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Stable expression of transfected *Torpedo* acetylcholine receptor α subunits in mouse fibroblast L cells

(flux analysis/single-channel recordings)

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ABSTRACT **_Torpedo californica_ electric organ cDNA libraries were constructed in λgt10 and λgt11. Four acetylcholine receptor (AcChoR) subunit cDNA clones were isolated and shown to contain the entire coding region for each of the subunits. When _in vitro_ synthesized AcChoR mRNA was microinjected into _Xenopus laevis_ oocytes, functional cell surface AcChoRs were expressed. A very simple and fast ^{22}Na-uptake experiment was performed on batches of microinjected oocytes to identify oocytes that were expressing large quantities of functional cell surface AcChoRs for use in single-channel recordings. In addition to the transient expression system, DNA-mediated cotransformation is described, which is a method for stably introducing AcChoR cDNAs into the chromosomes of tissue culture cells. Because the AcChoR is composed of four different subunits, it is necessary to integrate four cDNAs into the chromosomes of the same cell before stable expression of a completely functional receptor complex can be established. We show that 80% of the cells that integrated the selectable marker gene into their chromosomes also integrated all four AcChoR cDNAs. When _Torpedo_ α-subunit cDNA inserted into an appropriate expression vector was introduced into cells by transfection, α-subunit protein was synthesized that migrated on $NaDodSO_4$/polyacrylamide gels with the same molecular mass as native _Torpedo_ α subunits and expressed antigenic determinants similar to those of native _Torpedo_ α subunits.**

The nicotinic acetylcholine receptor (AcChoR) is the postsynaptic receptor at skeletal neuromuscular junctions and is the best characterized ionic-channel protein in vertebrates. It is an oligomeric, intrinsic membrane glycoprotein consisting of four subunits (α, β, γ, δ) with the stoichiometry $\alpha_2\beta\gamma\delta$. The molecular weights of the subunits are ≈40,000, 50,000, 60,000, and 65,000, respectively. From biochemical studies, the AcChoR is known to contain two binding sites for acetylcholine (AcCho) and shows allosteric transitions in binding affinity that accompany open and closed states of the transmembrane ionic channel. Electrical recordings of AcCho-induced currents have measured the permeability properties of the channel and have given details of the kinetics of receptor activation (for reviews, see refs. 1–5). The cDNA sequences for each of the four subunits of AcChoR from *Torpedo californica* (6–8) and several other species have been obtained (reviewed in ref. 9). The primary structure of the AcChoR has been derived from the cDNAs, but considerable controversy and much speculation remains about the topology of folding, about the three-dimensional structure of the oligomer, and about the location of important functional domains. Specific biochemical modifications and analyses continue to be applied as well as site-directed mutagenesis techniques (10, 11) to define these regions.

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The goal of many of the site-directed mutagenesis experiments is to localize specific structures and assign them to a specific function. For most of these studies, a system that expresses fully assembled and functional AcChoR is necessary. The system used thus far has been transient expression in *Xenopus laevis* oocytes. The major advantages of this method are the rapidity of the assay (protein can be analyzed 2–3 days after the introduction of RNA) and the ease of performing certain types of structure–function analyses. Other studies, such as determining the specific interaction of certain proteins with the AcChoR or looking at various steps in the biosynthesis and assembly of the subunits cannot be conducted in oocytes. A system more amenable to many cell biological questions is stable expression of AcChoR in established cell lines. In this paper, we describe the construction of *T. californica* electric organ cDNA libraries, the isolation and characterization of the four AcChoR subunit cDNA clones, transient expression of functional cell surface AcChoRs in *Xenopus* oocytes, and the stable expression and partial characterization of *Torpedo* AcChoR α subunits in mouse fibroblast L cells.

