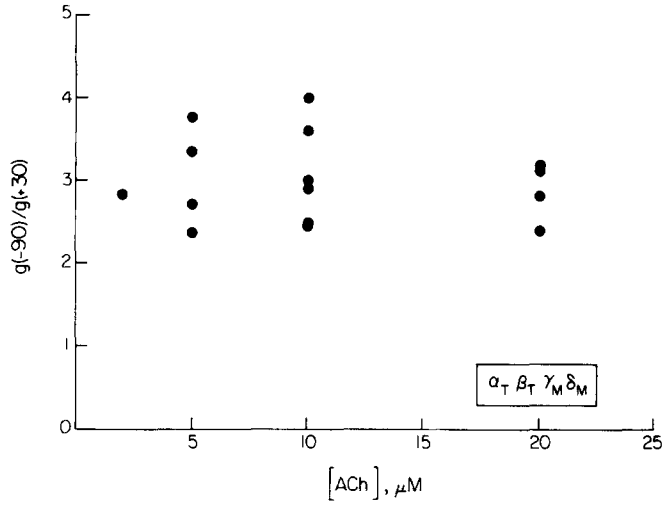

FIGURE 4. Voltage sensitivity vs. ACh concentration for 5 oocytes injected with the $\alpha_T\beta_T\gamma_M\delta_M$ combination. Each oocyte was tested at several ACh concentrations.

with the presence of two bound agonist molecules than with a single one. The slope decreased slightly for the combinations that yielded the smallest conductance per oocyte (Fig. 8). The least-squares linear fit to the data in Fig. 8 has a correlation coefficient of only 0.37; if one omits the combination $\alpha_T\beta_M\gamma_M\delta_T$ (which gave the lowest conductances), the correlation coefficient is 0.54. We doubt that this trend represents a real change in functional stoichiometry; it seems more likely that at the higher ACh concentrations necessary to test these combinations, the dose-response relation was distorted by desensitization, open-channel blockade, or partial saturation.

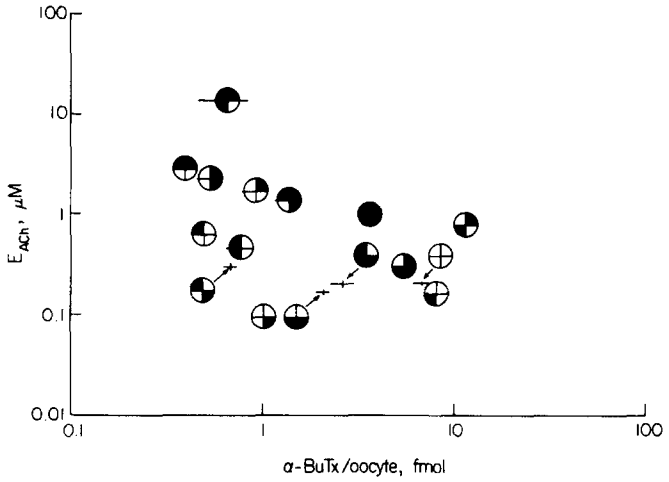

FIGURE 5. Scatter plot of assembly vs. E_{ACh} for all 16 combinations. For the meaning of the symbols, see Fig. 2. In this and subsequent figures, the SEM is shown where it exceeds the size of the symbol.

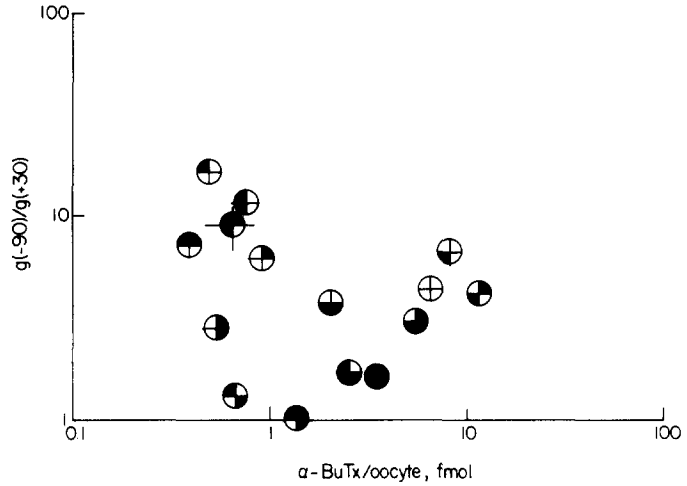

FIGURE 6. Scatter plot of voltage sensitivity vs. assembly. For the meaning of the symbols, see Fig. 2.

