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Altered Patterns of *N*-Linked Glycosylation of the *Torpedo* Acetylcholine Receptor Expressed in *Xenopus* Oocytes

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Summary. The nicotinic acetylcholine receptor (AChR) from *Torpedo* electroplax is an oligomeric transmembrane glycoprotein made up of four highly homologous subunits in a stoichiometry of $\alpha_2\beta\gamma\delta$. The role of *N*-linked glycosylation of the AChR has been studied in several cell lines and these studies have suggested that the addition of carbohydrate may be important for receptor expression. While *Xenopus* oocytes have proven to be an invaluable tool for studying the AChR, little is known about *N*-linked glycosylation of the oocyte-expressed receptor. The present report demonstrates that the oocyte-expressed AChR is glycosylated and contains the same number of oligosaccharide residues per subunit as the native receptor. However, unlike the native *Torpedo* receptor which contains both high mannose and complex oligosaccharides, the oocyte-expressed AChR contains only high mannose oligosaccharide modifications. However, as has been well documented, the *Torpedo* AChR expressed in oocytes is fully functional, demonstrating that the precise nature of the oligosaccharide modification is not critical for receptor function.

The role of the oligosaccharide component of the AChR in receptor function was examined using tunicamycin (TM) to inhibit *N*-linked protein glycosylation. TM treatment resulted in a 70–80% inhibition of AChR expression in oocytes. Functional, unglycosylated receptors were not expressed; receptors expressed in TM-treated oocytes were functional wild-type, glycosylated AChR, formed only during the initial 12 hr of TM exposure. These data suggest that while glycosylation of the oocyte-expressed *Torpedo* AChR is required for assembly of subunits into a functional receptor, as has been demonstrated in other cells, oocyte modification of normal *Torpedo* glycosylation patterns does not affect receptor function or assembly.

Key Words *Xenopus* oocytes · acetylcholine receptor · glycosylation · biosynthesis · tunicamycin

Introduction

The nicotinic acetylcholine receptor (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* (for reviews, *see* Popot & Changeux, 1984; McCarthy et al., 1986). The receptor is a transmembrane glycoprotein oligomer composed of four highly homologous subunits in a stoichiometry of $\alpha_2\beta\gamma\delta$. The subunits are arranged around a central aqueous core, believed to make up the ion channel, with each α subunit containing a binding site for receptor agonists and antagonists. The four AChR subunits have been cloned from *Torpedo* (Noda et al., 1982; Claudio et al., 1983; Noda et al., 1983) and the primary amino acid sequences have been deduced from the cDNA nucleotide sequences. Fully functional *Torpedo* AChRs have been reconstituted in *Xenopus* oocytes injected with RNA transcribed in vitro from the corresponding cDNAs (Mishina et al., 1985; White et al., 1985). The oocyte-expressed AChR is biochemically, pharmacologically and functionally indistinguishable from the native *Torpedo* receptor (Mishina et al., 1985; Sakmann et al., 1985; White et al., 1985; Buller & White, 1988). *Xenopus* oocyte expression of the *Torpedo* AChR has been thoroughly characterized and some of the post-transcriptional factors involved in receptor biosynthesis and expression in oocytes have been identified (Buller & White, 1988).

One post-transcriptional event that may play a role in protein function is the addition of oligosaccharide residues. Potentially, glycosylation can serve many functions including determination of protein structure, protein stability and turnover, cellular recognition and antigenicity, and intracellular transport and targetting (for review, *see* Olden, Parent & White, 1982). In many cases, inhibition of glycosylation has profound effects on protein biosynthesis and function. Secretion and intracellular processing of the vesicular stomatitis virus glycoprotein is inhibited when normal cellular glycosylation pathways are impaired (Morrison, McQuain & Simpson, 1978; Machamer, Florkiewicz & Rose, 1985). Similarly, assembly of the constitutive α and β subunits of thyroid-stimulating hormone is depen-

dent on subunit glycosylation (Weintraub et al., 1980). In addition, secretion of both IgE and fibronectin is blocked by inhibitors of cellular glycosylation (Olden et al., 1982). However, there are also instances where inhibition of glycosylation is without effect. Biosynthesis and function of both the S49 lymphoma cell β-adrenergic receptor (George, Ruoho & Malbon, 1986) and the Hep G2 cell asialoglycoprotein receptor (Brietfeld, Rup & Schwartz, 1984) are unaffected by inhibition of glycosylation. Thus, the role of the oligosaccharide moiety is highly dependent on the specific protein of interest.

Analysis of the carbohydrate content of the purified *Torpedo* AChR has revealed that each subunit is glycosylated (Lindstrom, Merlie & Yogeeswaran, 1979; Vandlen et al., 1979; Nomoto et al., 1986), a finding in agreement with the presence of potential sites for *N*-linked glycosylation within the deduced amino acid sequences of each subunit. The role of the *N*-linked oligosaccharide modification of the *Torpedo* AChR in receptor biosynthesis, assembly, expression and function is not completely known. However, studies on the biosynthesis of mammalian muscle AChR suggest an important role for *N*-linked glycosylation in regulating the normal expression of cell surface AChRs, since inhibition of glycosylation reduces cellular levels of the AChR (Prives & Olden, 1980; Merlie et al., 1982; Prives & Bar-Sagi, 1983).

The present investigation sought to use the *Xenopus* oocyte expression system to study glycosylation of the *Torpedo* AChR. *Xenopus* oocytes are capable of translating a remarkably diverse range of foreign RNAs (Gurdon et al., 1971; Colman, 1984). Additionally, oocytes perform many biosynthetic modifications required for biological activity of expressed proteins. Indeed, the capacity of the oocyte to glycosylate foreign proteins synthesized in response to mRNA microinjection has been well documented (Lane, 1983; Colman, 1984; Soreq, 1985). However, no detailed characterization of the role of glycosylation in oocyte expression of the *Torpedo* AChR has been reported. We sought to determine the nature of oocyte glycosylation of the *Torpedo* AChR and compare it to glycosylation of the native receptor. We also wanted to determine the role of glycosylation in oocyte expression of functional AChR. The results presented here demonstrate that the oocyte-expressed *Torpedo* AChR is glycosylated in a manner different from that seen in the native *Torpedo* receptor. Furthermore, while the precise nature of the oligosaccharide modification does not appear to be critical for oocyte expression of functional AChRs, the absence of glycosylation prevents receptor expression in *Xenopus* oocytes.

