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Functional Acetylcholine Receptors Expressed in *Xenopus* Oocytes after Injection of *Torpedo* β, γ, and δ Subunit RNAs Are a Consequence of Endogenous Oocyte Gene Expression

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SUMMARY

The nicotinic acetylcholine receptor (AChR) is an oligomeric transmembrane glycoprotein consisting of four homologous subunits in a stoichiometry $\alpha_2\beta\gamma\delta$. *Xenopus* oocytes were used to study the effects of selectively deleting the α subunit of the *Torpedo californica* AChR on functional receptor expression. Oocytes microinjected with only *Torpedo* β, γ, and δ subunit RNAs showed small acetylcholine-elicited currents. These "α-less" AChRs were pharmacologically similar to the wild-type (i.e., *Torpedo* $\alpha_2\beta\gamma\delta$) receptor. Actinomycin D, which blocks endogenous RNA transcription, completely inhibited the expression of α-less but not wild-type receptor. Coinjection of antisense RNA to the α subunit of the *Xenopus* muscle AChR with *Torpedo* β, γ, and δ subunit RNAs significantly reduced expression of the α-less AChRs without altering expression of wild-type receptors. These results indicate that *Xenopus* oocytes express low levels of AChR α subunit mRNA that, when translated, can lead to the formation of functional *Xenopus-Torpedo* AChR hybrids. These results strongly suggest that, unless the potential contribution of endogenous subunits can be determined, caution must be exercised when analyzing the effects of subunit deletions on multisubunit protein expression in *Xenopus* oocytes.

Xenopus oocytes have proven to be a valuable tool in the study of ion channel structure and function (1). A wide variety of ion channels and receptors have been successfully expressed in *Xenopus* oocytes that were microinjected with both naturally occurring mRNAs and synthetic RNAs derived *in vitro* from cloned cDNAs. The utility of this expression system lies in the relative paucity of endogenous ion channels and neurotransmitter receptors in the oocyte membrane.

The nicotinic AChR is a ligand-gated, nonselective, cation channel that mediates synaptic transmission at the vertebrate neuromuscular junction and the electroplax of electric fish such as *Torpedo californica* (2, 3). The receptor is an oligomeric transmembrane glycoprotein composed of four highly homologous subunits in a stoichiometry $\alpha_2\beta\gamma\delta$. Functional AChRs have been expressed in *Xenopus* oocytes, that were microinjected with RNA synthesized *in vitro* from subunit cDNAs (4, 5). Subunit deletion studies have demonstrated that oligomerization and functional AChR formation in oocytes do not require microinjection of all four subunit RNAs (5, 6), suggesting that at least some of the subunits share functional homology. Analysis of subunit deletion studies has assumed that *Xenopus* oocytes do not express endogenous AChR subunits. In the present report we demonstrate functional AChR expression in *Xenopus* oocytes injected with RNAs coding for the *Torpedo* β, γ, and δ subunits. In addition, the data in this report provide evidence suggesting that *Xenopus* oocytes express low levels of the mRNA coding for the α subunit of the *Xenopus* AChR.

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Materials and Methods

Plasmids. Full-length *Torpedo* AChR subunit cDNAs (7) inserted into vector pSP64T (8) were generously provided by D. Hartman and T. Claudio (Yale University) and full length *Xenopus* muscle AChR α subunit cDNA (9) in vector pGEM3 was the generous gift of S. Burden (Massachusetts Institute of Technology).

***In vitro* transcription.** Plasmids containing AChR subunit cDNAs were lineralized by digestion with *Xba*I. Full length AChR subunit transcripts were generated using SP6 RNA polymerase, as described (5, 10). *In vitro* transcribed RNA was resuspended in sterile distilled H_2O at the appropriate concentration and stored at −70° until used.

Translation in oocytes. Adult female *Xenopus laevis* (Xenopus One, Ann Arbor, MI) were anesthetized by immersion in 0.17% tricaine, and a portion of the ovarian lobe was removed. The isolated tissue was dissociated using collagenase (Type IA, Sigma), and the remaining follicle cell layer was manually removed (5). Isolated, follicle-free, stage V and VI oocytes were microinjected with 50 nl of subunit-specific

ABBREVIATIONS: AChR, acetylcholine receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; WT, wild-type; ACh, acetylcholine; *d*TC, *d*-tubocurerine; GABA, γ-aminobutyric acid.

