The synaptic form of acetylcholinesterase (AChE) in skeletal muscle CoQ-AChE derives from two separate genes encoding the catalytic and the non-catalytic collagenic tail (CoQ) subunits, respectively. CoQ-AChE expression is regulated by muscle activity; however, how this regulation takes place in skeletal muscle remains poorly understood. In this study, we overexpressed or knocked down CoQ expression in skeletal muscle and found that the level of this non-catalytic component by itself was sufficient to change the levels of total AChE activity by promoting assembly of higher order oligomeric forms including the collagen-tailed forms. These results initially suggested that CoQ could be limiting in the assembly of synaptic CoQ-AChE during development and differentiation. We then determined the levels of CoQ protein and CoQ mRNA during primary quail muscle cell development and differentiation in culture (QMCs) and as a function of muscle activity. Surprisingly, we found disassociation between transcription and translation of the non-catalytic subunit from its assembly into CoQ-AChE. Furthermore, we found that the vast majority of the steady state CoQ molecules in mature quail muscle cultures are not assembled into CoQ-AChE, suggesting that they are either rapidly degraded or have alternative function(s).

Acetylcholinesterase (AChE) is the enzyme that rapidly terminates neurotransmission at central and peripheral cholinergic synapses. In skeletal muscle, the synaptic form of AChE consists of three catalytic tetramers attached to a non-catalytic collagen-like tail (CoQ) referred to as CoQ-AChE or synaptic AChE (for reviews see Refs. 1–3). This molecule is highly expressed at the neuromuscular junction (NMJ), where its levels are regulated by the presence of the nerve and cell membrane depolarization (4–9). The direction of the regulation appears to be species-specific (for a review see Ref. 3). Avian systems are the only ones where AChE transcription (11) and enzymatic activity (12) increase after skeletal muscle denervation. This matter is still unresolved because of muscle lengthening after denervation that is enough to increase AChE transcription and enzymatic activity (13).

The CoQ and AChE subunits are encoded by two separated genes (14). Co-expression of catalytic and non-catalytic subunits in COS-7 cells (14), as well as microinjection of AChET and ColQ transcripts in X. laevis oocytes (15), results in the production of globular and collagen-tailed AChE forms whose distributions suggest that the pattern of forms expressed is determined by the molecular ratio of the catalytic and non-catalytic subunit mRNAs.

The regulation of mammalian CoQ has been studied during differentiation of the C2/C12 mouse muscle cell line in culture (16, 17) and in differentiated adult rat muscle in vivo (15, 18, 19) with emphasis on the transcriptional control elements responsible for muscle-type-specific expression. However, all these studies measured enzyme activity and/or transcript levels rather than the actual CoQ protein. Both in culture and in vivo, expression of the CoQ-AChE form is increased by membrane depolarization and decreased when membrane depolarization is blocked (20–23). When scorpion venom (ScVn) is added to primary quail muscle cultures (QMCs) to maintain sodium channels in the open state, thus chronically depolarizing the membrane, the levels of AChE mRNA decrease significantly (24). In contrast, there is a large increase in the intracellular ColQ-AChE pool as well as an increase in cell surface enzyme activity and AChE clustering (24). Conversely, when tetrodotoxin (TTX) is added to QMCs to maintain sodium channels in the closed state, thus blocking membrane depolarization and spontaneous contraction, the levels of AChE mRNA increase (24). Under this condition, there is a significant decrease or elimination of CoQ-AChE expression as well as a decrease in cell surface enzyme activity and AChE clusters (24). In both conditions, the levels of AChE catalytic subunit mRNA correlate with total AChE activity but not with the levels of CoQ-AChE. Given this evidence suggesting that CoQ-AChE catalytic activity levels in skeletal muscle cells are not regulated simply by the availability of catalytic subunits, one possible mechanism for regulating enzyme expression could be by regulating levels of the non-catalytic CoQ subunit as, suggested by the co-expression studies in COS-7 cells (14) and frog oocytes (15).

