The α7 nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel that modulates neurotransmitter release in the central nervous system. We show here that functional, homo-oligomeric α7 nAChRs can be synthesized in vitro with a rabbit reticulocyte lysate translation system supplemented with endoplasmic reticulum microsomes, reconstituted into planar lipid bilayers, and evaluated using single-channel recording techniques. Because wild-type α7 nAChRs desensitize rapidly, we used a nondesensitizing form of the α7 receptor with mutations in the second transmembrane domain (S2/T and L9/T) to record channel activity in the continuous presence of agonist. Endoglycosidase H treatment of microsomes containing nascent α7 S2/T/L9/T nAChRs indicated that the receptors were glycosylated. A protease K protection assay revealed a 36-kDa fragment in the ER lumen, consistent with a large extracellular domain predicted by most topological models, indicating that the protein was folded integrally through the ER membrane. α7 S2/T/L9/T receptors reconstituted into planar lipid bilayers had a unitary conductance of ~50 pS, were highly selective for monovalent cations over Cl−, were nonselective between K+ and Na+, and were blocked by α-bungarotoxin. This is the first demonstration that a functional ligand-gated ion channel can be synthesized using an in vitro expression system.

The nicotinic acetylcholine receptor (nAChR) is a member of a superfamily of ligand-gated ion channels that also includes GABA receptors, serotonin (5-HT)1 receptors, glycine receptors, and an invertebrate glutamate-gated chloride channel (1). Nicotinic receptors are located at the neuromuscular junction and in the central and peripheral nervous systems. Muscle-type nAChRs are pentamers of homologous subunits in the stoichiometry of αβγδε (or αββββ) arranged around a central pore (2). Neuronal nAChRs also form pentameric complexes (3, 4) from various combinations of the 11 neuronal nAChR genes (α2-α9 and β2-β4) that have been identified to date (5). α7, α8, and α9 nAChRs are blocked by the snake peptide toxin α-bungarotoxin (α-BTX), which also blocks muscle and *Torpedo* nAChRs but not other subtypes of neuronal nAChRs (5). α7 nAChRs are the most abundantly expressed nicotinic receptor subunit in the central nervous system (6) and are important in neuronal development, hippocampal function, and the modulation of fast neurotransmission (7, 8). α7 nAChRs are highly calcium-permeable (9, 10), and calcium influx through presynaptic α7 nAChRs modulates the release of excitatory neurotransmitters (11, 12).

During biosynthesis of nAChRs, the polypeptide is translocated into the endoplasmic reticulum (ER) membrane. The ER contains enzymes necessary for signal sequence cleavage (13) and other post-translational modifications required for correct subunit folding, assembly, and ligand-binding site formation. These modifications include core glycosylation (13) and disulfide bond formation (14, 15). In addition, ER and cytoplasmic chaperone proteins are thought to be involved in the maturation of muscle-type and α7 nAChRs (16–19). Based on current topological models, nicotinic receptor subunits have four putative transmembrane domains, a large, glycosylated N-terminal extracellular domain that contains the agonist-binding site (14, 15), and a short extracellular C terminus (1). The second transmembrane domain (M2) from each of the five subunits is postulated to line the ion-conducting pore (21).

The subunit composition of nAChRs containing the α7 gene product is not completely clear. The injection of α7 cRNA into *Xenopus* oocytes results in the formation of ACh-gated ion channels without requiring the co-expression of other neuronal nAChR subunit cRNAs (22), suggesting that α7 nAChRs are homo-oligomeric. However, *Xenopus* oocytes also express low levels of endogenous nAChR α subunits, which can co-assemble with β, γ, and δ muscle-type nAChR subunits to form functional nAChRs (23). These α-subunits could, potentially, co-assemble with expressed α7 receptors in *Xenopus* oocytes as well. Based on co-immunoprecipitation experiments, native α7 receptors in rat brain appear to be homo-oligomeric (24), whereas native chick α7 subunits are thought to form both homo-oligomeric and hetero-oligomeric receptors, complexing with α8 (25–27) and other neuronal nAChR subunits (28). However, it is possible that the apparent homomeric α7 nAChRs in native tissues could represent heteromeric complexes containing yet unidentified nAChR subunits.

α7 nAChRs have been difficult to express in several mammalian heterologous expression systems. The folding, assembly, and subcellular localization of heterologously expressed α7 nAChRs is deficient in some cell lines, due to misfolding and trapping of proteins in the ER (29). For example, human α7 nAChRs have been expressed in HEK-293 cells (30), but attempts to express chick or rat α7 nAChRs in HEK-293 cells have not yet been successful (29, 31, 32).

