Dihydropyridine Receptor Regulation of Acetylcholinesterase Biosynthesis*

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The dihydropyridine calcium channel antagonist nifedipine causes marked reductions in the amounts of acetylcholinesterase (AchE) molecular forms in primary tissue cultures of avian pectoral muscle. These reductions are time-dependent, requiring passage of 3 h prior to any observable response, dose-dependent, with principal actions occurring in the 1–100 nM range, are greater on the 7 S and 19 S forms than on the 11.4 S form, and, based on susceptibility of AchE to irreversible inhibition by a cationic inhibitor, occur almost exclusively with intracellular AchE coincident with a 2-fold reduction in the rate of secretion. The effects are markedly more pronounced in skeletal muscle than in neurons and differ from those observed for verapamil, diltiazem, and the calcium ionophore A23187. These reductions are incompatible with accelerated protein degradation, alterations in post-translational processing and assembly in the Golgi complex, or enhanced loss of enzyme to the medium, but instead indicate that nifedipine causes a reduction in AchE biosynthesis. Since AchE forms are thought to arise from a single gene, these findings imply a linkage in skeletal muscle between transcription and post-transcriptional processing of mRNA and ligand occupation of the dihydropyridine receptor.

Synthesis of the different molecular forms of acetylcholinesterase (AchE) in skeletal muscle depends on contractile activity as well as a number of putative, but as yet unidentified, neurotrophic factors (1–4). AchE appears in muscle and neurons as a polymorphic family of globular catalytic species that are distinguishable from the larger and more asymmetric forms containing elongated, collagen-like tails (2, 3). In contrast to the globular forms, encompassing multimeric, membrane-, and cell-surface-associated intracellular species, the asymmetric forms are extracellular species noncovalently localized in the basal lamina of the neuromuscular junction (5, 6). The asymmetric forms attract interest because they appear coincident with innervation and disappear following denervation and blockade of contractile activity (4). Mobilization of Ca\(^{2+}\) and formation of second messengers appear to underlie at least part of these phenomena since, as seen in study of noncontracting cultures of rat and avian skeletal muscle, elevated concentrations of intracellular calcium mediated through the ionophore A23187, either alone (7) or in combination with phorbol ester activation of protein kinase C (8), promote the appearance of the asymmetric forms of AchE.

While membrane-active ionophores and membrane-permeable phorbol esters can be demonstrated to alter the presence of AchE in skeletal muscle, heterologous regulation of AchE biosynthesis through ligand-specific occupation of distinct receptors has not yet been demonstrated. Voltage-dependent calcium channels are implicated in a wide variety of cellular processes including calcium conductances, excitation-contraction coupling, and second messenger formation (9, 10), and therefore represent one plausible target for consideration. Skeletal muscle transverse tubules contain large numbers of calcium channels that are identified through their capacity to associate with a chemically heterogeneous family of ligand antagonists (9, 10). The ligands most extensively studied fall among the dihydropyridines, phenylalkylamines, and benzothiazepines, typified, respectively, by nifedipine, verapamil, and diltiazem. Dihydropyridines, in particular, associate in skeletal muscle at t-tubule sites with high affinity (K\(_D\) ≈ 1–10 \(\times\) 10^\(-9\) M) and cause inactivation of the slow L-type \(\mathrm{Ca}^{2+}\) channel and a reduction in \(\mathrm{Ca}^{2+}\) release from the sarcoplasmic reticulum (11). These actions lead to an overall reduction in intracellular \(\mathrm{Ca}^{2+}\) and are to be distinguished from those of ionophores that elevate intracellular \(\mathrm{Ca}^{2+}\) by promoting passive transfer of \(\mathrm{Ca}^{2+}\) into the cell.