Materials and Methods

Plasmids and In vitro Transcription

Full length *Torpedo* AChR subunit cDNA clones inserted into the pSP64T vector (Krieg & Melton, 1984) were used as described (Buller & White, 1988). The cDNA inserts in this vector are flanked by the 5′ and 3′ untranslated region of the *Xenopus* β-globin gene. Plasmid DNA was linearized by digestion with *Xba* I and transcribed in vitro using SP6 RNA polymerase as described (White et al., 1985; Buller & White, 1988).

Translation in *Xenopus* Oocytes

Oocytes removed from mature female *Xenopus laevis* (Xenopus One, Ann Arbor, MI) were dissociated in 2 mg/ml collagenase (Type IA, Sigma Chemical, St. Louis, MO) in Ca^{2+}-free OR-2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) and the remaining follicle layer was removed manually using watchmaker's forceps. Isolated stage V and stage VI oocytes were maintained in complete ND-96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6, 2.5 mM sodium pyruvate, 1000 U/ml penicillin, 0.1 mg/ml streptomycin). AChR subunit RNAs were dissolved in sterile distilled water at the appropriate concentrations and mixed together in a molar stoichiometry of $2\alpha:\beta:\gamma:\delta$. Fifty nl of the final RNA mixture (7.5 ng total AChR RNA per oocyte; Buller & White, 1988) was microinjected into the oocyte cytoplasm as previously described (White et al., 1985). Oocytes were incubated in complete ND-96 solution at 19°C until ready for use.

Electrophysiology

Electrophysiological responses to bath application of acetylcholine (ACh) were measured using a standard two microelectrode voltage clamp (Axoclamp 2A, Axon Instruments, Foster City, CA). Electrodes were filled with 3 M KCl and had resistances of 0.6–3.0 MΩ. The recording chamber was continuously perfused with ND-96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) containing 0.3 μM atropine to block endogenous muscarinic cholinergic receptors (Kusano, Miledi & Stinnakre, 1982) associated with any remaining follicle cells. Whole cell current elicited by bath application of 2 μM ACh was determined at a holding potential of −60 mV. Only healthy cells with a minimum resting potential of −30 mV were used.

Tunicamycin Treatment of Oocytes

Tunicamycin (TM; Boehringer Mannheim, Indianapolis, IN) was dissolved in sterile water and co-injected into the cytoplasm of oocytes with an equal volume of AChR subunit RNAs (7.5 ng total AChR subunit RNAs, mixed in a molar stoichiometry of $2\alpha:\beta:\gamma:\delta$). The final concentration of TM was 25 μg/ml. Oocytes were incubated in the presence of 2 μg/ml TM in complete ND-96 until ready for use. Preliminary studies had determined that 25 μg/ml was the maximal concentration of TM that could be used to inhibit *N*-linked glycosylation with minimal effects on cell viability or protein synthesis.

[^{125}I]α-Bungarotoxin Binding

Oocyte-expressed cell surface receptors were labeled with 1 nM [^{125}I]α-bungarotoxin (17 μCi/μg, NEN, Boston, MA) for 90 min at 25°C in ND-96 containing 1 mg/ml bovine serum albumin (BSA) and 0.1 mg/ml cytochrome *c*. Cells were washed extensively in the same buffer and counted in groups of 5 in a γ counter. To label total cellular binding sites, groups of 5–10 oocytes were homogenized in binding buffer (50 mM sodium phosphate, pH 7.2, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 1 mg/ml BSA, 0.1 U/ml aprotinin, 0.1 mg/ml cytochrome *c*). Homogenates were incubated at room temperature for 10 min and yolk proteins were removed by centrifugation. The resulting detergent extract was incubated with 1 nM [^{125}I]α-bungarotoxin for 90 min at 25°C. The reaction was terminated by filtration through Whatman DE81 filter discs (preincubated in binding buffer containing 1 nM unlabeled α-bungarotoxin). Filters were washed and counted in a γ counter. Nonspecific binding of [^{125}I]α-bungarotoxin was determined using uninjected control oocytes.

Immunoprecipitation

Microinjected oocytes were metabolically labeled in complete ND-96 solution containing 25 μCi/ml [^{35}S] methionine (>1000 Ci/mmol; New England Nuclear, Boston MA). Groups of 10–20 oocytes were homogenized in 20 mM Tris HCl, pH 8.0, 1 mM dithiothreitol (DTT), 1% Triton X-100, 2 mM EDTA and incubated for 10 min at room temperature. Detergent-insoluble yolk proteins were removed by centrifugation. SDS was added to the cleared extract to a final concentration of 1%. Extracts were heated to 90°C for 5 min, diluted with an equal volume of solubilizing buffer (300 mM NaCl, 100 mM Tris, pH 8.0, 10% SDS, 2% deoxycholate, 1% Triton X-100, 2 mM DTT, 4 mM EDTA, 2 mM PMSF) and incubated with anti-*Torpedo* AChR subunit antibodies (Claudio & Raftery, 1977) for 6–8 hr at 4°C. Immune complexes were precipitated for 30 min with formula fixed protein A-bearing *Staphylococcus aureus* (Pansorbin; Calbiochem, San Diego, CA) prepared according to the manufacturer's recommendations. Immunoprecipitated proteins were run on 10% SDS-polyacrylamide gels (Laemmli, 1970) and visualized by fluorography (EN^{3}HANCE; NEN).

Purification of AChR

From Torpedo

Electric organ tissue was dissected from freshly killed *Torpedo californica* (Pacific Biomarine, Venice, CA) and frozen in liquid N_2. Frozen tissue was thawed on ice and a Triton extract was prepared (Froehner & Rafto, 1979). The tissue was homogenized in 2 volumes (wt/vol) of buffer A (10 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaN_3, 0.01 U/mol aprotinin). The homogenate was passed through two layers of cheesecloth and the resulting filtrate was centrifuged at 27,000 × *g* for 45 min. The supernatant was discarded and the pellet was resuspended in buffer B (buffer A containing 1% Triton X-100) and stirred at 4°C for 90 min. Residual Triton-insoluble material was removed by centrifugation at 27,000 × *g*. The Triton extract was incubated for 2 hr at 4°C with α-cobratoxin agarose affinity resin (Sigma Chemical, St. Louis, MO) previously equilibrated with buffer B as described (Mosckovitz & Gershoni, 1988) and then packed into a 10-ml disposable column. The column was sequentially washed with 2–3 column volumes of buffer B (buffer B containing 1 M NaCl and buffer A containing 0.1% Triton X-100). Bound AChR was eluted with 0.8 column volumes of 1 M carbamylcholine in buffer A containing 0.1% Triton X-100. Carbamylcholine was removed by dialysis against buffer A containing 0.1% Triton X-100. Purified receptor was stored at −70°C. No degradation of subunits was observed with storage up to 2 mo.