Another powerful approach to determine whether α7 nAChRs...
can form functional, homo-oligomeric receptors is to express them in a cell-free system. Muscle-type nAChRs translated in the presence of ER microsomes have been studied biochemically (13, 33, 34) but have not been examined for functional channel activity. Our goal was to express chick α7 nAChRs in vitro, where the co-expression of other nAChR subunits is extremely unlikely, and to study their biochemical and functional properties. Our experimental strategy was to express receptors using rabbit reticulocyte lysates in the presence of ER microsomes, reconstitute the channels into planar bilayers, and record single-channel activity. This method has been used successfully to synthesize and reconstitute functional Shaker potassium channels (35), amiloride-sensitive sodium channels (36), and gap junction channels (37). In this paper, we show that α7 S2′/T′/L′/T″ α7 nAChR cDNAs expressed in vitro were glycosylated, were processed integrally though the membrane, and formed functional channels when reconstituted into planar lipid bilayers. These data also show that α7 nAChR subunits can form functional, homo-oligomeric channels.

**EXPERIMENTAL PROCEDURES**

cDNAs and In Vitro Transcription of cRNA—Chick wild-type α7 nAChR cDNA was a gift from Mark Ballivet (University of Geneva, Geneva, Switzerland). Wild-type and S2′/T′/L′/T″ α7 nAChR cDNAs cloned into the pAMV vector (38) under control of the T7 promoter were kindly provided by Purnima Deshpande, Dr. Henry Lester, and Dr. Cesar Labarca (California Institute of Technology). The numbering of the residues in the M2 domain follows the convention of Miller (39), where the N-terminal (cytoplasmic) residue of the M2 domain is denoted as 1. The Ser at the 2′ position and Leu at the 9′ position correspond to amino acids Ser246 and Leu247, respectively, in the chick α7 cDNA sequence (22). The plasmid templates were digested with NotI. Capped cRNA transcripts were generated from the cDNA templates with T7 RNA polymerase using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) according to the manufacturer’s instructions. The cRNA was resuspended in 100 mM KCl and stored at −70 °C.

**Microinjection of cRNA and Maintenance of Xenopus Oocytes**—Oocytes were surgically removed from female Xenopus laevis (Nasco, Fort Atkinson, WI) and treated with collagenase as described (40) to remove the follicular cell layer. Oocytes were injected with 20 ng of α7 cRNA and incubated at 19 °C for 2–5 days in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM Na-HEPES, pH 7.5) supplemented with 50 μg/ml gentamicin and 0.05% sodium pyruvate (41).

**Two-electrode Voltage Clamp of Xenopus Oocytes**—Two-electrode voltage clamp was performed with a GeneClamp 500 amplifier controlled by pCLAMP6 software (Axon Instruments, Foster City, CA). Microelectrodes were filled with 3 M KCl and had resistances of 0.5–2.1 MΩ. Oocytes were superfused with ND96, which contained 1 mM MgCl2, 1 mM EGTA, 10 mM Na-HEPES, pH 7.5) in the presence of and absence of ACh. Unless otherwise indicated, oocytes were voltage clamped at a holding potential of −60 mV. Currents from S2′/T′/L′/T″ receptors were filtered at 50 Hz and sampled at 500 Hz. Additional filtering was added to the traces for display purposes. To adjust for rundown during acquisition of dose-response data, the peak currents from repeated applications of a standard dose of ACh were used to normalize the test responses. Dose-response relationships were fitted to the Hill equation in Prism (GraphPad Software, San Diego, CA). Current-voltage relationships during the sustained maximal response to ACh were generated by applying a series of voltage steps (125 ms, 0 mV intervals) from the holding potential of −60 mV. Leak currents obtained in the absence of ACh were subtracted from ACh-evoked currents.

**Preparation of Endoplasmic Reticulum-derived Quail Oviduct Microsomes**—Endoplasmic reticulum microsomes were obtained from canine pancreas (Promega, Madison, WI) or were prepared from the oviducts of mature, laying Japanese quail (42). Before homogenization, the oviduct was minced, suspended in 5 volumes of TKMD buffer (50 mM Tris-HCl, pH 7.7, 25 mM KCl, 2.5 mM MgCl2, 3 mM dithiothreitol) plus 0.88 mM sucrose, homogenized, and centrifuged at 4000 × g in an HB4 rotor for 10 min. The supernatant was diluted with TKMD buffer to a final sucrose concentration of 0.6 M and layered onto a sucrose step gradient of TKMD buffer containing 1.5 M and 2.0 M sucrose. After centrifugation for 16–20 h at 100,000 × g in an SW28 rotor, the microsomes were harvested from the 1.5 M and 2.0 M sucrose interface and resuspended in 20 mM Na-HEPES. The volume was adjusted with 20 mM Na-HEPES to obtain an A260 reading of 0.6 in a sample containing 1% SDS. One-half volume of 750 mM sucrose, 20 mM Na-HEPES, pH 7.0 was added to the resuspended membranes, which were frozen in liquid nitrogen and stored at −70 °C.