To test this model in more detail, we cloned the quail CoQ (qColQ) from primary quail muscle cultures and produced antibodies against the qColQ protein. We found that overexpression...
pression of ColQ in QMCs and QM7 cells, a quail myoblast cell line, results not only in an increase in ColQ-AChE expression but also in a dramatic increase in total cell-associated AChE activity without affecting the levels of the monomeric and dimeric forms. Conversely, expression of ColQ-shRNAs resulted in a specific knockdown of the intracellular ColQ-AChE pool and cell surface AChE clusters without affecting the pool of catalytically active AChE monomers and dimers. Studies on the expression of catalytic and non-catalytic ColQ subunit during muscle development and differentiation as well as during experimentally manipulated changes in muscle activity clearly show a dissociation between RNA and protein expression on the one hand, and the assembly of ColQ-AChE on the other. In addition, our results show that many more ColQ subunits are expressed than will be assembled into complex ColQ-AChE forms emphasizing the importance of post-translational control mechanisms at the level of assembly in the regulation of COLQ-AChE in skeletal muscle.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture of Quail Skeletal Muscle**—Primary myoblasts were obtained from the pectoral muscles of 10-day-old quail embryos and plated at 5 × 10^4 cells/ml in Eagle’s Minimum Essential Medium (Invitrogen) supplemented with 2% chicken embryo extract, 10% horse serum (GemCell), and 0.1% Gentamicin (Invitrogen) (EMEM 210) (25). The cultures were fed with fresh medium on days 3 and 5 after plating. In addition, the medium was supplemented on day 3 with 10^-6 M cytosine-arabinoside (Sigma) to inhibit fibroblast proliferation. Cultures were maintained at 39 °C in a water-saturated incubator with 5% CO₂. For fluorescence microscopy, the QMCs were grown on collagen-coated glass coverslips.

**Drug Treatments**—To study the newly synthesized AChE, QMCs were treated with 10^-4 M diisopropylfluorophosphate (DFP, Sigma) in 20 mM phosphate-buffered saline (PBS, pH 7.4) for 10 min at room temperature, followed by three washes in PBS alone to remove unreacted DFP as previously described (26). The cultures were then returned to the incubator to recover in complete medium with or without additional treatments as described. When used, 5-day-old QMCs were pretreated with 20 μM ScVn (Sigma) or 5 μM TTX (Sigma) for 48 h before extraction and analysis.

**Analysis of AChE Oligomeric Forms and Assay of Enzyme Activity**—The oligomeric forms of AChE were extracted with borate extraction buffer (20 mM sodium borate pH 9.0, 0.5% Triton X-100, 5 mM EDTA, 1 mM NaCl, 0.5% bovine serum albumin) and the protease inhibitors leupeptin (1 μg/ml) and pepstatin (2 μg/ml). After centrifugation in a microcentrifuge, 100–150 μl aliquots of crude cell extracts pooled from triplicate samples were analyzed by velocity sedimentation at 36,000 rpm in an SW41 Sorvall Rotor for 16 h on 5–20% continuous sucrose gradients in extraction buffer without bovine serum albumin, except for those run to separate AChE tetramers (G4) from AChE-tailed tetramers (A14) in which 0.5% Brij-98 was used instead of 0.5% Triton X-100, and the samples were centrifuged at 40,000 rpm in an SW41 Sorvall Rotor for 22 h. The fractions were collected and assayed for AChE activity using the Ellman assay (27). The extraction buffer volumes as well as the sample sizes loaded on the sucrose gradients were constant for any given experiment. Thus, the activities on the gradients are quantitatively and qualitatively comparable.

**Cloning of Quail ColQ cDNAs**—Full-length Quail ColQ (qColQ) cDNA was cloned into pTarget (Promega) by RT-PCR from total RNA isolated from QMCs using primers based on the chicken ColQ sequence and RACE PCR (Invitrogen) to clone the 3’-end. The cloned cDNA was confirmed by DNA sequencing and alignment with human, mouse, rat, chicken, and Torpedo ColQ cDNAs, all available from the NCBI data base (supplemental Fig. S1). The qColQ cDNA used in all the experiments includes the endogenous leader sequences at the 5’-end and the entire coding region. The sequence most closely resembles the rat ColQ1 transcript (15) and appears to be the only one expressed in avian muscle (see text). The chicken AChE cDNA used in these studies was the generous gift of Dr. William R. Randall.

**Design and Cloning of qColQ shRNAs**—Based on the sequence of the cloned qColQ cDNA and using the online siDESING® center (Dharmacon), we designed and cloned in pSilencer 3.0-H1 (Ambion) three different qColQ shRNAs, qColQ shRNA1: GAATTCCTGGTATAATGG, qColQ shRNA2: TAA-TCCACCATATGGAGAA, and qColQ shRNA3: CTGCATCT-GCACTTCTTACT.

