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Subunit Requirements for *Torpedo* AChR Channel Expression: A Specific Role for the δ-Subunit in Voltage-Dependent Gating

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Summary. This study examines the subunit requirement for *Torpedo* acetylcholine receptor (AChR) channel expression and the influence of non-α-subunit deletions on single AChR-channel currents. *Xenopus* oocytes injected with subunit combinations deficient in single non-α-subunit mRNA transcripts display the following order of ACh sensitivity: β-less > γ-less > δ-less. Oocytes injected with only the α-subunit and one non-α-subunit display the order: αδ > αγ > αβ. These sequences indicate the effectiveness of non-α-subunit substitution is δ > γ > β. Single AChR-channel currents measured in oocytes deficient in either β or γ display conductance and voltage-sensitive burst kinetics similar to the wild-type channel. In contrast, the δ-less combination express channels with burst kinetics that are relatively faster and voltage insensitive. These results indicate that either a specific structural domain in the δ-subunit or its specific interactions with the α-subunit contribute to the voltage-dependent gating of the *Torpedo* AChR channel.

Key Words *Torpedo* AChR · *Xenopus* oocyte · expression · subunit function · single-channel currents

Introduction

The initial cloning of the nicotinic AChR from *Torpedo* electroplax (Noda et al., 1983) proved critical in the subsequent identification and cloning of nicotinic AChRs from muscle and central nervous tissue (Claudio, 1989; Heinemann et al., 1989; Numa, 1989; Galzi et al., 1991). Furthermore, through site-directed mutagenic studies it provided the first detailed model of ion permeation through a biologically relevant channel (Imoto et al., 1988). However, compared with our understanding of conductance mechanisms relatively little is known of the molecular processes that underlie AChR-channel gating.

Magleby and Stevens (1972) proposed that AChR-channel lifetime is rate limited by an agonist-induced conformational change that involves a dipole moment change, based on the observed voltage-sensitive kinetics of muscle end-plate currents and ACh-induced current fluctuations (Magleby & Stevens, 1972; Anderson & Stevens, 1973). Although a similar voltage sensitivity has been confirmed in numerous single-channel current measurements, its exact structural correlate has remained elusive (Sakmann et al., 1985; Mishina et al., 1986; Lo, Pinkam & Stevens, 1990, 1991; Yu et al., 1991). In contrast, the structural basis of voltage gating of the Na^+ and K^+ channel has been directly related to specific charged groups in a highly conserved transmembrane region (i.e., S4 region) of these channel proteins (Stuehmer et al., 1989; Papazian et al., 1991).

One complication in studying the AChR channel is that it is made up of four subunits α, β, γ and δ, with the stoichiometry of $\alpha_2\beta\gamma\delta$. Extensive homology between the subunits indicates they most likely evolved from a unique ancestral gene that underwent gene duplication and subsequent mutational divergence (Raftery et al., 1980; Numa, 1989). Although it is possible that an early form of the AChR channel functioned in a homo-oligomeric state (*see* Lunt, 1986), there is substantial biochemical and electrophysiological evidence (Kurosaki et al., 1987; Blount & Merlie, 1989) that indicates a minimal combination of different subunits is required to produce functional AChR channels. Presumably, evolution to the current hetero-oligomeric state has provided the AChR with a wider range of physiological functions, in which each subunit may play a specific role.

One strategy to examine subunit function has been to make chimeric AChRs formed from subunits of different organisms which express AChR channels with markedly different properties. Such studies on *Torpedo*-calf and *Torpedo*-mouse chimeras indicate that δ-subunit substitutions alone (Sakmann et al., 1985; Yu et al., 1991) and combinations of δ- and β-subunit substitutions (Yu et al., 1991) can strongly influence the voltage dependence of AChR-channel gating (*see also* Yoshii et al., 1987). Another

strategy has been to form AChR channels which are deficient in specific subunits and to compare their properties with wild-type AChR channels. In deletion studies a basic assumption is that the functional AChR is composed of five subunits and the omitted subunit is replaced by a remaining subunit. Such studies on mammalian muscle AChR channels have focused on the effects of γ-deletions (Jackson et al., 1990; Lo et al., 1990) and δ-deletions (Kullberg et al., 1990). The effects of β-deletions on single AChR-channel characteristics have not been reported.

In this study we demonstrate that ACh sensitivity can be generated in *Xenopus* oocytes injected with mRNA transcripts for only two *Torpedo* subunits, the α-subunit plus any one non-α-subunit, with the following order $\alpha\delta > \alpha\gamma > \alpha\beta$. This order is consistent with previous biochemical and binding studies on the assembly and agonist binding properties of α and non-α-subunit pairs (Blount & Merlie, 1989). In patch-clamp recording of these oocytes we have been unable to detect single ACh-sensitive channel currents presumably due to low densities of channel expression. However, single-channel current measurements from oocytes deficient in only one non-α-subunit indicate that when the β- or γ-subunit is omitted, channel conductance and voltage-dependent kinetics are largely unaffected. In contrast, the δ-less AChR channel displays burst kinetics that are relatively faster and voltage insensitive compared with wild-type AChR channels. We propose that either a specific structural domain in the *Torpedo* δ-subunit or its specific interactions with the α-subunit contribute to the voltage-dependent gating of the AChR channel. A preliminary account of these results has been previously presented (Golino & Hamill, 1991).

Materials and Methods

Preparation of mRNA

Torpedo-specific mRNAs were synthesized in vitro using the Promega (Madison, WI) Riboprobe system with SP6 polymerase from agarose purified cDNAs (linearized with Xba I), which were provided to us by Dr. Toni Claudio of Yale University. The mRNA sizes and quality were verified by formaldehyde gel electrophoresis (Sambrook, Frisch & Maniatis, 1989). The cDNA authenticity for each clone was verified by their specific restriction digest patterns against restriction maps provided to us by Dr. Claudio.

Preparation of Oocytes

Mature female frogs (*Xenopus laevis*) were obtained from Xenopus I (Ann Arbour, MI) and kept as pairs in aquaria under a controlled winter-light cycle. The isolation and preparation of oocytes was as described previously (Methfessel et al., 1986). Oocytes were surgically removed from frogs anesthetized by submerging them in 200 ml of water to which 250 mg of ethyl 3-aminobenzoate methanesulfonic acid (Aldrich Chemical) had been added. After isolation, type V and VI oocytes (Dumont, 1972) were sorted out and stored overnight in Barth's medium (in mM: NaCl 88, KCl 1, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $NaHCO_3$ 2.4, and Tris/HCl 5, pH 7.4, osmolarity 200 mOsm) containing 75 μg/ml gentamycin sulfate at 18°C.