**In Vitro Transcription, Translation, and Membrane Processing**—Coupled in vitro transcription/translation was performed using the Promega TNT kit. For analysis of translation and processing efficiency, reaction mixtures (25 μl) contained 12.5 μl of rabbit reticulocyte lysate, 20 μl reaction buffer, 20 μM amino acids minus methionine, 10 units of RNasin (Promega), 0.5 μl of T7 RNA polymerase, and 10 μCi of [35S]methionine (Amersham Pharmacia Biotech) with or without 1 μg of α7 S2′/T′/L′/T″ α7 nAChR cDNA. Reactions were also supplemented with 1 mM dithiothreitol and 1 mM oxidized glutathione. Some reactions contained microsomes from canine pancreas (1.5 μl/25-μl reaction) or quail oviduct (1.0 μl/25-μl reaction). Reactions were incubated for 90 min at 30 °C and stopped by placing on ice. After reserving 3 μl of the reaction mixture for gel analysis (labeled M in Figs. 2–4), the membranes were pelleted by centrifugation for 25 min at 14,000 rpm in a microcentrifuge, washed twice in solution D′ (160 mM KCl, 20 mM MOPS, pH 7.4), and resuspended in water. Samples were heated for 30 s at −95 °C in SDS-PAGE sample buffer and electrophoresed on 10% SDS-PAGE (38) and visualized by fluorimetry (Promega). Gels were treated with Fluor-Hance (Research Products International, Mount Prospect, IL), dried, and exposed to Kodak X-Omat film at −70 °C.

**Endoglycosidase H Treatment**—A coupled transcription/translation reaction (37.5 μl) was performed as described above in the presence of α7 S2′/T′/L′/T″ α7 nAChR cDNA and canine pancreatic microsomal vesicles. The membranes were pelleted (15 min at 14,000 rpm), resuspended in 50 mM sodium acetate, 1% (w/v) β-mercaptoethanol, pH 6.0, and divided into equal fractions. Endoglycosidase H (1 milliunit; Roche Molecular Biochemicals) or carrier buffer (25 mM EDTA, 0.05% sodium azide, 0.1% SDS, 50 mM NaH2PO4, pH 7.0) was added, and the samples were incubated for 2 h at 37 °C. The reaction was stopped by chilling on ice. SDS-PAGE sample buffer was added, and the samples were heated at −95 °C for 30 s and analyzed by SDS-PAGE.

**Protease K Analysis**—Pelleted microsomal membranes from a 50-μl coupled transcription/translation reaction were resuspended in 30 μl of reaction buffer (160 mM NaCl, 5 mM CaCl2, 50 mM Tris-Cl, pH 7.5). Each aliquot was treated with 0.1 mg/ml proteinase K, 0.1 mg/ml proteinase K plus 1% Triton X-100, or water for 45 min on ice. The reactions were stopped by adding 2 mM phenylmethylsulfonyl fluoride and 1 μl of SDS-PAGE sample buffer. The reactions were heated immediately at −95 °C for 30 s and analyzed by SDS-PAGE.

**Sucrose Density Gradient Analysis**—Coupled transcription/translations containing [35S]Met were performed in the absence or presence of canine pancreatic microsomes as described above. Mixtures (25 μl) from translations performed in the absence of microsomes were diluted to 40 μl in a final concentration of 10 mM NaH2PO4, 50 mM NaCl, 1 mM EDTA, 0.1% SDS, and 100 μCi of [35S]methionine. Reactions performed in the presence of microsomes were scaled to a final volume of 100 μl and centrifuged to pellet the microsomes. The microsomes were washed and resuspended in 40 μl of solution D′ and solubilized in 0.5% Triton X-100 for 60 min on ice. Samples were layered on top of 5-ml linear 5–20% (w/v) sucrose gradients containing 0.2% Triton X-100, 10 mM NaH2PO4, 50 mM NaCl, and 1 mM EDTA, pH 7.0 (44). Sucrose gradients run in parallel contained the standard proteins ovalbumin (3.6 S), bovine serum albumin (4.2 S), human gamma globulin (7 S), and catalase (11 S). The gradients were centrifuged for 12 h at 300,000 × g at 4 °C in a SW50.1 rotor. Fractions were collected from the top of the gradients, and the sucrose concentration of each fraction was determined by refractometry. Proteins from each fraction were separated by SDS-PAGE and visualized by fluorimetry (35S-labeled protein) or Coomassie Blue staining (standard proteins). Gels or autoradiograms were digitized, and the relative amount of protein in each band was analyzed by densitometry using NIH Image software.

