Cell Surface Acetylcholinesterase Molecules on Multinucleated Myotubes Are Clustered over the Nucleus of Origin

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Abstract. Multinucleated skeletal muscle fibers are compartmentalized with respect to the expression and organization of several intracellular and cell surface proteins including acetylcholinesterase (AChE). Mosaic muscle fibers formed from homozygous myoblasts expressing two allelic variants of AChE preferentially translate and assemble the polypeptides in the vicinity of the nucleus encoding the mRNA (Rotundo, R. L. 1990. *J. Cell Biol.* 110:715–719). To determine whether the locally synthesized AChE molecules are targeted to specific regions of the myotube surface, primary quail myoblasts were mixed with mononucleated cells of the mouse muscle C2/C12 cell line and allowed to fuse, forming heterospecific mosaic myotubes. Cell surface enzyme was localized by immunofluorescence using an avian AChE-specific monoclonal antibody. HOECHST 33342 was used to distinguish between quail and mouse nuclei in myotubes. Over 80% of the quail nuclei exhibited clusters of cell surface AChE in mosaic quail–mouse myotubes, whereas only 4% of the mouse nuclei had adjacent quail AChE-positive regions of membrane, all of which were located next to a quail nucleus. In contrast, membrane proteins such as Na+/K+ ATPase, which are not restricted to specific regions of the myotube surface, are free to diffuse over the entire length of the fiber. These studies indicate that the AChE molecules expressed in multinucleated muscle fibers are preferentially transported and localized to regions of surface membrane overlying the nucleus of origin. This targeting could play an important role in establishing and maintaining specialized cell surface domains such as the neuromuscular and myotendinous junctions.

T HE establishment of specialized cell surface membrane domains in eukaryotic cells requires that the expressed proteins be correctly targeted to and retained at their appropriate sites of function. In multinucleated skeletal muscle fibers, which in the adult can attain lengths of many millimeters and contain many hundreds of nuclei, this problem is accentuated by the existence of multiple specialized regions such as the neuromuscular and myotendinous junctions. Some proteins, such as acetylcholine receptors (AChRs)[1] and acetylcholinesterase (AChE), are highly concentrated at sites of nerve muscle contact, which account for <0.1% of the total cell surface area of the muscle fiber (for review see Fambrough, 1979; Salpeter, 1987). Studies of adult skeletal muscle have shown that the transcripts encoding synaptic components such as AChRs (Merlie and Sanes, 1985; Fontaine et al., 1988; Goldman and Staple, 1989; Brenner et al., 1990) and AChE (Jasmin, B. J., R. K. Lee, and R. L. Rotundo, manuscript submitted for publication) selectively accumulate in the subsynaptic region of the muscle fiber. This restricted expression of mRNAs encoding proteins destined for the overlying synaptic membrane suggests that skeletal muscle fibers are functionally compartmental-

ized and that individual nuclei are responsible for the expression and organization of cell surface as well as intracellular proteins within a restricted region of the fiber.

Muscle fibers do not require functional innervation nor insertion into tendons in order to exhibit specialized membrane domains. Evidence for functional compartmentalization in skeletal muscle fibers independent of innervation has been obtained using tissue-cultured muscle cells (for review see Hall and Ralston, 1989). In situ hybridization studies using exonic and intronic probes for ACh receptor subunit mRNAs show that only a subset of nuclei within a given myotube express the transcripts (Harris et al., 1989; Bursztajn et al., 1989; Horovitz et al., 1989; Tsim et al., 1992), whereas transcripts encoding another cell surface protein, AChE, are more widely distributed in the fiber and expressed by a majority of the nuclei (Tsim et al., 1992). However, in both cases, the transcripts were found predominantly within a 5–10-μm radius of the nucleus expressing them. A correlation between the distribution of nuclei in myotubes with overlying AChR clusters has been reported (Bruner and Bursztajn, 1986; Englander and Rubin, 1987) where it was postulated that the nuclei accumulated beneath the clusters after their formation. Using mouse–human mosaic myotubes, Pavlath et al. (1989) have shown that several proteins including a resident Golgi enzyme, a myofibrillar protein,

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1. *Abbreviations used in this paper:* AChE, acetylcholinesterase; AChR, acetylcholine receptor.

and a cell surface adhesion molecule were restricted to regions of the fiber near the nucleus encoding them. Expression of a LacZ fusion protein containing a nuclear localization signal in mosaic mouse C2/C12 myotubes resulted in preferential accumulation of this marker in the nuclei expressing the construct and adjacent nuclei (Ralston and Hall, 1989*a*). In primary quail mosaic myotubes expressing two allelic variants of the AChE polypeptide (Rotundo et al., 1988), it was possible to show that the AChE mRNAs were preferentially translated on the RER surrounding the nucleus of transcription and that the polypeptide chains were locally assembled and processed in that RER and Golgi complex (Rotundo, 1990). In contrast to AChE, which localizes to cell surface clusters associated with patches of extracellular matrix material (Wallace, 1989), membrane proteins, which do not interact with other cellular elements, can diffuse over the entire surface of the myotubes regardless of nucleus of origin (Ralston and Hall, 1989*b*). Recent studies using a temperature-sensitive mutant of vesicular stomatitis virus, whose G protein is retained in the RER at the restrictive temperature, to infect tissue-cultured myotubes show that the G protein is selectively transported to the region of the cell surface overlying the RER of translation when the cells are transferred to the permissive temperature (Metsikko et al., 1992). Moreover, these authors showed that when myotubes were simultaneously coinfected with Semliki forest and vesicular stomatitis viruses, their coat glycoproteins were translated, processed, and transported to the cell surface in nonoverlapping nuclear domains, providing strong evidence for vectorial transport of membrane proteins from the intracellular organelles surrounding individual nuclei to the overlying region of the plasma membrane in multinucleated skeletal muscle fibers. Together, these studies suggest that synaptic components expressed by individual nuclei in multinucleated skeletal muscle fibers can be selectively targeted and localized to regions of the cell surface nearest to the nucleus of origin.

In the present studies we show that cell surface AChE clusters form primarily on regions of the muscle membrane overlying nuclei, suggesting that each nucleus is responsible for organizing plasma membrane and extracellular matrix molecules on the overlying region of its cell surface. These cell surface AChE clusters are resistant to extraction with high salt and detergents but can be removed by treatment with collagenase. Using quail–mouse hybrid myotubes and avian-specific anti-AChE antibodies, we show that AChE molecules expressed by a given quail nucleus are selectively targeted to and retained on the region of the fiber surface overlying that nucleus. Together, these observations provide evidence for the existence of cell surface nuclear domains in skeletal muscle fibers and suggest that the localization of AChE molecules at the neuromuscular junction in vivo arises from the selective expression and targeting of this protein from nuclei located in the subsynaptic sarcoplasm to the overlying synaptic basal lamina.

Materials and Methods

Tissue Culture of Primary Quail Muscle and Mouse C2/C12 Cells

Cells were grown on scratched collagen-coated glass coverslips to promote alignment of myoblasts during the period of cell fusion and differentiation. This procedure results in the formation of linear arrays of myotubes, which in turn facilitates analysis of the distribution of nuclei and cell surface clusters of antigens. Acid-washed glass coverslips were coated with concentrated rat tail collagen and baked at 60°C for 48 h before brushing with a piece of triple-0-grade steel wool. The coverslips were then placed in 35-mm tissue culture dishes and sterilized by UV irradiation for 30–60 min before use.