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RNAs at the appropriate concentration and molar stoichiometry. Injected oocytes were maintained in a physiological saline solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6), supplemented with 2.5 mM sodium pyruvate, 1000 units/ml penicillin, and 0.1 mg/ml streptomycin, until ready for use.

Electrophysiology. Electrophysiological responses to bath application of ACh were measured using a standard two-microelectrode voltage clamp (Axoclamp 2A, Axon Instruments). Electrodes were filled with 3 M KCl and had resistances of 0.5–5 MΩ. The recording chamber was continually perfused with physiological saline supplemented with 0.3 μM atropine. Unless otherwise noted, the holding potential was −60 mV. Only healthy oocytes with a resting potential more negative than −30 mV were used in this study.

Results

Microinjection of *Xenopus* oocytes with 15 ng of *in vitro* transcribed RNA coding for *Torpedo* AChR subunits mixed in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ (termed WT) resulted in the expression of functional AChRs on the cell surface. Bath application of 2 μM ACh to voltage-clamped oocytes elicited a characteristic inward current that desensitized in the continued presence of agonist (Fig. 1A). Oocytes injected with 15 ng of RNA coding for the *Torpedo* β, γ, and δ subunits mixed in equimolar stoichiometry ("α-less" receptors) did not respond to bath application of 2 μM ACh (Fig. 1B). However, when higher concentrations of ACh were applied, small, rapidly desensitizing currents were observed (Fig. 1, C and D). These currents were not due to endogenous muscarinic ACh receptor activation (11), because they were not blocked by 1 mM atropine (data not shown). Uninjected oocytes did not respond to bath application of up to 1 mM ACh (Fig. 1, E and F), demonstrating that the observed ACh-elicited currents were not endogenous to the oocytes but were related to the microinjection of *Torpedo* AChR subunit RNAs. Although there was variability in the size of the α-less receptor-mediated currents from donor to donor, they were observed consistently in oocytes from numerous donors.

The presence of ACh-elicited currents in the absence of microinjected RNA coding for the α subunit presented several intriguing possibilities. Dunn and Raftery (12) have suggested the possibility of ligand binding sites on non-α AChR subunits. Alternatively, a recent report on the presence of endogenous voltage-dependent Na^+ channels, previously believed to be nonexistent in oocytes (13), suggested the possibility of low level expression of (at least) the AChR α subunit in oocytes. Such an RNA species would code for an α subunit protein that could lead to the formation of a *Xenopus-Torpedo* hybrid AChR. These possibilities were investigated by comparing the properties of WT and α-less receptors expressed in oocytes.

WT and α-less receptor-mediated currents had reversal potentials of approximately −5 mV (data not shown), suggesting similar ionic conductances for both receptors. ACh dose-response curves were generated for both types of receptors. In order to compare WT and α-less AChRs over the same range of ACh concentrations, the number of functional WT receptors on the surface of the oocyte was reduced by injection of only 0.1 ng of total subunit-specific RNAs [WT RNA in a subsaturating, limiting RNA concentration (10)] and then treatment with 100 nM α-bungarotoxin to block most of the surface receptors before assay. This protocol reduced the number of functional WT receptors on the surface to levels that produced macroscopic currents comparable to α-less receptor-mediated

