Localization of the CGRP Receptor Complex at the Vertebrate Neuromuscular Junction and its Role in Regulating Acetylcholinesterase Expression

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SUMMARY:

Calcitonin gene-related peptide (CGRP) is released by motor neurons where it exerts both short and long term effects on skeletal muscle fibers. In addition, sensory neurons release CGRP on the surrounding vasculature where it is in part responsible for local vasodilation following muscle contraction. While CGRP binding sites have been demonstrated in whole muscle tissue, the type of CGRP receptor and its associated proteins or its cellular localization within the tissue have not been described. Here we show that the CGRP binding protein referred to as the calcitonin receptor-like receptor (CRLR) is highly concentrated at the avian neuromuscular junction together with its two accessory proteins, receptor activity modifying protein (RAMP1) and CGRP-receptor component protein (RCP), required for ligand specificity and signal transduction. Using tissue cultured skeletal muscle we show that CGRP stimulates an increase in intracellular cAMP that in turn initiates downregulation of acetylcholinesterase (AChE) expression at the transcriptional level, and, more specifically, inhibits expression of the synaptically-localized collagen-tailed form of the enzyme. Together, these studies suggest a specific role for CGRP released by spinal cord motoneurons in modulating synaptic transmission at the neuromuscular junction by locally inhibiting the expression of AChE, the enzyme responsible for terminating acetylcholine neurotransmission.
INTRODUCTION:

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide expressed in a wide variety of neurons in the central and peripheral nervous systems, including motoneurons (reviewed in Jansen and Fladby, 1990; Changeux et al., 1992; Hall and Sanes, 1993; Sanes and Lichtman, 1999), where it is released upon stimulation (Sala et al., 1995). In skeletal muscle, CGRP has been shown to potentiate muscle contraction (Lu et al., 1993), increase the rate of acetylcholine receptor (AChR) desensitization (Miles et al., 1987; 1989; Mulle et al., 1988; Hoffman et al., 1994), increase the numbers of AChR (Buffelli et al., 2001), decrease the levels of acetylcholinesterase (AChE)(Hodges-Savola and Fernandez, 1995; Fernandez and Hodges-Savola, 1996; Fernandez et al., 1999), and locally increase the rate of blood flow following muscle contraction (Arden et al., 1994; Yamada et al., 1997a, 1997b). While the presence of CGRP in nerve terminals at the neuromuscular junction has been well documented (Peng et al., 1989; Uchida et al., 1990; Matteoli et al., 1990; Sakaguchi et al., 1991), the localization of CGRP receptors at the synapse has been more difficult to demonstrate because the only available probe was the receptor ligand itself, CGRP. Using $^{125}$I-CGRP as a probe, high affinity CGRP receptors were shown to be expressed on the surfaces of tissue-cultured myotubes (Jennings and Mudge, 1989), and present on whole muscle membranes isolated from embryonic or newly hatched chicks (Roa and Changeux, 1991), however the latter studies could not differentiate between CGRP receptors on skeletal muscle versus those on the surrounding vasculature. In one autoradiographic study (Popper and Micevych, 1989), a correlation between $^{125}$I-CGRP and AChE histochemical staining was observed in low power photomicrographs of rat bulbocavernosus muscle, suggesting an accumulation of CGRP receptors at sites of nerve-muscle contact.
contact. However, in these studies it was not possible to differentiate between CGRP receptors located in blood vessels or Schwann cells and those on muscle fibers.

Activation of the CGRP receptor increases intracellular cAMP (Van Valen et al., 1990; Asahina et al., 1995; McLatchie, 1998; Evans et al., 2000) with a concomitant activation of protein kinase A (PKA) (Chang et al., 1993; Maggi et al., 1995; Disa et al., 2000). In skeletal muscle, transcripts encoding the regulatory subunit of cAMP-dependent PKA, Ria, as well as the protein, are concentrated at the neuromuscular junction (NMJ) where they co-localize with AChR, while the transcripts encoding the catalytic subunit Cα are present in innervated and non-innervated regions of the fibers (Imaizumi-Scherrer et al., 1996). In addition, two PKA anchoring proteins, D-AKAP1 and D-AKAP2, have also been localized to the NMJ in adult skeletal muscle (Perkins et al., 2001).

