Equilibrium Properties of Mouse-\textit{Torpedo} Acetylcholine Receptor Hybrids Expressed in \textit{Xenopus} Oocytes

Kiyonori Yoshii, Lei Yu, Katharine Mixter Mayne, Norman Davidson, and Henry A. Lester

From the Division of Biology, California Institute of Technology, Pasadena, California 91125

\textbf{A B S T R A C T} This study used messenger RNA encoding each subunit (a, \(\beta\), \(\gamma\), and \(\delta\)) of the nicotinic acetylcholine (ACh) receptor from mouse BC3H-1 cells and from \textit{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 a, \(\beta\), \(\gamma\), and \(\delta\) 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 \(^{125}\text{I}\)-bungarotoxin to the oocyte surface, and (b) ACh-induced conductance, measured under voltage clamp at various membrane potentials. All combinations yielded detectable assembly (50-fold range among different combinations) and ACh-induced conductances (>1,000-fold range at 1 \(\mu\)M). 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 \(^{125}\text{I}\)-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 \(\mu\)M. Matched responses and geometric means are used for describing the effects of changing a particular subunit (mouse vs. \textit{Torpedo}) while maintaining the identity of the other subunits. A dramatic subunit-specific effect is that of the \(\beta\) subunit on voltage sensitivity of the response: \(g_{\text{ACH}}(-90 \text{ mV})/g_{\text{ACH}}(+30 \text{ mV})\) is always at least 1, but this ratio increases by an average of 3.5-fold if \(\beta_M\) replaces \(\beta_T\). Also, combinations including \(\gamma_T\) or \(\delta_M\) usually produce greater receptor assembly than combinations including the homologous subunit from the other species. Finally, \(E_{\text{ACH}}\) is defined as the concentration of ACh inducing 1 \(\mu\)S/fmol at \(-60 \text{ mV}\); \(E_{\text{ACH}}\) is consistently lower for \(\alpha_M\). We conclude that receptor assembly, voltage sensitivity, and \(E_{\text{ACH}}\) are governed by different properties.

Address reprint requests to Dr. Henry A. Lester, Division of Biology, 156-29, California Institute of Technology, Pasadena, CA 91125. Dr. Yoshii’s present address is Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan. Dr. Mayne’s present address is Dept. of Microbiology, University of Alabama, Birmingham, AL 35294.
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 pGEM 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 electroph-
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 Biotech).

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 spin 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 MgCl₂, 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 CaCl₂, 1 mM MgCl₂, 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 MgCl₂, 5 mM HEPES, pH 7.5, and 1 mg/ml bovine serum albumin. They were then transferred to the
same solution containing 5 nM [125I]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. \(\alpha\)-BuTx, \(\alpha\)-bungarotoxin. The line is a least-squares fit to the data, constrained to pass through the origin, and has a slope of 1.07 \(\mu\)S/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 \(\alpha\)-bungarotoxin per oocyte. There is a >30-fold variation in the number of binding sites per receptor.

