Heparin is capable of solubilizing a subset of collagen-tailed (A12) acetylcholinesterase (AChE) molecules from skeletal muscle fibers, but cannot detach AChE from the synaptic basal lamina (Rossi, S. G., and Rotundo, R. L. (1993) J. Biol. Chem. 268, 19152–19159). In the present study, we used tissue-cultured quail myotubes to show that, like adult fibers, neither heparin- nor high salt-containing buffers detached AChE molecules from cell-surface clusters. Prelabeling clustered AChE molecules with anti-AChE monoclonal antibody 1A2 followed by incubation in heparin-containing medium showed that there was no reduction in the number or size of pre-existing AChE clusters. In contrast, incubation of myotubes with culture medium containing heparin for up to 4 days reversibly blocked the accumulation of new cell-surface AChE molecules without affecting the rate of AChE synthesis or assembly. Newly synthesized A12 AChE becomes tightly attached to the extracellular matrix following externalization. However, in the presence of heparin, blocking the initial interactions between A12 AChE and the extracellular matrix results in release of AChE into the medium with a t½ of ~3 h. Together, these results suggest that once A12 AChE is localized on the cell surface, initially attached via electrostatic interactions, additional factors or events are responsible for its selective and more permanent retention on the basal lamina.

The asymmetric collagen-tailed (A12) acetylcholinesterase (AChE) molecule is the predominant oligomeric form of this enzyme at the neuromuscular junction, where it is attached to the synaptic basal lamina (reviewed by Taylor (1991) and Massoulie et al. (1993)). The molecular mechanisms underlying the highly selective targeting and retention of this synaptic component at the appropriate location on the cell surface are still not clearly understood, but probably involve a combination of transcriptional, post-transcriptional, and post-translational events. In tissue-cultured skeletal muscle fibers, the AChE catalytic subunits are locally translated and assembled around the nuclei encoding their transcripts (Rotundo, 1990), and the newly synthesized AChE oligomers are selectively localized to regions of the cell surface over the nucleus of origin (Rossi and Rotundo, 1992). The levels of AChE mRNA, studied in tissue-cultured cells, appear to be regulated post-transcriptionally (Fuentes and Taylor, 1993). In vivo, transcripts encoding AChE are highly concentrated at the vertebrate neuromuscular synapse (Jasmin et al., 1993), suggesting that, like the tissue-cultured myotubes, AChE oligomers are locally transcribed, translated, assembled, and selectively localized to the overlying synaptic basal lamina. The inability of conventional extraction procedures to remove the junctional AChE molecules, such as high ionic strength buffers, polyanions, and chaotropic agents, suggests that the enzyme is covalently attached to the extracellular matrix (Rossi and Rotundo, 1993).

A likely candidate molecule involved in localizing AChE to the neuromuscular junction is a heparan sulfate proteoglycan (HSP). A12 AChE binds specifically to heparin and sulfated glycosaminoglycans (Bon et al., 1978; Vigny et al., 1983; Brandan et al., 1985; Brandan and Inestrosa, 1986) and can be solubilized from muscle with heparin (Torres and Inestrosa, 1983; Barat et al., 1986) or other polyanions (Pérez-Tur et al., 1991a). Electron microscopy of negatively stained aggregates of A12 AChE and polyanionic components of the extracellular matrix from Torpedo electric organs shows that the distal regions of the collagen-like tail are involved in the binding (Bon et al., 1978). Brandan and Inestrosa (1984) demonstrated that only the A12 AChE form binds to heparin-agarose, indicating that binding is dependent on the noncatalytic collagen-like tail subunit, and only heparin is able to displace the bound AChE. In contrast, however, heparin does not detach A12 AChE from the synaptic basal lamina (Rossi and Rotundo, 1993).

Although widely distributed throughout the extracellular matrix surrounding muscle fibers, HSP also extends into the junctional region, where its density is increased severalfold on the synaptic basal lamina (Bayne et al., 1981; Anderson and Fambrough, 1983; Sanes et al., 1986). The deposition of HSP is spatially and temporally correlated with AChR aggregation in developing myotubes and at developing neuromuscular synapses in culture (see Anderson and Fambrough (1983) and Bayne et al. (1984); reviewed by Hall and Sanes (1993)). Furthermore, there is a positive correlation between the formation of acetylcholine receptor (AChR) clusters and subsequent accumulation of extracellular matrix components, including HSPs, in cultured muscle cells (Bayne et al., 1984; Swenarchuk et al., 1990). Agrin, a protein originally isolated from basal lamina-rich extracts of Torpedo electric organs, induces the clustering of AChR, AChE, butyrylcholinesterase, the cytoplasmic AChR-associated 43-kDa protein, and HSP on chick myotubes in culture (Wallace et al., 1985; Nitkin et al., 1987; Wallace, 1989; Lieth and Fallon, 1993). However, in myotubes pretreated with...
Inhibitors of protein synthesis, aggregates of HSP and AChE were not detected even though agonin continued to induce the formation of ACHR aggregates (Wallace, 1989). These observations indicate that the formation of HSP and AChE clusters are downstream events from the initial ACHR clustering and suggest that they may possibly be linked.