Injection of Oocytes

Injections of mRNAs were accomplished with a model NA-1 microinjector (Sutter Instrument, San Rafael, CA) fitted with injector pipettes cleaned and siliconized (DePamphilis et al., 1988) prior to pulling with a Sutter micropipette puller Model P-87. The pipette tips were broken to 15–20 μm diameter on a heat-cleaned wire with the aid of a Narishige Type MF-83 Microforge. A buffer layer of autoclaved Fluorinert FC-40 (Sigma) was used between the RNA solutions and the light mineral oil (Sigma) used to drive the injections. The mRNAs were injected in a total volume of 50 nl with concentrations of either 500 or 50 ng/μl. The concentrations of the individual subunit-specific mRNAs injected are shown in the Table. At the higher concentrations, the mRNAs tended to precipitate out after storage at −80°C. These mRNA mixtures were routinely heated for 10 min at 68°C, without quick cooling, to melt the precipitates which would otherwise clog the injection pipette, after determining that this treatment resulted in no loss of biological activity (*data not shown*). The injected oocytes were incubated at 18°C in Barth's medium which was changed daily. Testing was begun after three days and continued up to six days after injection. Activity increased slightly or not at all over that time period.

Whole-Cell Screening of Oocytes

Injected oocytes were initially screened for AChR-channel activity in a chamber perfused with Ringer's solution (in mM: NaCl 115, $CaCl_2$ 2.5, and HEPES (NaOH) 10, pH 7.2, 240 mOsm) with 5 μM atropine added to block the endogenous muscarinic response. Electrodes filled with 3 M KCl were used to record voltage and pass current in voltage-clamp experiments. However, most screening was carried out with a single electrode under current clamp. Only cells with an initial resting potential more negative than −25 mV and input resistances >1 MΩ were tested for their ACh sensitivity. Typically, a few minutes after electrode penetration the membrane potential recovered to a more negative value of between −40 to −50 mV at which point the oocyte was hyperpolarized further to −100 mV using an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) and ACh sensitivity then tested. There was no consistent difference in the resting potential or input resistance of oocytes injected with different concentrations or combinations of mRNA transcripts. Particular types of subunit comparisons of (e.g., β-less, γ-less, and δ-less) were always made on the same batch of oocytes and tested on the same day to reduce variability due to oocyte conditions. ACh was pressure-applied to the oocyte using an adjustable pipetter fitted with a 1–200 μl pipette tip. We preferred this rapid focal method over the slower method of bath perfusion (Kurosaki et al., 1987) because the latter method gave smaller peak responses presumably due to the more pronounced effects of desensitization. However, one disadvantage of the focal method is that the

Table. Concentration of subunit-specific mRNAs injected in ng/μl[a]

Combination	Subunit			
	α	β	γ	δ
$\alpha_2\beta\gamma\delta$	200 (20)	100 (10)	100 (10)	100 (10)
$\alpha_2\beta\gamma$	250 (25)	125 (12.5)	125 (12.5)	—
$\alpha_2\beta\delta$	250 (25)	125 (12.5)	—	125 (12.5)
$\alpha_2\gamma\delta$	250	—	125	125
$\alpha_2\beta$	333	167	—	—
$\alpha_2\gamma$	333 (33.3)	—	167 (16.7)	—
$\alpha_2\delta$	333 (33.3)	—	—	167 (16.7)
$\beta\gamma\delta$	—	167	167	167
α	500	—	—	—
β	—	500	—	—
γ	—	—	500	—
δ	—	—	—	500

[a] The final concentration of injected mixtures was either 500 or 50 ng/μl.

ACh concentration around the oocyte is not defined. In order to standardize ACh applications we stopped bath perfusion during testing, set the bath level so it just covered the top of the oocyte and kept the ACh pipetter out of the bath between applications to prevent ACh leakage. For each application the ACh pipetter was brought within 100 μm of the oocyte and 10 μl of the ACh-Ringer's solution puffed directly onto the top of the oocyte. This procedure maximized the concentration of ACh around the oocyte and resulted in a response waveform with a rapid increase to a peak which then decreased over a slower time period (*see* Fig. 1*A*). The decay of the response reflects a combination of the effects of ACh diffusion and desensitization. In between testing different ACh concentrations, the bath was rapidly perfused with Ringer's solution to remove ACh.

Patch-Clamp Recordings from Oocytes

Single-channel currents were recorded from cell-attached patches of oocytes after removal of their follicular and vitelline layers (Methfessel et al., 1986) using standard patch-clamp techniques (Hamill et al., 1981). The pipette solution for most experiments contained (in mM): KF 80, KCl 20, EGTA (KOH) 10, and HEPES (KOH) 10, pH 7.2, 217 mOsm, with 1 μM ACh added freshly for each experiment; in some experiments KF was substituted with KCl with no effect on channel characteristics. To reduce spontaneous activity of the endogenous mechanosensitive (MS) channel the oocytes in some experiments were bathed in a hypertonic solution (in mM: K^+ aspartate 200, KCl 20, $MgCl_2$ 1, EGTA 10, and HEPES (KOH) 10, pH 7.4, 475 mOsm) during recording. Membrane potential was expressed with respect to the reversal potential of the MS channel activated on the patch by application of suction, which under symmetrical K^+ concentrations is 0 mV. The temperature of the bath was reduced to 15°C to slow AChR-channel kinetics and to improve resolution.

Single-Channel Data Acquisition and Analysis

Single-channel events were recorded using an EPC-7 (List-Adams, NY) and stored on videotape (Neuro Data Instruments, NY). For subseqent analysis the data were played back through an 8-pole Bessel filter (Frequency Devices, MA) filtered at 4 kHz. Continuous data records were digitized by an analog-to-digital converter (Labmaster, Scientific solutions, OH) at 10 kHZ and stored on a PC-clone computer.

Single-channel analysis and histogram construction were carried out with the pClamp programs (Axon Instruments, Foster City, CA). Because of the fast gating kinetics of the *Torpedo* AChR channel and limited frequency response we chose to study burst durations rather than the briefer openings and closings during the burst. We defined a burst as a series of openings and closings in which the closed time never exceeded 300 μsec. In practice the event frequency was usually so low (approx. 1–10 sec^{-1}) that there was no ambiguity in identifying individual bursts. In constructing burst histograms all bursts were considered but when fitting the histogram the underestimated bins were ignored. A single exponential was found adequate in describing the burst durations for all subunit combinations.

Results

Whole Oocyte ACh Sensitivity

The ACh sensitivity of oocytes was highly dependent on the concentration and types of subunit-specific mRNAs injected. For example, oocytes injected with all subunits (wild type) and tested with two-electrode voltage clamp could express currents as large as 2 μA in response to 1 μM ACh. In contrast, many of the subunit-deficient combinations showed no response to 1 μM ACh applied under the same recording conditions. In these less-sensitive oocytes higher ACh concentrations (>10 μM) were required to induce significant currents. However, similar ACh concentrations applied to wild-type injected oocytes typically produced currents so large (i.e., >10 μA) they could not be clamped. While reducing the holding potential from −100 to −30