From Oocytes

Oocytes (75–100) were injected with AChR subunit RNAs and metabolically labeled with 25 μCi/ml [^{35}S]methionine for 18–24 hr. Oocytes were homogenized in 1 ml of extraction buffer (40 mM Tris HCl, 2 mM EDTA, 1% Triton X-100, pH 8.0, 1 mM DTT, 1 mM PMSF) and incubated at 4°C for 30 min. Triton-insoluble material was removed by centrifugation. The resulting Triton extract was incubated for 2 hr at 4°C with 200 μl α-cobratoxin affinity resin equilibrated with extraction buffer. Bound receptor was purified by batch elution with 1 M carbamylcholine in extraction buffer containing 0.1% Triton X-100 following washes with 1 M NaCl and 0.1% Triton X-100 as described above.

Glycosidase Analysis of AChR Subunits

Affinity-purified *Torpedo* AChR or immunoprecipitated [^{35}S]methionine labeled oocyte-expressed subunits were treated with either endo-β-*N*-acetylglucosaminidase H (Endo H; Boehringer Mannheim) in 80 mM sodium citrate, pH 5.5 and 0.3% SDS, or with endo-β-*N*-acetylglucosaminidase F (Endo F; Boehringer Mannheim) in 50 mM sodium acetate, pH 5.0, 50 mM EDTA, 1% β-mercaptoethanol, 0.5% Triton X-100 and 0.15% SDS. Reactions were incubated at 37°C for 15 hr. Proteins were separated on 10% SDS-polyacrylamide gels and visualized by staining with Coomassie blue (*Torpedo* subunits) or by fluorography (oocyte-expressed receptor).

Inhibition of Protein Synthesis and *N*-Linked Glycosylation

Oocytes were microinjected with AChR subunit RNAs alone or RNA mixed with TM as described above and incubated in groups of 5–10 cells with 50 μCi [^{3}H]leucine (140 Ci/mmol; Amersham, Arlington Heights, IL) or 100 μCi [^{3}H]mannose (40 Ci/mmol, New England Nuclear) for 12 hr. Incorporation of [^{3}H]leucine or [^{3}H]mannose into protein was determined by precipitation of protein in 10% trichloroacetic acid followed by liquid scintillation counting.

Results

Oligosaccharide Composition of the Oocyte-Expressed AChR

Torpedo electric organ AChR and oocyte-expressed AChR were purified by α-cobratoxin affinity chromotography. A comparison of the affinity-purified AChR from *Torpedo* and oocytes is shown in Fig. 1.

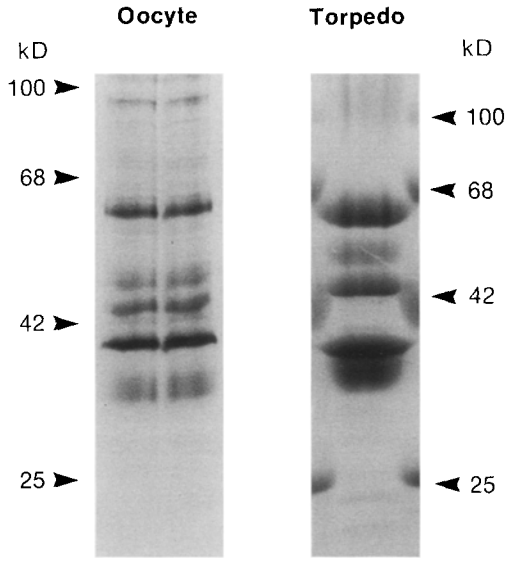

Fig. 1. Comparison of native *Torpedo* and oocyte-expressed affinity-purified AChRs. Oocytes were injected with AChR subunit RNAs and labeled with [^{35}S]methionine. Oocyte-expressed AChR and native *Torpedo* AChR were purified from Triton-extracts by α-cobratoxin affinity chromatography as described in Materials and Methods. Purified proteins were run on 10% SDS-polyacrylamide gels. Oocyte-expressed AChR subunits were visualized by subsequent fluorography. *Torpedo* AChR subunits were visualized by Coomassie blue stain. The relative mobilities of the molecular weight markers are indicated by arrowheads

Table. Apparent molecular weight determinations of native *Torpedo* and oocyte-expressed AChRS

	Oocyte	*Torpedo*
α	37,500	37,500
β	43,000	42,000
γ	45,000	50,000
δ	58,000	61,000

Molecular weights were determined by running the affinity purified oocyte-expressed and native *Torpedo* AChR on 10% SDS-polyacrylamide gels. Subunits were visualized by staining with Coomassie blue (*Torpedo* AChR) or by fluorography (oocyte-expressed AChR) and compared to the mobility of molecular weight standards (*see* Fig. 1). Shown are the means of 2–3 determinations. Standard errors were less than 3% of the mean mol wt.

While the α and β subunits from oocytes were similar in size to the corresponding *Torpedo* subunits, the γ and δ subunits expressed in oocytes were smaller than their native *Torpedo* receptor counterparts. The Table presents the molecular weight determinations of the AChR subunits (averaged from 2–3 separate determinations). The γ subunit expressed in oocytes is approximately 5 kDa smaller than that seen in *Torpedo* and the δ subunit is approximately 3 kDa smaller in oocytes than the native *Torpedo* δ subunit.