In quail skeletal muscle cultures, the A₁₂ AChE form is clustered on the upper surface of the myotubes, where it can be removed using purified collagenase (Rossi and Rotundo, 1992). However, the mechanism of attachment of the clustered A₁₂ AChE has not been analyzed in detail. In this study, we show that heparin does not detach A₁₂ AChE previously clustered on the surface of myotubes. In contrast, heparin reversibly blocks the accumulation of newly synthesized A₁₂ AChE in a time-dependent manner. Furthermore, we show that only the newly synthesized A₁₂ AChE molecules can be solubilized from the cell surface. In untreated cultures, these molecules become tightly linked to the extracellular matrix, whereas in the presence of heparin, they are readily removed. This study suggests that HSPs are involved in the initial targeting of AChE to specialized regions of the cell surface, but that once localized, more permanent mechanisms of attachment are formed.

### Experimental Procedures

Preparation of Tissue-cultured Muscle—Pectoral muscle cells from 10-day-old quail embryos were grown on scratched collagen-coated coverslips (25-mm diameter) in Eagle's minimal essential medium supplemented with 2% chick embryo extract, 10% horse serum, and 50 μg/ml gentamicin (EMEM 210) as described previously (Rossi and Rotundo, 1992). Cultures were fed on the third day and every other day thereafter. When used, 0.1–1 mg/ml heparin (Sigma, H-3393; M, 9,000–21,000) was dissolved directly in the medium, and the cultures were fed at the indicated times. Other test compounds added to the culture medium included polyaspartate (Sigma), Dextran sulfate (Sigma), and sodium chloride (NaClO₃; Fluka Chemical Co.) at the indicated concentrations. In some experiments, differentiated myotubes were incubated in serum-free defined medium (Bottenstein and Sato, 1979) modified for muscle culture (Rubin, 1985). Sulfate-free defined medium (Imai et al., 1994) was prepared using 50 × Eagle's minimal essential medium amino acid stock solution and 100 × vitamin supplement (both from Life Technologies, Inc.) and Earle's salts as recommended by the manufacturer, substituting sodium chloride for magnesium sulfate.

Cell-surface AChE Activity Assay—Cell-surface AChE activity was determined using a modification of the method of Johnson and Russell (1975) for whole cultures (Rotundo and Fambrough, 1980). Cultures were rinsed twice with Hank's balanced salt solution (HBSS) and twice with PBS, and the incubation was initiated by addition of 1 ml of buffer/substrate mixture/dish. The buffer/substrate mixture consisted of 0.6 mM unlabeled acetylcholine in PBS and 0.1 μCi of [³H]acetylcholine (DuPont NEN; specific activity = 73.7 mCi/mmol). All incubations were done at 4°C to prevent secretion of AChE from the intracellular pool. At the indicated times, 20-μl aliquots from each culture were removed and mixed with 0.3 ml of 50 mM glycine HCl buffer (pH 2.5) with 2 mM NaCl in scintillation vials, and the labeled acetylcholine was counted by addition of 5 ml of ACS scintillation fluid containing 20% 1-butanol (v/v). Under these assay conditions, the assay was linear for >1 h. To measure the effect of heparin on cell-surface AChE activity during development in culture, three cultures per group were washed three times with 2 ml of PBS and placed on ice, and the reaction was started by addition of 1 ml of buffer/substrate mixture. After a 1-h incubation, 100-μl aliquots were removed and counted.

Alternatively, AChE dimeric forms expressed on the cell surface were analyzed by velocity sedimentation following protection with the water-soluble reversible AChE inhibitor BW284C51 and irreversible inactivating cell surface AChE with disulfopropyl fluoroisocyanate (DFP) as described previously (Rotundo, 1984b). Under these conditions, >80% of the total cell-surface AChE is protected by BW284C51 (data not shown).

Extraction and Analysis of Cell-associated and Secreted AChE Forms—Muscle culture AChE was extracted using borate extraction buffer containing 30 mM borate buffer (pH 9.0), 5 mM EDTA, 0.03–1 M NaCl, 0.2% Triton X-100, 0.5% bovine serum albumin, 2 mM benzamidine, 5 mM N-ethylmaleimide, and 0.7 mM bacitracin, with or without 0.5–1 mg/ml heparin. Triton X-100 was omitted from the solution when live cells were treated with extraction buffers to test removal of surface AChE. Alternatively, cultures were washed with PBS and extracted with PBS containing either additional NaCl or polyamines at the indicated concentrations. Low salt extraction buffer (LSB) consisted of borate extraction buffer with 30 mM NaCl, and high salt extraction buffer (HSB) contained 1.0 M NaCl.