Several ways that cholinergic transmission can potentially be modulated include altering the numbers of receptors at the synapse, their gating properties, or the rate of neurotransmitter degradation. Studies using tissue cultured skeletal muscle have consistently shown that CGRP increases expression of AChR mRNA and protein with a consequent increase in AChR on the cell surface (New and Mudge, 1986; Jennings and Mudge, 1989; Fontaine et al., 1986, 1987; Laufer and Changeux, 1987). This increase appears to be mediated by increased levels of intracellular cAMP (Laufer and Changeux, 1987, 1989). The results of studies on the effects of CGRP and increasing cAMP on the expression of AChE in tissue-cultured skeletal muscle, on the other hand, have been more variable. Skeletal muscle cells express several forms of AChE, including globular and collagen-tailed forms, all of which are catalytically active (reviewed in Massoulié et al., 1993; Rotundo and Fambrough, 1994). However, only the latter are concentrated at the neuromuscular synapse (Hall, 1973) where they are attached to the synaptic
basal lamina (Hall and Kelly, 1971; Lwebuga-Mukasa et al., 1976; McMahan et al., 1978; Rossi and Rotundo, 1993). Treatment of rat or mouse myotubes with CGRP results in a decrease in AChE expression (Boudreau-Larivièrè and Jasmin, 1999; Choi et al., 2001; da Costa et al., 2001), whereas treatment of chicken myotubes with CGRP in one laboratory showed no change in the expression of catalytically active AChE while actually showing an increase in total AChE protein and mRNA (Choi et al., 1996;1998). These changes were also mediated by a cAMP-dependent mechanism and depended on the cAMP response element (CRE) of the AChE gene (Choi et al., 2001). The reasons for these observed differences in AChE regulation by CGRP between mammalian and avian species are not yet clear.

Recently, the complex of proteins forming the CGRP receptor has been elucidated (McLatchie et al., 1998; Evans et al., 2000). Activation of the receptor by CGRP results in increased levels of intracellular cAMP, suggesting a G-protein-coupled receptor. We now know that the CGRP receptor is unique among G-protein-coupled receptors in that it is a complex composed of at least three proteins. The ligand binding protein named calcitonin receptor-like receptor (CRLR) has the stereotype structure of a 7 transmembrane receptor, but is inactive when expressed in cells alone (Chang et al. 1993; Fluhmann et al., 1995). CRLR requires two additional accessory proteins for function; the receptor activity modifying protein (RAMP1) acts as a molecular chaperone and is required for routing of CRLR to the cell surface as well as pharmacologic specificity (McLatchie et al., 1998), and the CGRP receptor component protein (RCP) is required for coupling the receptor the cellular signal transduction pathway (Evans et al., 2000; Prado et al., 2001).

While studies from many laboratories have clearly shown that CGRP has effects on skeletal muscle at the cellular, molecular and physiological levels two laboratories have recently
reported that αCGRP-null mice are devoid of an abnormal skeletal muscle phenotype (Lu et al., 1999; Salmon et al., 1999). The studies by Lu et al. were particularly detailed in that they examined not only the morphology of the NMJ but also the appearance of specific synaptic components such as the AChR, the receptor density, the localized expression of synapse-specific genes and AChR subunits in the adult and during early development, synapse elimination, and even reinervation following nerve crush. In all cases the CGRP-null mice appeared identical to the wild type animals. Thus CGRP may not be necessary for normal development, or, alternatively, and as has happened in the cases of many experimentally generated gene deficient strains of mice, the normal function has been taken over by another protein. Another possibility discussed by Lu et al., 1999, is a compensatory role that αCGRP may be playing in these αCGRP null mice. Another interpretation is that CGRP functions not in the development of the neuromuscular synapse but in the regulation of specific synaptic proteins once the synapse has formed. In this case its role would be modulatory rather than regulatory, a possibility that was not examined in the studies described above.