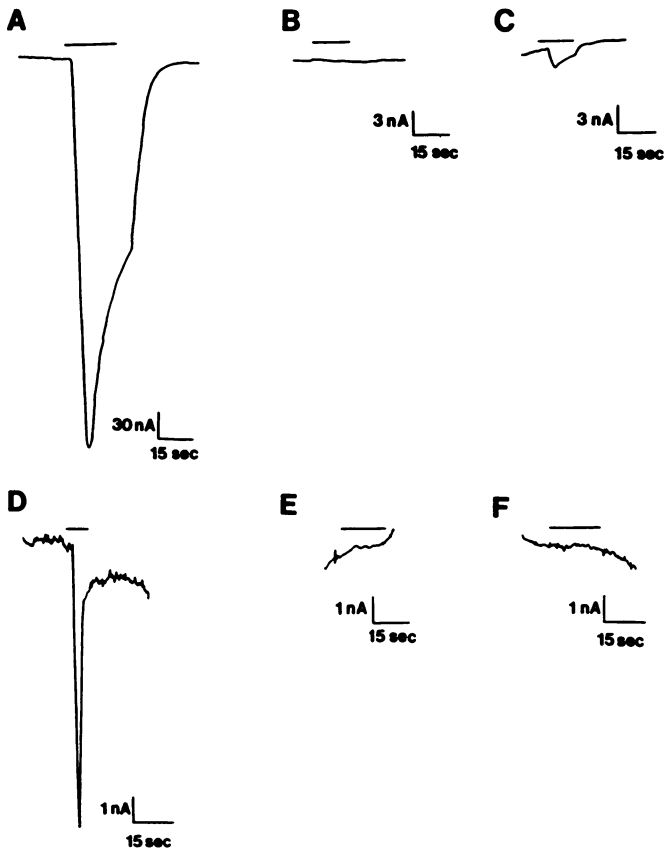

Fig. 1. Expression of functional AChRs in *Xenopus* oocytes. Oocytes were microinjected with either 15 ng of subunit-specific AChR RNAs mixed in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ (WT) or 15 ng of subunit-specific RNAs mixed in a molar stoichiometry of $\beta\gamma\delta$ (α-less). Oocytes were incubated for 24 (WT) or 48 (α-less) hr and then voltage clamped and the current response to bath application of ACh was measured. A, Response of WT oocyte to 2 μM ACh. B, Response of α-less oocyte to 2 μM ACh. C, Response of α-less oocyte to 20 μM ACh. D, Response of α-less oocyte to 200 μM ACh. E, Response of uninjected oocyte to 200 μM ACh. F, Response of uninjected oocyte to 1 mM ACh.

currents. These conditions are termed WT_{BTX}. In addition, all dose-response experiments were performed using oocytes from the same frog to eliminate any donor variability. Electrophysiological responses to bath application of varying concentrations of ACh were determined in oocytes expressing either WT, WT_{BTX} or α-less receptors. Analysis of the dose-response curves on double logarithmic coordinates (Fig. 2A) revealed that WT receptors had an apparent Hill coefficient of approximately 1.6, in agreement with previous reports (5, 14). The apparent Hill coefficient for the α-less receptor was only 0.47. However, this decrease in the apparent Hill coefficient is most likely due to a flattening of the dose-response curve at the high concentrations of ACh necessary to elicit detectable α-less responses. A log-log plot of the fractional activation (I/I_{max}) for a receptor that requires m ACh molecules to open versus [ACh] will necessarily flatten as saturation is approached:

$$I/I_{max} = [1 + (K_d/[ACh]^m]^{-1}$$

The value of n, the apparent Hill coefficient, which is operationally defined as the slope of the curve on log-log coordinates, will, therefore, depend on [ACh]. At low concentrations (i.e., [ACh] $\ll K_d$), the apparent Hill coefficient is approximately