This study examines the influence of the dihydropyridine antagonist nifedipine on biosynthesis of AchE molecular forms in skeletal muscle and in neurons. Results obtained with nifedipine are compared with those obtained for verapamil, diltiazem, and the calcium ionophore A23187. Particular attention focuses on the dose and time dependences of these actions in monolayers, resolution of intracellular forms of the enzyme, and accompanying alterations in secretion. AchE forms are examined in primary cultures of avian pectoral muscle because the kinetics of synthesis, degradation, and transport of AchE in this tissue are well documented (2, 12–14), providing a suitable frame of reference for interpreting changes in the amounts and distribution of AchE forms. Avian neural retina serves as the source of neurons; this tissue arises as an extension of the forebrain, follows an orderly program of growth and differentiation (15), and provides a rich source of glial-free neuronal cells (16). The influence of nifedipine on intracellular AchE forms is resolved by employing a rapid, irreversible, cationic methylphosphonate inhibitor of AchE, β-(trimethylammonium)ethyl methylphosphonofluoridate (β-(TMA)ethyl-MPF) (17). By virtue of its cationic charge, low concentrations of β-(TMA)ethyl-MPF are expected to be cell-impermeant and therefore to react with cell surface forms of the enzyme, whereas higher concentrations might be expected to penetrate the cell and to react with intracellular forms of AchE. β-(TMA)ethyl-MPF is ad-
of time allowed for spontaneous hydrolysis of any residual inhibitor, and for "aging" of the inhibited esterase, a phenomenon which renders the enzyme refractory to reactivation (19). This inhibited serum was employed without further treatment; culture medium prepared with this inhibited serum is referred to as the conditioned medium.

Preparation of Drug Solutions—Solutions of nifedipine or verapamil in polyethylene glycol, and A23187 or diltiazem in phosphate-buffered saline, pH 7.0, were prepared 1 day before use. These solutions were protected from light and applied to cultures in the dark.

Sucrose Density Centrifugation—Monolayers were harvested and homogenized in 0.01 M sodium phosphate buffer containing NaCl (1 N), PIGTA (0.01 M), Triton X-100 (5%), bacitracin (1 mg/ml), benzamidine HCl (2.5 mM), leupeptin (40 mg/ml), pepstatin A (20 mg/ml), and aprotonin (5 mg/ml) (20, 21). The supernatant derived from a low-speed spin (13,200 × g, 20 min) was layered over a linear 5 to 20% sucrose gradient. N-Ethylmaleimide (5 mM) was added to the gradient aliquot prior to ultracentrifugation. Sedimentation markers (3 S, carbonic anhydrase; 11.4 S, catalase; 16 S, β-galactosidase) were added with the sample to calibrate the gradient. Following ultracentrifugation (Beckman SW-27 rotor; 40,000 rpm; 22 h), gradients were fractionated by upward extrusion into 40 fractions of 20 drops each and analyzed for AcChE. AcChE was determined by measuring hydrolysis of acetylthiocholine iodide (5 × 10⁻⁴ M) in the presence of 5,5'-dithiobis(2-nitrobenzoic acid) (3 × 10⁻⁴ M) by the method of Ellman et al. (22). The muscle and neuronal cultures contained less than 1% butyrylcholinesterase, as indicated by measurement of enzyme activity in the presence of 5-bis-(4-amino)benzammonium phenylpentanoic dibromide (BW282c51), a specific inhibitor of butyrylcholinesterase (21).

RESULTS

Physical Properties of AchE Molecular Forms in Primary Culture of Chick Skeletal Muscle and Neural Retina—AchE in primary culture of embryonic chick skeletal muscle appeared as three principal molecular forms characterized by sedimentation coefficients of 7 S, 11.4 S and 19 S (Fig. 1A). A 5 S species was also evident and migrated as a small shoulder associated with the predominant 7 S form. Primary cultures of neural retina contained the 5 S, 7 S, and 11.4 S molecular forms of AchE, which have been shown to contain no AchE (18).