Reversal potential. The reversal potential for the agonist-induced currents ranged between −2 and −9 mV for all the combinations tested. There was little or no significant difference among the combinations.

Effects of Individual Subunits: Quantitative Measures

A major purpose of this study is to decide whether the identity of any particular subunit (*Torpedo* vs. mouse) determines a property of the ACh receptor complex. To address this question in a quantifiable way, we introduce several simple measures. The first, the subunit-specific T/M ratio, compares two hybrids that

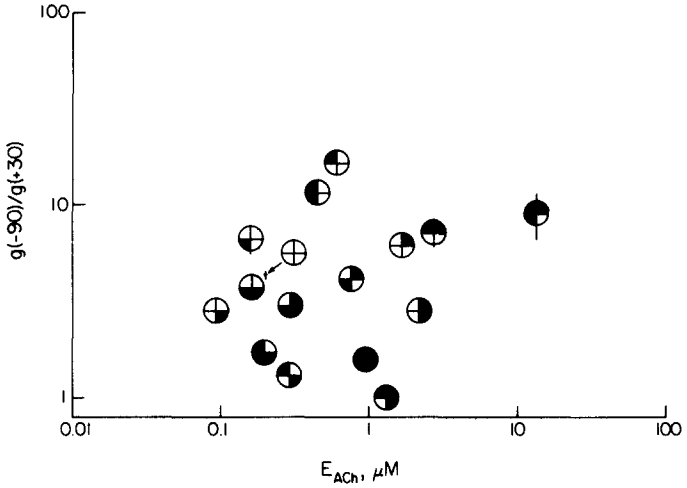

FIGURE 7. Scatter plot of voltage sensitivity vs. E_{ACh}. For the meaning of the symbols, see Fig. 2.

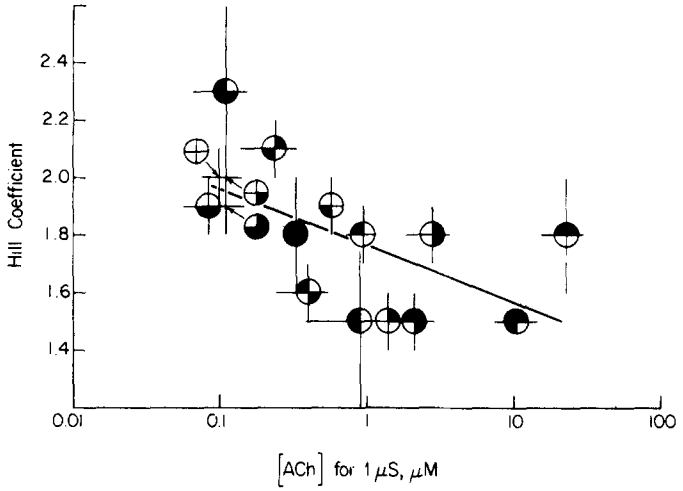

FIGURE 8. Scatter plot of Hill coefficient vs. ACh concentration inducing 1 μS. Note that the abscissa refers to the conductance per oocyte, rather than per femtomole as in previous figures. For the meaning of the symbols, see Fig. 2.

differ by only one subunit. This quantity is simply the ratio between the values for *Torpedo* and mouse. To provide an unbiased measure over the entire dynamic range, we will actually be dealing with logarithms of this ratio and abbreviate it qS. Thus, the qS for assembly associated with the $\alpha_T\beta_T\gamma_T\delta_{T,M}$ pair is -0.195. The qS values for assembly in this study range from -1.25 for the $\alpha_T\beta_M\gamma_T\delta_{T,M}$ pair to 1.1 for the $\alpha_T\beta_M\gamma_{T,M}\delta_M$ pair, and include several values close to zero, e.g., the $\alpha_{T,M}\beta_M\gamma_T\delta_T$ pair, that differ by <20%. We generalize this measure by averaging over the 8-qS values for each subunit; the resulting parameter is the subunit average [qS(subunit)]. This measure (Table II) shows that the identity of the α subunit had no consistent effect upon assembly: $qS(\alpha) = -0.03$. The same lack of effect was noted for β, because $qS(\beta) = -0.005$. In both cases, there were equal numbers of positive and negative values. The *Torpedo* γ subunit appeared to give better assembly, however: $qS(\gamma) = 0.49$ and all eight values were >0. Finally, the mouse δ subunit seems to produce better assembly: $qS(\delta) = -0.45$, with six of eight values negative. The final measure, the global average $qS(\alpha\beta\gamma\delta)$,