**RNA Isolation and RNase Protection Assay (RPA)**—Total RNA was obtained from 1-, 2-, 3-, 4-, 5-, and 7-day-old QMCs and QM7 cells treated or not with ScVn and TTX using TRIzol (Sigma) following the manufacturer’s suggested protocol. To quantitate the AChE mRNA, we used a 302-nucleotide probe (AChE 302) designed and used previously in our laboratory (24). To create specific probes to quantitate ColQ mRNA, we used several restriction fragments within the coding region of the quail ColQ cDNA. These fragments were subcloned in pGEM-4Z and used to transcribe in vitro probes for RNase protection assays following the protocol in molecular cloning (28). A 490-nucleotide (ColQ 490) fragment from nucleotide 880 to the end of the qColQ coding sequence gave the best signal and was used in all subsequent experiments. All RPAs were done incubating 2.5 × 10^5 cpm of each antisense probe, ColQ 490 and AChE 302, with 20–60 μg of total RNA or 0.5, 16.6, or 50 pg of synthetic ColQ and AChE cRNA as standards to determine precisely the numbers of ColQ and AChE mRNA molecules in the samples. The protected RNAs were electrophoretically separated in denaturing acrylamide gels following the protocol in molecular cloning (28). After resolving, the gels were exposed to a PhosphorImager screen (Amersham Biosciences), the images were acquired with a Storm 840 PhosphorImager scanner (Molecular Dynamics), and the data analyzed using Image-Quant software (Amersham Biosciences).

**Cell Transfections**—QMCs, QM7, and HEK-296 cells were transfected with plasmids encoding qColQ (pTarget-qColQ), 4 W. R. Randall, personal communication.
Assembly of ColQ Acetylcholinesterase

**FIGURE 1. Overexpression of ColQ is sufficient to increase catalytically active ColQ-AChE whereas its absence results in ColQ-AChE loss.** Panels A and B, active AChE forms expressed in QM7 cells previously transfected with qColQ (A, solid circles) or qColQ shRNA (B, solid circles). Controls consisted of transfection with an empty vector alone (A, open circles) or EGFP shRNA (B, open circles). Panels C and D, active AChE forms expressed in QM7 cells previously transfected with chAChE alone (C, open circles) or together with qColQ (C, solid circles) or qColQ with either qColQ shRNA (D, solid circles) or EGFP shRNA (D, open circles). To analyze the different AChE forms expressed total cell extracts from three cultures were pooled for each gradient, fractionated by velocity sedimentation, and their enzymatic activity assayed. G1, G2, G4, A12, A4+G4, and A8 refer to AChE monomers, dimers, tetramers, collagen-tailed tetramers, two tetramers attached to ColQ, and the synaptic ColQ-AChE, respectively. The expression of ColQ affects only the higher order AChE oligomeric forms while leaving the monomeric and dimeric forms unchanged.

**RESULTS**

**Overexpression of ColQ Is Sufficient to Increase ColQ-AChE Expression**—To test whether additional non-catalytic ColQ subunits expressed in muscle are sufficient to increase ColQ-AChE, we overexpressed qColQ in primary QMCs. Exogenous qColQ was shown to enhance expression of the ColQ-AChE and potential intermediates (A4 and A8) in QMCs (Fig. 1A and supplemental Fig. S2). However, the increment in ColQ-AChE levels did not correlate with a detectable down-regulation of the globular forms pool (G1 and G2), as reported in previous over-expression studies in frog oocytes (15). Likewise, when qColQ was co-expressed with chAChE in cultured QM7 cells, a quail muscle cell line that does not express ColQ-AChE and only very low levels of globular AChE, it induces expression of the ColQ-

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**Development of Anti-qColQ Polyclonal Antibodies**—The anti-qColQ polyclonal antibodies were raised against the whole quail ColQ protein at ProteinTech Group INC (Chicago, IL). We received two affinity-purified rabbit anti-qColQ polyclonal antibodies designated 3414-1 (αColQ1) and 3414-2 (αColQ2). Both antibodies were shown to be highly specific by Western blot analysis and immunofluorescence (see “Results”).