Measurement of AchE Secretion Two types of experiments were conducted concerning the influence of nifedipine on secretion of AchE from primary chick pectoral muscle cultures. One set of experiments examined the effects on AchE secretion after long-term treatment (24 h) with nifedipine. The medium covering the cultures was removed and replaced in the dark with fresh medium in the absence or presence of nifedipine (10⁻⁴ M); after 24 h the drug-containing medium was removed, replaced with a "conditioned" medium (described below), and enzyme activity in the medium was monitored for the subsequent 8-10 h. In the other set of studies, the time course for appearance of AchE in the medium was monitored immediately after replacing the complete culture medium with the conditioned medium containing the designated concentration of nifedipine. The secretion studies utilized 35- or 60-mm culture dishes. When the smaller dishes were employed, three dishes were removed at the designated time and the medium covering each dish was isolated and assayed for AchE. When the larger dishes were employed, an aliquot of the medium from each dish was withdrawn at the designated time, replaced with an equal volume of fresh conditioned medium, and assayed for AchE. In both cases, each reported value represents the mean ± S.D. averaged over three separate culture dishes. The small dishes allowed an estimate of variation among the individual dishes, while the large dishes afforded an estimate of any variation in measuring the small amounts of enzyme activity secreted. Both methods afforded similar results.

Cholinesterase activity associated with the culture medium was eliminated by treatment of the horse serum with cyclohexyl methyolphosphonofluoridate, an irreversible organophosphate inhibitor (19). Serum was titrated with cyclohexyl methylphosphonofluoridate until greater than 95% of the initial activity was lost. Typically, this required an inhibition of 10⁻⁵ M. The inhibited serum was allowed to stand at 37 °C for at least 3 h prior to use. This procedure

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Molecular forms of acetylcholinesterase extracted from monolayer cultures of avian skeletal muscle and neural retina. Density gradient profiles for AchE extracted from monolayer cultures of pectoral muscle (A) and neural retina (B). Three principal molecular forms of AchE in primary avian skeletal muscle appear as 7 S (peak I), 11.4 S (peak II), and 19 S (peak III) forms. A 5 S form migrates with the 7 S form. Neural retina contains the 5 S, 7 S, and 11.4 S molecular forms. The arrow indicates the position of the calibration markers (3.3 S, carbonic anhydrase; 11.4 S, catalase; 16 S, β-galactosidase). The light side of the gradient appears to the left of the figure.
forms of AchE (Fig. 1B). The 19 S form was conspicuously absent from neural retina.

Compartmentalization of AchE in skeletal muscle and neural retina was assessed by measuring accessibility of the different molecular forms to irreversible inhibition by the cationic methylphosphonate inhibitor β-(TMA)ethyl-MPF present in the concentration range 10^{-10} to 10^{-6} M (Fig. 2). More than 90% of the initial 7 S AchE activity remained in skeletal muscle after treatment with β-(TMA)ethyl-MPF in the concentration range 10^{-10} to 10^{-7} M (Fig. 2A). Activity of 7 S AchE fell sharply at β-(TMA)ethyl-MPF concentrations above 10^{-7} M. The 11.4 S form was not appreciably inhibited at β-(TMA)ethyl-MPF concentrations less than 10^{-9} M. The extent of inhibition increased with increasing concentrations up to 10^{-7} M, at which it levelled at 40-50% of the initial enzyme activity. Complete inhibition of both the 7 S and 11.4 S forms was observed after treatment with 10^{-5}Mβ-(TMA)ethyl-MPF. The 19 S form displayed an exquisite sensitivity to the presence of inhibitor and fell sharply after treatment with concentrations of β-(TMA)ethyl-MPF in the range 10^{-10} to 10^{-6} M; nearly complete inhibition of 19 S AchE occurred at 10^{-7} M, a concentration that was 100-fold lower than required for inhibition of the 7 S and 11.4 S forms. Because 19 S AchE was present in only small amounts, measurement of this form engendered a greater uncertainty than seen for the other more abundant forms. These data revealed that 7 S AchE was inaccessible to lower concentrations of inhibitor, suggesting that this AchE molecular form existed predominantly as an intracellular species. In contrast, 11.4 S AchE appeared to be partitioned equally between intracellular and extracellular localizations. The sensitivity of the 19 S form to presence of low concentrations of inhibitor was compatible with an extracellular localization.

In neural retina approximately 90% of the initial 7 S and 50% of the initial 11.4 S AchE remained after pulse inhibition by β-(TMA)ethyl-MPF in the concentrations range 10^{-9} to 10^{-7} M (Fig. 2B). At concentrations higher than 10^{-6} M, complete inhibition of both the 7 S and 11.4 S forms was observed. These results were virtually identical with those found for the corresponding forms in skeletal muscle. That is, the AchE molecular forms common to skeletal muscle and neural retina showed common cell compartmentalization.