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Summary of Some Parameters for Each of the 16 Combinations</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>RNAs per oocyte</th>
<th>(E_{\alpha\text{Ch}}) at -60 mV</th>
<th>(g(0))</th>
<th>Hill coefficient</th>
<th>(K_{\text{EC50}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha\beta\gamma\delta)</td>
<td>FMol</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td></td>
</tr>
<tr>
<td>MMMM</td>
<td>6.49±0.67 (5)</td>
<td>0.20±0.006 (5)</td>
<td>4.3±0.2 (4)</td>
<td>2.0±0.1 (4)</td>
</tr>
<tr>
<td>MMTT</td>
<td>0.49±0.04 (5)</td>
<td>0.61±0.015 (5)</td>
<td>16.0±2.2 (4)</td>
<td>1.8±0.1 (4)</td>
</tr>
<tr>
<td>MTMM</td>
<td>8.06±0.05 (5)</td>
<td>0.16±0.002 (8)</td>
<td>6.6±1.1 (4)</td>
<td>1.9±0.1 (4)</td>
</tr>
<tr>
<td>TMMM</td>
<td>1.01±0.08 (5)</td>
<td>0.09±0.004 (8)</td>
<td>2.8±0.2 (4)</td>
<td>2.0±0.2 (4)</td>
</tr>
<tr>
<td>MMTT</td>
<td>0.91±0.14 (5)</td>
<td>1.57±0.089 (8)</td>
<td>6.1±0.6 (4)</td>
<td>1.5±0.1 (4)</td>
</tr>
<tr>
<td>MTTM</td>
<td>0.76±0.12 (5)</td>
<td>0.45±0.075 (8)</td>
<td>11.0±0.9 (4)</td>
<td>1.5±0.3 (4)</td>
</tr>
<tr>
<td>MTMT</td>
<td>2.02±0.27 (5)</td>
<td>0.16±0.009 (8)</td>
<td>3.7±0.3 (4)</td>
<td>1.9±0.1 (4)</td>
</tr>
<tr>
<td>MTMM</td>
<td>0.67±0.05 (5)</td>
<td>0.29±0.025 (8)</td>
<td>1.3±0.1 (4)</td>
<td>1.6±0.1 (4)</td>
</tr>
<tr>
<td>TMMT</td>
<td>0.39±0.04 (5)</td>
<td>2.75±0.14 (4)</td>
<td>7.1±1.1 (4)</td>
<td>1.8±0.2 (4)</td>
</tr>
<tr>
<td>TTMN</td>
<td>11±1.48 (5)</td>
<td>0.76±0.066 (8)</td>
<td>4.1±0.3 (4)</td>
<td>2.1±0.1 (4)</td>
</tr>
<tr>
<td>TTTM</td>
<td>0.53±0.08 (5)</td>
<td>2.20±0.13 (8)</td>
<td>2.8±0.3 (4)</td>
<td>1.8±0.1 (4)</td>
</tr>
<tr>
<td>MTTT</td>
<td>2.53±0.37 (5)</td>
<td>0.20±0.016 (4)</td>
<td>1.7±0.1 (4)</td>
<td>2.3±0.3 (4)</td>
</tr>
<tr>
<td>TMTT</td>
<td>0.65±0.18 (5)</td>
<td>13.5±0.71 (8)</td>
<td>8.9±2.5 (4)</td>
<td>1.5 (1)</td>
</tr>
<tr>
<td>TTMM</td>
<td>1.36±0.21 (5)</td>
<td>1.33±0.099 (8)</td>
<td>1.0±0.1 (4)</td>
<td>1.5±0.1 (4)</td>
</tr>
<tr>
<td>TTTM</td>
<td>5.45±0.64 (5)</td>
<td>0.29±0.02 (8)</td>
<td>3.0±0.1 (4)</td>
<td>1.9±0.1 (4)</td>
</tr>
<tr>
<td>TTTT</td>
<td>3.48±0.27 (11)</td>
<td>0.96±0.044 (11)</td>
<td>1.6±0.2 (4)</td>
<td>1.8±0.2 (4)</td>
</tr>
</tbody>
</table>

\(n\) is given in parentheses.
average number of α-bungarotoxin-binding sites per oocyte for the various combinations, ranging from 0.39 fmol for the αTβMγMδT hybrid to 11.4 fmol for the αTβMγTδM 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, (αβγδ)M—yielded signals too large for reliable clamping (>5–10 μA); others, such as αTβMγTδT, yielded signals too small for accurate measurement (<5 nA). This range of >10³ 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.
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.
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 4](image)

**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.

![Figure 5](image)

**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
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 $\alpha_T\beta_M\gamma_T\delta_{T,M}$ 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 $\alpha$ subunit had no consistent effect upon assembly: qS($\alpha$) = $-0.03$. The same lack of effect was noted for $\beta$, because qS($\beta$) = $-0.005$. In both cases, there were equal numbers of positive and negative values. The *Torpedo* $\gamma$ subunit appeared to give better assembly, however: qS($\gamma$) = $0.49$ and all eight values were $>0$. Finally, the mouse $\delta$ subunit seems to produce better assembly: qS($\delta$) = $-0.45$, with six of eight values negative. The final measure, the global average qS($\alpha\beta\gamma\delta$),

![scatter plot](image)

**Figure 8.** Scatter plot of Hill coefficient vs. ACh concentration inducing $1\mu S$. 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.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>qS</th>
<th>Number &gt; 0 (of 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>$-0.03$</td>
<td>4</td>
</tr>
<tr>
<td>$\beta$</td>
<td>$-0.005$</td>
<td>4</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>$0.49$</td>
<td>8</td>
</tr>
<tr>
<td>$\delta$</td>
<td>$-0.45$</td>
<td>2</td>
</tr>
</tbody>
</table>

**Global average**

| qS($\alpha\beta\gamma\delta$) | $-0.001$ | 18 of 32 |

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

\( \alpha_M \) consistently gives the lowest \( E_{\text{ACH}} \). 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 \( E_{\text{ACH}} \) (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 \( \alpha \) subunit: in all eight comparisons, \( \alpha_M \) produced a lower \( E_{\text{ACH}} \) than did \( \alpha_T \), by an average factor of 6.7.

\( \beta_M \) 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 \( \beta \) subunit: the highest voltage sensitivities are all associated with \( \beta_M \). Also, combinations of \( \beta_M \) with \( \delta_T \) were more voltage sensitive than those of \( \beta_M \) with \( \delta_M \).