In the present studies we use immunofluorescence microscopy to show that all components of the CGRP receptor, CRLR, RCP, and RAMP1 are present at the avian neuromuscular junction. Using tissue-cultured quail skeletal muscle, we show that CGRP increases intracellular cAMP levels with a consequent decrease in total AChE expression as well as a complete loss of the synaptic form of the enzyme. This decrease is due to a decrease in AChE mRNA levels and rate of AChE translation. Thus CGRP appears to inhibit the expression of AChE at the transcriptional level, and, at the adult NMJ, may be involved in attenuating AChE expression through a similar mechanism.
EXPERIMENTAL PROCEDURES:

Tissue culture of embryonic muscle and drug treatments:

Primary quail skeletal muscle cells were cultured on collagen-coated 35 mm dishes in 2 ml of Eagle’s Minimum Essential Medium supplemented with 10 % horse serum, 2 % chicken embryo extract, and 10 µg/ml of gentamicin (EMEM 210)(Rotundo, 1984a). Cultures were fed on the third day and every other day thereafter. When used, CGRP or forskolin (both from Sigma, St. Louis, MO) were added directly to the culture medium at the concentrations and for the times indicated in each experiment. The cAMP analogs 8br-cAMP and db-cAMP, as well as IBMX, an inhibitor of phosphodiesterase, and dd-forskolin, an inactive analogue of forskolin used as a control, were purchased from Sigma (St. Louis, MO). These pharmacological agents were dissolved directly in the culture medium, usually for 24 or 48 hours, as indicated in each experiment.

Analysis of AChE oligomeric forms and assay of enzyme activity:

Total AChE activity was measured by scraping the cells into 500 µl borate extraction buffer per culture dish (20 mM borate buffer, pH 9.0, 1 M NaCl, 5 mM EDTA, 0.5 % Triton X-100, 5mg/ml bovine serum albumin, 2 mM benzamidine, 5 mM N-ethyl maleimide, 1 µg/ml pepstatin A, 2 µg/ml leupeptin)(all from Sigma, St. Louis, MO) followed by 20 min centrifugation at 12,000 rpm. 20 µl aliquots of supernatant were assayed for AChE activity using the radiometric method of Johnson and Russell (1975) as previously described (Rotundo, 1984a). To analyze AChE forms, 100-200 µl of supernatant were layered on 5-20% sucrose gradients in 20 mM borate buffer pH 9.0, 1M NaCl, 0.5% Triton X-100, and 2mM EDTA and centrifuged in a Beckman SW41 rotor for 16 hours at 36K rpm at 4°C. The fractions were assayed for AChE activity using the colorimetric reaction of Ellman (Ellman et al., 1961) or the radiometric assay.
Cell surface AChE was assayed in whole cultures using the modified radiometric assay as described previously (Rotundo and Fambrough, 1980). To measure the rates of AChE synthesis, muscle cultures were incubated in the presence or absence of forskolin or CGRP for 24 hours, and the total myotube AChE inhibited by incubation with 10 µM diisopropylfluorophosphate (DFP), a membrane permeable irreversible AChE inhibitor, in phosphate buffered saline (PBS) for 10 min. Fresh media containing the same drugs was then added back to the cultures and the newly synthesized AChE assayed 2 hours later.

**Immunofluorescence localization of AChE and CGRP receptor complex components:**

Antibodies: Rabbit anti-RCP (R83)(Evans et al., 2000; Prado et al., 2001), rabbit anti-RAMP1 (OA-350) and rabbit anti-CRLR (OA910)(both the generous gifts from Dr. Kevin Oliver; see Evans et al., 2000 and Prado et al., 2001, for details), plus pre-immune rabbit serum (PI) were used to labeled the CGRP receptor complex at a 1:1000 dilution. FITC-conjugated donkey anti-rabbit IgG and FITC-conjugated rabbit-anti mouse IgG were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA. The mouse monoclonal anti-avian AChE 1A2 (Rotundo, 1984b) was used at a concentration of 20 µg/ml to directly label the enzyme at the neuromuscular junction. Rhodamine-conjugated α-BTX was used to label AChR.