Primary quail skeletal muscle cultures were made from myoblasts obtained from pectoral muscle of 10-d embryos and grown in MEM with 10% horse serum, 2% chick embryo extract, and 50 μg/ml gentamicin (EMEM 210) for 7 d (Rotundo and Fambrough, 1980*a*). All culture media supplies were obtained from Gibco-BRL, Grand Island, NY. Cytosine arabinoside (1 μM final concentration; Sigma Chemical Co., St. Louis, MO) was added from days 3 to 5. Low-serum medium (2% horse serum and 2% embryo extract in EMEM) was used to feed the quail myotubes the day before extraction of AChE and analysis of oligomeric forms by velocity sedimentation to increase the asymmetric collagen-tailed forms.

Mouse-derived C2/C12 cells were passaged as mononucleated cells in DME supplemented with 20% FBS and 0.5% chick embryo extract (growth medium). Differentiation was initiated by replacing the growth medium with fusion medium consisting of DME supplemented only with 2% horse serum, which induces the cells to pull out of the cell cycle and fuse to form multinucleated myotubes.

Quail–Mouse Mosaic Muscle Fibers

Mosaic quail–mouse myotubes were grown by plating primary quail cells on collagen-coated coverslips and then adding C2/C12 mouse cells grown under growth conditions. Generally, 6 × 10⁴ primary quail muscle cells were cultured on the coverslips overnight in EMEM 210. The next day, 1–2 × 10⁴ C2/C12 cells were added to the cultures in growth medium. After 24 h the medium was replaced with fusion medium and the cells were allowed to differentiate overnight. Cultures were then fed with fusion medium containing cytosine arabinoside and refed every 48 h thereafter. The cells were then used for experiments 4 d after onset of cell fusion (day 7 of culture).

Immunofluorescence Localization of AChE and AChR Clusters

AChE clusters were localized by indirect immunofluorescence using the anti-avian AChE monoclonal antibody 1A2 (Rotundo, 1984*a*) followed by fluorescein-conjugated affinity-purified rabbit anti-mouse IgG (FITC-RAM; Cappel Laboratories, Cochranville, PA). AChR clusters were visualized using tetramethyl rhodamine–conjugated α-bungarotoxin (TRITC α-Btx; Molecular Probes, Inc., Junction City, OR). mAb No. 24 against avian Na+/K+ ATPase was the generous gift of Dr. Douglas M. Fambrough (Johns Hopkins University, Baltimore, MD).

For labeling, cultures were rinsed with HBSS and incubated with PBS (pH 7.4) containing 10% horse serum (PBS/HS) and the monoclonal antibody at a concentration of 20 μg/ml for 60 min. Cultures were then rinsed three times with PBS/HS and incubated with fluorescein-conjugated second antibody at 10 μg/ml. In some experiments, cells were preincubated with 1 μg/ml TRITC α-Btx in PBS/HS for 30 min at 37°C to label AChRs. The cells were then rinsed three times with PBS/HS, then PBS alone, followed by fixation with 4% phosphate-buffered paraformaldehyde. After fixation, the cultures were rinsed in PBS and incubated with 1 μg/ml HOECHST 33342 to label the nuclei. Coverslips were then mounted in bicarbonate-buffered glycerol containing 1 mg/ml phenylenediamine and viewed with a Universal microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence with narrow band filters for HOECHST dyes, fluorescein, and rhodamine. Photographs were taken on (Tri-X; Eastman Kodak Co., Rochester, NY) film and developed with Diafine (Acufine, Inc., Chicago, IL).

For histochemical localization of AChE, the method of Koelle and Friedenwald (1949) was used after the fixation and washing step, and the cells were incubated overnight at 5°C. The coverslips were then washed and processed as described above.