To quantitate newly synthesized AChE, three 35-mm cultures dishes per group were rinsed three times with HBSS, followed by a 10-min incubation with 10 μM DFP in HBSS to irreversibly inhibit all AChE activity. The cultures were then rinsed with HBSS and returned to the culture incubator for 24 h in modified defined medium with or without heparin. To identify secreted AChE forms, 200-μl aliquots of medium from each dish were analyzed by velocity sedimentation. The three cultures per group were then rinsed with HBSS and extracted in a total volume of 600 μl of borate extraction buffer containing 0.5% Triton X-100 and 1 M NaCl (HSB) to determine total cell-associated AChE forms. The pooled culture extracts were homogenized and centrifuged for 20 min in a microcentrifuge (4°C, 14,000 rpm). The AChE dimeric forms were resolved by velocity sedimentation on 5–20% sucrose gradients for 16 h at 32,000 rpm in an SW 50.1 rotor. Fifteen-drop fractions were collected, and AChE activity was assayed by a modification of the radiometric method of Johnson and Russell (1975) as described previously (Rotundo and Fambrough, 1979).

### Results

Neither High Salt- nor Heparin-containing Buffers Release AChE from the Extracellular Matrix of Cultured Quail Muscle Cells—The asymmetric AChE form accumulates in clusters on the upper surfaces of myotubes in culture (Rossi and Rotundo, 1992), possibly attached to heparan sulfate proteoglycan-like molecules. To determine whether high salt- or heparin-containing buffers could detach clustered AChE molecules from the extracellular matrix, 7-day-old quail muscle cultures were incubated for 1 h in either LS and HS with or without 0.5 mg/ml heparin. The cultures were rinsed with PBS, and the AChE clusters were visualized using anti-AChE mAb I2 (Rotundo and Fambrough, 1993), which is known to recognize high salt- or heparin-containing buffers. Eluted AChE clusters were not detected even though agrin continued to induce the formation of AChR aggregates (Wallace, 1989). These observations are consistent with our findings on intact adult muscle fibers (Rossi and Rotundo, 1993). Neither heparin nor high salt buffers could remove the AChE molecules from cell-surface clusters, indicating that, even in culture, the standard high salt- or heparin-containing buffers cannot solubilize the matrix-bound AChE (Fig. 1). In addition, we have attempted to remove the cell-surface AChE with chaotropic agents. Extraction buffers containing 1% sodium dodecyl sulfate or 4 M urea, concentrations that still leave the cells attached to the substratum, do not remove AChE (data not shown).

To quantitate the amount of cell-surface AChE remaining after the different extraction procedures, three cultures per group were incubated for 1 h with PBS alone, PBS plus 1.0 mg/ml heparin, PBS with 1 M NaCl, or PBS with 1 M NaCl and 1.0 mg/ml heparin; and the remaining cell-surface AChE activity was assayed. Control cultures were incubated with PBS.
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TABLE I

Cell-surface AChE activity following incubation of intact cells with high ionic strength buffers or heparin

Six cultures per group were incubated for 1 h in the indicated extraction buffer, rinsed with PBS alone, and assayed for cell-surface AChE activity as described under “Experimental Procedures.” The results are expressed as total cell-surface AChE activity (cpm) and as percentage control (PBS alone) 17,700 ± 800 100.0 ± 4.7
PBS + 1 mg/ml heparin 17,600 ± 700 99.1 ± 3.8
PBS + 1× NaCl 16,800 ± 1,900 95.0 ± 10.5
PBS + 1× NaCl + 1 mg/ml heparin 17,800 ± 800 100.6 ± 4.4

TABLE II

Detergent-, high salt-, and polyanion-containing buffers do not detach clustered AChE

Seven-day-old cultured myotubes were extracted for 1 h using PBS, LSB, HSB, LSB plus 0.5 mg/ml heparin, or PBS plus either 1 mg/ml dextran sulfate or 1 mg/ml polyaspartate. LSB and HSB contained 0.5% Triton X-100. AChE was then localized by indirect immunofluorescence using mAb 1A2, and the nuclei were stained using Hoechst 33342. The total number of myotube nuclei and AChE clusters on muscle fibers in five fields per slide was summed. Three slides were analyzed per group, and the results are expressed as the means ± S.E. Neither high salt-, detergent-, nor polyanion-containing buffers caused any detachment of clustered AChE.

| Treatment                  | No. of nuclei | No. of clusters | Clusters/nucleus |
|----------------------------|---------------|-----------------|------------------|
| PBS                        | 375           | 119             | 0.32 ± 0.05      |
| LSB (30 mM NaCl)           | 303           | 112             | 0.36 ± 0.11      |
| HSB (1 M NaCl)             | 242           | 96              | 0.41 ± 0.04      |
| LSB + 0.5 mg/ml heparin    | 123           | 44              | 0.36 ± 0.02      |
| PBS + 1 mg/ml dextran sulfate | 408         | 121             | 0.32 ± 0.08      |
| PBS + 1 mg/ml polyaspartate | 449          | 131             | 0.36 ± 0.14      |

The results show that neither heparin nor high concentrations of NaCl removed catalytically active AChE molecules from the cell surface (Table I). In addition, other polyanion-containing buffers have also shown to solubilize at least a few asymmetrical AChE from adult chicken muscle (Pérez-Tur et al., 1991a). To determine whether any of these buffers could detach AChE clusters from quail myotubes, the number of clusters per nucleus was quantitated following solubilization. The results, shown in Table II, indicate that AChE was not detached even after 1 h of extraction using high salt- or polyanion-containing buffers.