TABLE II

Subunit Effects on Assembly: Surface [^{125}I]α-Bungarotoxin Binding, T/M

Subunit	qS	Number > 0 (of 8)
α	−0.03	4
β	−0.005	4
γ	0.49	8
δ	−0.45	2
Global average $qS(\alpha\beta\gamma\delta)$	−0.001	18 of 32

See text for definition of qS.

includes all 32 pairs. For the α-bungarotoxin–binding measurements, qS($\alpha\beta\gamma\delta$) is very close to zero, showing no preferential incorporation of *Torpedo* or mouse proteins.

α_M consistently gives the lowest E_{ACh}. Fig. 2 presents dose-response relations for some of the combinations. Dose-response data were averaged for several oocytes injected with each combination to yield a value for E_{ACh} (Table I); this measure was examined for individual subunit effects by calculated qS values (Table III). The most dramatic effect is clearly that of the α subunit: in all eight comparisons, α_M produced a lower E_{ACh} than did α_T, by an average factor of 6.7.

β_M consistently gives the highest voltage sensitivity. As noted above, most of the combinations display a voltage-sensitive response (Fig. 3): for only one case, $\alpha_T\beta_T\gamma_M\delta_T$, the ratio $g(-90\text{ mV})/g(+30\text{ mV})$ does not differ significantly from unity. Table IV presents the qS ratios for voltage sensitivity and also arranges the various combinations in order of decreasing voltage sensitivity. Clearly the most consistent correlation is with the β subunit: the eight highest voltage sensitivities are all associated with β_M. Also, combinations of β_M with δ_T were more voltage sensitive than those of β_M with δ_M.

TABLE III

Subunit Effects on E_{ACh}, T/M

Subunit	qS	Number > 0 (of 8)
α	0.83	8
β	−0.34	2
γ	−0.10	2
δ	0.40	7
Global average qS($\alpha\beta\gamma\delta$)	0.20	18 of 32

All Four Subunit RNAs Are Required for Substantial Responses

Actually, there are not just 16 possible combinations, but 80, because each subunit could be selected from mouse (M) or *Torpedo* (T), or omitted entirely (0). We have not tested all 64 additional combinations involving one, two, or three omitted subunits, or even all 32 combinations involving only one omitted subunit. The available data all suggest, however, that omission of even a single subunit RNA leads to rather inefficient assembly, so that the data reported with a complete set of subunits in this article would not be distorted by such incomplete receptor complexes.

Omission of δ. Several studies have reported that $\alpha\beta\gamma$ combinations induce functional responses in oocytes (Mishina et al., 1984; White et al., 1985; Boulter et al., 1986). White et al. (1985), using quantities of RNA and ACh concentrations similar to those in the present study, found that the combination $\alpha_T\beta_T\gamma_T\delta_0$ produced ~3% the agonist-induced conductance of $\alpha_T\beta_T\gamma_T\delta_T$ and an even smaller percentage of $\alpha_T\beta_T\gamma_T\delta_M$. Therefore, any δ_0 complexes would have contributed an insignificant amount of conductance to the macroscopic measurements reported here. However, Mayne et al. (1987) report that $\alpha_T\beta_T\gamma_T\delta_0$ assembles much less efficiently than combinations including a δ subunit, so that the ACh-induced conductance per α-bungarotoxin site is ~20% that of $\alpha_T\beta_T\gamma_T\delta_T$.