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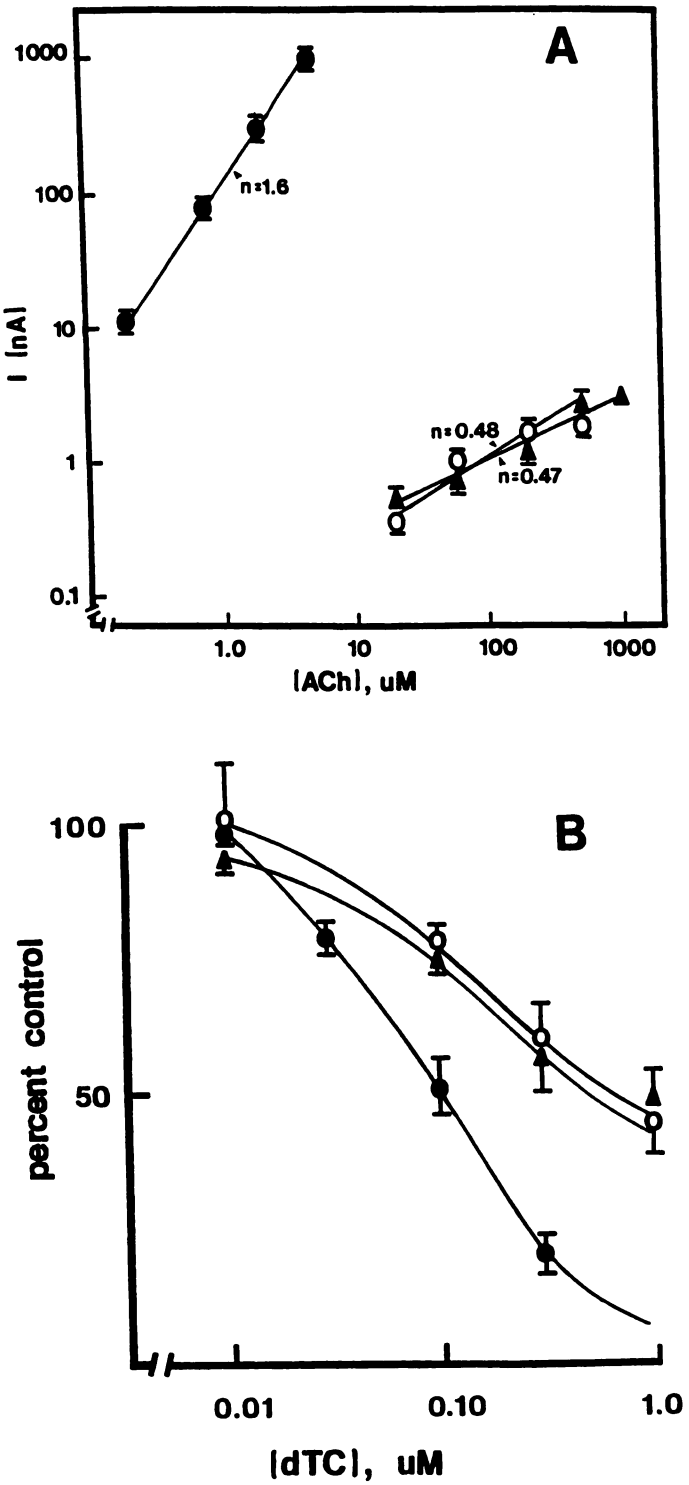

Fig. 2. α-less AChRs are pharmacologically similar to WT AChRs. A, Oocytes were injected with either WT or α-less RNAs and then treated as described in the legend to Fig. 1. Oocytes were also microinjected with 0.1 ng of RNA in a molar stoichiometry of $\alpha_2\beta\gamma\delta$, incubated for 48 hr, and then treated with 100 nM α-bungarotoxin for 60 min before voltage clamping (termed WT_{BTX} oocytes). The dose response for WT (●), α-less (▲), and WT_{BTX} (○) oocytes was then determined. Data were fit by linear regression, and the slopes (*n*, the apparent Hill coefficient) are indicated. Each *point* represents the mean ± standard error of 6–10 oocytes taken from the same frog. B, *d*TC inhibition curves for WT (●), α-less (▲), and WT_{BTX} (○) oocytes. Current responses were elicited by bath application of either 2 μM (WT) or 200 μM (α-less and WT_{BTX}) ACh. Data are presented as the percentage of the current response in the absence of *d*TC. Each *point* represents the mean ± standard error of 6–10 oocytes taken from the same frog.

m, whereas at high ACh concentrations it approaches 0. This idea was supported by the demonstration that oocytes injected with WT RNA but treated with α-bungarotoxin before voltage clamping (WT_{BTX} receptors) had an apparent Hill coefficient of 0.48 over the same concentration range in which the α-less responses were obtained, similar to the value obtained for the α-less receptors (Fig. 2A).