**Influence of Nifedipine on Appearance of AchE Molecular Forms in Skeletal Muscle and Neural Retina—Nifedipine caused measurable reductions in the amounts of AchE in primary culture of pectoral muscle. The effects were time-dependent, as measured over the duration 0-48 h (Fig. 3A), and dose-dependent, over the range 10^{-9} to 10^{-4} M (Fig. 4A). With regard to the time dependence, the presence of nifedipine (10^{-5} M) caused graded, pronounced reductions in the 7 S and 19 S forms of AchE. These reductions approached 50% loss after 10 h and remained essentially unchanged through the subsequent 48 h of observation. The 11.4 S form underwent no measurable reduction during this time.

With respect to the dose dependence of these reductions, greater than 75% of the 7 S and 19 S forms remained after treatment with 10^{-6} M nifedipine; more than 50% of these forms remained after treatment with higher nifedipine concentrations in the range 10^{-5} to 10^{-4} M (Fig. 4A). In contrast to 7 S and 19 S AchE, the 11.4 S form of the enzyme was relatively unaffected by treatment with nifedipine over the concentration range 10^{-9} to 10^{-6} M; measurable reductions of 11.4 S AchE were observed only at the highest concentrations employed, 10^{-5} to 10^{-4} M.

Several features of these data are noteworthy. Loss of 7 S and 19 S AchE required passage of 9 h prior to observations of any discernible reductions of AchE. The concentration

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**Fig. 2.** Compartmentalization of the molecular forms of AchE in cultured skeletal muscle and neural retina. Compartmentalization of AchE forms in muscle and neuron was assessed by measuring accessibility of the different molecular forms to inhibition by the irreversible methylphosphonate inhibitor β-(TMA)ethyl-MPF. Monolayers were pulse-inhibited with the indicated concentrations of β-(TMA)ethyl-MPF at room temperature for 10 min, washed three times to remove any unreacted inhibitor, and harvested and examined on sucrose density gradients as described under "Experimental Procedures." A presents results for inhibition of skeletal muscle monolayers by β-(TMA)ethyl-MPF present at concentrations in the range 10^{-10} to 10^{-6} M. Data are presented as the percent activity present in untreated cultures and represent the mean ± S.D. averaged over four independent sets of cultures. AchE: 7 S (●), 11.4 S (○), and 19 S (■). B presents results obtained after inhibition of neural retina monolayers by β-(TMA)ethyl-MPF present at concentrations in the range 10^{-9} to 10^{-5} M. Data are presented as the percent activity present in untreated cultures and represent the mean ± S.D. averaged over three separate sets of cultures. AchE: 7 S (●), 11.4 S (○), and 19 S (■).

**Fig. 3.** Time dependence of nifedipine-induced alterations in the molecular forms of AchE in primary cultures of chick skeletal muscle and neural retina. Cultures of skeletal muscle (A) and neural retina (B) were treated with nifedipine (10^{-6} M) for the indicated times, washed, harvested, and the molecular forms of AchE resolved by sucrose gradient centrifugation. Data are presented as percent activity present in untreated cultures and represent the mean ± S.D. averaged over at least three independent determinations. AchE: 7 S (●), 11.4 S (○), and 19 S (■).
treatment with A23187, but sharply declined during the subsequent 9 h. The transient increase in 19 S AchE was similar to that reported for cultured rat (7) and avian (8) skeletal muscle. It was significant that the A23187-induced alterations in the molecular forms of AchE were neither loss of spontaneous contractile activity nor any visible morphological or cytochemical alterations. This latter observation contrasts with reports that all classes of calcium channel antagonists, including dihydropyridines, block the depolarization-activated contraction of cultured mouse muscle (23, 24).

In neural retina the time (Fig. 3B) and dose dependences (Fig. 4B) for reduction of 7 S and 11.4 S AchE forms were quite shallow and contrasted with the more striking time and dose dependences obtained for skeletal muscle. Nifedipine concentrations less than 10⁻⁶ M caused no discernible loss of the 7 S AchE and loss of only 20% of 11.4 S AchE. The time and dose dependence for these effects reveal a markedly lower sensitivity of neural retina to nifedipine than skeletal muscle.