**In Vitro Transcription/Translation for Reconstitution into Planar Lipid Bilayers**—Coupled transcription/translations for the incorporation of microsomal vesicles into planar lipid bilayers were performed as described above except that amino acids minus cysteine were added to provide the full complement of all unlabeled amino acids, and all volumes were scaled up to make a final volume of 50 μl. After translation, the membranes were pelleted by centrifugation for 15 min at 9,000 rpm. The supernatant was removed immediately, and the pellet was washed twice with solution D′ and resuspended thoroughly by gentle trituration in 5 μl of 250 mM sucrose.
and 20 mM HEPES, pH 7.4. Bilayer experiments were generally performed on the same day as translations.

**Single Channel Recording of Cell-free Expressed nAChRs Reconstituted into Planar Lipid Bilayers**—Synthetic lipids (1-palmitoyl-2-oleoyl phosphatidylethanolamine and 1-palmitoyl-2-oleoyl phosphatidylserine; Avanti Polar Lipids, Alabaster, AL) were resuspended in n-decane at concentrations of 15 mg/mL and 5 mg/mL, respectively. Planar lipid bilayers were formed by applying the decane solution across a 200-μm hole in a polyvinylidifluoride partition separating two aqueous chambers denoted *cis* and *trans*.

Endoplasmic reticulum-derived microsomal vesicles from *in vitro* translations were incorporated into planar lipid bilayers by applying them directly onto the *cis* face of the bilayer with a fire-polished glass probe (45). To promote the fusion of vesicles with the bilayer, 0.5 mM CaCl$_2$ was present on the *cis* side. Acetylcholine was added to both chambers to activate all incorporated nAChRs regardless of membrane orientation. For each 50-μL translation, three to five bilayer experiments could be performed, each of which lasted about 1 h. Bilayers were voltage-clamped with a Warner Instruments patch-clamp amplifier (Hamden, CT). Voltages were assigned as *cis* relative to *trans*, with *trans* corresponding to the luminal side of the ER (the extracellular face). Data were filtered at 200 Hz using a 4-pole Bessel low pass filter, digitized at 1 kHz, and analyzed off-line using in-house analysis programs written in A xoBasic (Axon Instruments, Foster City, CA).

**RESULTS**

**Selection of a Nondesensitizing Mutant of the α7 nAChR**—The goal of these experiments was to characterize the biochemical and functional properties of α7 nAChRs synthesized *in vitro* and reconstituted into planar lipid bilayers. To study the activity of ligand-gated ion channels in a planar lipid bilayer, agonist is continually present to evoke channel opening events. Because wild-type α7 nAChRs desensitize very rapidly in the continued presence of ACh, we selected a nondesensitizing form of the α7 nAChR so that channel activity could be observed for extended periods of time. This receptor has a serine to threonine mutation at the 2 position and a leucine to threonine mutation at the 9 position (α7 S2T/L9T nAChR). Fig. 1A shows that wild-type α7 nAChRs activated rapidly and desensitized completely within 1 s of ACh application. In contrast, α7 S2T/L9T nAChRs (Fig. 1B) did not desensitize during a 30-s application of ACh, behavior similar to that of α7 L9T nAChRs described by Revah et al. (46). Like wild-type and α7 L9T nAChRs (47), α7 S2T/L9T nAChRs were completely inhibited by α-BTX (Fig. 1B). Fig. 1C shows the ACh dose-response characteristics of wild-type and α7 S2T/L9T nAChRs. As was seen with α7 L9T nAChRs (46), the EC$_{50}$ of α7 S2T/L9T nAChRs (14.1 μM) was much lower than that of wild-type α7 nAChRs (345 μM). Fig. 1D shows that Ca$^{2+}$ permeates through α7 S2T/L9T nAChRs. In the absence of any permeant ions in the bath, no ACh-evoked currents were observed. In the presence of 10 mM Ca$^{2+}$ (and no other permeant ions), robust ACh-evoked currents were recorded. Thus, α7 S2T/L9T nAChRs displayed the nondesensitizing kinetics necessary to sustain channel activity in planar lipid bilayers in the continued presence of agonist. In addition, α7 S2T/L9T nAChRs displayed sensitivity to α-BTX, high potency of ACh, and Ca$^{2+}$ permeability, as expected.