Immunofluorescence localization and analysis: 10 µm cryostat sections of adult quail posterior latissimus dorsi (PLD) muscle were labeled for one hour with the primary antibody against one of the components of the CGRP complex together with rhodamine-conjugated α-BTX. The sections were washed 3 times with PBS containing 10% horse serum (PBS/HS) followed by the addition of FITC-conjugated donkey anti-rabbit second. After fixation with 4% paraformaldehyde, the sections were mounted and viewed with a Leica DMR-A microscope equipped with a Princeton Instruments cooled CCD camera and the images analyzed using Metamorph software.
RNase protection assay:
Quantitation of AChE mRNA was measured as described in Rossi et al. (2000). Briefly, aliquots of RNA isolated from triplicate 60 mm cultures, incubated for 24 hours in the presence or absence of forskolin, were hybridized overnight at 45°C with antisense $^{32}$P-labeled probe (Sambrook et al., 1989). The probe consisted of a 302 nucleotide segment transcribed from quail AChE cDNA starting 46 nucleotides upstream from the ATG translation start site, subcloned in pGEM-4Z and linearized with HindIII. Unlabeled sense AChE transcript was used as a standard. Hybridized samples were digested with 6 U RNase ONE (Promega, Madison, WI), ethanol precipitated and electrophoresed on denaturing polyacrylamide urea gels. The protected RNA was quantified using a Molecular Dynamics PhosphorImager and Image Quant software.

cAMP accumulation assay:
The production of cAMP was assayed using the method of Salomon et al. (1974) as modified (Sarkar and Dickerson, 1997; Evans et al., 2000). Quail myotube cultures (4-7 days old) were incubated overnight in EMEM 210 supplemented with 1µCi/ml $[^{3}H]$-adenine (New England BioLabs, Beverly, MA). The following day the cells were preincubated with 0.2 mM isobutylmethylxanthine (IBMX), an inhibitor of phosphodiesterase, for 30 minutes prior to the addition of the indicated drugs or agonists for an additional 30 minutes in the presence of IBMX. The reaction was terminated by removing the media and extracting the cells in ice cold 5% trichloroacetic acid (TCA). $[^{3}H]$-ATP and $[^{3}H]$-cAMP were separated by sequential chromatography through Dowex column (ATP counts), and alumina column (cAMP counts) and the percent conversion of ATP to cAMP measured as the ratio of cAMP counts / (cAMP counts + ATP counts).
RESULTS:

Components of the CGRP signaling complex are localized at the neuromuscular junction

While it is clear that CGRP can regulate the expression of AChR and AChE in muscle, and that CGRP is present in motoneurons where it accumulates in nerve terminals, the presence of CGRP receptors at the neuromuscular junction has not been demonstrated. To determine whether the CGRP receptor complex was present in adult quail muscle, 10 µm cross sections of posterior latisimus dorsi (PLD) and anterior latisimus dorsi muscles were labeled with rhodamine α-Btx to visualize the AChR and anti-AChE monoclonal antibody 1A2 to visualize the enzyme (Figure 1). Since the CGRP receptor complex consists of three proteins, CRLR or CGRP receptor-like receptor, RAMP1 or receptor activity modifying protein, and RCP, the CGRP-receptor component protein required to activate the signaling pathway, we labeled each individually using the appropriate antibody described under Experimental Procedures, followed by FITC-conjugated second antibody. For each set of experiments at least two microscope slides each with 2-3 cryostat sections of muscle were used for each antibody. Each section has at least 15-20 neuromuscular junctions so that more than 50 NMJs were viewed for each antibody-AChR co-localization. This experiment was repeated three times, and groups for all four antibodies as well as preimmune serum and second antibody alone were included in each experiment. On all sections examined the indicated protein co-localized with the AChR and AChE at all the synapses. Thus the three components of the CGRP receptor complex, detected by indirect immunofluorescence using specific antibodies, are all concentrated at the neuromuscular junction where they co-localize with AChE and AChR (Figure 1).

(Insert Figure 1 about here)
CGRP and forskolin stimulate cAMP synthesis in quail skeletal muscle.