Differences in glycosylation of the native receptor and the receptor expressed by oocytes may account for observed differences in molecular size of the γ and δ subunits. To test this possibility, Endo H and Endo F sensitivities of oocyte-expressed AChR subunits and native *Torpedo* receptor subunits were compared. *N*-linked protein glycosylation begins with the transfer of a preformed core oligosaccharide from a dolichol-phosphate lipid carrier to the asparagine residue of a growing polypeptide chain (for review *see* Kornfeld & Kornfeld, 1985). This transfer occurs co-translationally in the endoplasmic reticulum (ER). Modification of the core oligosaccharide by enzymes localized to the ER and the Golgi creates a range of diverse carbohydrate modifications, falling into at least two categories. High mannose carbohydrate residues are formed exclusively in the ER and contain only mannose residues. Complex oligosaccharides, on the other hand, require Golgi processing and contain a number of carbohydrate moities in addition to mannose. Endo H recognized *N*-linked oligosaccharides containing five or more mannose residues (high mannose type) while Endo F recognizes all types of *N*-linked oligosaccharides (both high mannose and complex types) and cleaves at the site of the Asn-GlcNAc linkage (Tarentino, Trimble & Maley, 1978; Elder & Alexander, 1982). Thus, differential patterns of sensitivity to these two glycosidases can provide information on the carbohydrate composition of the glycoprotein.

Oocytes were microinjected with AChR RNAs and metabolically labeled with [^{35}S]methionine. AChR subunits were immunoprecipitated with anti-*Torpedo* subunit antibodies and treated with Endo H or Endo F. For comparison, affinity-purified *Torpedo* AChR was also treated with Endo H or Endo F. Figure 2 shows the results of treatment of AChR subunits with Endo H and Endo F. Endo H digestion of oocyte-expressed, immunoprecipitated AChR subunits resulted in an increase in the electrophoretic mobility of all four subunits (Fig. 2*A*). Treatment of the oocyte-expressed AChR subunits with Endo F had a similar effect on the mobility of the subunits. No differential sensitivity to Endo H and Endo F was observed; identical changes in subunit molecular weight were observed after treatment with both glycosidases. These data suggest the presence of high mannose forms of oligosaccharides but no complex oligosaccharides on the oocyte-expressed receptor subunits. Based on an approximate molecular weight of 2500–3000 for a sugar moeity, the alterations in apparent molecular

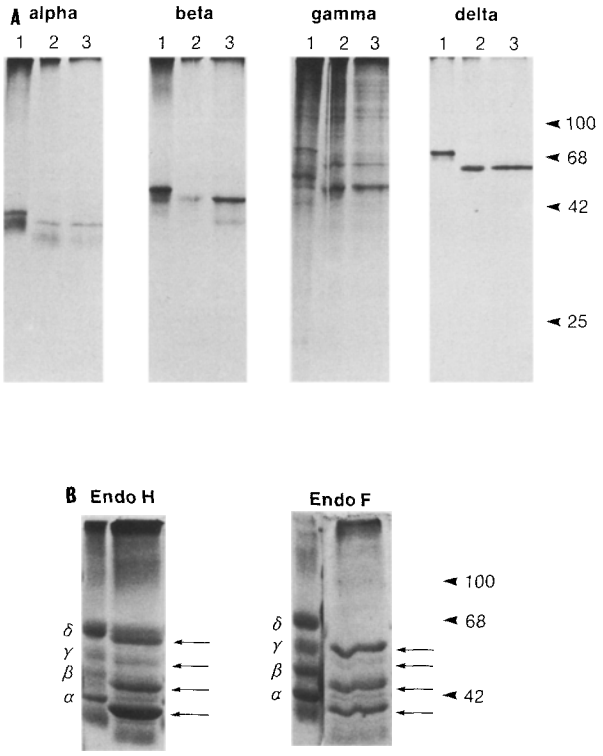

Fig. 2. (*A*) Endo H and Endo F digestion of oocyte-expressed AChR subunits. Oocytes were injected with AChR subunit RNAs and incubated in the presence of [^{35}S]methionine. AChR subunits were immunoprecipitated with anti-α, anti-β, anti-γ, or anti-δ antibodies as indicated. Lane *1*: untreated immunoprecipitated subunit. Lane *2*: immunoprecipitated subunit after incubation with 2 mU Endo H. Lane *3*: immunoprecipitated subunit after incubation with 120 mU Endo F. Treated and untreated control subunits were run on 10% SDS-polyacrylamide gels and visualized by fluorography. Mobilities of the molecular weight markers are indicated by arrowheads on the right. (*B*) Endo H and Endo F treatment of *Torpedo* AChR subunits. *Torpedo* AChR was purified by α-cobratoxin affinity chromatography. Purified receptor subunits were incubated with 0.5 mU/μg Endo H or 400 mU/μg Endo F as indicated. Endoglycosidase-treated AChR subunits (right lane of each panel) were compared to untreated control AChR subunits (left lane of each panel). The positions of the subunits before glycosidase incubation is indicated on the left of each panel. Arrows show the positions of the AChR subunits after incubation with Endo H or Endo F. The relative mobilities of the molecular weight markers is indicated by the arrowheads on the right

weights of the AChR subunits after endoglycosylase treatment are consistent with the notion that the α and β subunits each contain one oligosaccharide residue, the γ subunit contains two oligosaccharide residues and the δ subunit contains three oligosaccharide residues, in agreement with previous studies on purified *Torpedo* AChRs (Nomoto et al., 1986).

Digestion of affinity-purified *Torpedo* AChR with Endo H also resulted in an increase in electrophoretic mobility of all four subunits (Fig. 2*B*), indicating that all four subunits contain Endo H-sensitive, high mannose oligosaccharide residues. However, while digestion of native *Torpedo* subunits with Endo H and Endo F produced similar changes in the molecular weights of α and β, the γ and δ subunits also contained Endo H-resistant oligosaccharides, susceptible to cleavage by Endo F. These data indicate that all four *Torpedo* subunits contain high mannose oligosaccharides. However, unlike the oocyte-expressed subunits, the γ and δ subunits also contain complex, Endo F-sensitive oligosaccharide residues. Additional analysis revealed that the *Torpedo* γ subunit was also sensitive to sialidase treatment while the oocyte-expressed γ subunit was resistant to digestion with sialidase (*data not shown*). These data suggest that the *Torpedo* γ subunit contains sialic acid, a component of complex oligosaccharides, while the oocyte-expressed γ subunit does not contain this modification.