Omission of β or γ. In the present study, the rather low agonist-induced conductances for some combinations (such as $\alpha_T\beta_M\gamma_T\delta_T$) and the rather low assembly for some combinations (such as $\alpha_T\beta_M\gamma_M\delta_T$) lead to the question, can function or assembly be detected in the absence of β or γ subunits? In one experiment, we tested the combinations $\alpha_T\beta_T\gamma_M\delta_M$, $\alpha_T\beta_M\gamma_M\delta_M$, $\alpha_T\beta_0\gamma_T\delta_T$, and $\alpha_T\beta_0\gamma_M\delta_M$. For the former two combinations, ACh (10 μM) induced conductances of 3–4 μS/fmol at −60 mV; however, the two combinations lacking β yielded little or no detectable assembly (<0.10 fmol) and little or no detectable ACh-induced conductances (<0.2 μS).

We also compared the combinations $\alpha_T\beta_M\gamma_T\delta_T$ and $\alpha_T\beta_M\gamma_0\delta_T$. α-Bungarotoxin binding was 1.80 ± 1.1 and 0.15 ± 0.06 fmol, respectively (mean ± SEM, five oocytes). The conductance induced by ACh (20 μM, −60 mV) was 25.8 and 1.5 μS, respectively. Furthermore, the $g(-90)/g(+30)$ ratios were 10.2 and 1.3, respectively. Boulter et al. (1986) also reported only very small responses with $\alpha_M\beta_M\gamma_0\delta_M$. Thus, there seems to be little contribution by γ_0 combinations to the

TABLE IV

Subunit Effects on Voltage Sensitivity, g(−90)/g(+30), T/M

(*A*) Subunit average qS:

Subunit	qS	Number > 0 (of 8)
α	−0.10	1
β	−0.55	0
γ	0.05	6
δ	−0.02	4
Global average qS(αβγδ)	−0.15	12 of 32

(*B*) Rank order:

	Decreasing sensitivity															
α	M	M	T	T	M	T	M	T	M	T	M	T	M	T	M	T
β	M	M	M	M	M	M	M	M	T	T	T	T	T	T	T	T
γ	M	T	T	M	T	M	M	T	T	T	M	M	T	T	M	M
δ	T	T	T	T	M	M	M	M	M	M	M	M	T	T	T	T

macroscopic conductances. Nonetheless, it must be pointed out that the functional efficiency, in microsiemens per femtomole, seems to be little decreased for the few receptors that are correctly assembled in the absence of the γ subunit (Mayne et al., 1987).

Omission of α. Mayne et al. (1987) report no detectable binding or function when the α subunit is omitted.

α alone. We tested oocytes injected with α_T, α_M, or α_{chick} RNA without other subunit RNAs. In some cases, ACh concentrations of ~500 μM induced detectable conductances (Yu, L., unpublished data). However, no responses were detected at ACh concentrations <50 μM as employed in the present study.

Dissociation Constant for d-Tubocurarine

For five of the combinations, we used the dose-ratio method to measure the apparent dissociation constant for the competitive inhibition by *d*-tubocurarine

(Fig. 9). This method involves assessing the parallel shift in dose-response relations at various inhibitor concentrations; its use for the nicotinic ACh receptor has been frequently discussed (Jenkinson, 1960; D. Armstrong and Lester, 1979; Krouse et al., 1985). Combinations including δ_M showed a small (30%) decrease