Several reports have shown that CGRP is capable of regulating AChR and AChE expression in skeletal muscle through a cAMP-dependent signaling pathway. To determine whether CGRP can stimulate the accumulation of cAMP in quail muscle cultures, 4 day old myotubes were incubated with varying concentrations of CGRP or forskolin, an activator of adenylyl cyclase, and cAMP accumulation assayed. Figure 2 shows that incubation with CGRP or stimulation of adenylyl cyclase by forskolin resulted in a dose dependent accumulation of intracellular cAMP. Addition of SQ22536, a specific inhibitor of adenylyl cyclase, for 30 minutes followed by 30 µM forskolin resulted in a complete inhibition of cAMP accumulation, indicating that the increased levels of cAMP by forskolin in our cultures are due to adenylyl cyclase stimulation (results not shown).

(Figure 2 about here)

Forskolin and cAMP analogs decrease total AChE synthesis and block expression of the collagen-tailed AChE form.

To determine whether increasing intracellular cAMP altered the synthesis of AChE forms, total enzyme activity in 7 day quail muscle cultures was irreversibly inhibited with DFP (see Experimental Procedures), followed by culturing in EMEM 210 for 24 hrs in the presence of the cAMP analogs 8br-cAMP (100 µM) or db-cAMP (100 µM), forskolin (50 µM), IBMX (100 µM), the inactive forskolin analog dd-forskolin (50 µM), or normal medium. After 24 hrs, the cells were rinsed with PBS and lysed in 500 µl borate extraction buffer, centrifuged, and 200 µl of the supernatants loaded on 5-20% sucrose gradients to analyze AChE forms by velocity
sedimentation. Fractions were collected from the bottom of the gradient and assayed for AChE activity. Figure 3 shows that any treatment that increases effective intracellular cAMP levels results in a decrease of all newly synthesized AChE oligomeric forms. The effects, however, were most pronounced for the collagen-tailed (A12) AChE form whose expression was completely eliminated.

(Insert Figure 3 about here)

**Increasing cAMP levels decreases the expression of AChE molecules on the muscle cell surface**

One of the predicted consequences of decreasing expression of the collagen-tailed AChE form, which is the form that attaches to the extracellular matrix, would be a decreased accumulation of cell surface AChE. To determine whether inhibiting expression of the collagen-tailed AChE resulted in changes in cell surface AChE, seven day old muscle cultures were incubated for 48 hrs in complete medium with or without 10 µM forskolin and the cell surface AChE assayed as described (Rotundo and Fambrough, 1980). This assay uses 3H-acetylcholine phosphate buffered saline, pH 7.4, added directly to washed cells on the culture dish to measure hydrolysis by enzyme molecules with their catalytic sites on the surface of the cell. Figure 4A shows that the expression of AChE activity is attenuated in forskolin-treated cultures. When the collagen-tailed AChE form aggregates on the surface of quail myotubes in culture it forms clusters of enzyme molecules that associate with AChR and several other membrane-bound and extracellular matrix molecules (Rossi and Rotundo, 1992; 1996). To determine whether the decreased cell surface enzyme activity, on forskolin-treated myotubes was also reflected in a decrease in clusters of AChE, we quantified the number of AChE clusters per nucleus on
cultures treated for 48 hrs with forskolin compared to untreated cultures. The AChE was localized by indirect immunofluorescence using mAb 1A2 and FITC-conjugated second antibody, followed by Hoechst 33342 to stain the nuclei. Three muscle cultures per group were quantified by counting the number of AChE clusters on myotubes and the number of myonuclei in the same fibers in 10 random fields per dish. The number of AChE clusters per nucleus on forskolin-treated cultures was ~70% of the number of AChE clusters per nucleus on normal cultures (Figure 4B), suggesting also that the number of nuclei expressing AChE decreased after activation of the cAMP pathway.

*(Insert Figure 4 about here)*

**Long term forskolin treatment reversibly down regulates AChE activity**

To determine the half maximal forskolin concentration needed to inhibit AChE synthesis, triplicate 6 day quail muscle cultures were treated overnight with increasing concentrations of forskolin and total AChE activity assayed. The dose-response curve shows that the half maximal effect of forskolin on AChE activity is obtained at ~10 µM (Figure 5A). Long term forskolin treatments (24 hrs) were required to obtain a reduction of AChE activity while a 4 hr treatment had no effect, as shown in Figure 5B. These results suggested that the mechanism of forskolin action on AChE regulation probably involved the transcription of AChE mRNA rather than a translational control (see next section). To determine whether the effect of forskolin on AChE activity was reversible, we treated 4 day old muscle cultures with forskolin for 24 hrs and then either fed the cultures with normal medium or continued forskolin treatment for one more day. The reversibility of the inhibition of AChE synthesis by increased accumulation of cAMP is shown in Figure 6.
Forskolin inhibits the synthesis of AChE by reducing AChE mRNA levels.

