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Golgi apparatus in chick skeletal muscle: Changes in its distribution during end plate development and after denervation

(acetylcholine receptor/neuromuscular junction/synaptic plasticity)

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ABSTRACT **In the course of studies about the cellular and molecular mechanisms of motor end plate formation, the distribution of the Golgi apparatus (GA) has been investigated by immunofluorescence methods in chick skeletal muscle in primary culture and in innervated muscles of 15-day-old chicks. By using a monoclonal antibody directed against the GA, we confirmed the known distribution of the GA in myogenic cells: a juxtanuclear polarized organization in myoblasts and a perinuclear nonpolarized distribution in myotubes. In contrast, the innervated anterior latissimus dorsi muscle of "young adult" chicks displayed a focal distribution of GA that appeared restricted to areas located underneath the motor end plates identified by α-bungarotoxin fluorescent labeling of the acetylcholine receptor. Five days after denervation of anterior latissimus dorsi muscle, a striking reorganization and expansion of the GA was observed. The GA now showed a perinuclear distribution in close association with every nucleus of the muscle fibers as observed in myotubes. The focal distribution of the GA in innervated muscle fibers and its remodeling upon denervation are interpreted in terms of a model of local synthesis, processing, and routing of acetylcholine receptor to the end plate and of regulation of these processes by functional motor innervation.**

A critical process in the development of the neuromuscular junction in vertebrates is the clustering and accumulation of the nicotinic acetylcholine receptor (AChR) at the level of the postsynaptic domain starting at early stages of development up to a few weeks after birth. In mononucleated differentiated myoblasts, significant levels of AChR are detected by α-bungarotoxin labeling (1). After fusion of myoblasts into myotubes, a significant burst of AChR biosynthesis occurs, yielding an increase in surface density that reaches 100–500 α-bungarotoxin sites per μm^2 with an even distribution over the membrane surface (for review, see refs. 2–6). Upon innervation, AChR rapidly accumulates under the exploratory motor nerve endings and becomes immobilized, and several of its functional properties along with its metabolic turnover are modified (3, 4, 7, 8).

The molecular mechanisms involved in the accumulation, maintenance, and renewal of AChR at the level of the neuromuscular junction are currently being investigated in several laboratories (reviewed in refs. 5 and 7). For example, Merlie and Sanes (9) have reported that end plate-rich sections of mouse diaphragm muscle contain significantly higher steady-state levels of α- and δ-subunit mRNA compared to extrajunctional areas. Furthermore, *in situ* hybridization experiments performed by Fontaine and coworkers (10) have shown that in innervated 15-day-old chick muscle, only the subsynaptic nuclei express significant levels of α-subunit mRNA. Such a restricted pattern of α-subunit gene expression results from the activity-dependent repression of α-subunit gene transcription in extrajunctional nuclei during motor end plate formation as revealed with genomic probes, including strictly intronic ones (11). Thus, a mechanism of local synthesis and focal insertion of AChR is likely to be involved in the maintenance of high AChR concentration at the end plate level (3, 4, 12).

A fundamental issue raised by these observations thus becomes the intracellular routing and targeting of the AChR to the end plate in skeletal muscle. In this context, several lines of evidence suggest that during its intracellular transport, the AChR is conveyed to the Golgi apparatus (GA). Glycosylation of AChR subunits has been demonstrated (13, 14) and is probably carried out by enzymes located within the GA (15). However, despite its likely involvement in AChR processing (16, 17), the precise location and organization of the GA in adult skeletal muscle have not been established. By using a monoclonal antibody to decorate the GA, we now examine the distribution of this organelle in chick skeletal muscle during development and after end plate formation. Since denervation increases the number of extrajunctional nuclei expressing the AChR α-subunit gene (10), we also examine GA distribution in denervated muscle.

MATERIALS AND METHODS

Preparation of the Anti-Golgi Antibody. The CTR433 anti-Golgi antibody used in the present work was obtained from a library of monoclonal antibodies raised against centrosomes isolated from human KE37 lymphoblastic cells.¶ This antibody reacts with a Triton-X-100-extractable antigen in the Golgi domain of cultured cells from many mammalian species.