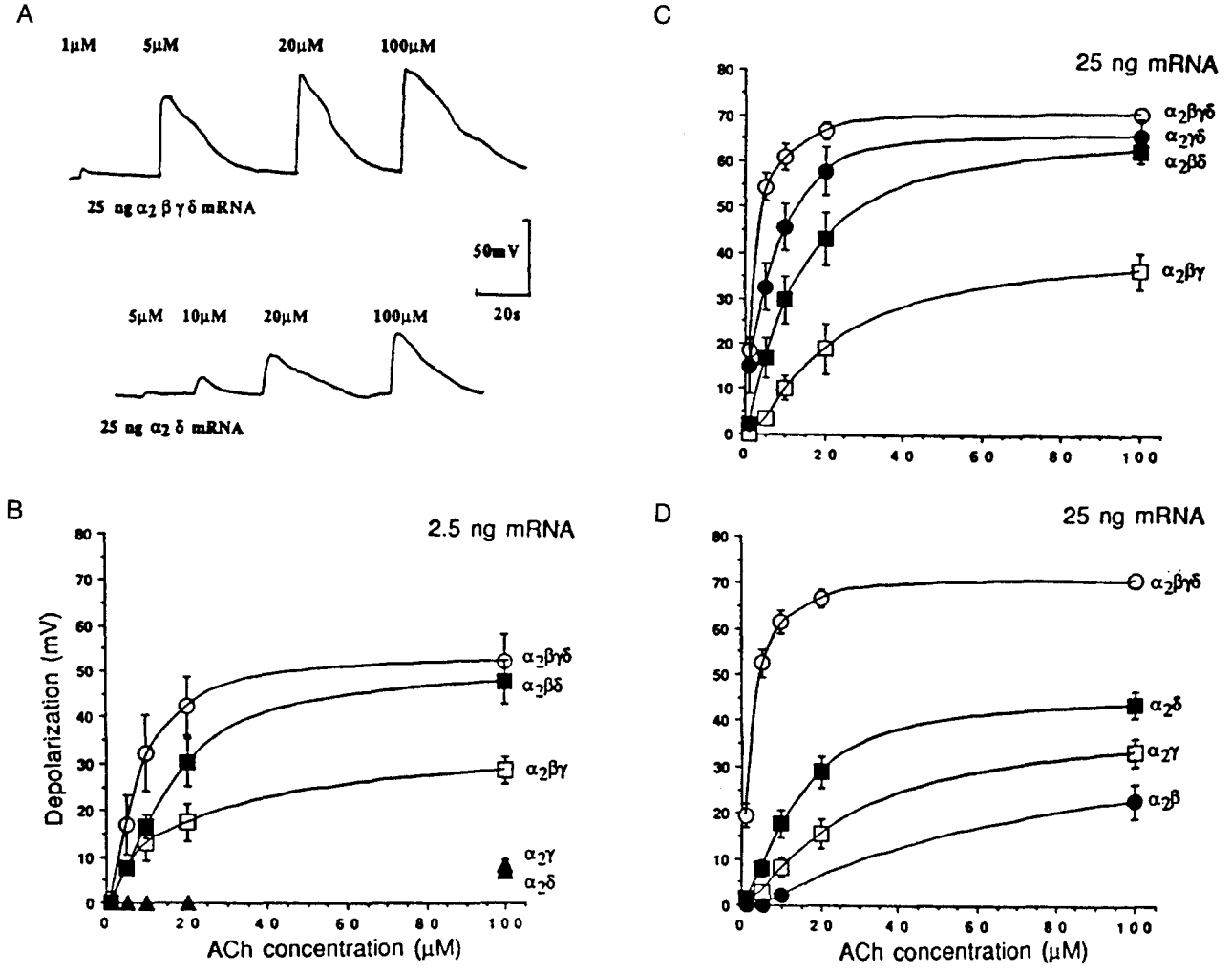

Fig. 1. Whole oocyte ACh sensitivity following injection of different AChR subunit transcript combinations. (*A*) The response to ACh applications of two oocytes injected with 25 ng of mRNA with molar stoichiometries of $\alpha_2\beta\gamma\delta$ (top trace) and $\alpha_2\delta$ (lower trace). The recordings were carried out three days after mRNA injection, and both oocytes were current clamped at −100 mV. (*B*) The response of oocytes injected with 2.5 ng total mRNA in various subunit stoichiometries to application of different concentrations of ACh. (*C* and *D*) Similar to *B* except the oocytes were injected with 25 ng total mRNA. The data points represent the mean ± SEM for 20 to 80 oocytes. The curves drawn through the data points have not been fitted by any model owing to uncertainties in exact ACh concentration and the effects of desensitization.

mV reduced the currents and improved the clamp, the same procedure reduced sensitivity for detecting currents in subunit-deficient oocytes. Since we found no single voltage-clamp condition which allowed all subunit combinations to be tested over the necessary ACh-concentration range (1–100 μM) we used current-clamp recording to screen ACh sensitivity. This technique, although less quantitative, did allow ranking of a wide range of ACh sensitivities under similar recording conditions (*see* Materials and Methods).

Figure 1*A* illustrates the ACh-induced depolarizations produced in two different oocytes. One oocyte (top trace) had been injected three days earlier with a total of 25 ng of mRNA with the molar stoichiometry of $\alpha_2\beta\gamma\delta$; the other oocyte (lower trace) had been injected with a total of 25 ng of mRNA with the molar stoichiometry of $\alpha_2\delta$. Figure 1*B–D* summarizes the results obtained on oocytes injected with different concentrations of AChR subunit-specific subscripts. Comparison of Fig. 1*B* and *C* indicates that oocytes injected with 25 *versus* 2.5 ng total mRNA gave larger responses to the same activating concentration of ACh. The higher mRNA concentrations as well as higher test ACh concentrations were required to detect as well as rank the ACh sensitivities of oocytes injected with only two types of subunits (i.e., *compare* Fig. 1*B* and *D*).

The graphs indicate that the ACh sensitivity of subunit combinations deficient in single non-α-subunits was β-less > γ-less > δ-less and for combinations deficient in two non-α-subunits it was $\alpha_2\delta$ > $\alpha_2\gamma$ > $\alpha_2\beta$. Note that the combination $\alpha_2\delta$ was on average more sensitive than $\alpha_2\beta\gamma$. However, interpretation of this last result is complicated because the concentration of α-subunit transcript varied between the combinations (*see* the Table). Evidence indicates the concentration of α-subunit is rate limiting in the synthesis of AChRs (Buller & White, 1988).

The curves shown in Fig. 1 are not amenable to quantitative analysis because the exact ACh concentration around the oocyte at the time of the peak response was unknown (*see* Materials and Methods). Furthermore, desensitization and nonlinearities in the voltage responses most likely distort the high end of the ACh concentration-response curves. Although it is also possible that subunit-specific effects on desensitization might distort the ranking of some combinations, we feel this did not occur since none of the curves in Fig. 1 showed crossovers with increasing ACh concentration. Regardless of the quantitative details, the results shown in Fig. 1 indicate significant AChR expression in oocytes injected with subunit combinations that were previously thought incapable of forming functional AChR channels (e.g., Kurosaki et al., 1987). These observations have previously had implications concerning the assembly of the AChR, the specificity of subunit interactions and the normal arrangement of subunits in the wild-type receptor (*see* Claudio, 1989).

Apart from the subunit combinations described in Fig. 1 other combinations were also tested. For example, 22 out of 55 oocytes injected with the subunit combination $\beta\gamma\delta$ (i.e., α-less) were found to be ACh sensitive and responded to 100 μM with an average depolarization of 18 ± 3 mV (SEM), while 7 out of 30 oocytes injected with 25 ng of the δ-subunit were sensitive with an average response of 20 ± 7 mV. No ACh sensitivity was detected in 43, 27 and 20 oocytes injected with only α-, γ- or β-subunit transcripts, respectively.