Collagenase Treatment of Quail Muscle Cultures

Primary quail muscle cultures were grown on collagen-coated glass coverslips in 35-mm culture dishes as described above. 7-d cultures were rinsed with 2 × 2 ml 10 mM Hepes-buffered HBSS, pH 7.2 (Hepes/HBSS) containing 0.5 mg/ml BSA. Cultures were incubated in the 35-mm dishes in
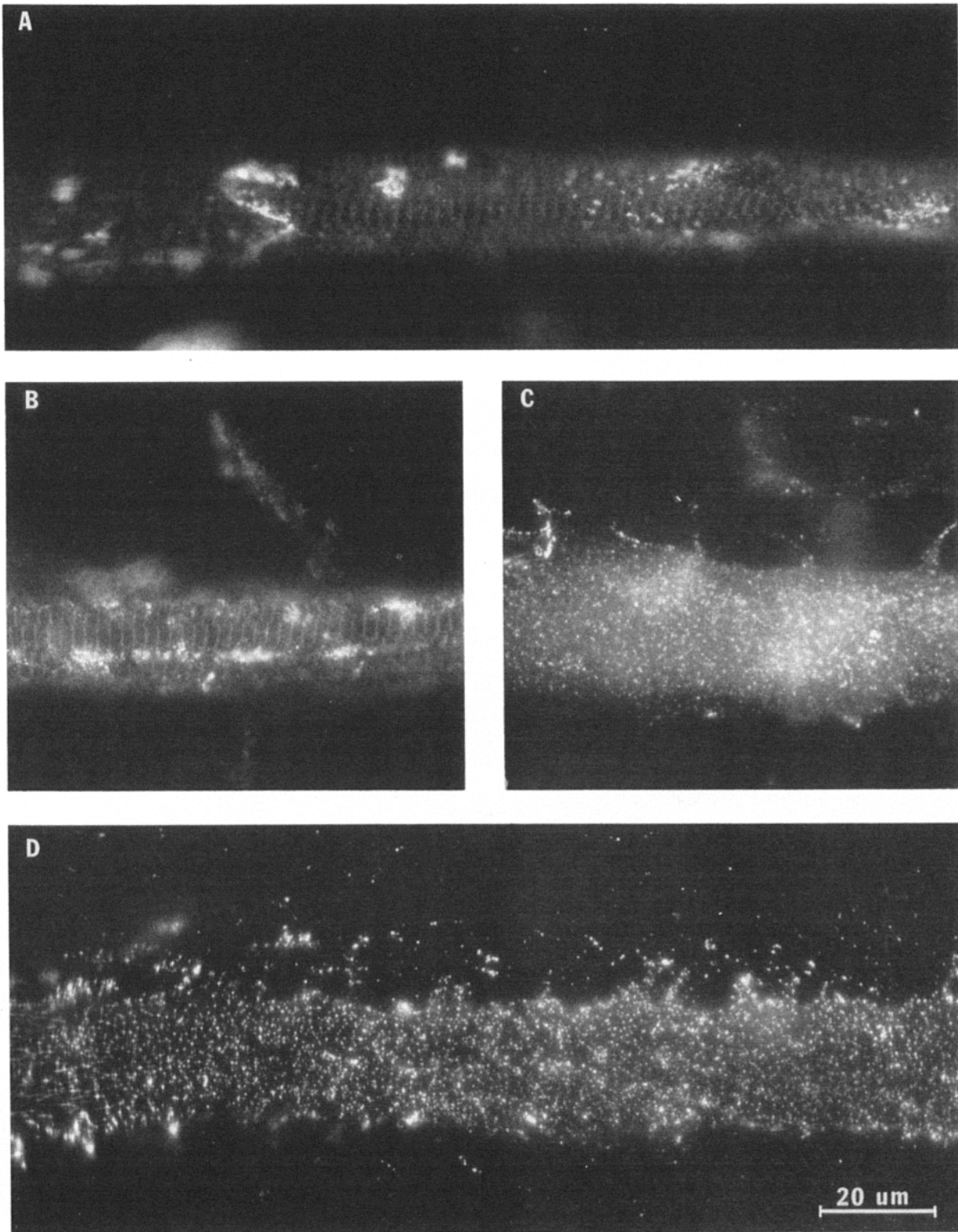
Figure 1. Differential distribution of AChE molecules on upper and lower surfaces of quail myotubes in culture. Primary quail myotubes were extracted with three 1-ml washes of extraction buffer consisting of 20 mM borate, pH 9.0, 1 M NaCl, 1% Triton X-100, and 1 mM EDTA, followed by three rinses with PBS and preincubation for 10 min in PBS containing 10% horse serum. The cultures were then incubated with 1A2 anti–avian AChE followed by FITC rabbit anti–mouse IgG as described in Materials and Methods. *A* and *B* show the distribution of AChE clusters on the upper surface of the myotubes. *C* and *D* show the diffuse punctate of AChE on the regions where the myotube is in direct contact with the substratum. *B* and *C* are, respectively, the upper and lower surfaces of the same region of myotube, which was thick enough to allow "optical sectioning" using a conventional microscope.
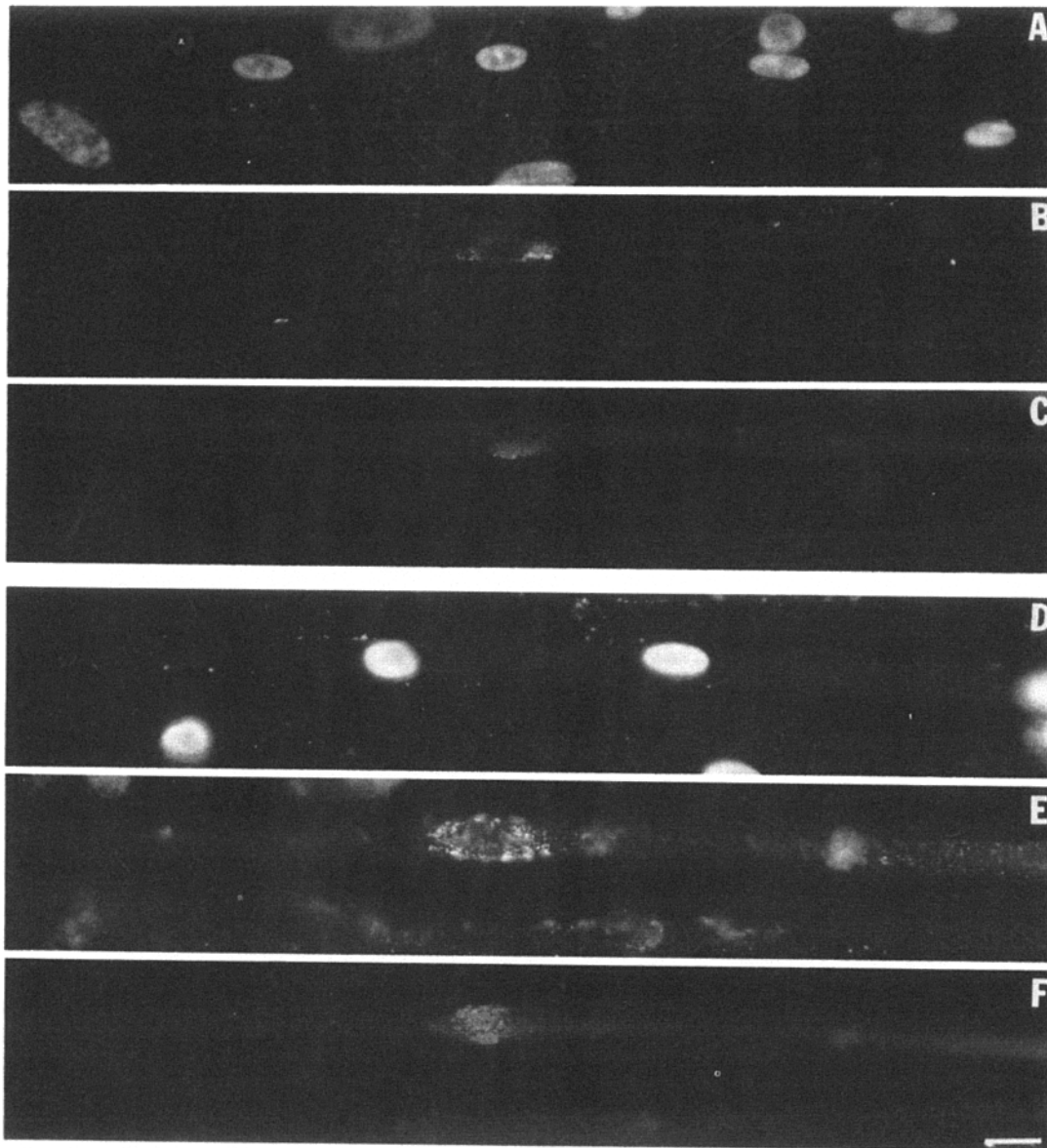
Figure 2. Localization of AChE and AChR clusters over nuclei in primary quail myotubes. Primary quail muscle cultures (7 d) were double stained using rhodamine-conjugated α-bungarotoxin to label AChRs, and with mAb 1A2 followed by FITC-conjugated rabbit anti–mouse IgG to label AChE. The cultures were fixed and incubated with the nuclear stain HOECHST 33342 before mounting in glycerol. *A* and *D* show the distribution of nuclei in individual quail myotubes. *B* and *E* show the distribution of cell surface AChE clusters in the vicinity of the quail nuclei. *C* and *F* show the localization of ACh receptor clusters. Bar, 25 μm.

500 μl Hepes/HBSS with or without 100–500 U/ml collagenase at room temperature on a rotary platform for 1–3 h. Three types of collagenase were used in these studies: purified bacterial collagenase type III (Advanced Biofactures, Lynnbrook, NY), *Clostridium histolyticum* collagenase type IV (C5138; Sigma Chemical Co.), and tissue culture–grade collagenase CLS IV (Worthington, Freehold, NJ). Details of individual experiments are given in Results. After collagenase treatment the cultures were rinsed with Hepes/HBSS and the AChE clusters localized by indirect immunofluorescence as described above. The coverslips were stained with HOECHST 33342 after the fixation step to visualize the nuclei.

Analysis of Cluster and Nuclear Distributions

AChE-rich clusters are defined as conspicuous accumulations of AChE on the upper surface of the myotubes. They are virtually absent from the regions of myotubes in contact with the substratum, where AChE immunofluorescence appeared as a very even punctate distribution. The distance between individual nuclei in myotubes was measured from center to center using a calibrated ocular micrometer and a 40× oil immersion objec-

tive. The distance between nuclei and AChE cluster was measured from the center of a nucleus to the nearest cluster. Quail and mouse nuclei can easily be distinguished by their relative sizes and the presence of clumped chromatin in the mouse nuclei.