Heparin Inhibits the Accumulation of Cell-surface AChE Enzyme Activity on Myotubes—Following cell fusion and formation of multinucleated myotubes, the cells begin to produce the collagen-tailed A12 AChE form capable of clustering on the cell surface. The clusters of AChE molecules begin to form around day 5 in culture, coincident with the onset of spontaneous contractile activity. To determine whether long-term exposure to heparin affected the accumulation of cell-surface AChE activity, muscle cultures were incubated for 1 h to 4 days in normal medium containing 0.5 mg/ml heparin. At the indicated times, the cultures were rinsed with PBS, and cell-surface AChE activity was assayed. Fig. 2A shows the linearity of cell-surface AChE activity with time for each group assayed on day 7. The results in Fig. 2B show the effect of long-term heparin treatment on cell-surface AChE activity during development of myotubes. There was a time-dependent inhibition of cell-surface AChE activity accumulation during exposure to heparin-containing medium that occurred coincident with the onset of A12 AChE form accumulation on day 5, suggesting that heparin blocks the attachment of the enzyme molecules to specific cell-surface sites. To determine whether only the attachment of newly synthesized AChE is affected by heparin, 5-day cultures were incubated with DFP for 10 min and returned to normal or heparin-containing medium for 2 days. The accumulation of newly synthesized cell-surface AChE was decreased by 30% in the presence of heparin (data not shown; however, see below and Fig. 7B).

Heparin Inhibition of Cell-surface AChE Cluster Formation—Since cell-surface AChE has been shown to form clusters that correlate with the localization of heparan sulfate proteoglycan on tissue-cultured myotubes and is colocalized with the same heparan sulfate proteoglycan concentrated at sites of nerve-muscle contact in adult muscle, we sought to determine whether heparin could inhibit the formation of AChE clusters. Tissue-cultured myotubes grown on collagen-coated glass coverslips were incubated in the presence of heparin at concentrations ranging from 0.1 μg/ml (0.01 M) to 1.5 mg/ml (100 μM) (based on an average M, of 15,000) from days 5 to 7. The clusters were visualized by indirect immunofluorescence, and the number of AChE clusters per nucleus was determined. Fig. 3 shows that heparin inhibited the formation of AChE clusters in a concentration-dependent manner, with half-maximal inhibition near 10 μg/ml or ~1 μM.

Since the inhibition of cell-surface AChE activity and lack of cell-surface AChE cluster formation could also result from an inhibition of AChE biosynthesis, we measured the effect of heparin on newly synthesized AChE. Six-day-old muscle cultures were reincubated in complete medium with or without 1 mg/ml heparin for 24 h. The cultures were then treated with DFP in HBSS to inhibit all AChE, followed by washing and recovery for 2 h in complete medium with or without heparin. Under these conditions, the rate of appear-
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The presence of heparin in the medium prevented the formation of aChR clusters (Fig. 4A). In contrast, heparin had no effect on the spontaneous formation of aChR clusters (Fig. 4B), indicating that the heparin effects are specific for AChE. Note that, in these cultures, the density of aChR clusters is about an order of magnitude lower than that of the AChE clusters, a ratio that we have consistently observed in our quail muscle cultures.

To determine whether heparin could disperse or release previously clustered AChE molecules, 6-day-old myotubes were incubated with anti-AChE mAb 1A2 for 2 h, washed in complete medium, and incubated for 24 h in the presence or absence of complete medium containing 1 mg/ml heparin. The cultures were rinsed and incubated with fluorescein isothiocyanate-conjugated second antibody on day 7, and the number of AChE clusters per nucleus was quantitated. Our results show that heparin did not remove AChE once localized to clusters since the number of clusters per nucleus in cultures incubated in heparin-containing medium from days 6 to 7 following mAb 1A2 addition on day 6 is virtually identical to that in the day 6 controls (Fig. 5).

Heparin Blocks AChE Cluster Formation Is Reversible—Long-term heparin treatment of cultured muscle cells reduced the accumulation of cell-surface AChE. If heparin acted by blocking the attachment of A12 AChE to a heparan sulfate-like proteoglycan, the observed effects should then be reversible upon removal of heparin from the medium. Cultures treated from days 3 to 6 with heparin-containing medium were rinsed extensively to remove the polyanions and incubated in normal medium from days 6 to 7. The results, shown in Fig. 6, indicate that the heparin effect was at least partially reversible since the clustering of AChE molecules reappeared upon removal of heparin.