WT receptor-mediated currents were sensitive to inhibition by *d*-TC, with an IC_{50} of approximately 90 nM (Fig. 2B). α-less and WT_{BTX} receptors were also inhibited by *d*TC, but the curves for both receptors were shifted to the right, with an IC_{50} of approximately 1 μM for both receptors (Fig. 2B). Correction of the IC_{50} values for all three types of receptors for the concentrations of ACh used in the determinations (15) revealed that the K_i values for *d*TC for all three receptors were essentially identical (approximately 40 nM).

Both WT and α-less receptors were also sensitive to inhibition by α-bungarotoxin (Fig. 3). In addition, both WT and α-less receptors were blocked by 10 μM lidocaine in a use-dependent manner (data not shown). Taken together, all of these data indicate that the WT and α-less receptors share a similar pharmacological profile and, in fact, suggest that the two types of receptors are similar. It, therefore, seemed reasonable to hypothesize that the α-less receptor may actually contain α subunits supplied by the oocyte as the result of translation of endogenous *Xenopus* α subunit mRNA. To examine this possibility more directly, we studied the contribution of endogenous oocyte transcription to the formation of α-less receptors.

The dependence of WT, α-less, and WT_{BTX} receptor expression on endogenous oocyte transcription was determined by

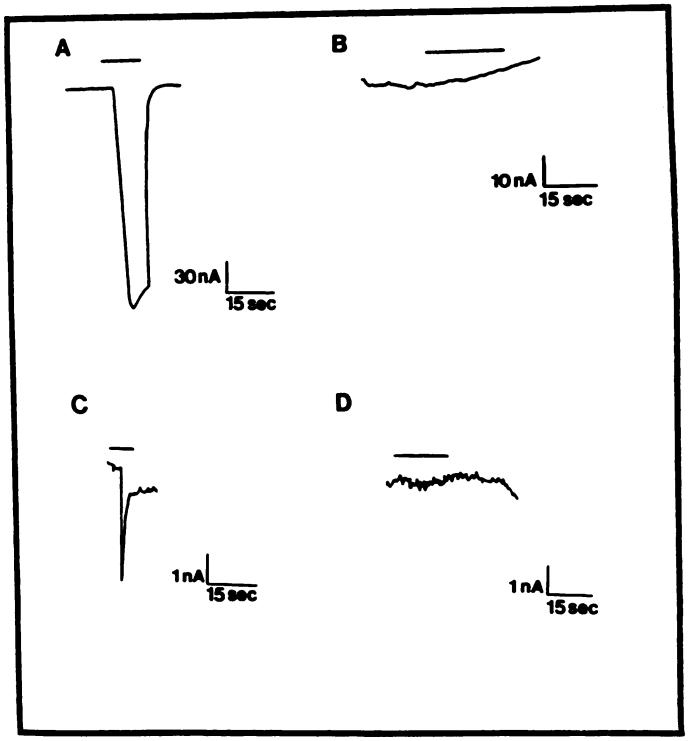

Fig. 3. α-bungarotoxin inhibition of WT and α-less AChRs. A, Response of WT oocyte to bath application of 2 μM ACh. B, As in A, but after treatment of the same oocyte with 100 nM α-bungarotoxin for 30 min. C, Response of α-less ooctye to bath application of 200 μM ACh. D, As in C, but after treatment of the same oocyte with 100 nM α-bungarotoxin for 30 min. Note that both types of receptors are blocked by α-bungarotoxin.

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injecting oocytes with the appropriate RNA mixture and then incubating the oocytes in the presence of 50 μg/ml actinomycin D to block endogenous transcription (16). This concentration of actinomycin D blocked the incorporation of [^{3}H]uridine into oocyte RNA by greater than 70% with no effect on either protein synthesis or cell viability (data not shown). Following a 48-hr incubation, WT_{BTX} oocytes were treated with 100 mM α-bungarotoxin, oocytes from all three groups were voltage clamped, and the expression of AChRs was determined by bath application of ACh. As shown in Fig. 4, actinomycin treatment had no effect on expression of either WT or WT_{BTX} receptors, demonstrating that the formation of these AChRs was independent of endogenous oocyte transcription. In addition, actinomycin had no effect on the shape of the ACh dose-response curve or the apparent Hill coefficient for either the WT (not shown) or WT_{BTX} (Fig. 5) receptors.