We had previously observed a positive correlation between the amount of globular AChE forms present, the AChE transcript levels, and the rate of AChE synthesis in our quail muscle cultures (Rossi et al., 2000). Because the globular AChE forms were decreased in forskolin-treated cultures (Figure 3), we expected to find decreased AChE transcript levels as well. Moreover, as mentioned above, long term forskolin treatments were required to have an effect on AChE activity, suggesting a possible regulation of AChE synthesis at the transcriptional level. Therefore, to examine this possibility, we measured AChE transcript levels using an RNase protection assay in muscle cultures incubated overnight with or without 10 μM forskolin. Our results indicate that after long term (overnight) forskolin treatment the levels of AChE transcripts decreased (Figure 7A and B), and the rate of AChE translation, measured by assaying newly synthesized AChE activity after DFP treatment following the procedure described under Experimental Procedures, decreased as well (Figure 7C).

Treatment of myotubes with CGRP inhibits AChE synthesis.

To determine the effects of CGRP on newly synthesized AChE activity and oligomeric forms, 6 day quail muscle cultures were DFP treated, and newly synthesized enzyme extracted in borate extraction buffer after 24 hrs incubation in medium with or without 30 μM CGRP or 10 μM forskolin. Long term CGRP treatments, like long term forskolin, decreased all AChE oligomeric forms, especially the collagen-tailed (synapse-associated) form (Figure 8). To
determine whether the effects of CGRP were directly on AChE synthesis, muscle cultures were treated with 30 μM CGRP overnight and total AChE activity assayed. A parallel set of cultures pre-treated with CGRP overnight were DFP-treated and the rate of synthesis measured. Figure 9 shows that total AChE activity (A) and rate of AChE synthesis (B) decreased when CGRP receptors were activated indicating the involvement of the cAMP signaling pathway in AChE regulation by CGRP.

(Insert Figures 8 and 9 about here)
DISCUSSION:

Here we present direct evidence for the localization of the CGRP receptor complex, CRLR and its accessory proteins, RAMP1 and RCP, at the neuromuscular synapse. The co-localization of these three proteins with other components of the neuromuscular junction, the AChR and AChE (Figure 1), and the catalytic and regulatory subunits of PKA (Imaizumi-Scherrer et al., 1996; Perkins et al., 2001), provide evidence that the entire signaling system necessary for transducing CGRP binding into increases in intracellular cAMP is specifically localized to this synapse. All these components are also expressed in aneural quail muscle cultures since CGRP is capable of stimulating adenylyl cyclase and eliciting an increase in intracellular cAMP (Figure 2).

Increasing intracellular cAMP levels has a pronounced inhibitory effect on the expression of all AChE oligomeric forms with the most striking effect on the expression of the collagen-tailed form that is concentrated at the neuromuscular junction (Figures 3 and 8). This observation suggests that cAMP is also affecting expression of the non-catalytic collagenic tail subunit, ColQ. These observations are consistent with observations from several groups on the effects of CGRP on AChE in mammalian skeletal muscle, both in vivo (Hodges-Savola and Fernandez, 1995; Fernandez and Hodges-Savola, 1996; Fernandez et al., 1999) and in vitro (Boudreau-Larivière and Jasmin, 1999; Choi et al., 2001; da Costa et al., 2001) where downregulation of the enzyme has been documented. In contrast, one laboratory has shown that CGRP and increasing cAMP levels had no effect on the expression of chicken AChE activity whereas it increased the expression of AChE mRNA and protein, representing an inactive form of the enzyme (Choi et al., 1996;1998 ). The reason for the increase of this catalytically inactive AChE, originally described as a rapidly turning-over intracellular pool in avian muscle cultures (Rotundo, 1988),
is unknown but could be a property unique to tissue cultured chicken muscle since it has not been observed in other species.