Tissue Preparation. Ten-day-old chicks had left anterior latissimus dorsi (ALD) muscle surgically denervated by cutting selectively the nervis latissimus dorsi. Contralateral muscles and muscles obtained from unoperated animals served as controls yielding identical results. Five days after the denervation procedure, left and right ALD muscles were excised and fixed with 3% paraformaldehyde/0.1 M phosphate buffer, pH 7.4, at 4°C. Subsequently, they were impregnated with 25% sucrose (wt/vol) and rapidly frozen in melting Freon R22 cooled by liquid nitrogen.

Frozen sections (4 μm) were obtained by cutting longitudinally the muscles in a cryostat (SLEE, London) at −20°C.

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Abbreviations: AChR, acetylcholine receptor; ALD, anterior latissimus dorsi; GA, Golgi apparatus.

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¶Bailly, E., Berges, J., Bordes, N., Celati, C. & Bornens, M., European Symposium on the Structure and Functions of the Cytoskeleton, April 13–16, 1988, Lyon, France.

The sections were recovered onto ovalbumin-coated glass slides, air dried, and stored at −70°C until immunofluorescence analyses.

Immunofluorescence of Frozen Sections. Antigens of the GA present in the frozen sections were detected by indirect immunofluorescence. After washing in phosphate-buffered saline (PBS), the sections were permeabilized with 0.1% Triton X-100 in PBS for 5 min and then thoroughly washed in PBS. Nonspecific binding was blocked by preincubation of the sections in PBS containing 5% decomplemented goat serum and 1% bovine serum albumin for 15 min. Sections were then incubated with the anti-Golgi antibody for 1–12 hr (undiluted culture supernatant). Tetramethylrhodamine-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used as a second antibody. Neuromuscular junctions were identified by fluorescein isothiocyanate-conjugated α-bungarotoxin (1 μg/ml; Sigma). This staining was further amplified by addition of an antitoxin antibody (5 μg/ml; kindly provided by R. Sealock, University of North Carolina, Chapel Hill).

Immunocytochemistry of Cells in Culture. Primary cultures of chick leg muscles were obtained and cultured as described (18). Myoblasts (1 day of culture) and myotubes (5–7 days) were fixed briefly in 3% paraformaldehyde, then fixed with methanol at −20°C, and processed for immunofluorescence experiments as described for cryostat sections.

HeLa cells were grown in Earle modified Eagle medium (EMEM) containing 1% nonessential amino acids and 2 mM glutamine and complemented with 10% fetal calf serum and antibiotics. Nocodazole (1 μM) (Janssen Pharmaceutica) or taxol (5 μM) (a generous gift from D. Guénard, Institut des Substances Naturelles du Centre National de la Recherche Scientifique, Gif-sur-Yvette, France) was added for 1 or 6 hr, respectively, in some experiments. These cells were fixed with methanol at −20°C for 6 min. They were then processed for immunofluorescence after immersion in PBS containing 0.1% Tween 20. Fixed cells were incubated for 30 min with the monoclonal antibody CTR433 together with affinity-purified rabbit antibodies raised against human β-galactosyltransferase (19, 20).

Micrographs were taken with a Leitz photomicroscope equipped with epifluorescence illumination (filters for rhodamine, fluorescein, and 4′,6-diamidino-2-phenylindole) and with plan ×63 (numerical aperture, 1.40) or ×100 (numerical aperture, 1.32) immersion optics.

For electron microscopic experiments, immunoperoxidase detection of the GA in chick myotubes was carried out as described in detail elsewhere (21). Pictures were taken with a Philips 410 electron microscope.

RESULTS

Characterization of the CTR433 Anti-Golgi Antibody. The CTR433 antibody used in the immunofluorescence studies was selected from a library of monoclonal antibodies raised against human centrosomes. Its specificity as a GA marker was assessed in human cells (HeLa cells) by comparison of its immunofluorescence localization with that of the enzyme β-galactosyltransferase, a *bona fide* marker of the transside of the GA (19, 20). Fig. 1 *A* and *B* show that the two stainings were congruent at the optical level. Furthermore, such congruence was conserved after dispersal of the GA within the cytoplasm following microtubule disruption by nocodazole (Fig. 1 *C* and *D*) or redistribution in paramarginal areas after taxol-induced assembly of the whole cellular pool of tubulin (Fig. 1 *E* and *F*).