Single-Channel Recording

Following oocyte screening for each subunit combination, the most sensitive oocytes were patch clamped. We chose the cell-attached recording mode to study the kinetics of AChR channels because in initial experiments on outside-out patches we found channel activity faded before sufficient events over a number of voltages could be collected. One problem faced in cell-attached recording from *Xenopus* oocytes is distinguishing AChR-channel currents from the spontaneous activity of endogenous mechanosensitive (MS) channels. Presumably, the spontaneous activity seen in both uninjected and injected oocytes arises from cell swelling or pressure applied during the sealing procedure. Because the MS channel is a multiple-conductance state channel (Methfessel et al., 1986) and can display at least three different conductance levels with quite variable kinetics as illustrated in Fig. 2*A*, it is a potential source of confusion in analyzing exogenous AChR channels. Furthermore, even though the identity of MS-channel currents can be verified by the application of suction (Fig. 2*B*) and their conductance and kinetic characteristics differ from the wild-type *Torpedo* AChR channel (Fig. 2*C*), MS-channel activity becomes a more serious source of confusion in studying subunit-deficient AChR channels of unknown characteristics (e.g., *see* Kullberg et al., 1990). We have found that bathing the oocyte in hypertonic Ringer's is effective in reducing MS-channel activity in uninjected oocytes. Furthermore, in ACh-sensitive oocytes it reduces the contamination of AChR-channel recordings by MS-channel currents (*c.f.* Fig. 2*D* and *E*). We have routinely used this procedure in our characterization of single AChR channels.

Figure 3*A* shows superimposed current traces of AChR-channel currents recorded from a cell-attached patch for a wild-type injection. Figure 3*B* illustrates amplitude and burst-duration histograms constructed from such events. The amplitude distributions were well described by single Gaussians that gave current amplitudes of 8.1 pA at −75 mV and 20.6 pA at −175 mV. We have seen no evidence of smaller amplitude events or multiconductance state behavior in current-amplitude distributions even at the most hyperpolarized potentials (i.e., at −200 mV). The single-channel conductance of the AChR channel on this patch estimated from *i-V* relations was 110 pS. This value is similar to previous estimates for the *Torpedo* channel made in the presence of high (100 mM) external K^+ (Sakmann et al., 1985). The burst durations of wild-type AChR channels were fitted by single exponentials (initial underestimated bins were ignored in the fits) and gave time constants of 0.24 msec at −75 mV and 0.55 msec at −175 mV, respectively. Similar kinetics have been reported previously for wild-type *Torpedo* AChR channels (Sakmann et al., 1985; Yu et al., 1991).

As ACh sensitivity decreased in oocytes with specific subunit omissions so did the probability of recording single-channel activity. However, in the case of the β-less and γ-less combinations there was still a good probability (>50% of patches) of obtaining single-channel activity on sensitive oocytes.

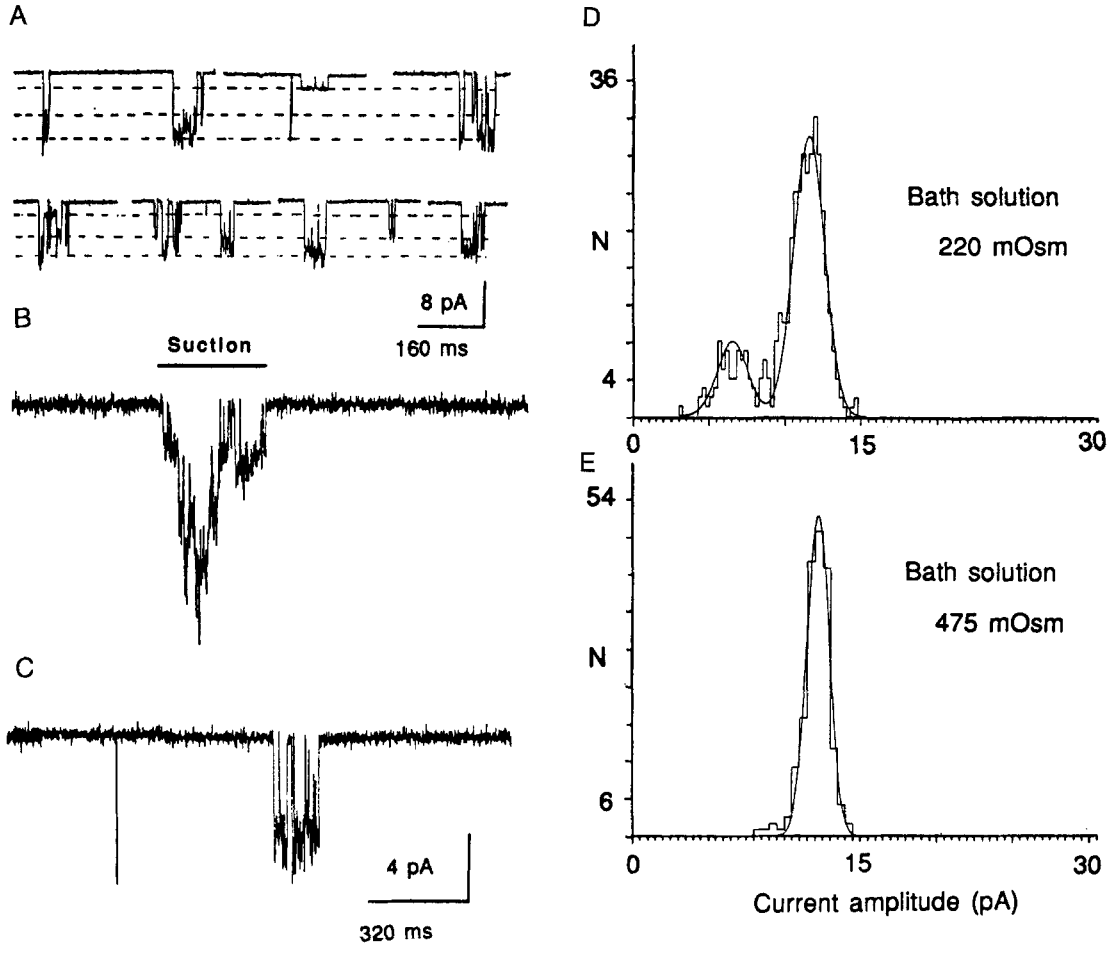

Fig. 2. Comparison of the properties of endogenous MS and exogenous AChR-channel currents. (*A*) Spontaneous, single-channel currents recorded from an uninjected oocyte at patch potentials of −150 mV (upper trace) and −130 mV (lower trace). The broken lines illustrate three distinct current levels. (*B*) The currents shown in *A* are presumed to be mechanosensitive because they were increased in frequency by brief application of suction (20 mm Hg) to the patch pipette. (*C*) Comparison of the kinetics of an AChR-channel current (on the left) and a presumed MS-channel current (on the right) recorded from an ACh-sensitive oocyte which had been injected with *Torpedo* subunit mRNAs. ACh (1 μM) was included in the pipette solution to activate the brief current events as shown on the left. (*D*) Current-amplitude histogram of two classes of currents (similar to those shown in *C*) recorded from an oocyte in a bathing solution with an osmolarity of 220 mOsm. The smaller amplitude (mean 6.5 pA) represents presumed spontaneous MS-channel activity, and the larger amplitude class (mean 11.5 pA) represents ACh-sensitive channel currents. (*E*) Histogram made from recordings from the same patch 5–10 min after bathing the oocyte in a solution with an osmolarity of 475 mOsm. Under the hypertonic conditions the presumed MS-channel activity disappeared but the ACh-sensitive currents remained (mean amplitude 12.2 pA).