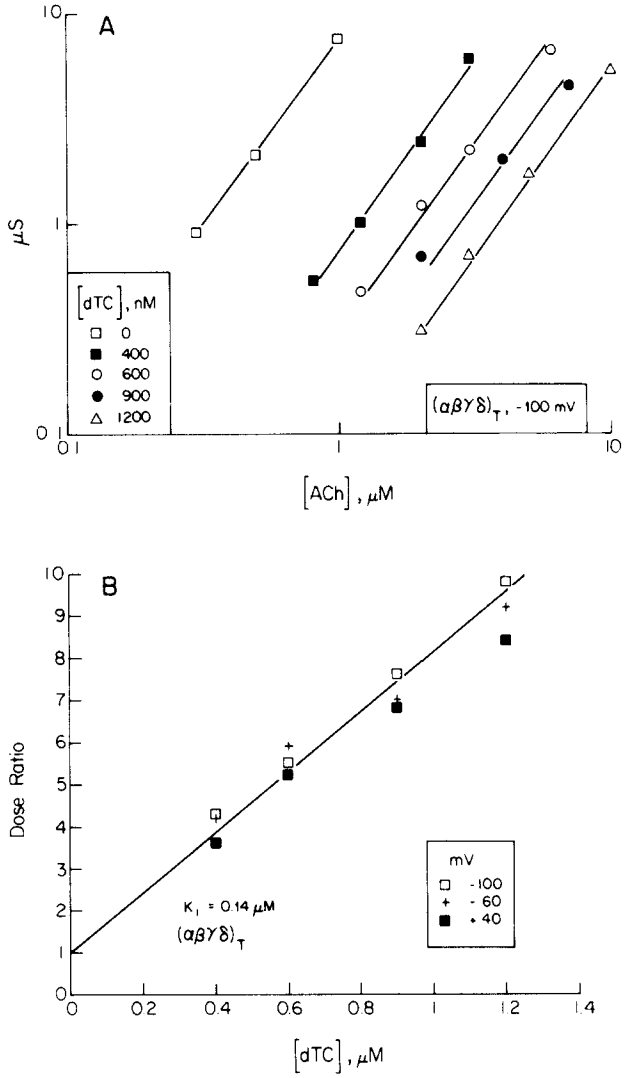

FIGURE 9. Dose-ratio analysis of inhibition by *d*-tubocurarine (dTC). (*A*) Parallel shift of dose-response curves. (*B*) Dose-ratio plots at several voltages.

in the dose-ratio slope at −100 mV, probably because δ_M produces channels with a greater lifetime (Sakmann et al., 1985; Leonard, R. J., L. Yu, C. Labarca, N. Davidson, and H. A. Lester, unpublished data) and thus a greater sensitivity to open-channel block by *d*-tubocurarine. Therefore, Table I presents the values for potentials between −60 and +40 mV. The differences in K_i cover a range of

3.5-fold, roughly equal to the range of variations in E_{ACh}. The two measures (K_i and E_{ACh}) do not seem to vary together, however. The dissociation constant for agonists often depends on voltage (Lester et al., 1978); we therefore recalculated the value of E_{ACh} for +30 mV. There was still no meaningful correlation with the K_i values.

DISCUSSION

In this study, we surveyed the properties of all 16 possible hybrid ACh receptors involving a complete set of α, β, γ, and δ subunits from two species. A major conclusion is that different subunits determine the greatest assembly of surface receptors (γ_T and δ_M), the lowest E_{ACh} (α_M), and the greatest voltage sensitivity (β_M). It must therefore be concluded that receptor assembly, E_{ACh}, and voltage sensitivity are governed by different properties.

E_{ACh} Is Roughly as Expected

We adapted the somewhat arbitrary response of 1 μS/fmol for comparing equipotent ACh concentrations among the hybrid combinations. Because the dose-response relations are nearly parallel in the range studied here, this choice for E_{ACh} does not strongly influence the conclusions concerning relative functional efficiencies. It should be pointed out that 1 μS/fmol = 3.3×10^{-3} pS/ACh receptor (assuming two α-bungarotoxin–binding sites per receptor). Assuming an open-channel conductance of 40 pS, this in turn corresponds to an average steady state open probability of 8×10^{-5}. We note that this value is roughly in agreement with expectations from physiological measurements of dose-response relations for mouse and *Torpedo* receptors. For a Hill coefficient of 2, an open probability of 8×10^{-5} corresponds to an agonist concentration of 9×10^{-3} times the half-maximal concentration. The lowest concentrations giving this value are in the range 0.1–1 μM (see Table I). Taking the value of 0.5 μM as an average, this would suggest that half-maximal activation would occur at ~50 μM ACh. This value is in good agreement with actual measurements on BC3H-1 cells (Sine and Taylor, 1980; Brett et al., 1986) and on *Torpedo* membrane fragments (Neubig and Cohen, 1980; Heidmann et al., 1983), although it should be pointed out that the half-maximal concentration is liable to be voltage sensitive in cases where the response is voltage sensitive (as observed here for nearly all combinations).