Assessment of the validity of the CTR433 antibody as a marker for the GA in chick myogenic cells was verified by using myoblasts and myotubes in culture. A polarized juxtanuclear labeling in each individual myoblast was observed

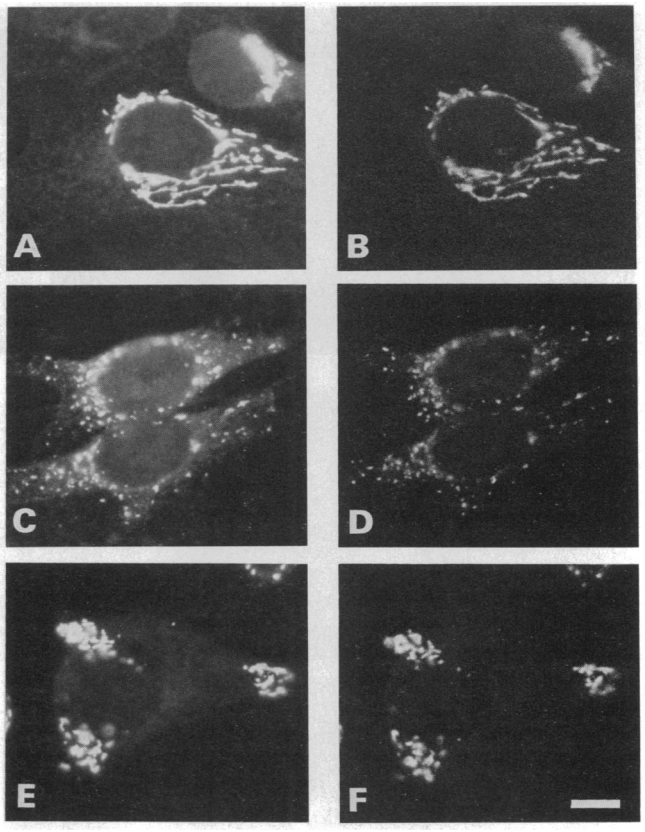

FIG. 1. Double-labeling experiments of HeLa cells. Left (*A*, *C*, and *E*) and right (*B*, *D*, and *F*) columns represent CTR433 anti-Golgi antibody and β-galactosyltransferase, respectively. The cells show GA staining after culture in EMEM (*A* and *B*) and after nocodazole (*C* and *D*) and taxol (*E* and *F*) treatment. Note the perfect codistribution of the two markers. (Bar = 10 μm.)

(Fig. 2*A*). Such distribution is consistent with the known polarity of these mononucleated cells (21, 22). In contrast, the GA labeling in myotubes (Fig. 2 *B* and *C*) corresponded to its characteristic perinuclear organization, which involves a physical association between the GA and the nuclear periphery (21, 23–25). At the electron microscopic level, the CTR433 labeling in myotubes was confined to the GA (Fig. 2*D*) and, furthermore, appeared specific for the medial compartment of the GA (Fig. 2 *E* and *F*).

Distribution of the GA in Innervated Chick Muscles. The distribution of the GA in innervated chick muscles was examined in ALD and posterior latissimus dorsi (PLD) muscles of 15-day-old chick (see ref. 10). The GA distribution in these "young adult" muscles appeared strikingly different from that of the cultured myotubes. As illustrated in Fig. 3 for ALD, the GA was restricted to discrete, highly focalized regions that appeared strictly codistributed with end plates revealed by fluorescein isothiocyanate conjugated α-bungarotoxin. No GA labeling was detected, within the resolution of our technique, close to or around nuclei in the extrajunctional areas of the muscle fibers. Similar observations were made on the PLD muscle. Thus, upon innervation, an extensive remodeling of GA distribution takes place.