In contrast, the expression of functional α-less AChRs was completely abolished by the actinomycin treatment (Fig. 4). The unique sensitivity of the α-less AChR to actinomycin strongly suggests that *Xenopus* oocytes express (at low levels) α subunit mRNA. This α subunit mRNA can be translated and the resulting *Xenopus* α subunit protein can form a hybrid receptor with the *Torpedo* β, γ, and δ subunits translated from the exogenous microinjected RNAs. The formation of hybrid receptors between subunits of different species has been well established (5, 14, 17).

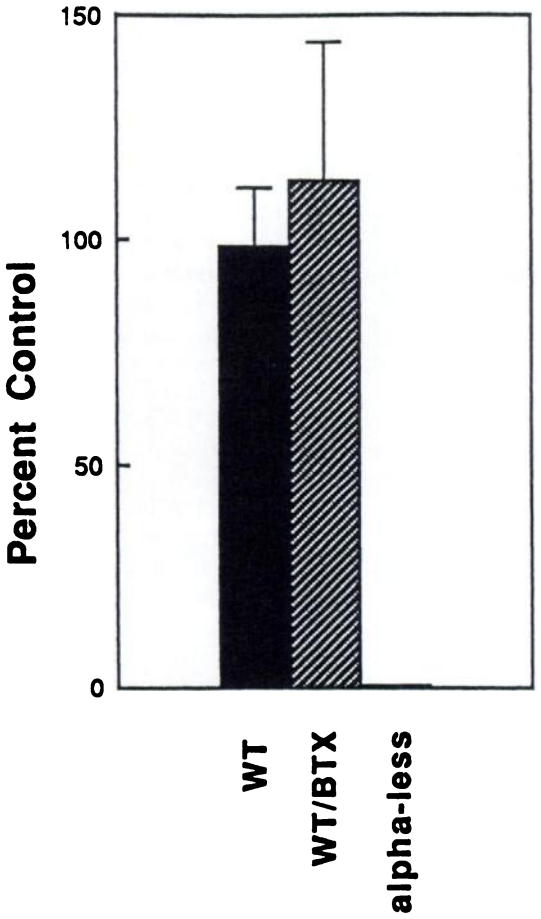

Fig. 4. Actinomycin blocks the expression of α-less but not WT or WT_{BTX} receptors. Oocytes were injected with the appropriate RNA mixture and then incubated for 48 hr in the presence of 50 μg/ml actinomycin D. In addtion, WT_{BTX} oocytes were treated with 100 nM α-bungarotoxin for 60 min before recording. Oocytes were then voltage clamped and the response to bath application of 2 μM (WT) or 200 μM (α-less and WT_{BTX}) ACh were measured. Control oocytes were treated identically, with the exception that actinomycin D was not present during the 48-hr incubation period. Results are presented as the mean percentage of control response ± standard error of 6–10 oocytes taken from the same frog. None of the α-less oocytes treated with actinomycin D responded to ACh. These results are representative of three separate experiments.