The regulation of AChE by CGRP in quail skeletal muscle cultures appears to be via activation of the adenylyl cyclase/PKA pathway, as has been shown in other systems, and most likely involves transcriptional controls. While there is no effect of increasing cAMP levels short term, within a few hours, there is a pronounced inhibition of AChE expression following a more prolonged overnight exposure to drugs that increase intracellular cAMP levels (Figures 5, 7, and 9). This inhibition of AChE expression most likely occurs as a consequence of decreased AChE mRNA transcription (Figures 7A and B) resulting in a proportional decrease in the rate of AChE translation (Figures 7C; 9A and B). This stands in contrast to the long term effects of CGRP and increased cAMP levels on expression of the AChR where mRNA and protein levels are both increased.

Since activation of the CGRP receptor results in a decrease in all AChE forms, along with a specific decrease in the collagen-tailed synaptic form of the enzyme, it would appear that one function of this pathway is to increase the sensitivity of this synapse to acetylcholine. One way to accomplish this would be to decrease the levels of AChE bound to the synaptic basal lamina and hence decrease the degradation rate of the neurotransmitter acetylcholine. This interpretation is strengthened by the observation that activation of adenylyl cyclase decreases the levels of cell surface AChE as well as the surface clusters of AChE molecules that consist primarily of the collagen-tailed AChE associated with AChRs (Figure 4). In addition, this hypothesis is consistent with studies from several laboratories focusing on the regulation of the nicotinic AChR in adult muscle as well as in culture (reviewed in Jansen and Fladby, 1990; Changeux et al. 1992; Hall and Sanes, 1993; Sanes and Lichtman, 1999). These studies indicate that CGRP
and/or increases in cAMP increase the synthesis of AChR and increase the numbers of receptors inserted into the plasma membrane (Buffelli et al., 2001). Increases in cAMP also appear to result in increased stability, and hence longer half-life, of the receptors once they are inserted into the synapse (Shyng, et al., 1991; Xu and Salpeter, 1995). These coordinated consequences of CGRP receptor activation, increases in AChR with a parallel decrease in AChE, provides a simple mechanism for increasing the sensitivity of the synapse to acetylcholine on an intermediate to long term basis.

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REFERENCES:


FIGURE LEGENDS:

FIGURE 1: Colocalization of the three CGRP receptor complex components and AChE with AChR at the neuromuscular junction. Frozen sections of adult quail muscle (10 µm) were stained with rhodamine α-Btx and either mAb 1A2 (AChE) or rabbit-anti rat CRLR, RAMP1 or RCP, followed by FITC-conjugated second antibody, to label the indicated protein. Negative controls consisted of incubation with pre-immune serum (PI) or with second antibody alone (not shown). The CGRP receptor, CRLR, and its two associated components, RAMP1 and RCP, are highly concentrated at the neuromuscular junction where they co-localize with AChE and AChR.

FIGURE 2: Stimulation of cAMP synthesis by CGRP and forskolin in cultured muscle cells. Muscle cultures were pre-incubated with 3H-adenine overnight, followed by 30 minutes in medium with 0.2 mM IBMX, and then incubated for 30 min in IBMX medium with the indicated concentration of CGRP or forskolin. cAMP production was assayed as described under Experimental Procedures. The muscle cells are capable of synthesizing cAMP and do so in response to CGRP.

FIGURE 3: Increasing cAMP levels inhibits the expression of collagen-tailed A12 AChE. Muscle cultures were first treated with DFP to inhibit all AChE, then with one of several different pharmacological agents that increase cAMP levels through different mechanisms. Cultures were incubated for 24 hours either with medium alone, the membrane permeable cAMP analogues 8bromo-cAMP (100 µM) or dibutyryl-cAMP (100 µM), the phosphodiesterase inhibitor IBMX (100 µM), the adenylyl cyclase activator forskolin (50 µM), or its inactive
analogue dideoxy-forskolin (50 µM). In all cases, treatments that increased cAMP levels inhibited expression of the collagen-tailed A₁₂ AChE form as well as resulting in an overall decreased expression of the globular G₁, G₂ and G₄ AChE forms.