Effect of Denervation on GA Distribution in Chick Muscles. Five days after sectioning of the nervis latissimus dorsi in 10-day-old chicks, ALD muscle showed intense AChR labeling of its plasma membrane (Fig. 4*A*). Such reappearance of extrajunctional AChR unequivocally showed that the muscle was denervated. In addition, we observed perinuclear AChR labeling at the level of nearly all muscle nuclei (Fig. 4 *A* and *C*). This labeling most likely corresponded to the

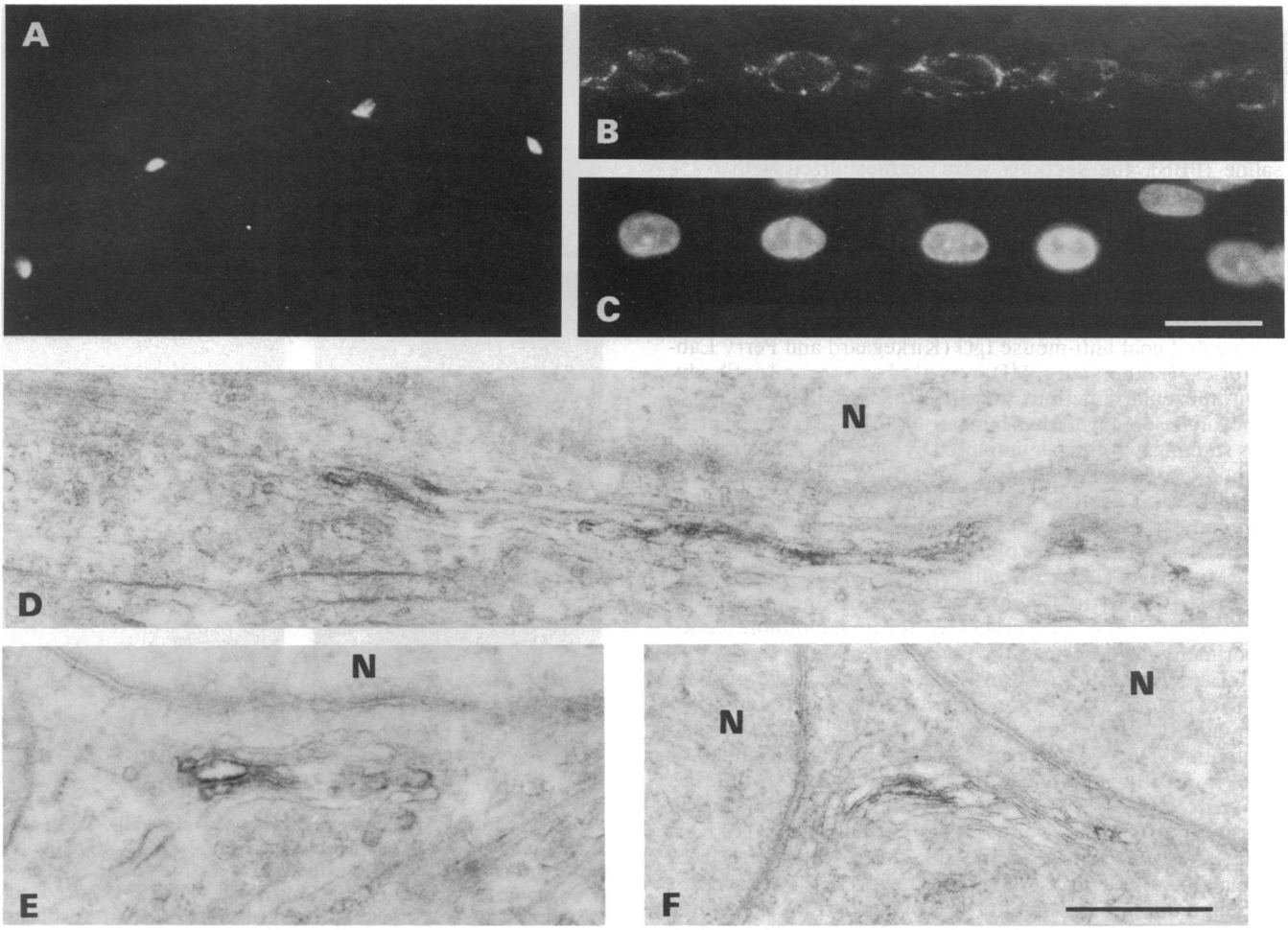

FIG. 2. GA distribution in chick myogenic cells using the CTR433 monoclonal antibody. (*A*) Labeling in myoblasts (1 day of culture). (*B*) Labeling in myotubes (7 days of culture). Compare the juxtanuclear (*A*) to the perinuclear (*B*) distribution. Note in *B* some longitudinal labeling in the sarcoplasm. (*C*) The nuclei of the myotube showing the perinuclear GA were labeled with 4′,6-diamidino-2-phenylindole. (Bar = 20 μm). (*D–F*) Immunoperoxidase localization of CTR433 GA labeling. Note that the labeling is strictly confined to the medial GA compartment. N, nuclei. (Bar = 1 μm.)

perinuclear endoplasmic reticulum where newly synthesized α subunits first acquire α-bungarotoxin binding sites (26, 27).