Blockade by d-Tubocurarine Is Roughly as Expected

There have been no previous quantitative electrophysiological studies of *d*-tubocurarine blockade at *Torpedo* ACh receptors. The K_i values that we found (0.08–0.27 μM for all five mouse-*Torpedo* combinations tested) are close to the ranges usually found for mouse muscle (0.04–0.15 μM; Pennefather and Quastel, 1981), frog muscle (0.39–0.43 μM; Jenkinson, 1960; Adams, 1975), and *Electrophorus* electroplaques (0.2 μM; Lester et al., 1975). It is unclear how the present values should be interpreted in terms of the existing binding studies on *Torpedo* electric organ (Neubig and Cohen, 1979) and BC3H-1 cells, the source of the mouse clones used here (Sine and Taylor, 1981), both of which suggest that the receptor has two distinguishable sites for competitive antagonist binding (see

discussions by Pennefather and Quastel, 1981; Krouse et al., 1985). Regardless of the detailed binding mechanism, however, we found only a modest range in K_i among the five combinations tested. It will be interesting to see how K_i varies for the other 11 combinations to be tested.

The β Subunit and Voltage Sensitivity

Another clear result of this study is that the $g(-90)/g(+30)$ ratio is at least unity; where present, voltage sensitivity is always in the same direction. Furthermore, the voltage sensitivity depends on the identity of the β subunit. The subunit qS was less than zero for all $\alpha\beta_{T,M}\gamma\delta$ pairs, yielding a subunit average qS of -0.55. Furthermore, all eight combinations containing β_M were more voltage sensitive than all eight combinations involving β_T. On the average, the mouse β subunit renders a combination 3.55 times more voltage sensitive than does the *Torpedo* β subunit.

According to present concepts, a voltage-sensitive ACh-induced conductance could arise from voltage sensitivity in at least one of three separate parameters: (*a*) the single-channel conductance, (*b*) the rate constant for channel closing, or (*c*) the rate constant for opening. One hopes for a decisive assignment of the voltage sensitivity to one of these three parameters. The first two of the parameters, and probably the third as well, can be assessed with single-channel recordings; these are now under way in our laboratory (Yu, 1987; Yu et al., 1987). Preliminary recordings are now available for about half the combinations in symmetrical solutions. The channel conductance is linear. Of the two rate constants, the closing rate depends more strongly on voltage.

In a study of some calf-*Torpedo* ACh receptor hybrids, Sakmann et al. (1985) found no evidence that the single-channel conductance is voltage sensitive. The combination $(\alpha\beta\gamma)_T\delta_{calf}$, but not the $\alpha_{calf}(\beta\gamma\delta)_T$ combination, showed the same channel duration (including voltage sensitivity) as the $(\alpha\beta\gamma\delta)_{calf}$ combination. Quantitative data were not reported for other combinations. On the basis of these data, it was suggested that the δ subunit governs the voltage sensitivity of the closing rate constant. Our data are not strictly comparable to the study cited, because (*a*) we studied mouse-*Torpedo* rather than calf-*Torpedo* hybrids, and (*b*) as noted above, our data do not address the channel duration alone. We do find a more subtle effect of the δ subunit on the equilibrium voltage sensitivity of the ACh-induced conductance. The subunit average qS (δ) for $g(-90)/g(+30)$ is nearly zero, with four positive and four negative values; but the four most voltage-sensitive combinations involving δ_M also involve β_M, and the four least voltage-sensitive combinations involving δ_M also involve β_T. This β-δ interaction is consistent with the suggestion that the β and δ subunits contact each other in the receptor oligomer (Karlin et al., 1983).

A final difference between the two studies is that we used ~10-fold more RNA per oocyte. We have found (unpublished results) that the combinations differ widely with respect to the amount of RNA that yields half-maximal assembly and response. Thus, we may have been able to detect responses from some combinations that might give undetectable responses with the smaller injections used by Sakmann et al. (1985). However, we have presented evidence that even the "worst" combinations were not substantially aided by endogenous subunits or by

incomplete complexes: all four subunit RNAs were required for substantial responses.