Changes in subunit mobility on SDS-polyacrylamide gels after enzymatic digestion was used to calculate the number of oligosaccharides contained in each *Torpedo* subunit. This analysis revealed that the α and β subunits each contain one high mannose residue, the γ subunit contains one high mannose and one complex oligosaccharide residue and the δ subunit contains one high mannose and two complex oligosaccharides. These data are in good agreement with other reports of the oligosaccharide composition of the *Torpedo* AChR (Nomoto et al., 1986). Taken together, these data suggest that while *Torpedo* and oocyte-expressed receptors contain the same number of oligosaccharide residues per subunit, the precise nature of the oligosaccharide modification differs, with oocytes apparently lacking the complex type of oligosaccharide modification. Additionally, the lack of complex oligosaccharide on the γ and δ subunits of the oocyte-expressed AChR has no apparent effect on receptor function since the oocyte-expressed receptor is functionally indistinguishable from the native *Torpedo* receptor (Mishina et al., 1985; Sakmann et al., 1985; White et al., 1985).

Role of Glycosylation in Oocyte-Expressed AChR Function

Tunicamycin (TM) was used to study the effects of inhibition of *N*-linked glycosylation on expression of the *Torpedo* AChR in *Xenopus* oocytes. TM, which inhibits the transfer of the core-oligosaccharide from the dolichol carrier to the growing polypeptide chain (Kuo & Lampen, 1974; Tkacz &

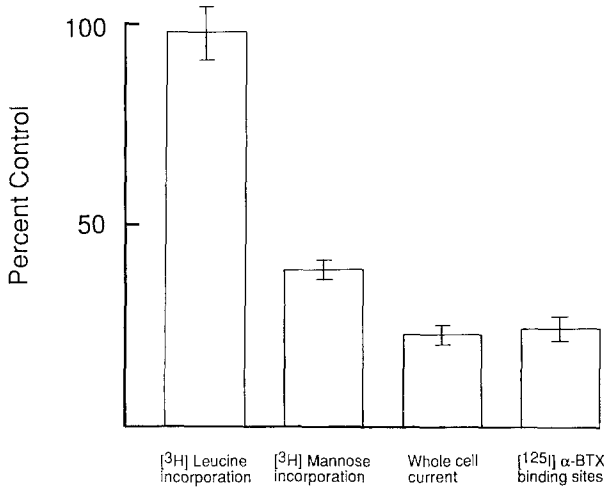

Fig. 3. Effects of tunicamycin on *N*-linked glycosylation, protein synthesis and AChR expression in *Xenopus* oocytes. Oocytes were microinjected with AChR subunit RNAs. Control cells were injected with RNA alone and TM-treated cells were injected with RNA plus 25 μg/ml TM as described in Materials and Methods. For analysis of effects on glycosylation and protein synthesis, groups of 5–10 oocytes were incubated in the presence of [^{3}H]leucine or [^{3}H]mannose for 12 hr. Incorporation of labeled precursor into acid precipitable counts was determined in control and TM-treated oocytes. The mean incorporation of labeled precursor in TM-treated cells is expressed as a percent of control incorporation ± SEM. To compare expression of functional AChR, control and TM-treated oocytes were injected with AChR RNA, incubated for 18–24 hr and then voltage clamped. The current response to bath application of 2 μM ACh was determined at a holding potential of −60 mV. The mean whole cell current response relative to untreated controls ± SEM of 6–10 oocytes is presented. Cell surface binding of [^{125}I]α-bungarotoxin in TM-treated and untreated control oocytes was determined as described under Materials and Methods. Results are presented as a percent of control [^{125}I]α-bungarotoxin binding sites ± SEM and are the mean of three groups of five oocytes

Lampen, 1975), prevents the formation of *N*-linked oligosaccharide modifications. Oocytes were microinjected with AChR subunit RNAs alone or mixed with TM. Inhibition of protein synthesis and *N*-linked glycosylation was determined by incorporation of [^{3}H]leucine and [^{3}H]mannose into acid-precipitable counts. As shown in Fig. 3, treatment of oocytes with 25 μg/ml TM had no effect on cellular protein synthesis but produced a 60–70% inhibition of [^{3}H]mannose incorporation, suggesting a selective effect on glycosylation. Expression of AChR on the oocyte cell surface was inhibited by TM treatment. Expression of functional receptors as well as expression of [^{125}I]α-bungarotoxin binding sites were both reduced to approximately 30% of control values. This suggests that the decreased expression was due to an inhibition of glycosylation since both [^{3}H]mannose incorporation and receptor expression were reduced by a similar magnitude with no effect on protein synthesis. In an attempt to achieve complete inhibition of glycosylation, higher concentrations of TM were used. However, at concentrations above 25 μg/ml, TM was toxic to the oocytes. In addition, as discussed below, further experiments determined that longer exposure to TM was required to achieve more complete inhibition.

To examine directly the effect of TM on AChR subunit glycosylation in oocytes, immunoprecipitation experiments were performed. Oocytes were injected with RNA alone or in combination with TM and incubated for 18 hr. At this time, groups of 15–20 oocytes were labeled for 4 hr with 25 μCi/ml [^{35}S]methionine and AChR subunits were immunoprecipitated using anti-*Torpedo* subunit antisera which recognize unglycosylated subunits (A.L. Buller & M.M. White, *unpublished observation*). As shown in Fig. 4*A*, subunits synthesized in the presence of TM have an increased electrophoretic mobility, corresponding to a decrease in molecular weight. The shift in molecular weight seen after TM treatment is identical to that seen after Endo H and Endo F treatment (Fig. 2*A*), demonstrating that both treatments produce completely unglycosylated subunits.

One notable feature of Fig. 4*A* is the virtual absence of any glycosylated subunits in TM-treated cells, a subunit whose existence is predicted by the partial inhibition of glycosylation seen earlier (*see* Fig. 3). One explanation for this finding is that there is a delay between the injection of TM and the onset of complete inhibition of glycosylation. To test this idea, oocytes were injected with RNA alone or in combination with 25 μg/ml TM and immediately incubated in the presence of [^{35}S]methionine for 8 hr. At this time, oocyte-expressed AChR subunits were immunoprecipitated. Figure 4*B* shows the results of this experiment for the α subunit. As can be seen, two α subunit species were immunoprecipitated by this protocol. One species corresponds to the unglycosylated species and the other corresponds to the fully glycosylated subunit. The latter is not seen when the labeling period began 18 hr after microinjection. These results suggest that assembled receptors detected by ligand binding and functional expression assays during the first 12 hr after treatment with TM are most likely glycosylated receptors. Furthermore, the formation of these glycosylated receptors does not occur at later time points due to more complete inhibition of glycosylation by TM. To further characterize the receptors expressed in TM-treated cells we performed experiments designed to compare the TM-treated and control cell expression of the AChR.