In the case of the δ-less combinations it was more difficult to find ACh-sensitive patches and only 7 out of 70 patches studied from 12 oocytes showed channel activity; of these only 4 patches gave sufficient events for detailed analysis over a number of voltages. So far we have not been able to characterize single-channel activity from oocytes in which two non-α-subunits were omitted.

Figure 4 compares amplitude and burst-duration histograms made at approximately similar patch potentials for channels measured in oocytes in which single non-α-subunits (i.e., δ-less, γ-less and β-less) were omitted from the injections. As in the case of the wild-type AChR channel the amplitude- and burst-duration distributions were described by single Gaussian and exponential fits, respectively. The average amplitudes of events were similar allowing for the differences in patch potentials. The burst-duration time constants for the β-less and γ-less combinations were also similar (0.52 and 0.61 msec) to the wild-type channel measured at the same potential, while the δ-less combination displayed a significantly briefer time constant of 0.21 msec.

Figure 5 shows the *i-V* relationships for different subunit combinations. Regression lines were fitted

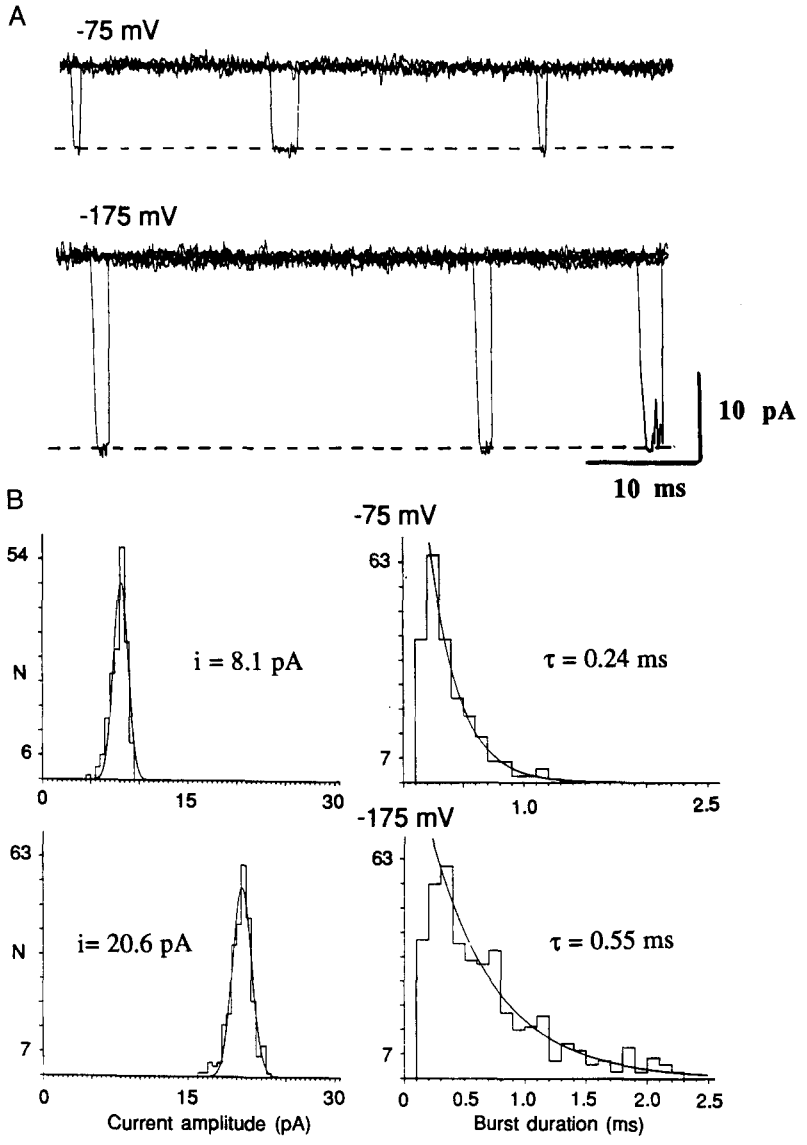

Fig. 3. The amplitude and kinetic behavior of AChR-channel currents recorded from an oocyte injected with all subunits. (*A*) Superimposed current traces recorded at patch potentials of −75 and −175 mV. The traces were selected for relatively long duration events and therefore do not illustrate the voltage dependence of open-channel lifetime. (*B*) Histograms of the amplitudes and burst durations of AChR-channel currents measured at −75 and −175 mV.

to the data so that they passed through 0 mV and gave single-channel conductances of 111, 106, 110 and 104 pS for the wild-type, γ-less, δ-less and β-less AChR channels, respectively. These fits indicate that the ion selectivity and conductance properties of the channels are largely unaffected by subunit omissions. However, real small differences between subunit combinations may have been concealed by variations in estimates made on different patches.

Figure 6 shows semilogarithmic plots of the time constant of burst durations obtained from the same patches described in Fig. 5. In the case of the wild-type, γ-less and β-less combinations the burst durations clearly increased with hyperpolarization and in all three cases the fitted regression lines indicated time constants of around 1 msec at −250 mV compared with around 0.15 msec at 0 mV. In contrast, the data for the δ-less combination indicated a time constant of about 0.2 msec at −250 mV, which was hardly different from the time constant of 0.15 msec predicted at 0 mV. The apparent differences in voltage sensitivity can be described by the relation

$$\tau_{(V)} = \tau_{(0)} \exp [V/H]$$

where $\tau_{(V)}$ is the time constant of the burst duration at patch potential V, $\tau_{(0)}$ is the time constant at 0 mV and H is the potential change required to produce an e-fold change in $\tau_{(V)}$. Both $\tau_{(0)}$ and H values for each combination are listed in Fig. 6. The wild-type, γ-less, and β-less combinations displayed similar H values of −137, −118, and −124 mV, respectively,