**Protein Precipitation from Sucrose Gradient Fractions and Western Blots**—Total proteins contained in 150-μl aliquots of the sucrose gradient fractions were precipitated by addition of 600 μl of methanol and thorough mixing. Then 150 μl of chloroform were added, the test tubes vortexed, 450 μl of water added, and the tubes vortexed again. The samples were then centrifuged for 5 min at full speed in a microcentrifuge. The aqueous phase was discarded, and the disc of protein washed with 650 μl of methanol. The protein precipitates were dissolved in SDS-PAGE sample buffer for electrophoresis and Western blotting. For analysis, primary QMCs treated with ScVn or TTX as described above, or HEK cells expressing qColQ, were extracted in radioimmuno precipitation assay buffer containing protease inhibitor mixture. After centrifugation, samples containing 50 μg of total protein were run in 10% reducing SDS gels as above. In all cases, the proteins were electrophoretically transferred onto a nitrocellulose membrane (Whatman) and blocked with 1% powdered skim milk (Carnation) for 1 h, incubated with rabbit anti-ColQ polyclonal antibody 3414-1 (1:5000), washed four times with 0.025% Nonidet P40 (Nonidet P-40) in PBS, incubated with peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoLabs) and developed with West Pico chemiluminescent substrate (Pierce). To detect AChE in QMC total cell extracts, AChE mouse 1A2 mAb (1.6 μg/ml) followed by peroxidase-conjugated goat anti-mouse antibody was used.

**Immunofluorescence**—Primary QMCs grown on glass coverslips were blocked for 20 min with 1% bovine serum albumin, then incubated with mouse anti-avian AChE antibody 1A2 for 1 h followed by 20 μg/ml Alexa 488-Donkey anti-mouse IgG (Molecular Probes) with 0.1 g/ml Hoechst 3342 dye to stain the nuclei. Between incubations, the cells were washed four times with HBSS. After the last wash, the cells were fixed with 4% paraformaldehyde in 20 mM PBS, pH 7, for 20 min. All images were acquired with identical exposure parameters using a Leica DMR-A or DMI 6000 fluorescence microscopes equipped with a Princeton Instruments MicroMax CCD camera using iSlidebook 4.0 software.
Assembly of ColQ Acetylcholinesterase

Acetylcholinesterase (AChE) (Fig. 1C) without consequentially decreasing the pool of monomeric (G1) and dimeric (G2) AChE forms when compared with QM7 cells that were only transfected with chAChE. Consistently, co-expression of qColQ with the AChE catalytic subunit also dramatically increased total AChE activity compared with cells expressing the AChE catalytic subunit alone.

Down-regulation of ColQ Subunits Results in Loss of the ColQ-AChE Form—To determine whether down-regulation of ColQ subunits has an effect on the expression of the different AChE forms, QMCS were transfected with qColQ shRNAs to knock down ColQ expression. When expression of qColQ molecules was inhibited in QMC cells by qColQ shRNAs, total AChE activity was dramatically decreased without any significant changes in the levels of globular G1 and G2 forms (Fig. 1B). Moreover, QMCS transfected with qColQ shRNAs showed a dramatic decrease in AChE clusters on the cell surface (Fig. 2). A similar effect was observed when chicken catalytic and quail non-catalytic AChE subunits were co-expressed together with qColQ shRNAs in QM7 cells (Fig. 1D).

ColQ Is Expressed in Mature Myotubes—The ColQ overexpression and down-regulation studies suggested that ColQ levels may be limiting during ColQ-AChE assembly. To test this hypothesis, and be able to determine the levels of endogenous ColQ in QMCS, we developed two rabbit polyclonal anti-ColQ antibodies designated αColQ1 and αColQ2. When tested by Western blot both antibodies recognized a single band of 48 kDa present only in total cell extracts from HEK-293 cells transfected with either full-length qColQ (Fig. 3A) or mColQ cDNA and total cell extracts from QMCS (data not shown). When tested by immunofluorescence using un-fixed cryo-preserved quail skeletal muscle cross-sections, anti-ColQ antibodies co-localized with acetylcholine receptor (AChR) at the NMJs and also labeled extra-junctional regions around a subset of muscle fibers (supplemental Fig. S3), regions not labeled by the fluorescent neurotoxin Fas2 (29), suggesting that the ColQ localized in extra-junctional regions may not be associated with AChE catalytic subunits. Using these antibodies we first determined the relative expression levels of ColQ and AChE catalytic subunit during QMC differentiation by Western blot (Fig. 3B). In parallel sets of QMCS, we determined the distribution of the different AChE forms during development by velocity sedimentation (Fig. 3, C and D). ColQ protein is undetectable in myoblasts and during early myoblast fusion and onset of muscle differentiation on day 2. Its levels start increasing progressively on day 3 together with muscle maturation. In particular, 4-day-old QMCS show detectable levels of ColQ while the levels of catalytically active ColQ-AChE are still very low. In fact, the low level of ColQ-AChE observed in 2-, 3-, and 4-day-old QMCS must likely reflect a residual amount contributed by the small number of mature cells isolated from the 11-day embryos during preparation of the culture. The assembled ColQ-AChE first appears around day 5 in culture, a full 48 h after the first appearance of the catalytic AChE and non-catalytic ColQ subunits proteins.