### Table III

<table>
<thead>
<tr>
<th>Subunit</th>
<th>( qS )</th>
<th>Number &gt; 0 (of 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>0.83</td>
<td>8</td>
</tr>
<tr>
<td>( \beta )</td>
<td>-0.34</td>
<td>2</td>
</tr>
<tr>
<td>( \gamma )</td>
<td>-0.10</td>
<td>2</td>
</tr>
<tr>
<td>( \delta )</td>
<td>0.40</td>
<td>7</td>
</tr>
<tr>
<td><strong>Global average</strong></td>
<td><strong>0.20</strong></td>
<td><strong>18 of 32</strong></td>
</tr>
</tbody>
</table>

### 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 \( \delta \).** 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 \( \alpha_T\beta_T\gamma_M\delta_T \) produced \( \sim 3\% \) 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 \( \delta_T \) complexes would have contributed an insignificant amount of conductance to the macroscopic measurements reported here. However, Mayne et al. (1987) report that \( \alpha_T\beta_T\gamma_T\delta_M \) assembles much less efficiently than combinations including a \( \delta \) subunit, so that the ACh-induced conductance per \( \alpha \)-bungarotoxin site is \( \sim 20\% \) 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 αTβMγTδT) and the rather low assembly for some combinations (such as αTβMγMδT) 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 αTβMγMδM, αTβMγMδM, αTβγTδT, and αTβγMδM. 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 αTβMγTδT and αTβMγ0δT. 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 αMβMγ0δM. Thus, there seems to be little contribution by γ0 combinations to the 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 αT, αM, or αchick 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 δm showed a small (30%) decrease in the dose-ratio slope at −100 mV, probably because δm 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 Ki cover a range of

![Figure 9](image-url)
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 $\alpha$, $\beta$, $\gamma$, and $\delta$ subunits from two species. A major conclusion is that different subunits determine the greatest assembly of surface receptors ($\gamma_7$ and $\delta_5$), the lowest $E_{ACh}$ ($\alpha_5$), and the greatest voltage sensitivity ($\beta_5$). 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 $\mu$S/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 $\mu$S/fmol = $3.3 \times 10^{-3}$ pS/ACh receptor (assuming two $\alpha$-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 $8 \times 10^{-5}$. 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 $8 \times 10^{-5}$ corresponds to an agonist concentration of $9 \times 10^{-8}$ times the half-maximal concentration. The lowest concentrations giving this value are in the range 0.1–1 $\mu$M (see Table I). Taking the value of 0.5 $\mu$M as an average, this would suggest that half-maximal activation would occur at ~50 $\mu$M 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 ($0.08-0.27$ $\mu$M for all five mouse-*Torpedo* combinations tested) are close to the ranges usually found for mouse muscle ($0.04-0.15$ $\mu$M; Pennefather and Quastel, 1981), frog muscle ($0.39-0.43$ $\mu$M; Jenkinson, 1960; Adams, 1975), and *Electrophorus* electroplaque ($0.2$ $\mu$M; 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 $\beta$ 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 $\beta$ subunit. The subunit $qS$ was less than zero for all $a\beta_T,\gamma \delta$ pairs, yielding a subunit average $qS$ of $-0.55$. Furthermore, all eight combinations containing $\beta_M$ were more voltage sensitive than all eight combinations involving $\beta_T$. On the average, the mouse $\beta$ subunit renders a combination 3.55 times more voltage sensitive than does the *Torpedo* $\beta$ 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 $a\beta_T,\gamma \delta_{\text{calf}}$, but not the $a\alpha_{\text{calf}}(\beta \gamma \delta)_T$ combination, showed the same channel duration (including voltage sensitivity) as the $a\beta \gamma \delta_{\text{calf}}$ combination. Quantitative data were not reported for other combinations. On the basis of these data, it was suggested that the $\delta$ 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 $\delta$ subunit on the equilibrium voltage sensitivity of the ACh-induced conductance. The subunit average $qS$ (b) for $g(-90)/g(+30)$ is nearly zero, with four positive and four negative values; but the four most voltage-sensitive combinations involving $\delta_M$ also involve $\beta_M$, and the four least voltage-sensitive combinations involving $\delta_M$ also involve $\beta_T$. This $\beta$-$\delta$ interaction is consistent with the suggestion that the $\beta$ and $\delta$ 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 that the coulombic interaction energy has the same sign for all of the 16 combinations (except for the single combination $\alpha_1\gamma\delta\tau$, for which the energy is presumably zero). A dipole moment (Magleby and Stevens, 1972a, 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* 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* 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 $E_{\text{ACH}}$**

The dose-response data show that all eight combinations involving $\alpha_M$ produced a lower $E_{\text{ACH}}$ than did all eight combinations involving $\alpha_T$. The average $q_S(\alpha)$ 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 $\alpha_{caf}$ for $\alpha_T$ 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 $\alpha_M$. 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.*

**References**


Dascal, N., T. P. Snutch, H. Lubbert, N. Davidson, and H. A. Lester. 1986. Expression and