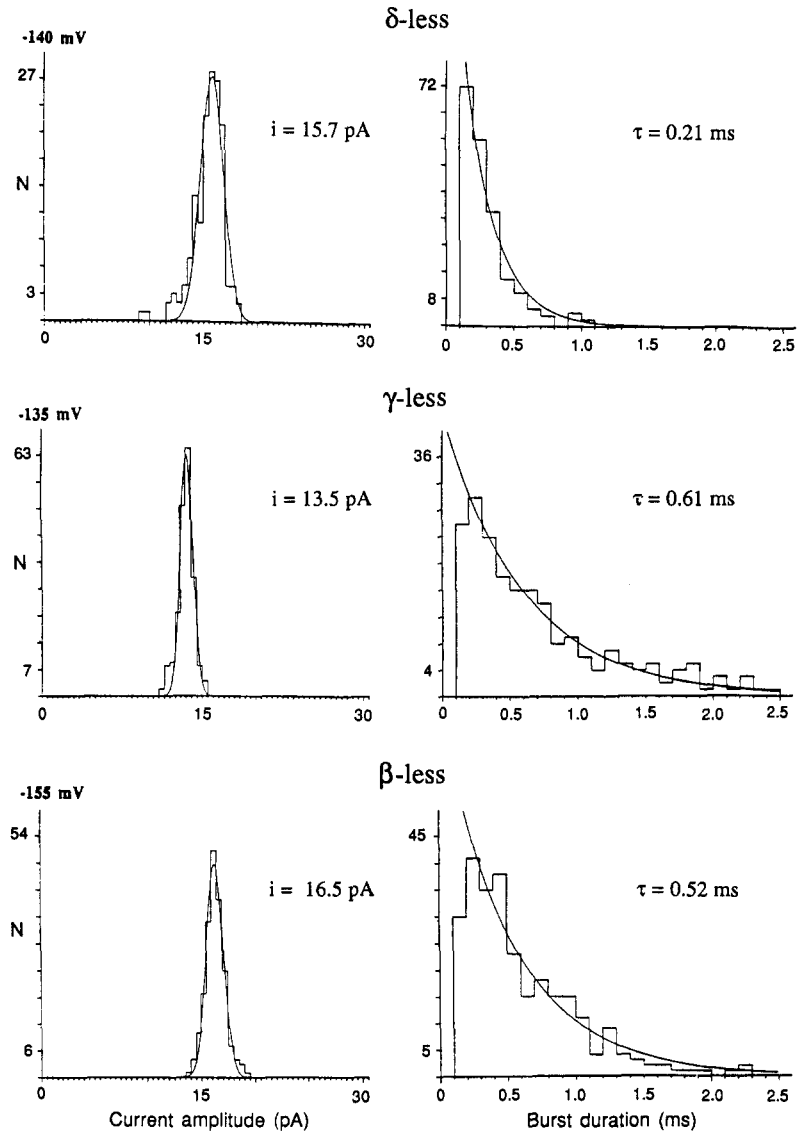

Fig. 4. Comparison of the single-channel currents of AChR channels formed in three different oocytes injected with subunit-transcript combinations deficient in either the δ-, γ- or β-subunit. The histograms were chosen from recordings made at similar patch potentials. Comparison of the histograms indicate that the major difference between the AChR channels was that the δ-less combination displayed significantly briefer burst durations than the γ-less and the β-less combination.

while the δ-less combination displayed a much weaker voltage sensitivity with an H value of −617 mV.

One possible explanation for the reduced voltage sensitivity displayed by the δ-less combination is that at depolarized potentials, where events are smaller, a more significant fraction are unresolved and the burst durations at these potentials are overestimated. In this case the $\tau_{(0)}$ value may be significantly smaller than the predicted 0.15 msec and as a consequence the voltage sensitivity steeper. The absence of the δ-subunit in this case would be to shift the burst durations to faster values without perhaps altering voltage sensitivity. At this point we cannot exclude this possibility. However, our estimates of time constants in all cases were based on exponential fits to events that were within our limits of resolution (i.e., >0.1 msec).

Discussion

Subunit Requirements

This study reports a number of new observations concerning the subunit requirements for forming functional *Torpedo* AChR channels. In the first place, we demonstrate that functional AChR channels can be formed by injection of only two subunits, an α-subunit and any one non-α-subunit. In order to see this activity relatively high (25 ng) total amounts of mRNA need to be injected and high (>10 μM) ACh concentrations are required to generate significant

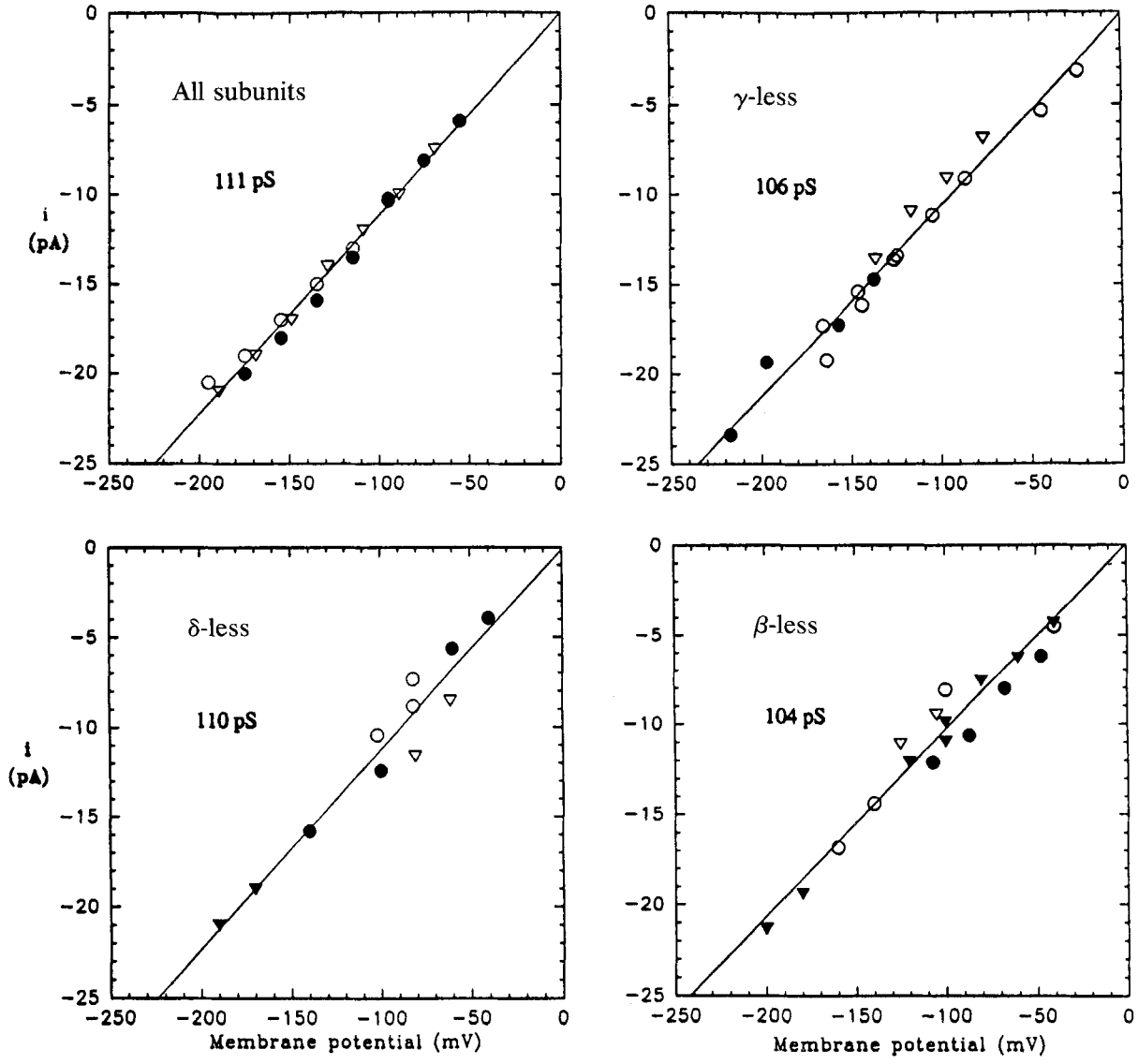

Fig. 5. Comparison of conductance properties of different combinations of AChR subunits. The data points were obtained from Gaussian fits to the amplitude histograms such as shown in Figs. 3 and 4. The different symbols represent different cells, which ranged between 3–4 for the different combinations. Regression lines were fitted through the data points with a forced 0-mV reversal potential to give the indicated values of single-channel conductance.