**In Vitro Translation and Processing of α7 S2T/L9T nAChRs**—To determine whether the S2T/L9T α7 nAChRs synthesized *in vitro* were full-length, glycosylated, and correctly folded, we evaluated a number of its biochemical properties. Fig. 2 shows a fluorogram of [35S]Met-labeled proteins generated from a coupled transcription/translation performed in the presence and absence of endoplasmic reticulum-derived microsomes purified from quail oviduct. In the absence of microsomes, a ~41-kDa protein and a 28 kDa protein were observed (lane 3). The primary sequence of wild-type α7 nAChRs indicates a nonglycosylated molecular mass of 54 kDa (22, 25), a value substantially higher than the ~41 kDa observed. Other nAChR α-subunits also run anomalously fast on SDS-polyacrylamide gels; nonglycosylated α subunits from *Torpedo* and mouse muscle nAChRs have calculated molecular masses of 50 kDa but migrate as 41–43-kDa proteins (33, 34, 48). Thus, the ~41-kDa protein expressed *in vitro* is likely to be the...
full-length α7 S2′/T/L9′ T nAChRs. The 28-kDa protein could be either a premature translation stop or a proteolytic degradation product.

In translation mixtures containing endoplasmic reticulum microsomes, a ~51-kDa product was observed in addition to the ~41-kDa protein (Fig. 2, lane 4). When the translation products were centrifuged to separate proteins associated with the microsomal membranes from those in solution, the 51-kDa product was predominantly associated with the membrane pellets (P, lane 5), whereas the 41-kDa product remained primarily in the supernatant (S, lane 6). These results suggest that the 51-kDa product was the membrane-processed form of the receptor and the 41-kDa protein was the unprocessed form. Similar results were obtained when canine pancreatic microsomes were used instead of avian oviduct microsomes, except that higher yields of processed protein were obtained with the canine pancreatic microsomes.

To test the hypothesis that the slower migration of the 51-kDa membrane-associated protein was due to glycosylation, the microsome-associated proteins were treated with endoglycosidase H (Endo H), which cleaves high mannose oligosaccharides from glycoproteins (49). Fig. 3 shows the two protein products of 41 and 51 kDa observed in unseparated translation mixtures (lane 2). The membrane pellets were resuspended and treated with either Endo H or carrier buffer. Endo H-treated, membrane-processed proteins had a molecular mass of 41 kDa (lane 5), identical to the size of the unprocessed proteins (lane 3), whereas the molecular mass of membrane-associated proteins treated with carrier buffer remained 51 kDa (lane 4). These data indicate that the membrane-associated α7 S2′/T/L9′ T receptors were glycosylated with high mannose oligosaccharides.

To determine whether the membrane-associated proteins were co-translationally inserted into the membranes with the transmembrane configuration expected for nAChRs, or were simply associated with the membranes in a nonspecific manner, membrane-associated proteins were treated with proteinase K, a nonspecific serine protease, in the presence and absence of detergent (Fig. 4A). A coupled transcription/translation of α7 S2′/T/L9′ T cDNA was performed in the presence of canine pancreatic microsomes (lanes 2–6). As before, the 51-kDa protein was found in the microsomal fraction (lane 4). Incubation of intact microsomal membranes with proteinase K produced a ~36-kDa fragment (lane 5), suggesting that a large part of the protein was protected from proteolysis by the membrane. In the presence of Triton X-100 to disrupt the membranes, proteinase K caused complete digestion of the membrane protein (lane 6), as expected. The size of the 36-kDa membrane-protected fragment was consistent with the expected size of the glycosylated extracellular N-terminal domain. Specifically, the mature N-terminal extracellular region plus the first transmembrane domain of the α7 nAChR has a calculated molecular mass of 27.5 kDa (22). The addition of carbohydrate to the three N-linked glycosylation sites in the N-terminal extracellular domain of α7 nAChRs (20) adds ~10 kDa, as evidenced by the apparent shift in gel migration (Fig. 3). Thus, the expected molecular mass for the glycosylated N terminus is ~37.5 kDa, a value similar to that of the ~36-kDa protein fragment observed. These results suggest that the nascent α7 nAChR proteins were co-translationally inserted across the membrane and folded into a transmembrane orientation with a large extracellular domain (Fig. 4B).

To determine whether the α7 subunits formed oligomeric assemblies, we performed sucrose gradient sedimentation analysis of in vitro translated α7 S2′/T/L9′ T nAChRs. On 5–20% linear sucrose gradients containing 0.2% Triton X-100, α7 S2′/T/L9′ T nAChRs translated in the absence of microsomes sedimented at 3.6 S (Fig. 5A). This value was similar to the sedimentation velocity of Torpedo α1 (50) and muscle α1 (51) nAChR subunits. The S2′/T/L9′ T nAChRs translated in the presence of microsomes formed multiple sizes of subunit com-
plexes ranging from 3.6 S to 11 S (Fig. 5B). The multiple sizes of these complexes suggest that some but not all of the α7 complexes synthesized in vitro form pentameric complexes. Similar results were observed with oocyte-expressed αI nACHRs, which form protein complexes composed of various numbers of subunits, but only those fractions corresponding to −10 S complexes bind α-BTX (52). Native Torpedo californica nACHR and α7 nACHR pentamers sediment at −9 and 10 S, respectively (44, 52).