MATERIALS AND METHODS

Materials. Restriction and other enzymes used in various cloning procedures were obtained from New England Biolabs (except where otherwise noted) and used according to the supplier's specifications. The phage cloning vectors λgt10 and λgt11, and the *Escherichia coli* strains $C_{600}rk^-mk^-$ *hfl* and LE392 were obtained from T. Huynh and R. Davis (12). The vector pSS-2 was constructed by S. Silverstein (Columbia University College of Physicians and Surgeons). This vector is basically a pBR322 derivative, PAT153, in which the 412-base-pair (bp) *Hin*dIII–*Bam*HI fragment was replaced by the 340-bp *Hin*dIII–*Pvu* II fragment from simian virus 40. The vector pVCOS was constructed by S. Goff (Columbia University College of Physicians and Surgeons) and contains 3 kilobases of Moloney murine leukemia virus DNA including the two long terminal repeats inserted into the cosmid pHC79 (13) at the *Eco*RI site. The *gag*, *pol*, and *env* genes of the virus were deleted from the *Pst* I site at the 5′ end of the *gag* gene to the *Hpa* I site at the 3′ end of the *pol* gene. The *Eco*RI site in pHC79 was destroyed by the addition of the Moloney sequences and a new *Eco*RI cloning site located between the long terminal repeats was introduced with a polylinker. Murine fibroblast L cells deficient in thymidine kinase and adenine phosphoribosyltransferase (Ltk^-Aprt^-) were obtained from R. Axel (Columbia University College of Physicians and Surgeons) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) calf serum and diaminopurine at 50 μg/ml. *Torpedo* AcChoR

Abbreviations: AcCho, acetylcholine; AcChoR, AcCho receptor; α-BTX, α-bungarotoxin; Ltk^-Aprt^-, L cells deficient in thymidine kinase and adenine phosphoribosyltransferase; pfu, plaque-forming units; UT, untranslated.

subunit-specific antisera were prepared in rabbits as described (14).

Construction of cDNA Libraries. Polyadenylylated mRNA was obtained from *T. californica* electric organ using the method of Chirgwin *et al.* (15). The polyadenylylated fraction of mRNA was obtained by passing the mRNA two times over an oligo(dT)-cellulose column. The first strand was synthesized using avian myeloblastosis virus reverse transcriptase and oligo$(dT)_{12-18}$- (Collaborative Research, Waltham, MA) primed mRNA in the presence of actinomycin D at 40 μg/ml and human placental RNase inhibitor at 300 units/ml (RNasin from Sigma). The second strand was synthesized with *E. coli* DNA polymerase I in the presence of RNase H (P-L Biochemicals) (16). The double-stranded cDNA was treated for 1 hr at 37°C with *Eco*RI methylase (in 100 mM Tris·HCl, pH 7.5/10 mM EDTA/6 μM *S*-adenosylmethionine), and the ends were made flush by filling in with the Klenow fragment of DNA polymerase I. *Eco*RI linkers (New England Biolabs) were phosphorylated with polynucleotide kinase, ligated to the termini of the cDNA, and digested with *Eco*RI. The cDNA was inserted into a unique *Eco*RI site in the phage cloning vector λgt10 (12), packaged, and grown in the bacterial strain. $C_{600}rk^-mk^-$ *hfl*. A λgt11 library was also constructed by ligating the double-stranded cDNA into the *Eco*RI site of this vector and growing the phage in the bacterial strain LE392 (17). Additional details of the construction of cDNA libraries will be presented elsewhere (18).

Screening and Subcloning. Probes used for identifying AcChoR subunit clones in the λgt10 library were as follows: a ^{32}P end-labeled synthetic 14-bp oligonucleotide sequence corresponding to the 5′-untranslated (UT) region of α (based on published DNA sequence data), nick-translated partial β and δ clones (19), and a nick-translated *Bgl* II–*Eco*RI fragment from the γ subunit (7). Screening of duplicate filters was carried out according to standard procedures (20). Clones were analyzed by restriction endonuclease mapping and partial DNA sequence analysis (21) to identify clones containing the entire structural gene region of each subunit. Clones from the λgt10 library containing the entire structural gene for the α, β, and δ subunits were subcloned into the *Eco*RI site of the pSS-2 vector. The γ-clone (7) was subcloned into the *Hin*dIII site of pSS-2. This clone extended from the *Alu* I site at −21 bp to the *Pvu* II site in the 3′-UT region. The α clone from the λgt10 library was also subcloned into the RI site of pVCOS.

Cotransformation. Cells were transfected with DNAs using the calcium phosphate precipitation procedure of Graham and van der Eb (22) as modified by Wigler *et al.* (23). pSS-2 -α (2.5 μg), -β (2.5 μg), -γ (2.5 μg), and -δ (2.5 μg) DNAs, the plasmid λAT3 (5 ng) (24), which contains the *Aprt* gene, and high molecular weight carrier DNA (10 μg) prepared from Ltk^-Aprt^- cells were introduced into 5×10^5 Ltk^-Aprt^- cells on 10-cm plates. $Aprt^+$ transformants were selected in DMEM containing azaserine at 4 μg/ml, adenine at 15 μg/ml, and 10% (vol/vol) calf serum as described (25). Eleven colonies were isolated using cloning cylinders and grown into separate cell lines. Other cells were transfected with 3 μg of pVCOS-α, 50 ng of λAT3, and 17 μg of high molecular weight carrier DNA, selected in medium containing azaserine and adenine followed by isolation of four individual colonies and a pool of three plates of colonies.