ColQ Follows the Expression Pattern of Other Muscle-specific Genes during Muscle Differentiation—During development, the ColQ-AChE enzymatic activity reaches a maximum around day 6–7 after plating (Fig. 3C). We then determined the pattern of ColQ mRNA expression in relation to AChE mRNA during development of QMCS. ColQ and AChE transcripts were first expressed at the time of myoblast fusion into multinucleated myotubes, about 48 h after plating, and increased during differentiation (Fig. 4). This expression pattern parallels that of the majority of muscle-specific proteins like the nicotinic AChR (30, 31) and some skeletal muscular myosin heavy and light chain (32–34). The levels of ColQ and AChE transcripts reach a maximum after the period of cell fusion (days 2–3) and begin to decrease following the onset of muscle contraction around day 4 in culture (Fig. 4). The observation that ColQ mRNA molecules are expressed early in development during periods when we could not detect any ColQ-AChE activity suggests the possibility of a regulatory step for ColQ-AChE expression at the translational and/or post-translational levels.

Muscle Activity Regulates ColQ Protein and mRNA Levels but They Are Not Correlated with COLQ-AChE Levels—In primary QMCS, the availability of catalytic subunits does not by itself account for the observed levels of ColQ-AChE induced by muscle activity. Therefore we determined whether the levels of ColQ and its transcripts correlated positively with ColQ-AChE levels induced by muscle activity as expected if ColQ is actually the limiting subunit for ColQ-AChE assembly (Fig. 1). Five day-old QMCS were treated with ScVn or TTX to increase or block membrane depolarization respectively. Increased muscle activity in the form of membrane depolarization decreases the level of total ColQ and AChE catalytic subunit proteins, deter-
mined by Western blot analysis (Fig. 5B), and both ColQ and AChE mRNAs, determined by RNase protection assay (Fig. 6), yet the catalytically active synaptic ColQ-AChE form increased (Fig. 5A). Conversely, decreased muscle activity in the form of membrane depolarization also decreased the levels of total ColQ and AChE catalytic subunit proteins (Fig. 5B) but increased both ColQ and AChE mRNAs (Fig. 6). In contrast, in this case the ColQ-AChE activity was down-regulated (Fig. 5A). These results show that muscle activity regulates the levels of ColQ and its transcript, but these changes are opposite to the expression of ColQ-AChE. Thus neither changes in ColQ nor changes in AChE levels can explain how increased muscle activity enhances ColQ-AChE expression, and vice versa. Clearly additional post-translational regulatory steps must be operating.

The Vast Majority of the ColQ Molecules Are Not Assembled into ColQ-AChE—Because muscle activity regulates both synaptic AChE and ColQ levels, but they do not correlate positively, we determined which fraction of the total intracellular ColQ is actually associated with the AChE catalytic subunits as ColQ-AChE forms. To this end, we fractionated all AChE forms from 6-day-old QMC total cell extracts by velocity sedimentation (Fig. 7A). After measuring enzymatic activity, the total protein from each AChE-containing fraction was precipitated, and
the relative amount of ColQ and AChE protein in each fraction detected by Western blot analysis. The majority of the intracellular pool of ColQ molecules is not assembled into ColQ-AChE (Fig. 7, B and C) suggesting that most of the ColQ molecules are either degraded after translation or have alternative function(s) different from interacting with AChE catalytic subunits.