oocyte responses. The order of sensitivity of these subunit combinations is $\alpha\delta > \alpha\gamma > \alpha\beta$. In the case of single non-α-subunit deletions we see the order β-less > γ-less > δ-less. Together, the above results indicate that the δ-subunit is the most effective and the β-subunit least effective in producing ACh sensitivity.

Much weaker functional expression is seen in oocytes injected with either the α-less combination $\beta\gamma\delta$ or the δ-subunit alone. Buller and White (1990) have previously reported weak AChR-channel activity in oocytes injected with *Torpedo* $\beta\gamma\delta$ and provided evidence that it arises from formation of chimeric AChRs composed of endogenous *Xenopus* α (Hartman & Claudio, 1990) and *Torpedo* $\beta\gamma\delta$. Our results would indicate that the *Torpedo* δ-subunit in combination with the *Xenopus* α-subunit may be sufficient to form functional chimeric AChR channels. In this study we cannot exclude the possibility that oocytes also express *Xenopus* β-, γ- and/or δ-subunits, which replace the missing *Torpedo* non-α-subunits. However, this seems unlikely since uninjected, sham injected or oocytes injected with high amounts (25 ng) of the *Torpedo* α-subunit did not express ACh sensitivity. If *Xenopus* β, γ-, or δ-subunits are expressed at all by the oocyte, they are in insufficient amounts to form detectable AChR-channel activity.

Comparison with Previous Studies

The differences we observe in non-α-subunit effectiveness in promoting ACh sensitivity in the whole oocyte may reflect subunit-specific effects on one or more of a number of different AChR properties, including AChR assembly, membrane insertion, agonist binding, desensitization and single-channel properties. In this study we directly address only the last possibility. However, a previous biochemical and receptor binding study by Blount and Merlie (1989) has reported the same order (i.e., $\delta > \gamma \gg \beta$) of efficiency for promoting subunit assembly and high-affinity agonist binding in α- and non-α-subunit pairs. At this stage no information exists on the role different non-α-subunits may play in AChR desensitization.

Our observations on the order of ACh sensitivity are consistent with those of Lo et al. (1990) who report the mouse AChR γ-less combination is four times more effective than the δ-less combination in inducing ACh sensitivity in *Xenopus* oocytes. The β-less combination was not tested. In contrast Kurosaki et al. (1987) investigating *Torpedo* subunit-deficient combinations observed the ranking: δ-less > γ-less > β-less (*see also* Yoshii et al., 1987). Furthermore, they observed no detectable response to 10 μM ACh for the combinations αδ, αγ, αβ, βγδ, βγ, βδ, γδ, α, β, γ, or δ. In another study on mouse AChR channels, Kullberg et al. (1990) reported the ranking δ-less ≫ γ-less with the β-less combination expressing little or no ACh sensitivity. Interestingly, it has been reported that the chimeric β-less, combination mouse αγ and rat ε can produce oocyte ACh sensitivity comparable to that seen with the wild-type AChR (Liu et al., 1990). At this stage the exact explanation(s) for the differences between studies is unknown. They do not appear to be due to species differences alone. However, there are a number of methodological differences in the amounts of total mRNA injected as well as in the methods of testing, recording and applying ACh that could play some role.

Single-Channel Characteristics

In terms of single AChR-channel characteristics we describe for the first time the properties of single AChR-channel currents in which the *Torpedo* β-subunit has been omitted. These channels display the same conductance and voltage-dependent burst kinetics as the wild-type and γ-less channels. In comparison the δ-less AChR channel displays burst durations which are relatively voltage independent being almost five times shorter than the other combinations at very hyperpolarized potentials but approximately the same duration at depolarized potentials.

Comparison with Previous Single-Channel Current Measurements

The single-channel characteristics of wild-type *Torpedo* AChR channels observed in this study are similar to those described previously (Sakmann et al., 1985; Imoto et al., 1988; Sine, Claudio & Sigworth, 1990; Yu et al., 1991). Furthermore, our results on the γ-less combination agree with the study of Lo et al. (1991) that measured the same conductance and gating properties for γ-less and wild-type mouse AChR channels. In another study on bovine AChR channels Jackson et al. (1990) also found that the γ-less and wild-type channel had the same conductance in the presence of Ca^{2+} but the γ-less channel was 20% larger than the wild-type in Ca^{2+}-free solutions. All our conductance measurements were made in the absence of Ca^{2+} and the presence of 11 mM EGTA. However, we cannot rule out that a similar smaller difference in conductance was concealed within the variabilities of our estimates. Kullberg et al. (1990) observed that the wild-type mouse AChR channel expressed in oocytes displays multiple-conductance states with a predominant 50-pS class and less frequently observed 12- and 25-pS classes. In comparison the δ-less AChR displays only a smaller (12-pS) conductance channel. Although similar multiple-conductance behavior has been reported in muscle membranes (e.g., Hamill & Sakmann, 1981) it was not detected in the present study possibly due to limits in resolution.

Comparison with Chimera Studies

Previous studies on chimeric AChRs provided the first evidence that the δ-subunit may play a specific role in voltage-sensitive channel gating. In one study on calf-*Torpedo* chimeras it was demonstrated that calf δ injected in combination with *Torpedo* αβγ-subunits produced AChR channels with calf-life voltage-sensitive kinetics (i.e., slower and more voltage sensitive than *Torpedo*) (Sakmann et al., 1985). In comparison, combinations of calf β or γ with *Torpedo* αγδ or αβδ, respectively, produced AChR channels that remained *Torpedo* like (Sakmann et al., 1985). In another study involving mouse-*Torpedo* chimeras, voltage sensitivity was initially estimated from the ratio of slope conductances measured in the whole oocyte at −90 and