DNA Analyses. A confluent 10-cm dish of cells was washed twice in phosphate-buffered saline, the cells were removed from the dish with trypsin, washed, and resuspended in 3 ml of 10 mM Tris·HCl, pH 7.5/5 mM EDTA/0.4 M NaCl. Then, 0.2% $NaDodSO_4$ and Pronase at 200 μg/ml were added, and the solution was gently rocked overnight at 37°C. Proteins were removed with successive phenol/chloroform, 1:1 (vol/vol), extractions, and the DNA was precipitated with 2.5 volumes of ethanol (20). DNA was digested with *Eco*RI, and 10 μg of DNA was loaded onto a 1% agarose gel. pSS-2 plasmid DNA containing α, β, γ, or δ inserts was digested with *Eco*RI, and 100 pg of DNA (representing ≈10 copies per cell) was loaded onto gels. Blots were done according to Southern (26). Four individual probes were made by nick-translating (27) α, β, γ, and δ inserts. Blots were hybridized in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) at 68°C and washed at 68°C with a final wash in 0.5× SSC.

Labeling and Immunoprecipitations. Confluent 10-cm dishes of cells were washed twice with phosphate-buffered saline, then incubated 2 hr at 37°C with medium containing all amino acids except methionine and supplemented with 10% (vol/vol) dialyzed fetal calf serum. Medium (4 ml) containing 1 mCi (1 Ci = 37 GBq) of [^{35}S]methionine (Amersham) was added, and cells were incubated for 20 min at 37°C. Cells were next washed and then lysed in 500 μl of lysis buffer [150 mM NaCl, 5 mM EDTA, 50 mM Tris·HCl (pH 7.4), 0.02% NaN_3, 0.5% Nonidet P-40, hemoglobin at 1 mg/ml] containing fresh 2 mM phenylmethylsulfonyl fluoride and 2 mM *N*-ethylmaleimide. Overnight incubations with antisera followed by precipitation with the Cowan I strain of *Staphylococcus aureus* (Pansorbin from Calbiochem–Behring) were essentially as described (28, 29). Antigens were dissociated from antibodies by the addition of 40 μl of 2× gel-loading buffer [4% (wt/vol) $NaDodSO_4$, 20% (vol/vol) glycerol, 0.125 M Tris·HCl (pH 6.8), 0.01% bromphenol blue] containing fresh 10 mM dithiothreitol. Samples were boiled 3 min, resins were removed by centrifugation, and the supernatants were loaded onto 10% discontinuous $NaDodSO_4$ gels (30). Gels were fixed for 30 min in a solution of 25% (vol/vol) methanol and 10% (vol/vol) acetic acid, then soaked in Amplify (Amersham) for 30 min, dried on a gel dryer, and put on film at −70°C with an intensifying screen.

Expression of AcChoR in Oocytes. SP6 mRNA transcripts (31) were made *in vitro* as described (32). Oocytes were obtained from *X. laevis* females (Nasco, Fort Atkinson, WI), prepared for microinjection as described (33), and microinjected with 50 nl of RNA at 0.125 mg/ml in water. Three days after cytoplasmic injections, oocytes were assayed for (*i*) the ability to bind α-bungarotoxin (α-BTX), (*ii*) AcCho-activated ^{22}Na uptake, and (*iii*) single-channel activity (34). The number of cell-surface AcChoRs capable of binding α-BTX was determined by incubating intact oocytes with 1 nM of ^{125}I-labeled α-BTX for 90 min at room temperature, washing in frog Ringer solution (33), then radioactivity in individual oocytes was measured by γ-counting. The ability of *Torpedo* AcChoRs expressed on the surface of injected oocytes to bind agonists and flux small cations was determined by ^{22}Na uptake assays (35). Tracer flux was initiated by adding 100 μl of frog Ringer solution containing 5 mM AcCho and 1 μCi of carrier-free ^{22}Na. After 15 min at 0°C, 1 ml of Ringer solution plus 1 mM *d*-tubocurarine was added to stop the uptake and prevent backward flux of ^{22}Na. Oocytes were washed with Ringer solution plus *d*-tubocurarine then transferred to individual tubes containing 0.5 ml of Ringer solution for γ counting. Currents for single-channel activity were recorded in standard *Xenopus* Ringer solution (33) as described (34). Additional details will be published elsewhere (18).