Examination of the GA in denervated ALD disclosed a striking development that paralleled the reexpression of AChR by extrajunctional nuclei. A perinuclear distribution of the GA at the periphery of every nucleus, an organization typical of myotubes (Fig. 2 *B* and *C*), was observed in denervated muscles (Fig. 4 *B* and *D*). Furthermore, double-labeling experiments demonstrated a codistribution of AChR and of the GA around the muscle nuclei (Fig. 4 *C* and *D*). Even the subjunctional nuclei displayed a perinuclear GA, contrasting with the focal distribution observed in innervated muscle (Fig. 3).

DISCUSSION

The data reported in this paper concern the location and organization of the GA in chick skeletal muscle at various developmental stages and after denervation using the monoclonal antibody CTR433. The validity of this antibody as a GA marker was evaluated in several ways. (*i*) In human cells, we saw superimposable stainings between CTR433 and an affinity-purified antibody directed against a *bona fide* GA marker: β-galactosyltransferase. (*ii*) We were able to observe with this antibody the reorganization of the GA known to occur in cultured human cells following nocodazole and taxol treatments. (*iii*) In chick myoblasts and myotubes, the respective juxtanuclear and perinuclear organization of the GA that we observed is consistent with the data presented in other reports using a variety of markers (21, 23–25, 36). (*iv*) Electron microscopic studies demonstrated that the CTR433 antibody recognizes specifically the medial compartment of the GA in myotubes. On the basis of these results, we feel confident that our anti-Golgi monoclonal antibody is a valid marker of the GA in skeletal muscle. By using this GA antibody, we show in the present work that in the innervated muscle, within the resolution of our techniques, the GA is a highly focalized organelle that is restricted to areas underneath end plates. Furthermore, we show that the GA can undergo major reorganization during muscle development and after denervation of the adult muscle. The extent of this reorganization indicates that the GA in skeletal muscle possesses a significant plasticity.

In innervated skeletal muscle, the focal "synaptic" organization of the GA is coherent with the state of subcellular anisotropy acquired upon innervation. At its strategic location in close association with the subsynaptic nuclei, the GA is most likely involved in the biosynthesis, processing, and targeting of the AChR (and eventually other synaptic proteins) toward the end plate and in its local accumulation and renewal (for discussion, see ref. 29). Also, the GA restricted size in the innervated muscle is coherent with the slow turnover of the AChR at the synapse (28). In contrast, in denervated muscle, the GA strikingly expands and becomes perinuclear and associated with all nuclei. This situation