Analysis of AChE Oligomeric Forms and Assay of Enzyme Activity

Muscle cultures were rinsed three times with HBSS and extracted in 500 μl borate extraction buffer (20 mM borate buffer, pH 9.0, 1.0 M NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% BSA, and protease inhibitors consisting of 2 mM benzamidine, 0.7 mM bacitracin, and 5 mM N-ethylmaleimide) for three 35-mm culture dishes (Rotundo, 1984b). The extracts were centrifuged for 20 min at 27,000 g. 200-μl aliquots of supernatant were analyzed by velocity sedimentation on isocratic 5–20% sucrose gradients made up in the same buffer minus serum albumin and protease inhibitors. The fractions were assayed for AChE activity using the radiometric assay of Johnson and Russell (1975) as previously described (Rotundo and Fambrough, 1979).
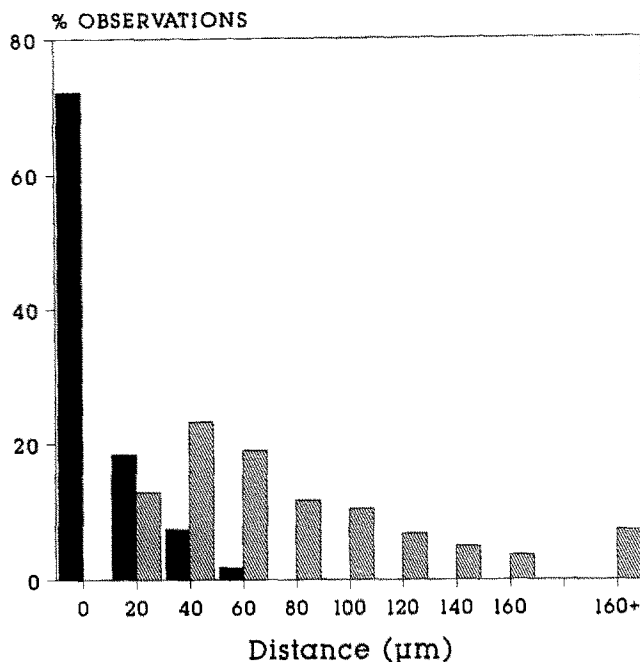
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Figure 3. Distribution of AChE clusters and nuclei in primary quail myotubes. The distances between individual nuclei and nuclei to nearest AChE cluster were measured using a calibrated ocular micrometer and plotted as percent of the total observations that fell within a 20-μm distance class. (*solid bars*) Nuclei-to-nearest AChE cluster; (*hatched bars*) nuclei-to-nuclei. The average diameter of a quail nucleus is 14.5 μm. In primary quail myotubes, 72% of all AChE clusters were localized directly over the nuclei; >85% occurred within one nuclear diameter distance. In contrast, the average distance between individual nuclei was 74 $\pm$ 24 μm ($n = 163$). Thus, AChE clusters are preferentially localized to the regions of the myotube surface overlying the nuclei.

Results

Clusters of AChE and AChR Are Localized over Nuclei in Primary Quail Myotubes

Primary quail myoblasts proliferate and fuse to form multinucleated myotubes during the first 3–5 d in culture, after which they become spontaneously contractile and clusters of AChR and AChE appear on the upper surface of the myotubes. The appearance of cell surface AChE clusters coincides with the expression of the collagen-tailed AChE form, approximately half of which is associated with the cell surface, where it is tightly bound to the extracellular matrix. Both the numbers of AChE clusters and levels of the asymmetric enzyme form continue to increase from days 5 to 9 in culture. That these cell surface clusters are associated with the extracellular matrix is suggested by the observation that they are resistant to extraction by high salts and detergents (Fig. 1). The relationship between cell surface AChE clusters and the underlying myotube nuclei was suggested in preliminary studies to determine the distribution of AChE on tissue-cultured skeletal muscle. Numerous clusters of AChE were distributed over the upper surface on intact myotubes, most frequently in the vicinity of nuclei. The AChE on the upper surface of the myotubes was usually confined to morphologically distinct clusters, in contrast to the diffuse punctate distribution on the lower surface, where the myotubes were in direct contact with the substratum (Fig. 1).

To determine the relationship between individual AChE clusters and the underlying nuclei, 7-d primary quail myotubes were labeled with anti-AChE mAb 1A2 followed by FITC-conjugated anti–mouse IgG and HOECHST 33342 to stain the nuclei (Fig. 2). In some experiments, TRITC-α-Btx was included in the first incubation to label the AChRs, which also occur in clusters. On primary quail myotubes we were able to detect AChRs in only ∼10–20% of the AChE clusters. This could reflect decreased numbers of receptors in the quail muscle cultures; however, we cannot exclude the possibility that this reflects the sensitivity of detection using TRITC-α-Btx. The distance between individual clusters and the center of the nearest nucleus was measured using a calibrated ocular micrometer and the internuclear distances were measured from center to center. Fig. 3 shows the distribution of internuclear distances compared with cluster-nucleus distances in primary quail myotubes. The average distance between myotube nuclei was 74 $\pm$ 24 μm, whereas 85% of the AChE clusters was localized either directly over or within 15 μm (one nuclear diameter) of the nearest nucleus. These observations indicate a high correlation between cell surface clusters and the underlying nuclei, and suggest the possibility that each nucleus is responsible for synthesizing and localizing the AChE molecules on the sarcolemma above it.

Collagenase Removes Cell Surface AChE Clusters; Clusters Contain the Collagen-tailed Asymmetric AChE Form

On tissue-cultured myotubes, the AChE concentrated at clusters is resistant to extraction by detergents even in the presence of high salt concentrations, suggesting that it is tightly associated with the extracellular matrix (Fig. 1). To determine whether the collagen-tailed form was associated with the cell surface clusters, primary quail myotubes were incubated with several different preparations of bacterial collagenase and the AChE clusters localized by indirect immunofluorescence. When possible, AChE clusters were quantitated and their size distributions determined by direct measurement of their longest dimension using an ocular micrometer.

In preliminary studies, cultures were incubated with several concentrations of each collagenase ranging from 100 to 500 U/ml in Hepes/HBSS for periods ranging from 1 to 3 h at room temperature. Three different preparations of collagenase of varying degrees of purity were tested, only one of which was completely free of contaminating proteases (type III; Advanced Biofactures). Only enzyme concentrations and incubation times that completely detached the cells from the substratum were capable of removing the AChE from the clusters. However, digestion with moderate concentrations of highly purified bacterial collagenase type IV (Advanced Biofactures) resulted in a time-dependent decrease in total numbers of AChE clusters per myotube nucleus as well as the absolute sizes of the clusters (Table I). Almost half of the clusters could be completely removed by collagenase from the myotube surface before the cells detached from the substratum, while the remaining clusters were markedly reduced in size and density. These studies together with the results presented in Fig. 1 suggest that the predominant, if not exclusive, AChE form in the cell surface clusters is the asymmetric collagen-tailed AChE form.

Table I. Disruption of Cell Surface AChE Clusters by Collagenase

Treatment	Nuclei	Clusters	Clusters/ Nucleus	Cluster Change	Cluster Size	Cluster Change
	n			%	(μm)	%
1-h control	938	189	0.22 ± 0.09		10.1 ± 6.0	
1-h collagenase	823	164	0.20 ± 0.02	−9	8.2 ± 3.8	−19
3-h control	701	182	0.25 ± 0.02		9.9 ± 5.8	
3-h collagenase	1215	171	0.14 ± 0.03	−44	6.8 ± 4.2	−30

Quail muscle fibers grown on collagen-coated coverslips were rinsed and incubated for the indicated times in Hepes-buffered HBSS with or without 500 U/ml purified bacterial type III collagenase (Advanced Biofactures) at room temperature. Cell surface AChE clusters were localized by indirect immunofluorescence and the nuclei stained with HOECHST 33342 as described in Materials and Methods. Three cultures were used for each group; 10 randomly selected fields per culture were counted using a 40× oil immersion objective. The lengths of the clusters were measured using a calibrated ocular micrometer. Longer periods of incubation or use of partially purified collagenase preparations resulted in detachment of cells from the coverslip (see Results). The number of nuclei refers to the total number of nuclei in multinucleated myotubes counted in the three cultures. The number of clusters are the total number associated with those myotubes. Where appropriate, values are expressed as the mean ± SD of three cultures.