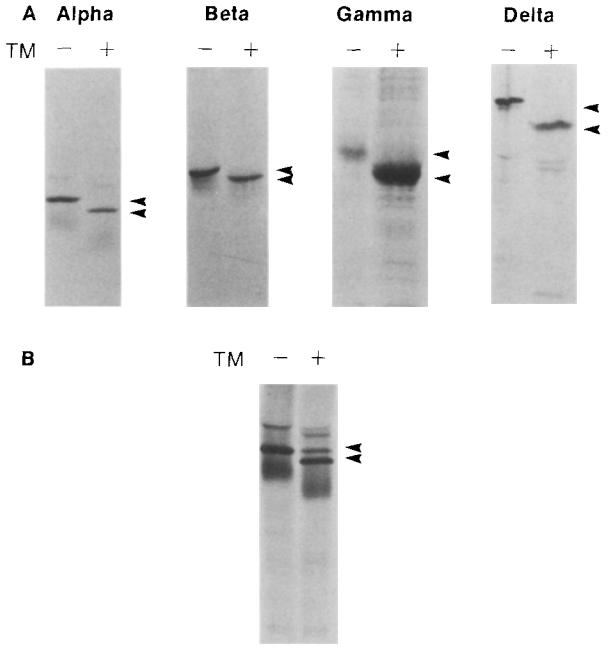

Fig. 4. (*A*) Effect of tunicamycin on glycosylation of AChR subunits. Oocytes were microinjected with AChR subunit RNAs alone or co-injected with 25 μg/ml TM, incubated for 18 hr and then exposed to a 4-hr "pulse" of [^{35}S]methionine. AChR subunits were immunoprecipitated with anti-α, anti-β, anti-γ or anti-δ antibodies as indicated. Samples were run on 10% SDS-polyacrylamide gels and immunoprecipitated proteins were visualized by fluorography. In each panel, the left lane is the subunit from control cells and the right lane is the subunit synthesized in the presence of TM. Arrows on the right of each panel represent the relative mobility of the wild-type, control subunit (upper arrow) and the subunit synthesized in the presence of TM (lower arrow). (*B*) Partial inhibition of glycosylation of AChR subunits by tunicamycin. Oocytes were microinjected with AChR subunit RNAs as described above and then metabolically labeled with [^{35}S]methione for 18 hr. The α subunit was immunoprecipitated and analzyed as described in *A* above. Arrows on the right indicate the positions of the fully glycosylated subunit (upper arrow) and the unglycosylated subunit (lower arrow)

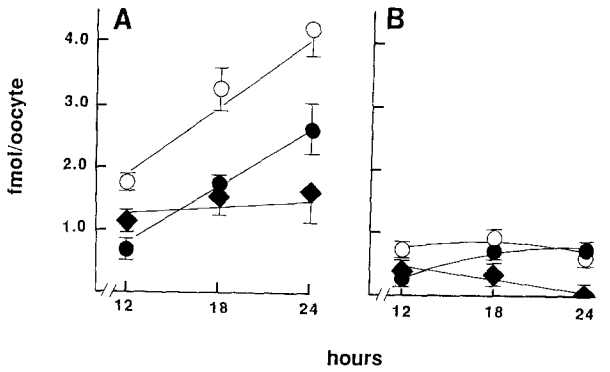

Fig. 5. Time course of [^{125}I]α-bungarotoxin binding sites in control and tunicamycin-treated *Xenopus* oocytes. Oocytes were microinjected with AChR subunit RNA alone or co-injected with 25 μg/ml tunicamycin. Oocytes were incubated for the times indicated and the binding of [^{125}I]α-bungarotoxin to cell-surface receptors (filled circles) and total cellular receptors (open circles) was determined in control (*A*) and TM-treated (*B*) oocytes. Each point represents the mean ± SEM for three to five separate groups of oocytes

Previous studies have shown that expression of AChRs in oocytes increases linearly with time (Buller & White, 1988). Figure 5*A* demonstrates that the appearance of [^{125}I]α-bungarotoxin binding sites on the cell surface as well as in detergent extracts (representing both cell surface and intracellular receptors) increase linearly with time in control, untreated cells. As reported previously, the size of the intracellular receptor pool, measured as the difference between total and cell surface binding sites is relatively constant in these cells. In TM-treated cells, however, the intracellular pool is gradually depleted as receptors move to the cell surface (Fig. 5*B*) and no new receptors are added to the intracellular pool after the initial 12 hr. These data suggest that the binding sites present in TM-treated cells reflect a population of glycosylated receptors synthesized during the initial 12 hr following microinjection of RNA and TM. This population is approximately 30% of control levels, a value in agreement with the inhibition of [^{3}H]mannose incorporation with TM treatment (*see* Fig. 3).

To examine the time course and stability of functional AChRs expressed in TM-treated cells, oocytes were injected with AChR RNA alone or in combination with TM. Control cells were incubated for 12 hr and TM-treated cells were incubated for 20 hr. Cells were then treated with 100 μg/ml cycloheximide to block new protein synthesis or with 100 nM α-bungarotoxin to block pre-existing surface receptors. Control cells in each group were not treated with either agent. Functional expression of AChR was monitored using voltage clamp in all groups for the following 8–10 hr. Figure 6*A* shows that control cells exhibit a linear increase in receptor expression that is blocked by treatment with cycloheximide, demonstrating that the continual increase in cell surface expression is dependent on protein synthesis. In addition, these results demonstrate the relatively stable nature of the oocyte-expressed receptor as little decrease in levels of receptor expression (approximately 25%) were seen during the course of cycloheximide treatment. Treatment of control cells with α-bungarotoxin blocked pre-existing surface receptors but did not prevent the expression of new AChR which were expressed at the same rate as in the untreated cells (determined by the slope of the time course). In contrast, TM-treated cells did not show an increase