To quantify the reversibility of long-term heparin treatment, muscle cultures maintained in heparin-containing medium for 2–3 days were rinsed and returned to normal medium for either 1 or 2 days. The cultures were then incubated with mAb 1A2 to localize and quantitate AChE clusters. As a control, AChE was immunolocalized immediately after heparin treatment (Table III). The rapid reappearance of AChE clusters following heparin removal suggests that the AChE attachment sites remained localized in the absence of AChE deposition while heparin was in the medium.

Attachment of Asymmetric AChE to the Cell Surface Is Reduced by Inhibition of Proteoglycan Sulfation—The A12 AChE form appears to bind only to sulfated proteoglycans (Bon et al., 1978). Therefore, if A12 AChE molecules are localized to specific regions of the cell surface by heparan sulfate-like proteogly-
Experiment 1), 10 mM sodium chlorate inhibited the normal sulfation (Higashiyama et al., 1993). As shown in Table IV (Experiment 2), and the effects were reversed by addition of 800 μM exogenous sodium sulfate. To determine whether the effects of sodium chlorate were due to decreased AChE synthesis, mature 6-day-old muscle cultures were incubated in complete medium with or without 10 mM sodium chloride for 24 h, and the rates of AChE synthesis were determined. After a 24-h incubation in 10 mM sodium chloride and in the continued presence of sodium chloride, the rate of AChE synthesis was (5.50 ± 0.09) × 10^3 cpm/2 h compared with (4.89 ± 0.08) × 10^3 cpm in control cultures. Thus, the observed decrease in AChE cluster formation is not due to a decrease in AChE synthesis in the presence of sodium chloride.

In the Presence of Heparin, Newly Synthesized Asymmetric AChE Is Secreted into the Medium—The A12 AChE form is normally associated with the cell surface of tissue-cultured myotubes, where it is tightly bound to the extracellular matrix. This form has not been previously observed secreted into the medium of muscle cultures, where only the soluble dimeric and tetrameric forms accumulate. To determine whether heparin could prevent the attachment of newly synthesized A12 AChE to the cell surface, muscle cultures were treated with DFP to irreversibly inhibit all AChE enzyme activity and incubated in defined medium devoid of serum or embryo extract esterases. The newly synthesized AChE forms associated with the cells or secreted into the medium over a 24-h period were analyzed by velocity sedimentation. In the presence of heparin, the collagen-tailed AChE accumulated in the medium (Fig. 7A) rather than on the cell surface (Fig. 7B). Incubation of muscle cultures with heparin-containing medium prevented the formation of cell-surface AChE clusters without apparent effect on the synthesis of AChE since the total amount of globular enzyme molecules synthesized following DFP treatment was not affected (Fig. 7). The small amount of asymmetric form associated with the cells incubated in heparin-containing medium most likely reflects the accumulation of the intracellular pool, which accounts for up to 50% of the total asymmetric AChE forms in quail muscle cultures (Rotundo, 1984b; Fernandez-Valle and Rotundo, 1989).

Asymmetric AChE Binds to Heparin in the Medium—To determine whether asymmetric AChE bound directly to heparin molecules present in the medium, we biotinylated the heparin used in our experiments using N-hydroxysuccinimide-(long chain)-biotin (Pierce) following the manufacturer’s recommended protocol such that only 1–2 biotin molecules would be attached to each heparin molecule. The biotinylated heparin was adsorbed onto avidin-conjugated agarose beads (Sigma), washed, and incubated with aliquots of isolated AChE oligomeric forms prepared by velocity sedimentation of tissue-cultured muscle cell extracts. This procedure ensured that only the newly assembled collagen-tailed AChE forms present in the intracellular pool (or the weakly attached externalized enzyme...
molecules present on the cell surface) would be isolated. The relative amounts of AChE bound were determined by direct enzymatic assay of the washed beads (Fig. 8). In concordance with the results of Brandan and Inestrosa (1984), there was selective binding of the A12 AChE form to heparin. The small amount of G2 binding probably reflects contamination of this fraction with the A8 or A4 forms, consisting of either two or four tetramers attached to collagen-like tail subunits. "Nonspecific" binding in this assay, as measured by the binding of the G2 AChE form, is ~8%. We can conclude that there is probably a direct interaction between the heparin and the A12 AChE form in these experiments.