Expression of AChE Oligomeric Forms and Distribution of AChE Enzyme Activity in Mosaic Quail-Mouse Myotubes

When primary quail and mouse C2/C12 cells are cocultured under conditions that promote differentiation of the mouse cells, ∼40% of the cells fuse to form heterospecific mosaic myotubes. After 7 d in culture, and treatment from days 3–5 with 1 μM cytosine arabinoside to reduce proliferating mononucleated cells, ∼50% of the nuclei are found in myotubes. The ratio of quail to mouse nuclei present in the myotubes depends on the initial density of each cell type. The nuclei of the two species can be readily distinguished after staining with HOECHST 33342 since the mouse nuclei are several times larger than the quail nuclei and contain characteristically clumped chromatin. The mosaic myotubes exhibit the morphological characteristics of primary myotubes and express AChE catalytic activity all along their length, as determined by enzyme histochemistry using the Koelle reaction (Fig. 4). Furthermore, all AChE oligomeric forms are expressed in these cultures, including the 16S mouse and 20S
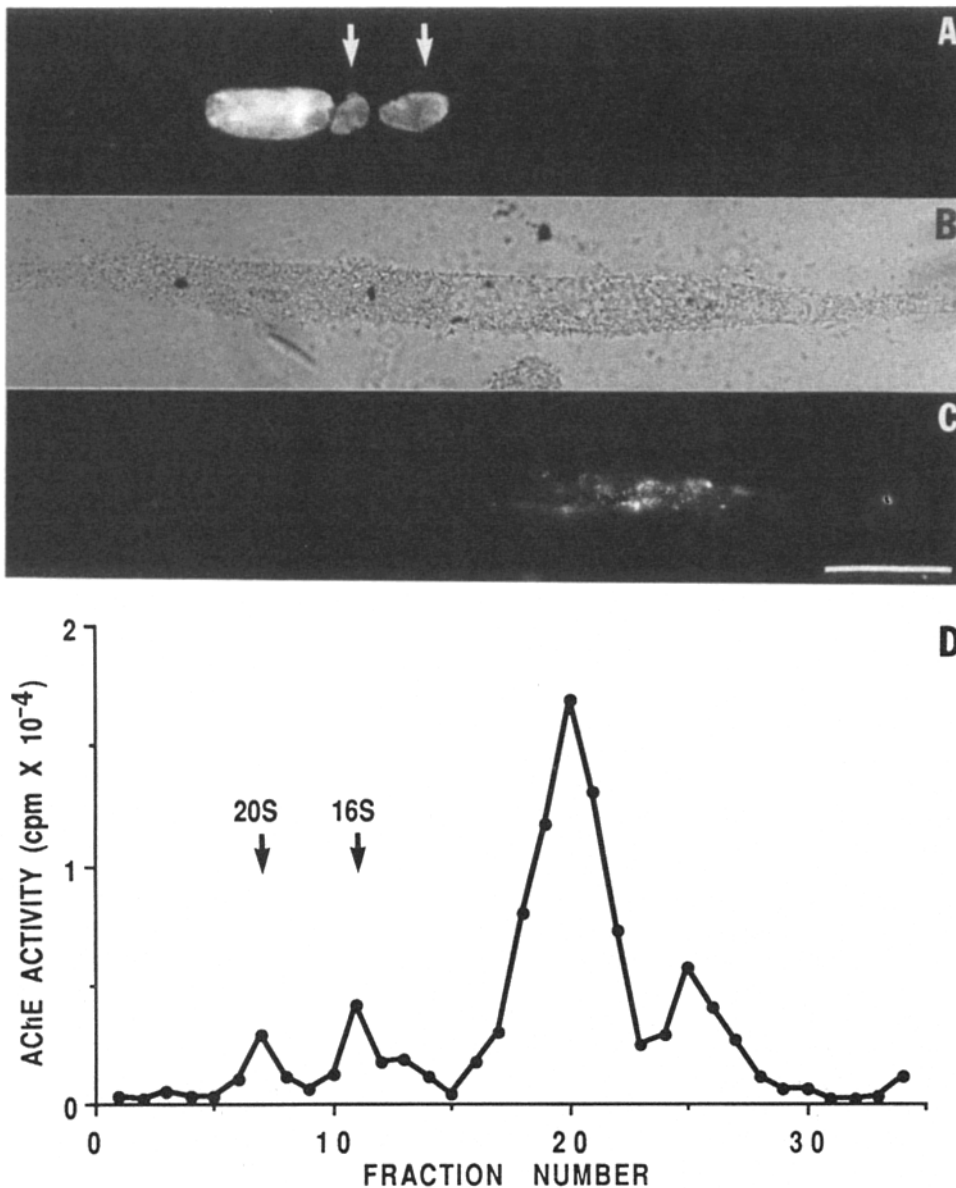