Functional Properties of α7 nACHRs Expressed In Vitro and Incorporated into Planar Lipid Bilayers—To determine whether the α7 nACHR protein translated in vitro formed functional ion channels, endoplasmic reticulum microsomes containing nascent α7 S2'TL9'T nACHRs were solubilized in 0.5% Triton X-100 and sedimented on 5–20% linear sucrose gradients containing 0.2% Triton X-100. The migration (in Svedberg units) of standard proteins run on parallel gradients is indicated with arrows. Sucrose concentrations for each fraction were determined by refractometry to confirm that paired gradients were identical. Image densities were determined from densitometric analysis of autoradiographs (35S-labeled α7 nACHR protein) or Coomassie Blue-stained gels (standard proteins). The 11 S peak in B indicates 35S-labeled protein that had sedimented to the bottom of the gradient.

Potential of −41 mV, a value close to the equilibrium potential for K⁺ (E_K) of −43 mV, indicating the expected K⁺-over-Cl⁻ selectivity of the reconstituted channels. The unitary conductance of 48 pS was similar to the 45 pS value reported for oocyte-expressed wild-type α7 nACHRs (46) and to the conductance of oocyte-expressed α7 S2'TL9'T receptors recorded from outside-out patches (−41 pS, data not shown).

Another distinctive property of both muscle-type and neuronal nACHRs is their inability to select between small monovalent cations such as Na⁺ and K⁺ (21, 53). After confirming that the incorporated channel was selective for K⁺ over Cl⁻ in asymmetrical KCl concentrations, currents were recorded in the presence of both Na⁺ and K⁺ to determine whether the reconstituted ion channel was equally permeable to Na⁺ and K⁺. Under these ionic conditions (see legend to Fig. 7), the reversal potential of a cation channel with equal permeabilities to Na⁺ and K⁺ would be −0 mV. Examples of single-channel recordings (Fig. 7A) and the current-voltage plot (Fig. 7B) are shown. As expected for a nonselective cation channel, the reversal potential was −2.0 mV, producing a calculated K⁺-to-Na⁺ permeability ratio (P_K⁺/P_Na⁺) of 1.2. The unitary conductance of the channel was 51 pS.

A distinguishing characteristic of the α7 neuronal nACHR is its sensitivity to block by α-BTX (22). Macroscopic currents evoked by acetylcholine in Xenopus oocytes expressing α7 S2'TL9'T receptors were blocked after a 30-min incubation by 100 nM α-BTX (Fig. 1B). To test the functional block of α7 S2'TL9'T receptors synthesized in vitro, α-BTX was added to an active
channel incorporated into a planar lipid bilayer. Channel activity stopped 20 min after the addition of 375 nM α-BTX to both sides of the bilayer (not shown). The slow onset of channel block was similar to the long incubations usually required for the α-BTX block of muscle-type nAChRs (54) and oocyte-expressed wild-type α7 receptors (22).

No similar channel activity was observed in control bilayer experiments using microsomes from quail oviduct or canine pancreas. The criteria for identifying nAChR-like channel behavior were: 1) cation selectivity, 2) lack of selectivity between Na\(^+\) and K\(^+\) (3 50 pS conductance, and 4) channel bursting kinetics. No channels with these characteristics were observed from microsome-processed α7 S2/T/L9 T receptors in the absence of agonist (11 experiments from 4 translations). In addition, no nAChR-like channel activity was observed following quail oviduct-processed translations of cDNA encoding the inward rectifier potassium channel (52 experiments from 37 translations). Finally, no nAChR-like channels were observed when quail oviduct microsomes that were not incubated with translation mixtures were incorporated into bilayers (319 experiments). These data suggest that the nAChR channel activity was a result of nascent protein synthesis and did not derive from channels present in the oviduct microsomes.

Occasionally, channels other than nAChRs were observed during the reconstitution experiments. We observed several Cl\(^-\)-selective channels as described previously (35), a few cation-selective channels with conductances of 20–38 or >100 pS, and one channel type with no selectivity among Cl\(^-\), Na\(^+\), and K\(^+\). These channels were observed in reconstitution experiments whether or not the microsomes had been incubated with translation mixtures, indicating that they were endogenous to the microsomal membranes.