**Transient Ionic Interactions between Cell-surface Asymmetric AChE and the Extracellular Matrix**—One prediction, based upon the above results, is that the most recently synthesized cell-surface AChE molecules should become tightly associated with the extracellular matrix shortly after externalization in untreated cultures, whereas in the presence of heparin, even the reduced numbers of molecules present on the surface would be only weakly associated with or possibly physically trapped within the matrix. To test this hypothesis, tissue-cultured myotubes, incubated in the presence or absence of 1 mg/ml heparin from days 5 to 7, were incubated with or without 10 μg/ml puromycin for 9 h. This protocol allows sufficient time to chase the intracellular pool of AChE oligomeric forms onto the cell surface (~3 h) (Rotundo and Fambrough, 1980) and allows time for dissociation and/or diffusion of the weakly or nonattached molecules into the medium. Cell-surface AChE was assayed on three dishes per group before and after extraction using the high ionic strength extraction buffer (HSB) to distinguish between electrostatically and tightly attached AChE molecules (Fig. 8). The high ionic strength extraction buffer did not remove significant amounts of cell-surface AChE activity (less than ±5%) from untreated myotubes when compared with unextracted control values (100 ± 3%), either with or without puromycin for 9 h, indicating that the surface AChE molecules, including those most recently externalized, are tightly attached to the extracellular matrix. In contrast, in heparin-treated cultures, where only half (51 ± 7%) of the control levels of AChE activity had accumulated on the cell surface, an additional 30% of the enzyme was removed by extraction with HSB. These results indicate that, in the presence of heparin, most of the surface AChE was electrostatically associated with the extracellular matrix and that only 22 ± 4% of the total surface activity was not extractable with HSB. Incubation of heparin-treated cultures with puromycin indicated that more than one-third of the cell-surface enzyme on heparin-treated myotubes consisted of newly synthesized molecules, that 60% of the previously synthesized AChE molecules were extractable with HSB (compare extracted and unextracted surface AChE activity in heparin-treated cultures), and that only 14% of the cell-surface AChE remained attached to the extracellular matrix of myotubes treated with heparin plus puromycin, followed by extraction with HSB (Fig. 9). These results indicate that most of the cell-surface AChE in heparin-treated myotubes was recently synthesized and high salt-extractable. Therefore, most of the molecules that are associated with the extracellular matrix of heparin-treated myotubes appear to be either weakly associated with components of the extracellular matrix or physically trapped between the matrix and the plasma membrane.

A second prediction based on our results would be that the small pool of “extractable” cell-surface A12 AChE molecules, those that have not yet formed tight associations with other molecules, would appear less stable in the presence of heparin, where they would be prevented from short-term interactions with the extracellular matrix. As can be seen in Fig. 9, this pool of extractable AChE molecules constitutes only a very small percentage of the total surface AChE activity. To estimate the relative association time of cell-surface A12 AChE molecules in the presence or absence of heparin, three myotube cultures per group were incubated in the same medium with or without puromycin for 3, 5, 7, or 9 h. Following protection of the cell-surface AChE with BW284c51, the “soluble” surface AChE forms were extracted with HSB and analyzed on sucrose gradients, and A12 AChE was quantitated (Fig. 10). In untreated controls, there was a small but detectable decrease in the...
amount of extractable A12 AChE during the puromycin chase period, suggesting that a portion of the matrix-associated molecules could maintain electrostatic interactions with the cell surface. In the presence of heparin, however, this small pool was unstable and could rapidly dissociate or diffuse away from the matrix. In the presence of puromycin, there was a rapid decrease in the amount of extractable A12 AChE with time in puromycin to levels <5% of control. This experiment indicates that most of the newly synthesized A12 AChE molecules could not maintain their association with the extracellular matrix in the presence of heparin.

### DISCUSSION

The synaptic basal lamina of skeletal muscle fibers contains higher concentrations of several identified molecules, including AChE and heparan sulfate proteoglycan(s), compared with non-innervated regions (reviewed by Massoulie et al. (1993) and Hall and Sanes (1993)). Of the several AChE oligomeric forms expressed in muscle, the asymmetric collagen-tailed, or A12, form appears to be the most abundant at the vertebrate neuromuscular junction. Its mechanism of attachment to the synaptic basal lamina is generally thought to be through electrostatic interactions with glycosaminoglycans such as heparan sulfate proteoglycan or dermatan sulfate proteoglycan (Bon

### TABLE IV

Sodium chlorate inhibits the formation of surface AChE clusters

| Treatment/days | Analysis | No. of nuclei | No. of clusters | Clusters/nucleus | Maximal accumulation |
|----------------|----------|---------------|----------------|-----------------|---------------------|
| Exp. 1         |          |               |                |                 |                     |
| Normal, 0–5    | Day 5    | 251           | 70             | 0.27 ± 0.04     | 34 ± 5              |
| Normal, 0–8    | Day 8    | 213           | 162            | 0.79 ± 0.13     | 100 ± 16            |
| 10 mM chlorate, 5–8 | Day 8    | 172           | 80             | 0.48 ± 0.10     | 61 ± 12             |
| Exp. 2         |          |               |                |                 |                     |
| SFDM* + sulfate, 5–7 | Day 7    | 332           | 115            | 0.36 ± 0.04     | 100 ± 11            |
| SFDM, 5–7      | Day 7    | 400           | 108            | 0.28 ± 0.07     | 78 ± 19             |
| SFDM + chlorate, 5–7 | Day 7    | 278           | 57             | 0.22 ± 0.05     | 61 ± 14             |
| SFDM + sulfate + chlorate, 5–7 | Day 7    | 324           | 95             | 0.29 ± 0.04     | 81 ± 11             |

* SFDM, sulfate-free defined medium.