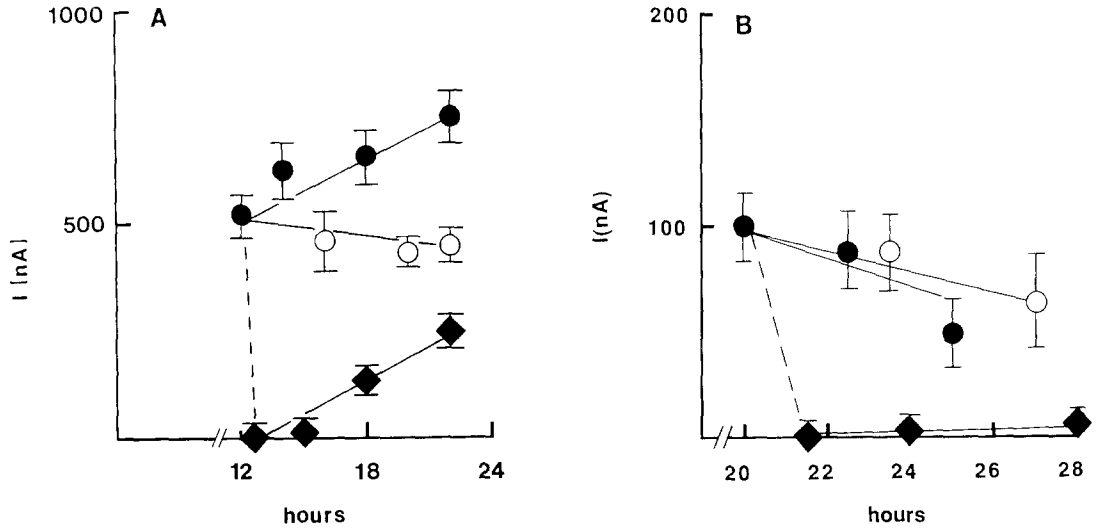

Fig. 6. Time course of functional AChR expression in control and tunicamycin-treated *Xenopus* oocytes. Oocytes were microinjected with AChR subunit RNA alone (*A*) or co-injected with 25 μg/ml tunicamycin (*B*). At the times indicated, oocytes were voltage clamped and AChR expression was assayed by bath application of 2 μM ACh at a holding potential of −60 mV. Filled circles: control untreated oocytes. Open circles: oocytes treated with 100 μg/ml cycloheximide at 12 (*A*) or 20 (*B*) hr. Filled diamonds: oocytes treated with 100 nM α-bungarotoxin for 60 min at 12 (*A*) or 20 (*B*) hr. Each point represents the mean ± SEM of 6–10 oocytes

in expression of functional receptors after the α-bungarotoxin treatment (Fig. 6*B*). Over the course of the experiments only approximately a 25% loss of functional surface receptors was seen in both control and cycloheximide-treated cells, indicating that the receptors expressed in TM-treated cells are similarly stable to those seen in the control cells. Unlike the control cells, however, treatment of TM cells with α-bungarotoxin to block surface expression revealed that no new receptors were inserted into the cell membrane. These results are consistent with the idea that the functional AChR expressed in TM-treated cells are fully glycosylated and are formed before the complete onset of TM-induced inhibition of *N*-linked glycosylation in oocytes.

Discussion

Xenopus oocytes have been used to study various aspects of the *Torpedo* nicotinic AChR. However, while oocytes glycosylate many of the foreign proteins they express, little is known regarding the precise nature of glycosylation of the *Torpedo* AChR expressed in *Xenopus* oocytes. The experiments presented here demonstrate that the oocyte-expressed AChR is indeed glycosylated, but that the pattern of glycosylation is unlike that of the native *Torpedo* AChR. Furthermore, our results show that the expression of functional AChR does not depend on exact duplication of native *Torpedo* AChR glycosylation patterns. However, although the exact nature of glycosylation of AChRs does not seem to affect the functional properties or biosynthesis of AChRs expressed in oocytes, our data show that glycosylation of the *Torpedo* AChR expressed in oocytes is required for expression of functional receptors.

Specific endoglycosidases were used to determine the nature of the carbohydrate moieties of the AChR subunits expressed in oocytes. While native *Torpedo* and oocyte-expressed AChR contained the same number of oligosaccharide residues per subunit, the composition of these oligosaccharides were strikingly different. Treatment of the oocyte-expressed AChR subunits with Endo H and Endo F revealed the presence of only high mannose type oligosaccharide modifications. The lack of Endo H-resistant carbohydrates and the insensitivity to Endo F digestion suggests that the *N*-linked carbohydrates on the subunits expressed in oocytes are not of the complex type. In contrast, however, the γ and δ subunits of the AChRs expressed in *Torpedo* electroplax (Nomoto et al., 1986) and rat muscle (Gu & Hall, 1988) were susceptible to both Endo H and Endo F treatments. Based on these data, we propose that the oocyte-expressed AChR subunits are modified exclusively by high mannose type oligosaccharides while the native *Torpedo* AChR subunits contain both high mannose and complex type oligosaccharides. This difference in oligosaccharide modification may account for the observed differences in apparent molecular weight of the affinity-purified γ and δ subunits from the two sources.

Incorrect glycosylation of proteins expressed in *Xenopus* oocytes from injected mRNA has been previously reported. Thornhill and Levinson (1987) have shown that the final maturation of the *Electrophorus* electroplax sodium channel expressed in oocytes, an event requiring Golgi-mediated processing of core sugars, is incomplete and no functional channels are detected on the cell surface. Human chorionic gonadotropin (hCG) expressed in *Xenopus* oocytes is also incorrectly glycosylated (Mous, Peeters & Rombauts, 1980). The oocyte-expressed hCGα does not contain terminal sialic acid residues present in the mature native protein. The oligosaccharide moieties on the low density lipoprotein receptor (LDL) expressed in oocytes are incompletely processed and a portion of the expressed protein remains Endo H sensitive while the native protein is completely insensitive to Endo H (Peacock et al., 1988). Similarly, oocytes fail to completely process the rat prostatic binding protein which lacks complex type carbohydrate residues characteristic of the native protein (Mous et al., 1982) and bovine rhodopsin exists in different glycosylated forms in oocytes (Khorana et al., 1988). Finally, altered glycosylation of *Drosophila Shaker* K^+ channels expressed in oocytes has been proposed as an explanation for differences in pharmacological sensitivity between oocyte-expressed and native channels (MacKinnon, Reinhart & White, 1988).