For comparison with nifedipine, the influence of the Ca²⁺ ionophore A23187 on AchE in skeletal muscle was examined over a duration of 0–48 h (Fig. 5). In contrast with the behavior observed for nifedipine, treatment of skeletal muscle cultures with A23187 resulted in reductions of all forms of AchE. Within experimental uncertainty, the time course for reduction in 7 S and 11.4 S AchE was approximately linear (Fig. 5). Amounts of 19 S AchE increased during the 1st h of treatment with A23187, but sharply declined during the subsequent 9 h. The transient increase in 19 S AchE was similar to that reported for cultured rat (7) and avian (8) skeletal muscle. It was significant that the A23187-induced alterations in monolayer activity occurred immediately after initiation of treatment, without any discernible requirement for a 3-h lag prior to evidence of any reduction.

Compartmentalization of Nifedipine Action in Skeletal Muscle and Neural Retina—β-(TMA)ethyl-MPF was employed as a probe for resolving the intra- and extracellular compartmentalization of AchE forms remaining after nifedipine treatment. Monolayer cultures of skeletal muscle that had been treated with nifedipine (10⁻⁶ M) for 24 h were pulse-inhibited at room temperature for 10 min with β-(TMA)ethyl-MPF (10⁻⁷ M), washed, and harvested, and the AchE molecular forms resolved on sucrose density gradients. Monolayer cultures of neural retina treated with nifedipine (10⁻³ M) for 48 h were examined in a similar manner. The results are presented in Fig. 6 as the percent activity of each molecular form remaining in cultures pulse-inhibited with β-(TMA)ethyl-MPF after treatment with nifedipine. Results with nifedipine and β-(TMA)ethyl-MPF alone are presented for comparison.

Treatment of skeletal muscle cultures with nifedipine resulted in a 38% loss of 7 S AchE; treatment with β-(TMA)ethyl-MPF alone resulted in a 16% loss (Fig. 6A). The 52% reduction of 7 S AchE after treatment with nifedipine followed by pulse-inhibition with β-(TMA)ethyl-MPF was the approximate sum of effects seen after the individual treatments. Similarly, for the 11.4 S and 19 S AchE forms, sequential treatment with nifedipine followed by pulse-inhibition with β-(TMA)ethyl-MPF resulted in reductions in these forms that were the sums of the individual treatments. In neural retina, as shown in Fig. 6B, the effects of sequential treatment with nifedipine and β-(TMA)ethyl-MPF on the 7 S and 11.4 S AchE molecular forms were approximately additive, and hence similar to those observed in skeletal muscle. Similar results were observed at nifedipine concentrations of 10⁻³ M. Hence, these effects on intra- and extracellular populations of AchE were independent of nifedipine concentration.

In all cases, the amount of enzyme available for inhibition by β-(TMA)ethyl-MPF after treatment with nifedipine was nearly identical with that available before nifedipine treatment. That is, the nifedipine-induced reductions of AchE occurred with a population of enzyme that was separate from the enzyme acted on by the cell-impermeant irreversible inhibitor. The reductions of AchE following treatment with β-(TMA)ethyl-MPF reflected direct inhibition of cell surface/accessible AchE. The reductions of AchE following treatment with nifedipine were therefore concluded to occur exclusively with intracellular AchE. Cell surface or extracellular popula-
The Influence of Nifedipine on Secretion of Skeletal Muscle AchE—The influence of nifedipine on secretion of AchE was examined by monitoring appearance of the enzyme in the culture medium. In one type of experiment, secretion was monitored after short-term treatment (24 h) with nifedipine (Fig. 7A), while in an alternative experiment secretion was monitored immediately after supplementation of the medium with nifedipine (Fig. 7B). In both cases secretion in the presence of 10^-7 M nifedipine was monitored over a time course of 8-10 h.