Origin of Voltage Sensitivity

Voltage-sensitive responses presumably have their origin in coulombic interactions. Several specific types of interaction can be envisioned. Among these are interactions between the dipole moment of the channel and the membrane field, between permeant ions and a barrier or binding site in the channel, or between ions bathing the membrane and binding sites on the receptor. At present, there is no strong basis for choosing among these possibilities. However, because the voltage sensitivity is also in the same direction, one might tentatively conclude

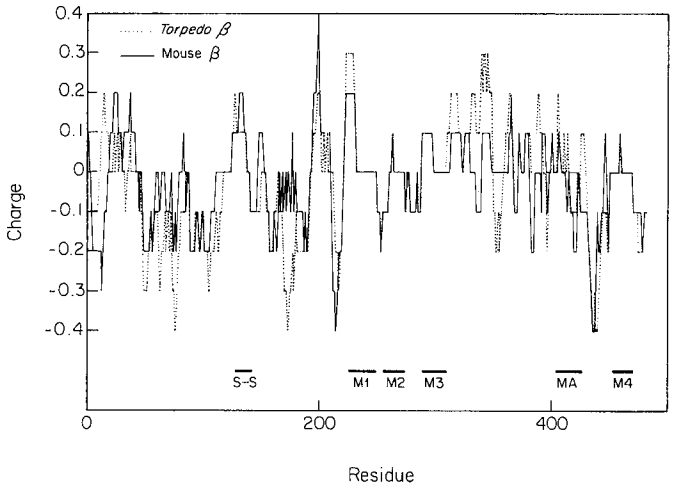

FIGURE 10. Charge distributions on mature mouse and *Torpedo* β subunits. The plot was generated by assigning a value of +1 to Lys and Arg, −1 to Glu and Asp, and 0 to other residues. The data were then subjected to a running average of 10 residues; gaps were then introduced to provide a good homology. The bars give positions of the disulfide bond in the putative extracellular region (S-S), putative α-helices M1–M4, and the putative amphipathic helix MA (Stroud and Finer-Moore, 1985).

that the coulombic interaction energy has the same sign for all of the 16 combinations (except for the single combination $\alpha_T\beta_T\gamma_M\delta_T$, for which the energy is presumably zero). A dipole moment (Magleby and Stevens, 1972*a*, *b*) could presumably have either sign, in disagreement with this idea. However, if the coulombic interaction involves an ion in the solution and a binding site on the receptor (C. M. Armstrong and Matteson, 1986), the energy would always have the same sign.

The most voltage-sensitive combination showed a ratio of 16 over a voltage range of 120 mV. This would correspond to an e-fold change per 43 mV. Such a variation could be caused by the motion of a single charge halfway through the membrane. The binding site might be either weaker or nearer the membrane surface for the more weakly voltage-dependent combinations.

Regardless of the detailed mechanism, it seems difficult to escape the conclusion that the voltage sensitivity difference between the mouse and *Torpedo* β subunits arises because of a difference in the charges on their amino-acid residues. One possibility is that the mouse and *Torpedo* subunits fold in exactly the same way, so that there are one or two regions with charge differences. Fig. 10, which presents a simple superposition of the charge distributions, discloses several such regions. A more subtle possibility is that the mouse and *Torpedo* β subunits fold differently owing to differences in nonpolar interactions, again resulting in three-dimensional structures with different charge distributions. This possibility would be more difficult to analyze from primary sequence data alone. The problem calls for further analysis using chimeric and mutant subunits (Imoto et al., 1986).

The α Subunit and E_{ACh}

The dose-response data show that all eight combinations involving α_M produced a lower E_{ACh} than did all eight combinations involving α_T. The average qS(α) was 0.83, corresponding to a ratio of 6.7. The Hill coefficient is near 2; for a given ACh concentration, the conductances differed by a geometrical mean ratio of 45. Of the three possible parameters described above, differences in single-channel conductance are likely to account for at most a factor of 1.3 (Sakmann et al., 1985; Imoto et al., 1986). Substitution of α_{calf} for α_T yields only a fourfold increase in channel duration (Sakmann et al., 1985). It is therefore difficult to avoid the prediction that kinetic measurements will reveal a much larger rate constant for channel opening in hybrids containing α_M. Because of the extensive homologies between α subunits from different species (Stroud and Finer-Moore, 1985), chimeric subunits should be especially interesting.

We thank Dr. J. P. Merlie for providing clones and Dr. Reid Leonard for discussion.

This research was supported by grants from the Muscular Dystrophy Association and from the National Institutes of Health (NS-11752).

Original version received 10 March 1987 and accepted version received 16 June 1987.

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