**DISCUSSION**

We have used a cell-free expression system consisting of an *in vitro* transcription and translation system derived from rabbit reticulocyte lysates and supplemented with ER microsomes to reconstitute functional α7 S2/T/L9 T nAChRs into planar lipid bilayers. We have shown that α7 S2/T/L9 T nAChRs synthesized *in vitro* were co-translationally processed into the ER-derived membranes and core glycosylated. When ER vesicles containing synthesized α7 S2/T/L9 T nAChRs were fused with planar lipid bilayers, functional ACh-gated ion channels were observed. The ion channels displayed the functional properties expected of the α7 nicotinic receptor: selectivity for K\(^+\) over Cl\(^-\), nonselectivity between Na\(^+\) and K\(^+\), the expected single channel conductance (~50 pS), and sensitivity to block by α-BTX. The nondenaturing properties of the α7 S2/T/L9 T nAChR allowed recording of the single channel activity for extended periods of time (over 30 min).

The observation that functional α7 S2/T/L9 T nAChRs could be synthesized *in vitro* confirms that other nAChR subunits are not required for the formation of functional α7 receptors, assuming that no endogenous nAChR subunits are present in quail oviduct ER microsomes. These data are consistent with the observation that α7 wild-type (22) and α7 S2/T/L9 T nAChRs (Fig. 1) require no additional nAChR subunits to form α-BTX-sensitive, ACh-gated channels when expressed in *Xenopus* oocytes. In addition, these data imply that maturation of carbohydrate moieties by the Golgi apparatus is not required for functional α7 channel activity.

Functional α7 S2/T/L9 T nAChR channels were observed when translation products were processed by quail oviduct ER microsomes (120 experiments from 32 translations) but not when processed by canine pancreatic ER microsomes (98 experiments from 27 translations). This difference was not due to the amount of protein produced, because the level of membrane-processed α7 protein was higher in translations containing canine pancreatic microsomes than in translations containing avian oviduct microsomes. Vesicle fusion with the planar lipid bilayer was achieved with both processing systems, as evidenced by the appearance of endogenous channels in both types of microsomes; the incorporation rate of channels present in the microsomal membranes (chloride channels and cation channels) was 24% for quail oviduct microsomes and 44% for canine pancreatic microsomes. Protein factors such as foldases or chaperonins that are necessary for nicotinic receptor maturation (16–19) may be more active in the oviduct microsomes than pancreatic microsomes. Perhaps additional factors or post-translational events that are required for functional expression of chick α7 nAChRs are more efficient in oviduct microsomes. Alternatively, commercial preparations of canine pancreatic ER microsomes may contain inhibitors that prevent the proper formation of functional α7 nAChRs channels.

The incorporation rate of the synthesized, reconstituted nAChR channels processed by quail oviduct microsomes was ~10%, similar to the incorporation rate of 12% for the Shaker potassium channel (35). The frequent appearance of endogenous channels suggests that vesicle incorporation into the bilayer was not the sole limiting factor for the low incorporation rate of functional α7 nAChRs. It seems likely that oligomerization may be a limiting step in the maturation of functional α7 channels *in vitro*, as it is in cultured muscle cells where only
30% of α1 nAChR subunits synthesized in the ER bind α-BTX or are assembled into heterotetramers (55). The multiple sizes of α7 nAChR subunit complexes from in vitro translations and from Xenopus oocytes (52) suggest that many of the subunits synthesized both in ovo and in vitro may be trapped in non-functional oligomers containing too few or too many subunits. It is also possible that improperly folded subunits may oligomerize with correctly folded subunits and prevent them from forming functional channels.

The assembly of α7 nAChRs expressed in Xenopus oocytes requires cytoplasm, a prolyl isomerase and chaperone protein (19), the concentration of which is estimated to be 4–8 μM in reticulocyte lysates (56). The co-expression of cloned cytoplasm A did not improve the rate of synthesis of functional α7 nAChRs in vitro (not shown), suggesting that the amount of cytoplasm in lysates was sufficient and that mechanisms other than prolyl isomerization were limiting.

An appropriate redox potential is critical for the correct formation of the α-BTX-binding site (33) as well as the correct folding and assembly (57) of muscle nAChRs. Most of the translation reactions were supplemented with equal amounts of dithiothreitol and glutathione to provide a redox gradient across the membrane. Under these conditions, the lumen of the ER vesicle has an oxidizing environment to promote the formation of disulfide bonds (58). We optimized these conditions for protein yield, but it is possible that the conditions were not optimal for proper assembly, folding, and/or α-BTX-binding site formation (33).