In the absence of nifedipine, AchE secretion into the medium was linear and was characterized by an average rate of 0.31 ± 0.09 h^{-1} (Fig. 7A). After treatment with nifedipine for 24 h, secretion remained linear but the rate was reduced to 0.21 ± 0.05 h^{-1}. When secretion was measured immediately after addition of nifedipine to the culture medium, appearance of AchE in the medium deviated from linearity, and was more pronounced at higher verapamil concentrations in the range 10^-7 to 10^-4 M. In untreated cultures secretion of AchE over 10 h occurred in a linear fashion at an apparent rate of 0.31 ± 0.09 h^{-1}. After treatment with nifedipine, the rate of secretion of AchE was determined to be 0.26 h^{-1}. Averaged over five separate sets of cultures the rate was calculated to be 0.21 ± 0.05 h^{-1}. The time course for secretion of AchE into the medium was monitored immediately after replacing the complete culture medium with the conditioned medium either free of or containing nifedipine (10^-5 M). In untreated cultures secretion of AchE over 10 h occurred in a linear fashion at an apparent rate of 0.31 h^{-1}. After initiating treatment with nifedipine the secretion of AchE occurred coincident with that seen for nifedipine and A23187 (Fig. 8). Treatment with verapamil and diltiazem, were examined and compared with that seen for nifedipine and A23187 (Fig. 8). Treatment with verapamil and diltiazem caused no marked losses of 7 S AchE and measurable but variable reductions, 39 ± 23 and 26 ± 27%, respectively, in 11.4 S AchE. Most noticeable were the losses in 11.4 S AchE of 78 ± 19 and 54 ± 30% following treatment with verapamil and diltiazem, respectively (Fig. 8, B and C), and therefore were comparable with those seen for nifedipine. These results represent the mean ± S.D. of seven to nine separate sets of cultures.

Treatment with diltiazem and A23187, as noted above for nifedipine, caused neither reductions in spontaneous contraction nor any morphological alterations in the muscle fibers. Verapamil, in contrast, not only blocked spontaneous contraction but also caused marked alterations in appearance of the fibers. When examined with phase-contrast microscopy before and after Giemsa staining, verapamil-treated fibers displayed a grannier appearance, an increased number of cytoplasmic vacuoles, loss of striations, and a disrupted, irregular alignment of nuclei. These observations were more pronounced at higher verapamil concentrations in the range 10^-9 to 10^-7 M, and were similar to those reported for rat skeletal muscle treated with D600, an analog of verapamil (25).
is elevated by treatment with the calcium ionophore A23187. As such, these data are most compatible with a dihydropyridine receptor-mediated reduction rather than an increase in intracellular Ca$^{2+}$.

Mechanism of Dihydropyridine Receptor-mediated Reduction in AchE—AchE exists as a polymorphic family of glycoprotein molecular forms. Employing the nomenclature of Massoulié and Bon (28), AchE molecular forms can be described as monomeric, dimeric, and tetrameric globular catalytically active species, denoted as G1, G2, and G4, respectively. In addition, there exists a unique family of asymmetric species containing the globular catalytic tetrameric units covalently linked to an elongated, fibrillar, collagen-like tail. The 19 S species in avian skeletal muscle, denoted A12 AchE, represents 3 tetrameric units linked to individual strands of the triple-stranded collagen-like tail; 12 S and 15.5 S forms containing 1 and 2 tetrameric units are also known. AchE molecular forms in chick skeletal muscle and neural retina display similar if not identical sedimentation coefficients and compartmentalization; hence their physical and cellular properties are similar. The reduction in AchE following nifedipine treatment must therefore arise from differences in activity of dihydropyridine receptors in skeletal muscle and neural retina. The possible actions of nifedipine with respect to reductions in AchE are discussed with reference to alterations in glycocalyx/assembly, secretion, degradation, and synthesis of the individual forms (2).