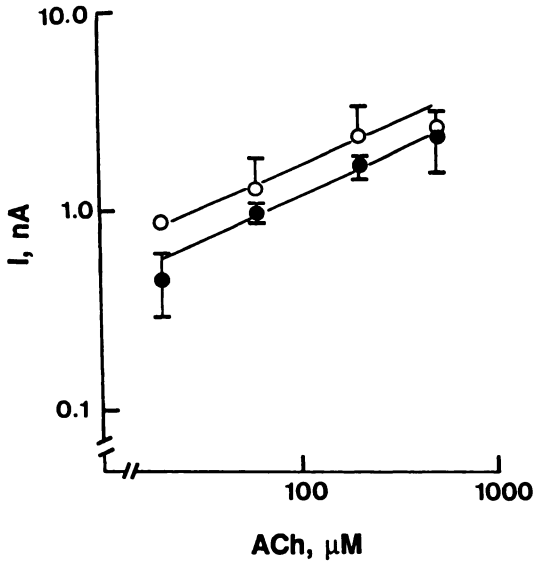

Fig. 5. Actinomycin D has no effect on the dose-response relationship in WT_{BTX} oocytes. Oocytes were injected with 0.1 ng of RNA in a molar stoichoimetry of $\alpha_2\beta\gamma\delta$ and incubated for 48 hr in the absence (○) and presence (●) of 50 μg/ml actinomycin D. Oocytes were then treated with 100 nM α-bungarotoxin for 60 min and then the response to bath application of various concentrations of ACh was determined. The mean ± standard error current responses of 6–10 oocytes are shown. The apparent Hill coefficients were 0.40 and 0.47 for oocytes incubated in the absence and presence of actinomycin D, respectively.

To further examine this possibility, endogenous mRNA translation was blocked using antisense RNA. Antisense RNAs injected into *Xenopus* oocytes have been used to block the translation of exogenous microinjected RNAs (18, 19). *Xenopus* AChR α subunit sense and antisense RNAs were synthesized from full length *Xenopus* α subunit cDNAs using SP6 or T7 RNA polymerases, respectively. Oocytes injected with *Xenopus* α sense RNA and *Torpedo* β, γ, and δ subunit RNAs (15 ng of total subunit RNAs in a molar stoichiometry of $\alpha_2\beta\gamma\delta$) expressed AChRs quantitatively similar to WT *Torpedo* AChRs (data not shown), demonstrating that *Xenopus-Torpedo* hybrid AChRs could form.

To determine the appropriate amount of antisense RNA to use, an estimation of the amount of α subunit RNA present in oocytes was made, based upon the previous demonstration of the effect of varying the amount of α subunit RNA on receptor expression (10). Calculations based on the minimum amount of α subunit RNA needed to obtain WT current responses the size of the α-less responses suggested that the α subunit mRNA is very scarce in the oocyte mRNA pool (0.001–0.01% of the pool). We used antisense RNA levels in 100–1000-fold excess of this amount. The large excess was used to control/compensate for errors in the assumptions used to calculate the amount of endogenous α subunit mRNA levels. Different amounts of antisense α subunit RNA were co-injected with 15 ng of a mixture of *Torpedo* β, γ, and δ RNAs. Oocytes were then assayed for the presence of functional AChRs after a 48-hr

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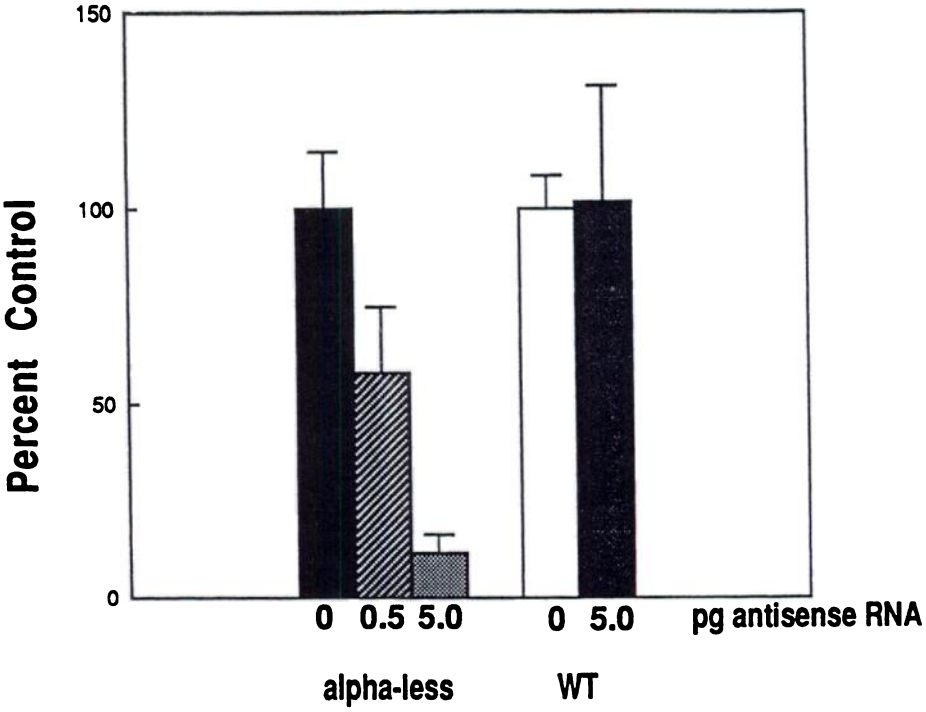