The cell-free expression approach offers several advantages over the isolation and reconstitution of channels from native tissues or the expression of cloned ion channels in heterologous systems. Regulatory molecules such as kinases and phosphatases are often closely associated with native ion channels reconstituted from plasma membranes (59–61). In contrast, ion channels newly synthesized in the ER are less likely to be assembled with regulatory protein complexes than ion channels purified from plasma membranes. Channels synthesized in vitro are dissociated from second messenger pathways in the host cell that can modulate the activity of the ion channel or activate other cellular components. In addition, the planar lipid bilayer technique offers precise control over the ionic conditions, isolation from other channels native to the host cell such as the Xenopus oocyte Ca2+-activated chloride channel (62), and the ability to co-reconstitute signaling pathways and modulatory proteins as desired. In addition, this in vitro translation and reconstitution approach can be combined with mutational analysis to evaluate structure-function relationships.

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REFERENCES

1. Karlin, A., and Akabas, M. H. (1995) Neuro 15, 1231–1244

2. Unwin, N. (1998) J. Struct. Biol. 121, 181–190

3. Anand, R., Conroy, W. G., Schoepfer, R., Whiting, P., and Lindstrom, J. (1991) J. Biol. Chem. 266, 11922–11928

4. Cooper, E., Couturier, S., and Ballivet, M. (1991) Nature 350, 235–238

5. McGehee, D. S. and Role, L. W. (1995) Annu. Rev. Biochem. 57, 521–546

6. Boyd, T. (1997) Crit. Rev. Biochem. 37, 299–318

7. Role, L. W., and Berg, D. K. (1996) Nucleic Acids Res. 24, 1022–1027

8. Reynolds, J. A., and Karlin, A. (1978) Biochemistry 17, 2035–2038

9. Perez, G., Lagrutta, A., Adelman, J. P., and Toro, L. (1994) J. Biochem. 66, 1022–1027

10. Revah, F., Bertrand, D., Galzi, J.-L., Devillers-Thiery, A., Mule, C., Hussy, N., Bertrand, S., Ballivet, M., and Changeux, J.-P. (1991) Nature 353, 846–849

11. Bertrand, D., Devillers-Thiery, A., Revah, F., Galzi, J.-L., Hussy, N., Mule, C., Bertrand, S., Ballivet, M., and Changeux, J.-P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1261–1265

12. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., and Numa, S. (1982) Nature 299, 793–794

13. Laemml, U. K. (1970) Nature 227, 680–685

14. Reynolds, J. A., and Karlin, A. (1978) Biochemistry 17, 2035–2038

15. Perez, G., Lagrutta, A., Adelman, J. P., and Toro, L. (1994) J. Biochem. 66, 1022–1027

16. Revah, F., Bertrand, D., Galzi, J.-L., Devillers-Thiery, A., Mule, C., Hussy, N., Bertrand, S., Ballivet, M., and Changeux, J.-P. (1991) Nature 353, 846–849

17. Bertrand, D., Devillers-Thiery, A., Revah, F., Galzi, J.-L., Hussy, N., Mule, C., Bertrand, S., Ballivet, M., and Changeux, J.-P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1261–1265

18. Maley, F., Trimble, R. B., Tarentino, A. L., and Plummer, T. H. (1989) Annu. Rev. Biochem. 58, 195–204

19. Paulson, H. L., Ross, A. F., Green, W. N., and Claudio, T. (1991) J. Cell Biol. 113, 1371–1384

20. Kriekemamp, H.-J., Maeda, R. K., Sine, S. M., and Taylor, P. (1995) Neuron 14, 635–644

21. Anand, R., Peng, X., and Lindstrom, J. (1993) FEBS Lett. 327, 241–246

22. Papke, R. L. (1993) Proc. Acad. Biol. Chem. 41, 509–531

23. Miledi, R., and Poter, L. T. (1971) Nature 233, 599–603

24. Merlie, J. P., and Lindstrom, J. (1983) Cell 34, 747–757

25. Kruse, M., Brunker, M., Esser, A., Szalay, A. G., Troschug, M., and Zimmermann, R. (1997) J. Biol. Chem. 272, 2584–2594

26. Gellman, S. M., and Prives, J. (1996) J. Biol. Chem. 271, 10709–10714

27. Hung, C., Sinkev, A. J., and Lodish, H. F. (1992) Science 257, 1496–1502

28. Wang, Y., Townsend, C., and Rosenberg, R. L. (1993) Am. J. Physiol. 264, C1473–C1479

29. Bielefeldt, K. and Jackson, M. B. (1994) J. Physiol. 475, 241–254

30. Chung, S., Reinhart, P. H., Martin, B. L., Brautigan, D., and Levitan, I. B. (1991) Science 253, 650–652

31. Barish, M. E. (1983) J. Physiol. 342, 309–325

32. Robinson, R. A., and Stokes, R. H. (1968) Electrolyte Solutions, pp. 445 and 492–494, Butterworths Publications, Ltd., London

33. Robinson, R. A. (1961) J. Phys. Chem. 65, 662–667