Glycosylation/Assembly—As measured through their acquisition of high mannose asparagine-linked oligosaccharides, the 7 S and 11.4 S molecular forms of AchE are identifiable in the rough endoplasmic reticulum as unique catalytically active species within 5 min of synthesis. Assembly and glycosylation of these forms in the Golgi complex are complete within the subsequent 45–60 min (14, 29). The 19 S form is assembled in the distal cisternae of the Golgi complex 90–120 min following appearance of the 11.4 S form in the rough endoplasmic reticulum (14). If nifedipine treatment of skeletal muscle were to lead to alterations in oligosaccharide processing of AchE in the Golgi complex, then it is predicted that reduction in 7 S AchE would be evident within 45–60 min of initiation of drug treatment, and the reduction of 19 S AchE would be evident within 1.5–2 h of treatment. The 3–4 h lag preceding any measureable reduction in AchE exceeds these times. Indeed, from the known transit times for these individual forms, the 3-h delay encompasses the time during which synthesis of 7 S AchE is already completed and during which the 19 S form is still resident in the Golgi complex. In addition, while synthesis and assembly of 7 S and 19 S AchE follow different time courses, the temporal dependence of the nifedipine-induced reductions is indistinguishable. The time course for the nifedipine-induced reductions of AchE is therefore not compatible with alterations in post-translational processing and assembly in the Golgi complex.

Secretion—AchE secretion in the presence of nifedipine, measured over 10 h, is characterized by two kinetically distinct components (Fig. 7B). The early component (<3 h) is coincident with secretion in the absence of nifedipine, while the later component (>3 h) is 2-fold slower. The observation of a reduction rather than an increase in rate of AchE secretion is sufficient to rule out enhanced loss of the 7 S and 19 S to the extracellular medium. The resolution of two clear components is compatible with a conversion between two distinct states. The conversion point at 3 h coincides with the onset of reductions in intracellular AchE, indicating a linkage between amounts of enzyme in the monolayer and the amounts of enzyme secreted. Moreover, since AchE secretion occurs from...
newly synthesized enzyme (12, 13), the reduction in AchE secretion implies a reduction in the amounts of new rather than old enzyme available for secretion.

Degradation—In tissue-cultured muscle two principal rates of AchE degradation are known. Intracellular forms of AchE undergo turnover with half-lives of 1.5 h, whereas cell surface membrane- and basal lamina-associated forms undergo turnover with half-lives in the range 40–50 h (12, 30). The loss of AchE can not represent an accelerated degradation of the slower turnover cell surface forms since the primary action of nifedipine occurs almost exclusively on intracellular AchE. With respect to the fast turnover rates, nifedipine-induced loss of AchE occurs only after passage of 3 h, a time encompassing two turnover half-lives, and therefore deviates significantly from the first-order kinetic behavior characteristic of intracellular AchE degradation (12). The loss of AchE following treatment with nifedipine differs both quantitatively and qualitatively from the pattern of AchE loss when protein degradation prevails, as observed after treatment of cultured muscle with A23187 (31–33). Finally, even though approximately 50% of 11.4 S AchE is intracellular, this form undergoes only small loss following nifedipine treatment. Overall, loss of the different molecular forms of AchE following treatment with nifedipine is not compatible with accelerated protein degradation.

Synthesis—The nifedipine-induced reductions are not compatible with alterations in post-translational processing and assembly in the Golgi complex, increased rates of secretion, or intracellular protein degradation. By exclusion, the evidence points to dihydropyridine receptor-mediated reductions in biosynthesis of the individual molecular forms of AchE, an action that is selective to the 7 S and 19 S forms. This conclusion is supported by the requirement for a 3-h duration prior to onset of reduction of 5/7 S and 19 S AchE, and prior to reduction in the rate of AchE secretion. This time coincides with the 3-h interval separating de novo synthesis and appearance of AchE and the nicotinic acetylcholine receptor in avian skeletal muscle (12, 13) and neurons (34). Since the 2-fold reduction in AchE secretion (Fig. 7) parallels the 50% loss in amount of AchE after long-term treatment with nifedipine (Fig. 3A), and on the basis of unaltered protein degradation, the rate of synthesis is concluded to be reduced approximately 2-fold.