### FIG. 7

**Newly synthesized asymmetric AChE is secreted into the medium in heparin-treated muscle cultures.** Six-day-old muscle cultures were treated with DFP to irreversibly inhibit all AChE molecules and allowed to recover for 24 h in defined medium containing 20 mg/ml bovine serum albumin. The AChE forms associated with the cells and secreted into the medium were analyzed by velocity sedimentation as described under "Experimental Procedures." A, AChE forms secreted into the medium in the presence (C) or absence (○) of heparin; B, AChE oligomeric forms synthesized by myotubes incubated in the presence (C) or absence (○) of heparin. Although the total amount of AChE synthesized during the 24-h period was not significantly different between the two groups, the accumulation of the asymmetric collagen-tailed form was attenuated in the presence of heparin. More important, in the presence of heparin, the collagen-tailed form of the enzyme was secreted into the medium rather than retained on the cell surface.

### FIG. 8

**Specific binding of quail asymmetric AChE to heparin.** Individual oligomeric forms of AChE were isolated from tissue-cultured quail muscle cultures by velocity sedimentation, and the peak fractions corresponding to each form were pooled for analysis and adjusted for sucrose concentration and similar levels of enzyme activity. Aliquots of the pooled fractions were incubated overnight with avidin-conjugated agarose beads previously saturated with biotinylated heparin. Each incubation mixture was prepared in quadruplicate, and NaCl was lowered to 200 mM. The beads were then washed with PBS containing 0.5% Triton X-100 and assayed for bound AChE. In these experiments, ~5% of the total A12 AChE was bound per 10 μl of biotin-heparin-agarose beads. The results show that the asymmetric AChE form synthesized by the tissue-cultured myotubes preferentially bound to the immobilized heparin. The small amount of binding observed for the G(4) tetramer probably reflects some contamination from the asymmetric A8 form, which is present in the cultures and cosediments with the G(4) form.
**Attachment of Collagen-tailed AChE in Skeletal Muscle**

**Fig. 9.** Newly synthesized cell-surface AChE on heparin-treated myotubes is extractable. Tissue-cultured myotubes were incubated in complete medium with or without heparin from days 5–7, and 10 μg/ml puromycin was added to the medium for an additional 9 h. At the end of the puromycin chase period, half the cultures in each group were extracted with HSB to remove electrostatically bound surface AChE, and all cultures were assayed for cell-surface AChE as described under “Experimental Procedures.” Results are expressed as percentage untreated controls (mean ± S.E.). In untreated myotubes, essentially all of the cell-surface enzyme was tightly attached to the extracellular matrix, and even the most recently synthesized molecules were unextractable as shown by the lack of reduced surface AChE in the presence of puromycin. In contrast, most of the cell-surface enzyme that had accumulated in the presence of heparin was extractable with HSB. The significant decrease in cell-surface AChE activity in the presence of puromycin and its solubility in high ionic strength buffer indicate that a large fraction of the externalized enzyme molecules are transiently associated with the extracellular matrix via electrostatic interactions.

**Fig. 10.** Analysis of extractable cell-surface asymmetric AChE in the presence or absence of heparin. Tissue-cultured myotubes were incubated in the presence or absence of heparin as described for Fig. 8, followed by incubation with 10 μg/ml puromycin for 3, 5, 7, or 9 h. At the end of the puromycin incubation, all cultures were treated with BW284c51 followed by DFP to inactivate intracellular AChE. Three dishes per group were extracted using HSB to remove electrostatically bound surface AChE. AChE forms were analyzed by velocity sedimentation; and A12 AChE was quantitated. Comparison of soluble cell-surface A12 AChE forms obtained from control and heparin cultures suggests that the rate of dissociation of the enzyme from the extracellular matrix is very slow (4%/h or less), if at all, in untreated cultures, whereas in the presence of heparin, dissociation is rapid, at least 15%/h.

Heparin is capable of solubilizing A12 AChE molecules from vertebrate muscle (Torres and Inestrosa, 1983; Brandan and Inestrosa, 1984, 1986; Barat et al., 1986; Pérez-Tur et al., 1991a) as well as from some neural preparations (Torres et al., 1983). This selective solubilization, together with the strong evidence for direct interactions between A12 AChE and proteoglycans, argues that they may play a role in the anchorage of asymmetric forms to the synaptic basal lamina.