RESULTS

cDNA Libraries and Clones. With the λgt10 library, the transformation efficiency was 2×10^7 plaque-forming units (pfu)/μg of double-stranded cDNA. Recombinants (1.5×10^5 pfu) were amplified to a titer of 5×10^9 pfu/ml. With the λgt11 library, the transformation efficiency was 2.6×10^6 pfu/μg of double-stranded cDNA, and the library contained 6.7×10^5 recombinants that were amplified to a titer of 8×10^9 pfu/ml

(6.2% of which contain inserts yielding a titer of phage with inserts of 5×10^8 pfu/ml). The λgt10 library was screened for AcChoR subunit cDNAs, and ≈0.25% of the plaques were positive for each subunit. Phage DNA was prepared, digested with *Eco*RI, and electrophoresed on 1% agarose gels. The inserts were isolated and mapped with various restriction enzymes to isolate full-length clones of α, β, γ, and δ subunits. From restriction map analyses, it was clear that all of the clones encoded the entire structural gene region of each subunit except possibly the 5′ end of the β clone. The 5′ end of this clone was sequenced (21) to determine its precise start. The clones have the following lengths: The α clone has a 5′-UT region ≈80 bp long and a 3′-UT region of ≈300 bp (clone, ≈1760 bp total). The β clone has a 5′-UT region of 2 bp and a 3′-UT region of ≈200 bp (clone, ≈1720 bp). The γ clone has a 5′-UT region of ≈10 bp and a 3′-UT region of ≈500 bp (clone, ≈2030 bp). The δ clone has a 5′-UT region of ≈40 bp and a 3′-UT region of ≈200 bp (clone, ≈1800 bp).

Expression of Functional Cell Surface AcChoRs in Oocytes. Although the results of the restriction endonuclease mapping and DNA sequence analyses were consistent with the clones being full length, it was necessary to determine that they could, in fact, encode subunits that would form functional AcChoR molecules. Receptor function was demonstrated by using the clones as templates to make SP6 mRNA transcripts *in vitro*, and then injecting the RNA into *Xenopus* oocytes and assaying for cell surface AcChoRs by ^{125}I-labeled α-BTX binding. Three of the four microinjected oocytes were capable of binding 2.5 fmol of ^{125}I-labeled α-BTX (1530, 1840, 100, and 1320 cpm; uninjected control oocytes bound 90, 70, 80, and 80 cpm), demonstrating that the subunits were made, then assembled into AcChoR complexes, and the complexes were inserted into the oocyte membrane. In addition to demonstrating toxin binding, we needed to demonstrate that these receptors would form channels that were permeable to small cations upon binding of their natural ligand, AcCho. The assay used for these studies was AcCho-activated ^{22}Na uptake (35). Again, three of the four injected oocytes responded in this experiment (3030, 2500, 2280, and 190 cpm; uninjected control oocytes took up 480, 300, 40, and 80 cpm). Of those that responded, the amounts of ^{22}Na taken up were ≈10 times the level of ^{22}Na uptake in noninjected oocytes or in injected but nonresponding oocytes. These results suggest that functional cell-surface AcChoR is made in quantities sufficient to obtain a surface density that will allow the activity of single channels to be recorded. Usually a receptor density of 10–100 AcChoRs per μm^2 is needed for single-channel studies. Approximately 7.5×10^8 molecules of functional AcChoR were expressed on the surface of each oocyte giving a density of ≈240 molecules per μm^2. With this level of expression, we could easily detect and, therefore, investigate the single-channel properties of the receptors. Oocytes that responded positively in the ^{22}Na-uptake experiments were used to obtain single-channel recordings with either a cell-attached patch (Fig. 1*A*) or an outside-out patch (Fig. 1*B*). Evidence that these were in fact AcChoR channels is as follows: (*i*) no channel activity was observed in the absence of AcCho in either the cell-attached or outside-out configurations, (*ii*) channels appeared upon the addition of AcCho to the outside-out patch, and (*iii*) channel activity desensitized completely at 100 μM AcCho and could be restored by washing out the AcCho. The conclusions from these studies are that the clones encode proper AcChoR subunit polypeptides, and the expressed receptors have the same properties as those in their native environment. Because transcripts from the clones function properly in the oocyte system, it suggests that these clones may also be expressed properly when introduced into mammalian cells.