AchE molecular forms in chick muscle and neurons appear to arise from allelic variants of a single gene (35, 36). The appearance of AchE from a single gene requires that the multiple forms of AchE arise either through alternative splicing of a single primary transcript or through differential promotion, or both, leading to formation of multiple transcripts (37). While alternative splicing has been deduced as one mechanism underlying AchE polymorphism in Torpedo (38–40), neither mechanism is yet known for regulation of AchE biosynthesis in skeletal muscle. If alternative splicing mechanisms prevail, then antagonist occupation of the dihydropyridine receptor can be concluded to alter AchE expression at post-transcriptional points such as mRNA processing and stability. If differential promoter mechanisms prevail, then antagonist occupation of the dihydropyridine receptor can be concluded to alter AchE expression at the level of transcription. Overall, any post-translational mechanisms must be subordinate to alterations occurring at transcriptional and post-transcriptional levels. While many examples of protein polymorphism through alternative splicing and differential promotion mechanisms are known (41–43), the cellular mechanisms that signal these processes have yet to be identified in molecular terms. The present results imply a linkage between transcription and post-transcriptional mRNA processing within the nucleus and ligand occupation of the dihydropyridine receptor at the t-tubule plasma membrane. Since dihydropyridines are known to cause a reduction in intracellular Ca2+ mobilization of Ca2+ subsequent to antagonist occupation of the dihydropyridine receptor represents one probable component in this mechanism. These results are specific to skeletal muscle since no such relationship is evident in neural retina and, by virtue of representing receptor-specific activity, differ from the more general examples of Ca2+ ionophore-mediated alterations in gene expression (44–48). Moreover, since dihydropyridine blockers in chick myotubes also cause up-regulation of the nicotinic acetylcholine receptor (49), these findings are of general significance to regulation of cholinergic proteins.

Precursor Relationships in AchE Synthesis—It is not known whether the asymmetric AchE forms undergo assembly from globular G1 or G4 units (14, 34, 50), and whether the G4 units within the A12 species are identical with the catalytically detected 11.4 S species. In this regard it is of interest that the 7 S, and 19 S forms show behavior common to one another but unique from that for 11.4 S AchE, analogous with observations characterizing reductions in AchE following denervation of rat anterior tibialis (4).

The observed loss of 19 S AchE with neither reduction nor increase in 11.4 S AchE is incompatible with a sequence in which synthesis of A12 AchE depends directly on availability of a common pool of tetrameric G4 AchE. Indeed, the parallel loss of 7 S and 19 S AchE (Figs. 3 and 4) is more compatible with assembly of A12 AchE from globular species that are physically distinct from the catalytically active 11.4 S form. Hence, these results argue against assembly of the asymmetric AchE molecular forms from fully processed G4 species that are identical with 11.4 S AchE. This conclusion implies that the individual molecular forms of AchE are synthesized and assembled independent of one another, and finds indirect support in observations that at least two forms of AchE in avian muscle, the 7 S and 11.4 S species, undergo no interconversion and retain distinct identities throughout their intracellular residence (29). In light of these considerations, the final identity of the asymmetric AchE molecular forms must be determined at a stage no later than post-transcriptional processing of mRNA.

Functional Significance of Dihydropyridine Receptor Regulation of AchE—The dose- and time-dependent reductions in AchE following treatment with nifedipine are unique from those seen for verapamil and diltiazem, are not evident in neurons, and are thus compatible with a dihydropyridine receptor-mediated phenomenon specific for skeletal muscle. Dihydropyridines bind in skeletal muscle at receptor sites localized within the transverse tubules of the junctional triad. A small fraction of these receptor sites operate as voltage-dependent slow calcium channels (61) while they, or a separate number, appear also to function as voltage sensors for excitation-contraction coupling (11, 24, 27, 52–54). Inositol 1,4,5-triphosphate is implicated in this process as a chemical second messenger linking t-tubule depolarization with calcium release from the sarcoplasmic reticulum (56–59).

As seen in noncontracting skeletal muscle treated with phorbol esters (8), calcium ionophores (7), and the Na+ channel activator veratridine (25), evidence is accumulating that the dependence of AchE biosynthesis on innervation and contractile activity is attributable to formation of intracellular second messengers in response to membrane electrical activity rather than contraction per se (60). The present studies provide a strong indication that the dihydropyridine receptor,
with an as yet incompletely defined role in excitation-contraction coupling, presents an abundant, localised, and highly coupled target for neuronal regulation of skeletal muscle. Evidence of an increasing number of endogenous ligands of neuronal origin that block both Ca\(^{2+}\) uptake and ligand association at voltage-dependent calcium channels serves to support such an outlook (61–65).

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