On the other hand, we have recently shown that heparin does not detach AChE from the neuromuscular junctions of adult fast and slow quail muscles or from rat muscles (Rossi and Rotundo, 1993). Furthermore, we demonstrated that essentially all of the immunohistochemically detectable enzyme localized on the synaptic basal lamina was tightly attached and could not be removed by high ionic strength buffers, detergents, or chaotropic agents such as guanidine HCl or urea. Only collagenase was able to detach the enzyme (Hall and Kelly, 1971; Betz and Sakmann, 1973; Rossi and Rotundo, 1993), indicating that the basal lamina-associated AChE is most likely covalently linked to one or more molecular components of the extracellular matrix. In this study, we show that, like the adult neuromuscular junction, short-term treatment (≤1 h) of tissue-cultured myotubes with heparin did not detach the AChE molecules associated with cell-surface clusters (Fig. 1), nor did it remove catalytically active enzyme molecules from the cell surface (Fig. 2 and Table I). Similar results were obtained using the standard high salt- or polyanion-containing buffers, which also were unable to solubilize the matrix-bound AChE (Tables I and II), whereas incubation of the cultures with purified collagenase removed the surface AChE (Rossi and Rotundo, 1992). These results support the idea that clustered AChE molecules on the surfaces of myotubes are attached in the same manner as at sites of nerve-muscle contact in vivo.

In contrast with the inability of heparin to extract previously clustered AChE molecules, long-term exposure to heparin in culture blocked the accumulation of catalytically active AChE on the myotube surface (Fig. 2) as well as the formation of new surface AChE clusters (Figs. 4 and 5). This effect was detectable only after days 3–4 in culture, coincident with the onset of A12 AChE expression and its deposition in clusters on the muscle cell surface. The normal increase in number of AChE clusters per nucleus was prevented when incubations were extended over periods of several days in heparin-containing medium. However, heparin did not prevent the spontaneous formation of AChR clusters, indicating that the effects are specific for AChE (Fig. 4). This heparin block was reversible (Fig. 6 and Table III), and newly synthesized AChE molecules continued to accumulate once heparin was removed from the medium, suggesting that heparin was interacting directly with the A12 AChE molecule, as shown in Fig. 8, rather than disrupting the binding sites for AChE on the extracellular matrix.

Although our present observations on tissue-cultured myotubes, as well as those in vivo (Rossi and Rotundo, 1993), may appear contradictory to published studies from other laboratories, they are complementary rather than mutually exclusive. The A12 AChE form is assembled intracellularly in the Golgi apparatus (Rotundo, 1984a) and must then be transported to the cell surface and secreted prior to attachment to the extracellular matrix. For this reason, a significant pool of intracellular A12 AChE molecules is usually found in mature muscle fibers (Younkin et al., 1982) as well as in cultured cells (Rotundo, 1984a; Brandan and Inestrosa, 1984). Once externalized, most of the A12 AChE molecules appear to accumulate either in clusters on tissue-cultured myotubes or on specialized regions of the muscle fiber surface in vivo. Therefore, the fraction of A12 molecules solubilized by heparin most likely corresponds to those molecules in the intracellular pool residing in...
the lumen of the Golgi apparatus as well as those that have been externalized but have not yet been strongly attached to the basal lamina. However, once attached, a large fraction of the A₁₂ AChE molecules can no longer be removed by high salt buffers or polyanions, indicating that the association is through more than simply electrostatic interactions. These are the A₁₂ AChE molecules that accumulate in cell-surface AChE clusters in culture or at the neuromuscular junction in vivo.

In addition to the tight association between the A₁₂ AChE molecules and the extracellular matrix, we also find that the A₁₂ AChE molecules undergo transient electrostatic interactions as well. In the presence of heparin, the newly synthesized A₁₂ AChE forms were secreted into the culture medium (Fig. 7) rather than accumulated on the extracellular matrix. Under normal culture conditions, only a small fraction of the total surface AChE appears to be electrostatically associated with the extracellular matrix and extractable with HSB (Fig. 9). When heparin is present to interact with the collagen-like tail of AChE molecules, HSB is unable to extract any of the AChE, indicating that the association is through electrostatic interactions. When heparin is present to interact with the collagen-like tail of AChE molecules, HSB is unable to extract any of the AChE, indicating that the association is through electrostatic interactions.

In summary, our results are consistent with earlier observations and suggest that a sulfated proteoglycan(s) is the target for localizing newly synthesized A₁₂ AChE molecules on the synaptic basal lamina. In the current model, HSP molecules would initially form cell-surface clusters, either spontaneously in nerve-free muscle cultures or induced at sites of nerve-muscle contact. These localized accumulations would then serve as attachment sites for the A₁₂ AChE molecules. This mechanism for localizing AChE at sites of nerve-muscle contact may explain why the appearance of AChE at ectopic synapses in vivo is such a late event compared with the appearance of AChRs (Limo and Slater, 1980) and how AChE can reaccumulate at the original synaptic sites following muscle regeneration in the absence of nerves (Anglister and McMahan, 1985), reinnervation of empty basal lamina sheaths (Anglister, 1991), and restoration of muscle activity by reinnervation through ectopic synapses (Weinberg and Hall, 1979). Furthermore, this model provides a mechanism for regulating the numbers and distribution of AChE molecules on the synaptic basal lamina, where the accumulation of HSP molecules at sites of nerve-muscle contact could act as a molecular “parking lot” for the subsequent insertion and removal of the A₁₂ AChE molecules at the vertebrate neuromuscular junction.

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