Cotransformation. To express functional *Torpedo* AcChoRs in mammalian cells, all four AcChoR cDNAs must be incorporated into the genome of the same cell, such that the four subunits can be expressed and assembled into proper AcChoR complexes. Because the stoichiometry of the α, β, γ, and δ subunits in the receptor complex is 2:1:1:1, incorporating similar copy numbers of each cDNA might also be important. To determine whether DNA-mediated cotransformation would be a suitable method for establishing such a system, the first questions we needed to address were: What is the efficiency of introducing five different genes or cDNAs into the same cell and, what is the copy number of each of these DNAs? The answers to these questions determined the suitability of using this method for such experiments and also told us how many colonies of cells had to be isolated in future experiments to assure that cell lines with the proper numbers of cDNAs would be obtained.

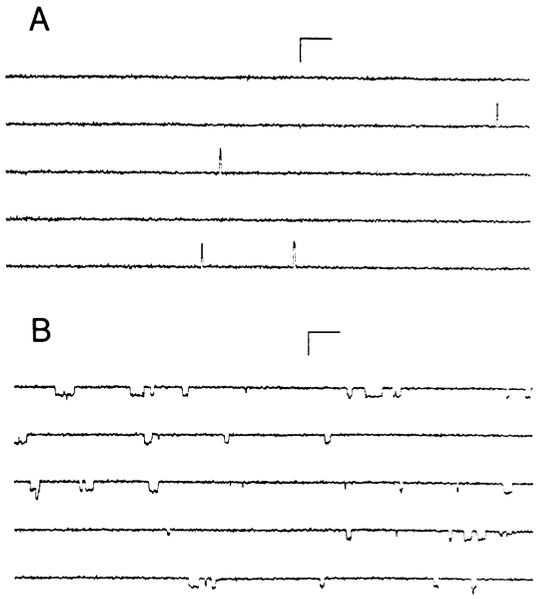

FIG. 1. Single-channel activity was recorded in standard *Xenopus* Ringer solution from oocytes expressing AcChoRs as determined by ^{22}Na-uptake studies. Records were obtained from a cell-attached patch (*A*) at 10°C and −70 mV and from an outside-out patch (*B*) at 10°C and −60 mV. Both recordings were filtered at 2200 Hz (low-pass, −3 dB). In *A*, the AcCho concentration in the pipette was 1 μM; in *B*, AcCho was added to a final concentration of 100 μM. The horizontal scale bar is 10 ms, and the vertical scale bar is 5 pA. The record shown in *B* was taken during the couse of AcCho mixing. At equilibrium, the channel activity disappeared, which is consistent with the onset of densensitization.

Ltk$^-$Aprt$^-$ cells were transfected with the four subunit cDNAs in the vector pSS-2 along with the *Aprt* gene. Cells were put into selective azaserine/adenine medium, and, after 12 days, 11 colonies expressing the Aprt$^+$ phenotype were isolated and grown into individual stable cell lines. DNA was isolated from each cell line, digested with the restriction enzyme *Eco*RI, electrophoresed on gels, blotted, and hybridized to four separate nick-translated α, β, γ, and δ cDNA probes. The results are shown in Fig. 2. Cell line 1 incorporated only the *Aprt* gene and none of the receptor cDNAs. Cell line 7 incorporated *Aprt* and the β and δ cDNAs. The other nine lines incorporated all five DNAs. Thus, 80% of the cells that incorporated the *Aprt* gene into their chromosomes also incorporated all four receptor subunit cDNAs. The amounts of each cDNA incorporated varied, however, but levels were from ≈1 to 20 copies of each per cell. In addition, half of these lines had approximately equal copy numbers of each cDNA (lines 2, 5, 6, 9, and 10). These results demonstrated that DNA-mediated gene transfer was a method that could be used to easily introduce the four AcChoR subunit cDNAs into the same cell in approximately equal copy numbers.