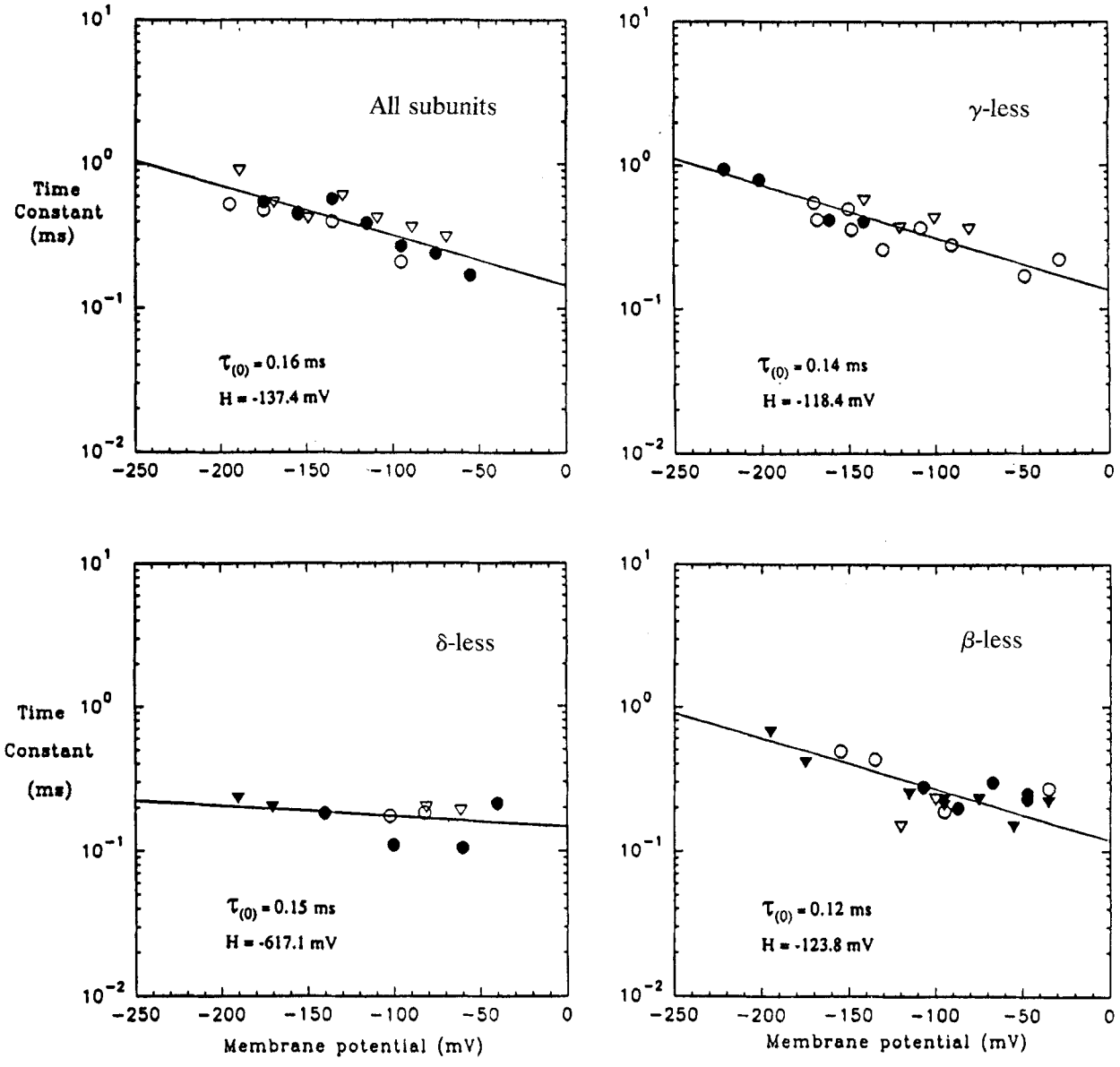

Fig. 6. Comparison of the voltage dependence of burst durations measured from AChR channels in oocytes injected with either all subunits or missing in one non-α-subunit. The data points were taken from burst-duration histograms constructed from the same events used in Fig. 5. Regression lines described by the relation $\tau_{(V)} = \tau_{(0)}\exp(V/H)$ were fitted through the data points, and the appropriate $\tau_{(0)}$ and H value for each combination is indicated.

30 mV (Yoshii et al., 1987). The most voltage-sensitive combination was *Torpedo* δ with mouse αβγ. However, in contrast to the calf-*Torpedo* chimera studies, the combination of mouse β with *Torpedo* αγδ was found to be more voltage sensitive than the combination mouse δ and *Torpedo* αβγ (Yoshii et al., 1987). To determine the mechanisms underlying the equilibrium responses, Yu et al. (1991) examined the single-channel properties of mouse-*Torpedo* chimeras and found that the mouse δ and *Torpedo* αβγ combination produced AChR channels with mouse-like voltage-dependent kinetics (i.e., again slower and more voltage dependent than *Torpedo*). However, the combination of mouse βδ with *Torpedo* αγ produced an even more pronounced voltage dependence even though the lifetime at −60 mV remained brief and more *Torpedo* like than mouse like. Unfortunately, the voltage sensitivity of single channels formed from mouse β and *Torpedo* αγδ were not reported (Yu et al., 1991) and therefore no firm conclusion can be made regarding the specific role of the β-subunit in the voltage dependence of equilibrium responses (Yoshii et al., 1987).

Molecular Mechanisms for δ-Subunit Effects on Voltage-Sensitive Gating

Without detailed knowledge of the quaternary structure of the closed and open conformations of the AChR channel it is difficult to speculate on the origin of voltage sensitivity of AChR-channel gating. However, one reason the *Torpedo* δ-subunit may have a stronger influence compared with other non-α-subunits is that it has more polar amino acids in its putative transmembrane regions (δ has 17 compared with 13 for γ and 12 for β). Note that unlike the S4 region of voltage-gated channels there are no charged groups in any of the putative transmembrane regions, M1, M2, M3 and M4, of the AChR subunits. Assuming the full voltage drop occurs across the membrane, then only the polar groups in these transmembrane regions would be sensitive to voltage changes. In this case movement of the δ-subunit during channel gating could result in a greater contribution to a dipole moment change during the rate-limiting conformational change (Magleby & Stevens, 1972). Furthermore, if the δ-subunit underwent a greater relative displacement perpendicular to the membrane field than other subunits its influence might be amplified. Although no direct information exists on subunit displacements during gating it is interesting that desensitizing concentrations of agonist appear to cause a quaternary rearrangement of the subunits, involving predominantly displacement of the δ-subunit and to a lesser extent the γ-subunit (Unwin, Toyoshiba & Kubalek, 1988). Finally, the mouse AChR which has significantly more polar residues in its putative transmembrane regions of δ as well as αβ and γ (*c.f.* Connolly, 1989; Numa, 1989) compared with *Torpedo* also displays significantly more voltage-sensitive channel gating (Yu et al., 1991).

An alternative explanation for the influence of the *Torpedo* δ-subunit on voltage-sensitive gating is that its specific interactions with the α-subunit produces a unique conformation that displays voltage sensitivity. In support of this type of explanation it has already been demonstrated that specific αδ interactions are required for the α-subunit to display the same high-affinity agonist binding as seen in the wild-type receptor but which is absent in the unassembled α-subunit or in αβ and αγ pairs (Blount & Merlie, 1989).

In conclusion, our study indicates a specific role for the δ-subunit in determining voltage-dependent gating of *Torpedo* AChR channels. However, evidence indicates that in other species, the β-subunit (Yu et al., 1991) as well as the γ-subunit, can also directly influence AChR-channel gating (Mishina et al., 1986; Lo et al., 1991). Understanding the exact mechanism(s) of these effects will require a more detailed physical picture of the gating process itself.

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