Fig. 6. *Xenopus* α subunit antisense RNA blocks expression of α-less but not WT receptors. Oocytes were injected with either WT or α-less RNA mixes alone or with the appropriate mix and *Xenopus* α subunit antisense RNA. Oocytes were then incubated for 48 hr and the response to bath application of 2 μM (WT) or 200 μM (α-less) ACh was determined. Results are presented as the mean percentage of control current response ± standard error of 6–10 oocytes from the same frog. In each case, the variability of the control group (no antisense RNA) is shown. These results are from oocytes from a frog that showed very robust α-less responses, with the actual mean values for the α-less currents being 35.4, 20.4, and 4.10 nA for 0, 0.5, and 5 pg of antisense RNA, respectively.

incubation. Oocyte expression of α-less receptors was significantly reduced by co-injection of either 0.5 or 5 pg of antisense RNA (Fig. 6). Lower doses of antisense RNA were without effect (data not shown). Injection of 5 pg of antisense RNA had no effect on expression of WT *Torpedo* AChRs (Fig. 6).

Discussion

In this report, we demonstrate the expression of functional AChRs in *Xenopus* oocytes when only the *Torpedo* β, γ, and δ subunit-specific RNAs are injected. Our results suggest that the expression of this α-less receptor is the result of endogenous α subunit RNA translation and the formation of a hybrid receptor composed of the *Xenopus* α and *Torpedo* β, γ, and δ subunits. Whether the *Xenopus* β, γ, and δ subunits are also transcribed in oocytes has not been determined. However, the lack of responsiveness of uninjected oocytes to high concentrations of ACh suggests either that only the *Xenopus* α subunit is expressed or that levels of expression of the other subunits in uninjected oocytes are so low that they are below the threshold for detection of functional receptors.

Although there are no reports of expression of α-less receptors in *Xenopus* oocytes, the concentrations of ACh used in the current study were higher than those used in earlier studies (6). In addition, enhanced levels of translation in oocytes obtained with the pSP64T vector (10) might enhance the likelihood of detecting α receptor species expressed at very low levels. Although the α-less receptor was expressed in oocytes at lower levels than the WT receptor, the α-less receptors were functional and pharmacologically similar to the WT AChR. Lower levels of expression of the α-less receptor were most likely due to the limited availability of the α subunit protein for assembly of the receptor complex (i.e., fewer receptors in the cell membrane), rather than any changes in the biophysical properties of the channel itself. Indeed, we have previously demonstrated the crucial role of the α subunit in controlling the levels of surface AChR expression in *Xenopus* oocytes (10).

Xenopus oocytes have been used extensively to study expression of multisubunit proteins. Functional $GABA_A$ receptors have been expressed in oocytes following microinjection of RNA coding for a single subunit (20). Although it is possible that homooligomeric $GABA_A$ receptors were formed in oocytes, the absence of any endogenous $GABA_A$ receptor subunits was not demonstrated. Recently, Parker, and Miledi (13) have demonstrated the presence of endogenous voltage-dependent, tetrodotoxin-sensitive, Na^+ channels in oocytes, channels previously believed not to be expressed in oocytes. This finding may present a difficulty in the interpretation of experiments using oocytes to express foreign sodium channel mRNA. We similarly suggest that caution be used in interpreting data on the effects of subunit deletions on foreign multisubunit protein expression in oocytes, unless the contribution of endogenous subunit expression has been determined.

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