Figure 4. Distribution of AChE activity by enzyme histochemistry and expression of AChE oligomeric forms in mosaic quail-mouse muscle cultures. The distribution of total quail plus mouse AChE activity in mosaic myotubes was determined using the histochemical procedure of Koelle and Friedenwald (1949). The cultures were first incubated with monoclonal anti-AChE antibody followed by FITC-conjugated rabbit anti-mouse IgG. The cultures were then fixed with 4% paraformaldehyde in PBS and incubated in the buffer-substrate mixture for AChE histochemistry. After rinsing, the cultures were also stained with HOECHST 33342 to show the localization of individual nuclei. (*A*) Distribution of quail and mouse nuclei. Arrows indicate the quail nuclei. (*B*) Distribution of total AChE in mosaic myotubes by enzyme histochemistry. The fine punctate reaction product shown is distributed throughout the muscle fiber. (*C*) Distribution of cell surface quail AChE (bar, 25 μm). (*D*) Distribution of avian and murine AChE forms extracted from 7-d muscle cultures and analyzed by velocity sedimentation. AChE activity was assayed using the radiometric method of Johnson and Russell (1975). Both the 20S quail and 16S mouse collagentailed forms are expressed in the mosaic muscle fibers as well as the globular dimeric and tetrameric forms.
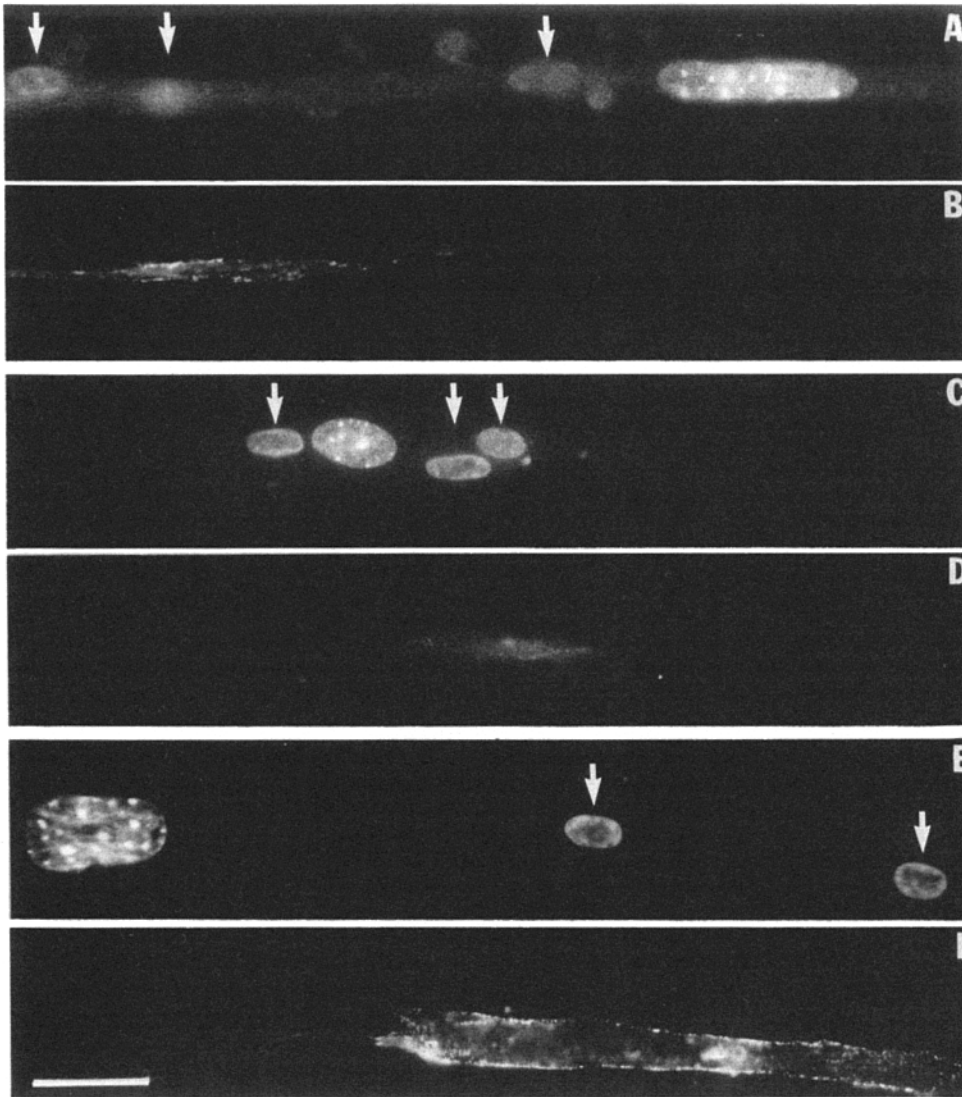
Figure 5. Localization of AChE clusters on mosaic quail–mouse myotubes. Mosaic quail–mouse myotubes were generated by co-culturing quail myoblasts with C2/C12 mouse myoblastic cells in fusion medium. The C2/C12 cells were added to the quail cells 24 h after initial culturing. Staining was done and measurements were made as described in Fig. 2. (*A*, *C*, and *E*) Distribution of nuclei. (*B*, *D*, and *F*) Distribution of AChE clusters. The mouse nuclei are large and characteristically stained with HOECHST while the quail nuclei indicated by the arrows are much smaller and more homogeneously stained. Note the accumulation of AChE in the vicinity of quail nuclei. Bar, 25 μm.

quail collagen–tailed forms of the enzyme, which are the predominant types localized in the clusters (Fig. 4 *D*).

Clusters of Avian AChE on Mosaic Quail–Mouse Myotubes Are Localized Exclusively over Quail Nuclei

To test the hypothesis that localized targeting of AChE molecules to the cell surface occurs in skeletal muscle fibers, we took advantage of the fact that primary quail myoblasts can fuse with mononucleated cells of the C2/C12 mouse muscle cell line to form heterospecific mosaic myotubes. The quail AChE can be readily detected using our monoclonal anti-avian AChE antibody 1A2, which does not cross-react with mouse AChE (Rotundo, 1984*a*). For the present studies, we introduced approximately equal numbers of quail and mouse nuclei in each myotube; under these conditions, ~80% of the quail nuclei exhibited clusters of cell surface AChE (Fig. 5). When quail AChE clusters did appear in the vicinity of mouse nuclei, there was always a quail nucleus nearby (Fig. 6). This observation is significant because it indicates that the mouse nuclei did not express factors that could inhibit the formation of avian AChE clusters in their vicinity.

The distances between individual nuclei and between nuclei and AChE clusters, regardless of species of origin, in quail–mouse mosaic myotubes were determined as described for primary quail myotubes. The average distance between nuclei in the mosaic myotubes was 64 ± 25 μm and showed a similar distribution to the internuclear distances observed in primary quail cultures (Fig. 7). Of the total number of AChE clusters counted, 96% were localized in the vicinity of a quail nucleus; of these, >80% were either directly over or within a 20-μm radius from the center of the quail nucleus. This highly circumscribed localization of AChE molecules occurred even in mosaic myotubes in which only one quail nucleus was present, clearly illustrating that the source of the enzyme was the underlying nucleus. This preferential accumulation of AChE molecules on the cell surface overlying the nucleus encoding them indicates that this protein is selectively targeted to and retained on the overlying specialized membrane domain.

Avian Na+/K+ ATPase in Mosaic Myotubes Is Not Restricted to Nuclear Domains

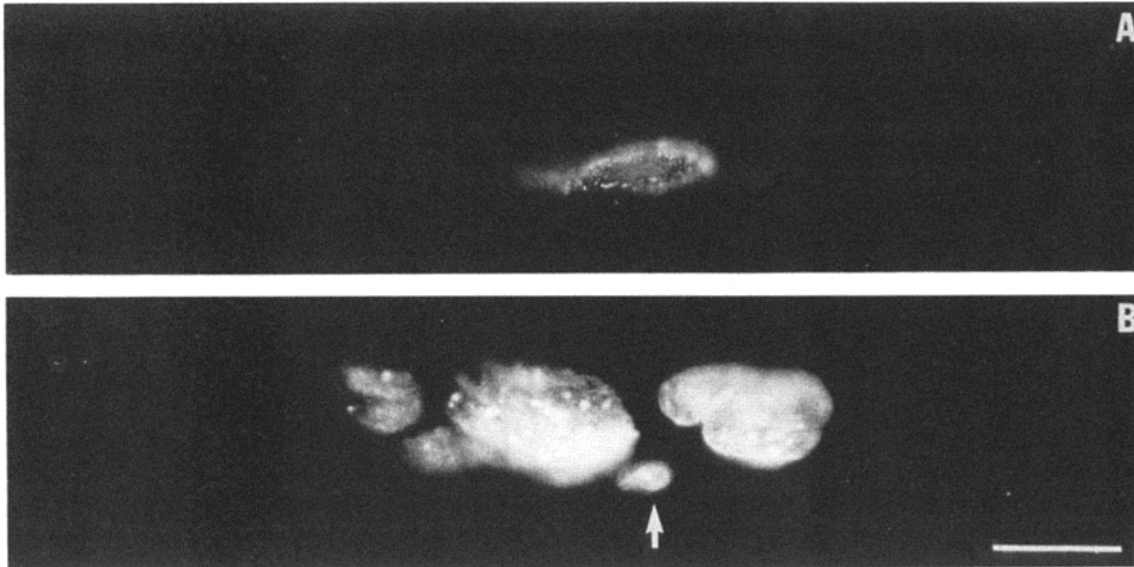In contrast to molecules such as the collagen-tailed form of

Figure 6. Mouse nuclei do not inhibit formation of AChE clusters over quail nuclei. Occasionally, clusters of AChE activity could be observed in the vicinity of mouse nuclei in mosaic myotubes. However, these accounted for only ∼4% of the cases; in every instance, there was a quail nucleus adjacent to the mouse nucleus. These occasional observations indicate that the mouse nuclei are not acting to inhibit the formation of quail AChE clusters on their overlying regions of membrane. (*A*) Cluster of AChE visualized by indirect immunofluorescence. (*B*) Mouse and quail nuclei stained with HOECHST 33342 in the same field. The arrow shows the position of the quail nucleus. Bar, 25 μm.