**FIGURE 4: Increasing cAMP reduces the levels of cell surface AChE and the accumulation of enzyme at cell surface clusters.** The collagen-tailed form of AChE accumulates on the cell surface where it co-localizes with nicotinic acetylcholine receptors and other synaptic components in cell surface clusters. Muscle cultures were incubated for 48 hrs. with medium containing 10 µM forskolin, or medium alone. A) Assay of cell surface AChE activity shows a 30% decrease compared to controls. B) The number of AChE clusters, expressed as AChE clusters per myotube nucleus, decreased by a similar amount as the cell surface AChE activity.

**FIGURE 5: Stimulation of adenylyl cyclase inhibits AChE synthesis.** A) Dose response curve for forskolin inhibition of AChE synthesis. Muscle cultures were incubated overnight with the indicated concentration of forskolin and total AChE activity assayed. Each point is the mean ± SEM of three cultures. The half maximal effect is at 10 µM forskolin. B) Muscle cultures were treated for 4 or 24 hours with 10 µM forskolin and total AChE activity assayed as described under Experimental Procedures. The effects of increasing cAMP on AChE expression appear only after longer incubation times, suggesting that there is no immediate effect on the translation of AChE mRNA. In the same cultures, increasing cAMP levels had no effect on total protein synthesis (see text).
FIGURE 6: The inhibition of AChE synthesis by forskolin is reversible. Groups of muscle cultures were incubated beginning on day 4 with medium plus or minus 10 µM forskolin. After 24 hours, a subset of cultures were rinsed with normal culture medium and incubation continued in the absence of forskolin. (●) untreated, (○) 10 µM forskolin, (▼) 10 µM forskolin 24 hrs, then normal medium. The effects of forskolin are completely reversible under these conditions. Each point is the mean ± SEM of three cultures.

FIGURE 7: Activation of adenylyl cyclase reduces AChE transcript levels and the rate of AChE translation. Muscle cultures were treated from days 6 to 7 with 10 µM forskolin, or kept in regular medium, and total RNA extracted on day 7 for RNase protection assay using the specific 5’ AChE mRNA probe. An additional set of time zero cultures were extracted on day 6. Parallel sets of cultures were used to assay total AChE activity. A) Phosphorimager exposure of RNase protection gel showing representative bands from control and treated cultures together with a set of pure AChE cRNA standards for quantitation. B) AChE mRNA levels in control and forskolin treated muscle cultures, expressed as pg mRNA per dish. C) Rate of AChE synthesis in control and forskolin treated cultures measured as the amount of AChE activity following 2 hours recovery from DFP treatment. Each bar in these graphs is the mean ± SEM of three cultures.

FIGURE 8: Activation of CGRP receptors or adenylyl cyclase preferentially inhibits the expression of the collagen-tailed AChR oligomeric form. Six day old quail muscle cultures were treated with DFP and allowed to recover overnight in the presence of either 3 x10^-7 M CGRP or 10 µM forskolin. The newly synthesized AChE was extracted and the oligomeric
forms analyzed by velocity sedimentation as described under Experimental Procedures. The peaks of AChE activity in control cultures are, from left to right, the A_{12} collagen-tailed form, a small peak of A_{8} collagen-tailed form, and the G_{4} tetramer and G_{2} dimer consisting of either 4 or 2 globular catalytic subunits respectively. Treatment with either CGRP or forskolin completely blocks the expression of the collagen-tailed AChE forms that normally accumulate at the neuromuscular synapse.

FIGURE 9: CGRP stimulation reduces the rate of AChE translation in cultured muscle cells. Muscle cultures were treated overnight with CGRP and the rate of AChE synthesis measured using the rate of recovery of AChE enzyme activity following DFP treatment. A) Total AChE levels were reduced by almost 50% in the cells, as usual for this treatment time, and the rate of AChE translation (B) was reduced proportionately. Each bar is the mean ± SEM of three cultures.
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Localization of the CGRP receptor complex at the vertebrate neuromuscular junction and its role in regulating acetylcholinesterase expression
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