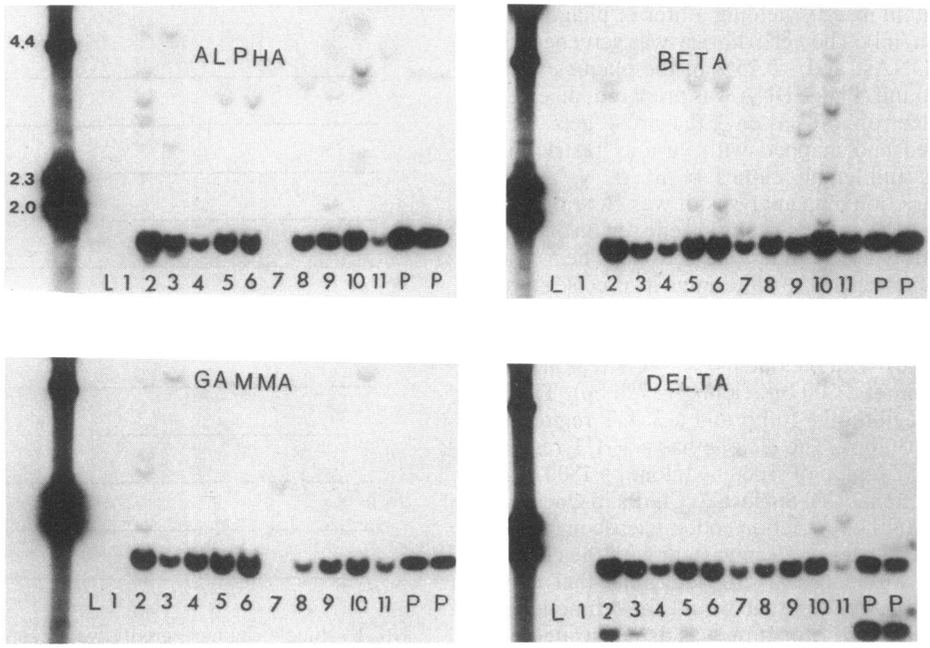

FIG. 2. DNA blots of 11 stable cell lines cotransfected with mixtures of *Torpedo* α, β, γ, and δ cDNAs plus an *Aprt* gene. L, untransformed Ltk$^-$Aprt$^-$ cellular DNA; P, plasmid DNA containing the cDNA from which the individual probes were made; lanes 1–11 represent individual transformed cell lines; the positions of the molecular weight standards (in kilobases) of λDNA digested with *Hin*dIII restriction enzyme are shown. Plasmid DNA (100 pg) (representing ≈10 copies per cell) was electrophoresed in the lanes marked P. Blots were hybridized to nick-translated α, β, γ, and δ probes.

Protein Expression. The α-subunit cDNA was inserted into the expression vector, pVCOS (from S. Goff). Ltk$^-$Aprt$^-$ cells were transfected with pVCOS-α and *Aprt* as described above for the pSS-2 vectors, and, after 11 days in selection, four colonies were isolated and grown into stable cell lines. Confluent dishes of cells were labeled with [^{35}S]methionine, solubilized, immunoprecipitated with anti-α-subunit antiserum, electrophoresed on discontinuous NaDodSO$_4$ gels, and autoradiographed. The results are shown in Fig. 3. α subunit is seen only in those lanes immunoprecipitated with anti-α-subunit antiserum and only in lanes containing cells transfected with pVCOS-α. There is no α protein seen in the untransformed Ltk$^-$Aprt$^-$ cells, and no α protein seen in transformed cell lines that are immunoprecipitated with preimmune serum. Thus (*i*) *Torpedo* α subunit can be expressed in mouse fibroblast cells (Ltk$^-$Aprt$^-$), (*ii*) the α subunits made in L cells and in *Torpedo* electric organ share at least one antigenic determinant, and (*iii*) the migration of *Torpedo* α subunits synthesized in L cells is identical to that of native *Torpedo* α subunits synthesized in *Torpedo* electric fish. This latter result suggests that the α subunit was made and processed correctly.