**DISCUSSION**

ColQ-AChE in skeletal muscle is regulated by activity in the form of membrane depolarization. However the regulation of the catalytic subunit alone cannot account for the levels of synaptic AChE induced by muscle activity. Initial studies on the co-expression of AChE and ColQ subunits in COS-7 cells and frog oocytes suggested that the presence of the non-catalytic ColQ subunit alone was sufficient to regulate the appearance of the ColQ-AChE forms (14, 15). In our studies, overexpression of additional non-catalytic subunits in cultured skeletal muscle cells also appeared to be sufficient to induce assembly of ColQ-AChE without affecting preexisting levels of AChE monomers and dimers (Fig. 1, A and C). This was consistent with the early observations of Krejci et al. (15) who found that the ratios of AChE to ColQ subunits expressed could affect the ratios of ColQ-AChE to globular AChE forms expressed. In addition, ColQ knock down caused a decrease in all intracellular collagen-tailed AChE forms including the synaptic ColQ-AChE (Fig. 2). Together, these results suggest that the abundance of AChE non-catalytic subunits could be limiting during assembly of the ColQ-AChE forms, thus raising the possibility that muscle activity regulated synaptic AChE levels by controlling ColQ and/or its assembly.
with catalytic subunits. In addition, they also support the hypothesis that a pool of rapidly turning over AChE catalytic subunits exists in QMCs that can be stabilized upon hetero-
oligomerization with the non-catalytic component. Our observations suggest that ColQ expression induces the assembly of synaptic ColQ-AChE and other tailed forms by interacting with the pool of rapidly turning over monomeric and dimeric sub-
units consistently detected in QMCs. While this is the first indication that the non-catalytic ColQ subunit is involved in post-
translational regulation of AChE expression in primary skeletal muscle cells, the regulation of AChE assembly is clearly more complex than subunit availability.

During myogenic differentiation of C2C12 cells in culture transcripts encoding the ColQ and AChE catalytic subunits increase together with the up-regulation of ColQ-1 transcript variant instead of ColQ-1a (35). However, these observations alone cannot fully explain the appearance of ColQ-AChE forms because the protein levels were not determined. In addition, in C2C12 myotubes the role of muscle activity in AChE regulation cannot be determined because these cells show poor, if any, spontaneous contractile activity. By determining the ColQ levels during primary QMC differentiation (Figs. 3 and 4), and when muscle activity was either enhanced or blocked pharmacologically (Figs. 5 and 6), we found that the ColQ subunit is indeed regulated by muscle activity in the form of membrane depolarization, but based on the pattern and levels of expres-
sion it does not appear to be limiting during ColQ-AChE assembly. In fact, treatment of the muscle cells with TTX resulted in a 2-fold increase AChE and a 10-fold increase in ColQ mRNAs (Fig. 6) with only a small decrease in ColQ protein (Fig. 5). Moreover, the ColQ transcripts and protein can be detected 48 h before the developmental appearance of the assembled ColQ-AChE (Fig. 3, C and D). Thus in both these cases, there is a clear dissociation between the appearance of detectable ColQ, its transcripts, and the assembled catalytically active ColQ-AChE form. These results suggest the possibility that translational and post-translational events in avian muscle regulate synaptic AChE levels in developing and mature muscle cells independently of ColQ and AChE transcript and protein levels. In addition, in QMCs, the newly synthesized AChE pool exists as both inactive and catalytic active molecules (36). How the ratio of active versus inactive molecules is established during myogenesis remains to be determined.

It appears that the vast majority of the intracellular pool of ColQ molecules are not assembled into ColQ-AChE (Fig. 7, B and C), emphasizing the idea that the ColQ subunit is not lim-
iting during ColQ-AChE assembly assuming that the associa-
tion with AChE catalytic subunits is the only function of ColQ in muscle fibers. In addition, this result, in contrast with the observation that ColQ overexpression in QMCs increases cat-
alytically active ColQ-AChE levels (Fig. 1A), suggests that most of the endogenous ColQ molecules are either rapidly degraded after translation or are not available for assembly due to differ-
ent compartmentalization. The third possibility is that they have an alternative function(s) in addition to interacting with AChE catalytic subunits. This last alternative would be sup-
ported by the immunofluorescence studies (supplemental Fig. S3) that show ColQ localized in extra-junctional regions of the muscle fibers where there is a paucity or complete absence of AChE catalytic subunits. Because ColQ mRNA has been detected in non-cholinergic tissues such as lung and testis (10) where there is little or no ColQ-AChE, and alternative function for ColQ is an attractive possibility. It will be very interesting to determine whether there are other binding partners for ColQ besides AChE and butyrylcholinesterase. Finding new potential ColQ binding partners would be the first step toward the elucidation of new ColQ functions. So far, our studies indicate that there is a poor correlation between AChE and ColQ RNA and protein on the one hand, and ColQ and AChE assembly on the other. Moreover, they strongly suggest that post-translational events related to the folding and assembly of ColQ with the AChE catalytic subunit are important regulatory steps during
synaptic ColQ-AChE biogenesis in skeletal muscle cells. This hypothesis is tested in a submitted report.\textsuperscript{5}

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