Thus, while oocytes do glycosylate many of the proteins they express, analysis of the oligosaccharide modification may reveal incomplete processing. It would appear from the examples cited above as well as the present data, that glycosylation steps requiring Golgi processing (i.e., transition from high mannose to complex oligosaccharide) is often incomplete or even absent. The reason for the lack of complex oligosaccharide formation in the oocytes is not clear and may be due to low levels of Golgi processing enzymes, difficulty in intracellular transport or alterations in the tertiary structure of the oocyte-expressed protein. It is possible that one component of this complex processing phenomenon may be extremely inefficient or saturated by the large amount of foreign protein that must be synthesized and subsequently modified. However, if this were always the case, a mixture of immature precursor and mature protein would be expected to be seen in the oocytes. While in some instances this is the case (e.g., bovine rhodopsin (Khorana et al., 1988), this is not so for the AChR expressed in oocytes. Only one species of each receptor subunit was seen, indicating a lack of heterogeneity in the oligosaccharide modification of the *Torpedo* AChR. Significantly, however, the oocyte-expressed AChR was fully functional.

While there are many reports of incorrect glycosylation of proteins expressed in *Xenopus* oocytes, only one other study contains information dealing with the question of what effect, if any, alterations in glycosylation has on the function of the expressed protein. Peacock et al. (1988) reported that a portion of the carbohydrate on the human LDL receptor is Endo H sensitive, while the receptor expressed in mammalian cells is completely resistant. However, these authors did not report to what extent this alteration occurred. The oocyte-expressed LDL receptor did exhibit the properties expected for a functional LDL receptor with two important exceptions. First, the internalized receptor was not degraded. Second, the oocyte-expressed receptors did not exhibit the temperature-dependent change in affinity for LDL seen with the native receptor. It is not clear whether or not these functional differences are due to the ill-defined improper glycosylation of the LDL receptors, or if it is due to some other property of *Xenopus* oocytes. Nonetheless, it is clear that LDL receptors expressed in oocytes are not quite the same functionally as the native receptor. In contrast, our results, in conjunction with results of functional studies from several laboratories (Mishina et al., 1985; Sakmann et al., 1985; White et al., 1985) show that despite the oligosaccharide modification, oocyte-expressed *Torpedo* AChRs appear to be functionally equivalent to the native receptor.

To examine the role of glycosylation in oocyte-expression of the AChR, tunicamycin was used to inhibit oocyte glycosylation of the AChR. TM prevents the formation of the oligosaccharide-lipid precursor and thus inhibits all forms of *N*-linked glycosylation (Kuo & Lampen, 1974; Tkacz & Lampen, 1975). Under conditions of specific inhibition of *N*-linked glycosylation by TM without concomitant effects on protein synthesis, TM treatment reduced AChR expression. No effect was seen on the synthesis of the individual subunits, and the inhibition of AChR expression was comparable to the level of inhibition of mannose incorporation. Our data suggest that unglycosylated subunits do not assemble into a functional and toxin binding receptor species and that the small amount of functional receptors expressed in TM-treated oocyte were, in fact, glycosylated. Several lines of evidence support this theory. First, the delayed onset of TM effects allow syntheis of some fully glycosylated as well as unglycosylated subunits during the initial exposure to TM. These glycosylated subunits are not formed at later times. Exposure of TM-treated oocytes to α-bungarotoxin to block the receptors expressed during the first 20-hr exposure to TM revealed that no new functional receptors were inserted on the cell surface. Additionally, unlike control cells, a gradual

depletion of the α-bungarotoxin binding sites in the intracellular receptor pool of TM-treated cells was observed as receptors moved to the cell surface and failed to be replaced by newly synthesized AChR. These data are consistent with other reports of the role of glycosylation in AChR function and supports the idea that unglycosylated receptor is not expressed. Previous studies by other investigators have shown that inhibition of protein glycosylation by tunicamycin, prevents the expression of cell surface AChR in both BC3H1 mouse (Merlie et al., 1982) and chick (Prives & Olden, 1980; Prives & Bar-Sagi, 1983) muscle cells. Indeed, in BC3H1 cells, the reduction in cell surface expression is apparently due to a failure of unglycosylated subunits to assemble into an oligomeric, α-bungarotoxin binding species. More subtle modification of oligosaccharide composition of the BC3H1 cell AChR by treatment with 1-deoxynojirimycin, an agent that prevents the trimming of the core oligosaccharide, alters the stability of the individual subunits (Smith et al., 1986). Mishina et al., (1985) have shown that mutation of the site of *N*-linked glycosylation of the α subunit is sufficient to prevent the appearance of α-bungarotoxin binding sites and ion channel formation. Our data strongly suggest that any functional AChRs detected after TM treatment are actually glycosylated receptors synthesized before TM completely blocks glycosylation.

Our data supports the work of Merlie et al. (1982) who have proposed that subunit glycosylation is required for receptor assembly and that the failure of oligomerization is responsible for the reduced levels of cell surface AChR expression. The apparent discrepancy between these results and the data of Prives and colleagues (Prives & Olden, 1980; Prives & Bar-Sagi, 1983) who reported cell surface expression of functional chick muscle AChR in the presence of TM, may be due to several factors including differences in cell culture systems and different receptor sources being studied. It should be emphasized that we also found AChR expression in TM-treated cells but, closer inspection of these receptors revealed that they were actually glycosylated. Given the delay in onset of TM's effects and the relatively stable nature of the AChR (Fig. 6; Devreotes & Fambrough, 1975; Patrick et al., 1977), it is possible that receptors observed in TM-treated chick muscle cells were formed prior to complete inhibition of glycosylation or wild-type glycosylated receptors formed in the presence of TM.

The present report demonstrates differences in the oligosaccharide composition of the γ and δ subunits of the *Torpedo* AChR expressed in oocytes and the native protein. However, since the oocyte-expressed AChR is fully functional, these differences in glycosylation are not critical for receptor function and may be the result of inefficient processing. However, the presence of carbohydrate modifications is a requirement for expression of functional *Torpedo* AChR in *Xenopus* oocytes.

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