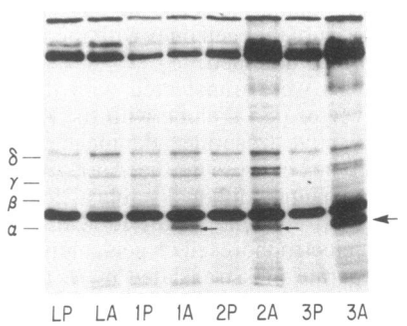

FIG. 3. Immunoprecipitation of *Torpedo* α-subunit protein stably expressed in Ltk$^-$Aprt$^-$ cells. Three different [^{35}S]methionine-labeled, immunoprecipitated stable Ltk$^-$Aprt$^-$ cell lines cotransformed with the *Torpedo* α cDNA-containing plasmid (pVCOS-α) and *Aprt* were electrophoresed on 10% NaDodSO$_4$/polyacrylamide gels and autoradiographed. Each lane represents approximately 7.5 × 10^6 cells labeled with 1 mCi of [^{35}S]methionine. L represents untransformed Ltk$^-$Aprt$^-$ cells; lanes 1–3 represent the three transformed cell lines; P represents preimmune serum; A represents anti-α-subunit antiserum. The positions of unlabeled *T. californica* electric organ AcChoR subunits are marked (α, β, γ, and δ). The arrows indicate the positions of *Torpedo* α subunits.

DISCUSSION

Several expression systems are available that can be used to study various properties of the AcChoR. The fastest and easiest system is probably transient expression of AcChoR after microinjection of AcChoR mRNAs into oocytes. We are interested, however, in certain properties of the receptor that can best be studied in a system where AcChoR is continuously expressed in an established tissue culture cell line such as the interaction between AcChoRs and nerve. A cultured fibroblast stably expressing AcChoR can easily be cocultured with nerve cells whereas an AcChoR-expressing *Xenopus* oocyte cannot. This system can also be used to determine the specific effect of the nerve on the AcChoR without the interference of other muscle-specific proteins. Other advantages of the stable expression system over the transient system include the following: (*i*) the ease of working with large quantities of identical cells for biochemical and pharmacological studies, (*ii*) certain biophysical techniques (such as single-channel recording using patch clamp techniques) are more easily performed on tissue culture cells than on oocytes, and (*iii*) the cell lines (unlike oocytes) are not seasonal.

To establish the stable system, it was necessary to first obtain the four AcChoR subunit cDNA clones. Two *T. californica* electric organ cDNA libraries were constructed: one in λgt10 and the other in λgt11. The λgt10 library was screened, and the four full-length AcChoR subunit cDNA clones were identified. When *in vitro* synthesized mRNA using these clones as templates was microinjected into

Xenopus oocytes, functional cell surface AcChoRs were formed. We know, therefore, that all the information required to make a correctly synthesized, processed, assembled, and functional AcChoR is encoded in these clones. In addition, a simple assay originally devised for tissue culture cells (35) was used on oocytes. This technique not only allows one to rapidly determine which microinjected oocytes are expressing cell surface AcChoRs, but it is gentle enough to allow the oocytes to then be patch clamped for single-channel analysis.

One method for stably introducing the AcChoR subunit cDNAs into cultured cells was explored: DNA-mediated gene transfer using calcium phosphate precipitation. One difficulty with establishing expression of a protein such as the AcChoR is the need to introduce four different cDNAs into the same cell. Using DNA-mediated gene transfer, we demonstrated that the cotransformation efficiency of introducing all four cDNAs plus the selectable marker gene was 80%. In addition, half of these cells took up approximately equal copy numbers of each cDNA. Cotransformation thus appeared to be an excellent method for solving the problem of stably introducing the four AcChoR cDNAs into the same cell. In addition, we were able to achieve protein expression of *Torpedo* α subunits in Ltk^-Aprt^- cells. The subunit migrated with the same molecular mass as native *Torpedo* α subunit and was recognized by polyclonal anti-α-subunit antiserum. Both results indicated that the *Torpedo* subunit had been correctly synthesized and processed in a mouse fibroblast cell.

Transient expression of fully assembled and functional AcChoR has been achieved in *Xenopus* oocytes with these clones (Fig. 1 and ref. 32) and other clones (36). In addition, transient expression of AcChoR subunits using some of these clones has also been achieved in yeast (37). In both the yeast system and the stable expression system described in this communication, the subunits appear to be correctly synthesized and processed. We will not know this for certain, however, until all of the subunits are expressed in the same cell and functional cell surface receptor complex is formed. Based on the successful expression of these clones in other systems (Figs. 1 and 3; ref. 37) and the successful stable expression of other proteins in cultured cells, it appears likely that functional *Torpedo* AcChoRs will be stably expressed in this system as well. Toward this goal, we have now stably introduced all four of the *Torpedo* AcChoR subunits cDNAs into three different muscle cell lines and/or mouse fibroblasts, and the subunits all appear to be properly synthesized and processed (T.C., unpublished data).

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