AChE, which are tightly associated with the extracellular matrix, integral membrane proteins are free to diffuse in the plane of the lipid bilayer unless specifically anchored to either intra- or extracellular structures. To determine whether integral membrane proteins were freely diffusible in our mosaic quail-mouse cocultures, we examined the cell surface distribution of the quail $Na+/K+$ ATPase under the same conditions used for observing the distribution of AChE clusters. As shown in Fig. 8, even a single quail nucleus present in a predominantly mouse myotube expressed sufficient $Na+/K+$ ATPase to diffuse over the entire surface of the myotube, clearly indicating the absence of diffusion barriers to integral membrane proteins.

Discussion

We have previously shown that mosaic myotubes made from homozygous quail myoblasts expressing two different allelic variants of AChE preferentially translate the polypeptides on the RER surrounding the nuclei expressing the transcripts (Rotundo, 1990). The polypeptides were also locally assembled in the RER into disulfide-bonded dimers and presumably processed in the Golgi region surrounding that nucleus. These studies indicated that the myotubes were functionally compartmentalized with respect to the expression and translation of mRNAs encoding this cell surface protein. Since clusters of AChE are found distributed over the entire length of tissue-cultured quail myotubes (Figs. 1 and 2) and the vast majority are localized directly over nuclei or within a 20-μm radius (Figs. 2 and 3), these observations suggested the possibility that AChE molecules expressed by a given nucleus were selectively targeted to the region of the cell surface overlying that nucleus.

In the present studies, we used mosaic quail-mouse myo-

tubes to test the hypothesis that localized clusters of AChE form over the nucleus encoding them. Our choice of quail-mouse mosaic fibers for studying AChE localization was based on the following observations. The primary quail myoblasts readily fuse with the mouse C2/C12 cells to form heterospecific myotubes (Fig. 4). Both cell types express all oligomeric forms of AChE (Inestrosa et al., 1982; Rotundo, 1984*b*) and continue to do so when cultured together (Fig. 4 *D*). The C2/C12 cells also express clusters of AChE on their surface that consist entirely of the 16S mouse collagen-tailed form (Inestrosa et al., 1982). In all species examined including electric rays (Bon et al., 1978; Vigny et al., 1983; Brandan et al., 1985), rat (Torres and Inestrosa, 1985), chicken (Ramirez et al., 1990), and quail (Rossi, S., and R. Rotundo, unpublished observation), the asymmetric collagen-tailed AChE form appears to be attached to the extracellular matrix via the glycan portion of a heparan sulfate-type proteoglycan, the glycan portion being the same across all species. Both quail and mouse nuclei expressed AChE and these molecules are distributed throughout the myotubes (Fig. 4). However, since our antibody is specific for the avian AChE, only the quail AChE clusters were detected by cell surface labeling (Figs. 4-6). Thus, the formation of AChE clusters strictly over quail nuclei, which express the quail AChE transcript, is evidence for the selective targeting of this protein to the overlying region of the membrane.

Although an alternative explanation that could account for these findings is suppression or inhibition of AChE cluster formation in the vicinity of the mouse nuclei, this possibility is unlikely for the following reasons. First, C2/C12 cells cultured alone form AChE clusters on their surface (Inestrosa et al., 1982). Second, the C2/C12 nuclei in mosaic myotubes express AChE and the enzyme is detected surrounding all the
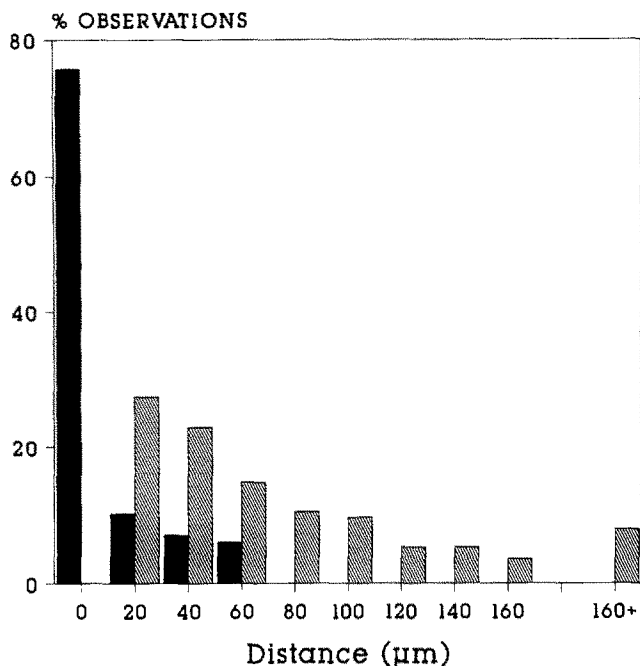
% OBSERVATIONS

Distance (µm)

Figure 7. Distribution of AChE clusters and nuclei in mosaic quail-mouse myotubes. The distances between nuclei or nuclei and AChE clusters refer to the distances to the nearest nucleus regardless of species of origin. Measurements were made as in Fig. 3. (solid bars) Nuclei-to-AChE cluster distance; (hatched bars) nucleus-to-nucleus distance. The distribution of AChE clusters and the average distance between nuclei were very similar between primary quail and mosaic myotubes (64 ± 25 µm); 76% of the AChE clusters were directly over quail nuclei and 83% were within one nuclear diameter. Only 4% of the AChE clusters were near mouse nuclei. In these cases, there was always a quail nucleus in close proximity. A total of 113 nuclei were measured in mosaic myotubes. We conclude that AChE clusters are restricted to the regions of the myotube surface overlying the nuclei expressing the AChE.

mouse nuclei (Fig. 4). Finally, in a small number of cases, clusters of quail AChE could be found adjacent to and slightly overlying mouse nuclei in mosaic myotubes (Fig. 6), yet in every one of those instances there was an adjacent quail nucleus. Since the C2/C12 cells are also capable of organizing AChE clusters on their surface when cultured alone (Inestrosa et al., 1982), we infer that selective targeting of mouse AChE molecules to the regions overlying their nuclei must also be occurring. Since AChE as well as AChR molecules are cotransported to the plasma membrane via a constitutive pathway in myotubes (Rotundo and Fambrough, 1980a,b; Porter-Jordan et al., 1986), the present studies suggest that a similar transport pathway is established from the Golgi region surrounding each nucleus to the overlying region of the plasma membrane in multinucleated skeletal muscle fibers.

Several lines of evidence from both in vitro as well as in vivo studies over the past few years have established that skeletal muscle fibers are compartmentalized with respect to transcription, translation, assembly, and localization of several classes of skeletal muscle proteins. Transcripts encoding all subunits of the nicotinic AChR (Merlie and Sanes, 1985; Fontaine et al., 1988; Goldman and Staple, 1989; Brenner et al., 1990) and the synaptic isoform of AChE (Jasmin,

B. J., R. K. Lee, and R. L. Rotundo, manuscript submitted for publication) have been shown to be selectively expressed at the neuromuscular junction in adult skeletal muscle. In tissue-cultured cells, transcripts encoding components of the myofibrillar apparatus and a resident Golgi enzyme have been shown to be locally translated and assembled in mosaic myotubes (Pavlath et al., 1989). Expression of transcripts encoding proteins with an "exposed" nuclear localization signal are preferentially transported into the nuclei encoding them and within a short distance away, whereas in the absence of this signal the molecules are free to diffuse over long distances within the myotubes (Ralston and Hall, 1989a). Furthermore, recent studies using the temperature-sensitive mutant of vesicular stomatitis virus G protein that remains in the RER at the restrictive temperature show that the G protein is transported to the overlying region of the plasma membrane when the block is released (Metsikko et al., 1992). These studies provide convincing evidence for a localized constitutive transport pathway leading from a particular nucleus to the local region of the cell surface.

The accumulation of AChE clusters on the surface of myotubes or at the neuromuscular synapse requires the presence of other plasma membrane and extracellular matrix molecules. In vivo, AChE at the neuromuscular junction consists predominantly of the asymmetric collagen-tailed forms (for review see Toutant and Massoulié, 1987; Rotundo, 1987), where it is attached to the synaptic basal lamina (McMahan et al., 1978). Collagenase has been shown to remove essentially all AChE associated with the synaptic basal lamina using enzyme histochemistry as an endpoint (Hall and Kelly, 1971; Betz and Sakmann, 1973), suggesting that the predominant AChE form at sites of nerve-muscle contact is the asymmetric collagen-tailed form. In tissue-cultured myotubes, the AChE clusters also appear to be associated with the extracellular matrix (Wallace, 1989; Fig. 1; Table I). Factors such as agrin, which induce formation of AChR clusters on myotubes (Godfrey et al., 1984), also induce accumulations of AChE (Wallace et al., 1985), heparan sulfate proteoglycan and the cytoplasmic 43-kD AChR-associated protein (Wallace, 1989), and several cytoskeletal proteins known to accumulate at the vertebrate neuromuscular junction including α-actinin, filamin, and vinculin (Shadiack and Nitkin, 1991). In fact, proteins expressed from nearby nuclei in quail-mouse mosaic cells can even cooperate to establish clusters of AChRs on the cell surface as shown in recent studies using a glycosaminoglycan-deficient mouse muscle cell line (Gordon et al., 1992). In contrast to proteins such as the AChR and AChE, membrane proteins not known to be restricted to specific cell surface domains in tissue-cultured skeletal muscle, including the Na+/K+ ATPase (Pumplin and Fambrough, 1983; Fig. 8) and transfected CD8 (Ralston and Hall, 1989b), are free to diffuse over the entire myotube surface and are not associated with the nucleus encoding that protein. Thus, a picture emerges whereby each nucleus is responsible for expressing many, if not all, of the proteins necessary for organizing specialized cell surface domains, including the necessary extracellular matrix components, on the overlying region of the cell surface.

These studies bear directly on the question of how AChE molecules, and other synaptic components, are targeted to the neuromuscular junction. In addition to AChE and
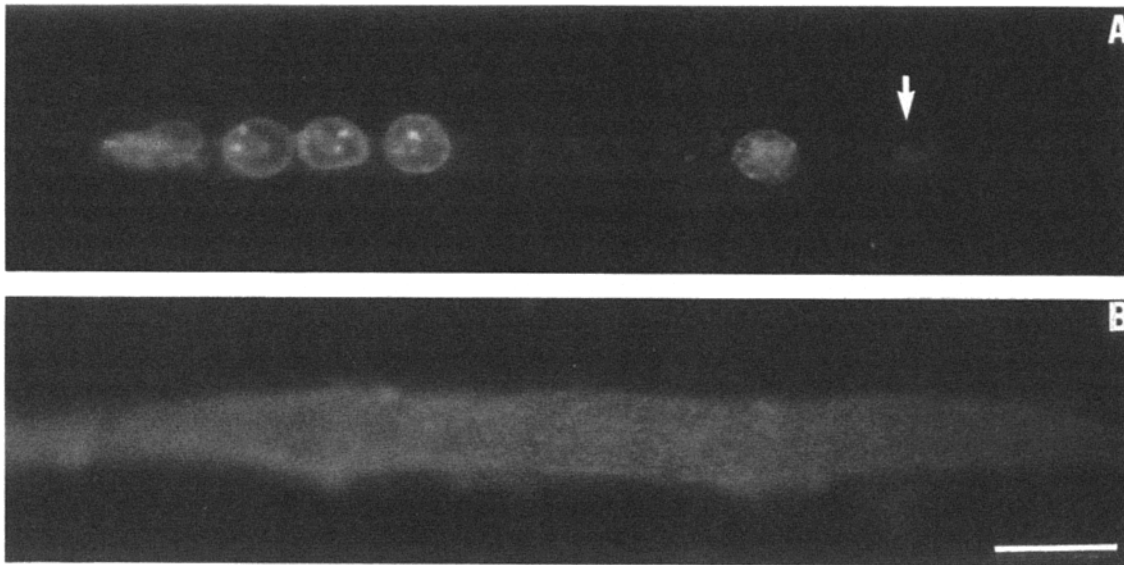
Figure 8. Distribution of cell surface avian Na+/K+ ATPase on mosaic quail–mouse myotubes. In the absence of specific mechanisms for restricting their movement, transmembrane proteins are free to diffuse in the plane of the lipid bilayer. In this experiment, mosaic quail–mouse myotubes were stained by indirect immunofluorescence with a monoclonal antibody specific for the avian Na+/K+ ATPase. *A* shows the distribution of nuclei in the mosaic quail–mouse myotube. *B* shows the distribution of Na+/K+ ATPase in the same myotube. In all cases, the avian Na+/K+ ATPase was distributed over the entire surface of the myotubes, regardless of nuclear composition. The presence of a single quail nucleus in a mixed myotube expressed sufficient ATPase to be detected over the entire myotube surface. The arrow points to the single quail nucleus in this fiber. Bar, 25 μm.

AChRs, several cell surface molecules expressed by muscle cells have been shown to accumulate at the neuromuscular synapse, including S-laminin (Hunter et al., 1989), heparan sulfate proteoglycan (Bayne et al., 1984), and voltage-dependent sodium channels (Betz et al., 1984; Beam et al., 1985). Several cytoskeletal and cytosolic proteins, which act in part to stabilize the membrane proteins at the neuromuscular junction, also accumulate in this region (for review see Bloch and Pumplin, 1988; Froehner, 1991). Additional specializations also occur in the subsynaptic domain of the skeletal muscle fiber, including unique arrays of specialized microtubules (Jasmin et al., 1990) and increased expression of certain specific Golgi antigens (Jasmin et al., 1989) in the innervated portions of the muscle fiber. Since AChE enzyme and its mRNA are highly concentrated at the neuromuscular junction (Jasmin, B. J., R. K. Lee, and R. L. Rotundo, manuscript submitted for publication), the enzyme preferentially translated on the RER surrounding the nucleus of transcription, and the polypeptide chains assembled and processed in that RER and Golgi region (Rotundo, 1990), it seems reasonable that selective transport to localized regions of the cell surface should also occur. The present studies suggest that once assembled, the AChE oligomeric forms are targeted to the overlying region of the cell surface, where they may be selectively retained by components of the extracellular matrix. Thus, compartmentalized transcription, translation, and processing alone are not sufficient to establish specialized cell surface domains; interactions with other cellular components must be necessary. These results further suggest that, in vivo, molecules expressed in the vicinity of the neuromuscular junction will be selectively targeted and retained at that specialized cell surface domain and that the spatial and temporal regulation of gene expression at the neuromuscular junction can regulate the local accumulation of synaptic components.

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