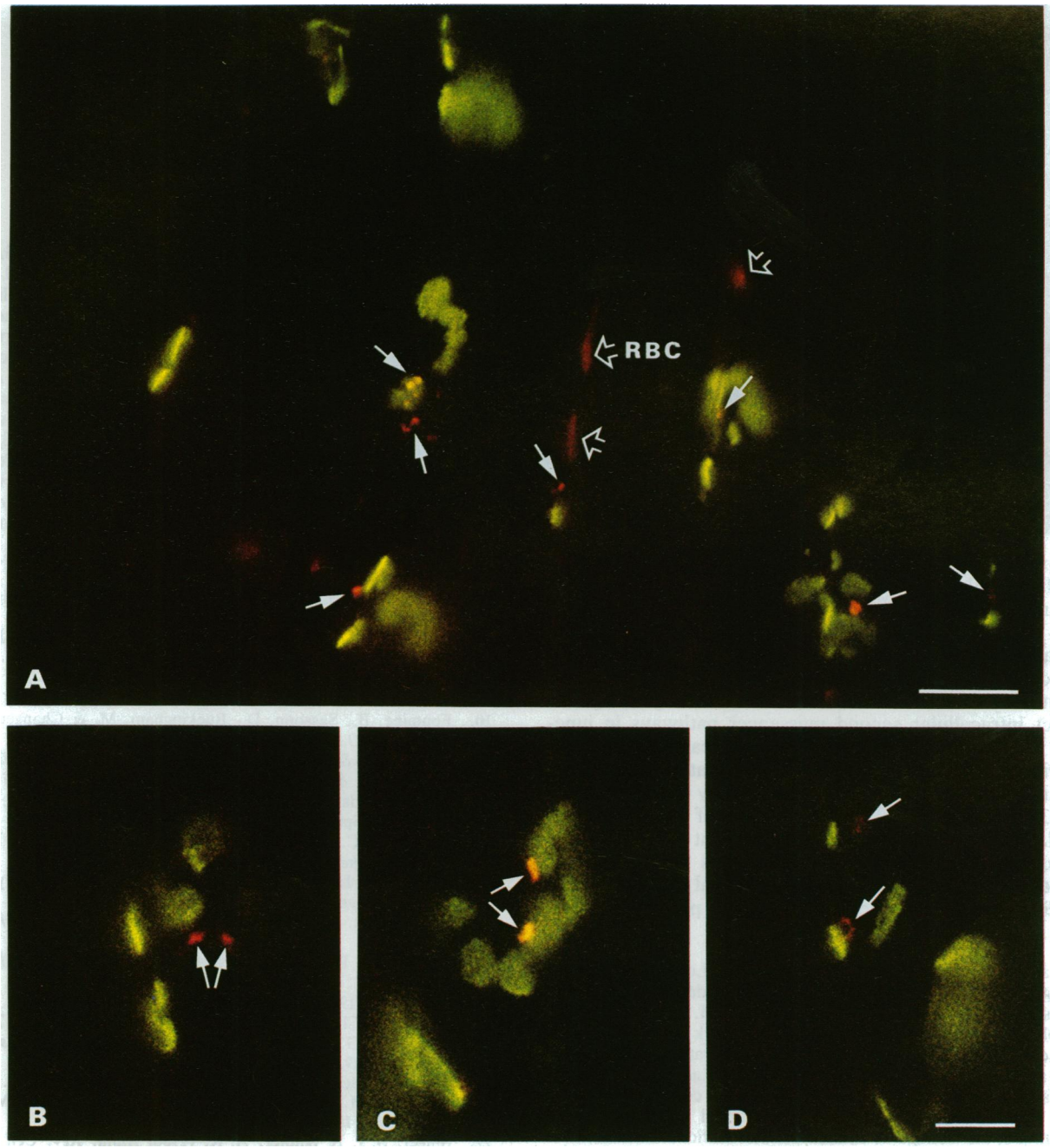

FIG. 3. Double-labeling experiments of cryostat sections of 15-day-old chick ALD muscle. Green and red fluorescence correspond to synapses identified by means of α-bungarotoxin labeling of AChR and to GA labeled with CTR433 antibody, respectively. Note the focal GA (small arrows) in the muscle fibers that codistributes with synapses and the absence of labeling elsewhere in the sarcoplasm. Diffuse red fluorescence in the center of the field is caused by red blood cells (RBC; large arrows). (*A*, bar = 20 μm; *D*, bar = 10 μm.)

resembles that seen in myotubes in which such GA organization may represent a prerequisite for allowing the establishment of muscle polarity by external clues, such as innervation. In these conditions, a uniform distribution of the AChR, which turns over more rapidly, all along the membrane surface is observed with a corresponding increased number of nuclei expressing the α-subunit gene (10, 11). Since these cells no longer show a polarity, the AChR molecules may be incorporated in the membrane by means of a delocalized mechanism of protein insertion (see ref. 24). Thus, it appears that motor innervation plays a determinant role in the distribution and organization of the GA in skeletal muscle. However, this conclusion does not seem valid for *Torpedo* (see ref. 27) since polarized embryonic electrocyte already presents, before innervation, a focal organization of its GA, which remains as such in the adult (B.J.J. and J.C., unpublished observations). Yet, plasticity and reorientation of the GA have also been reported in wounded cultures of fibroblasts and in natural killer cells (30, 31).

The characteristic synaptic organization of the GA in innervated skeletal muscle may be related to the metabolism and targeting of several other components of the postsynaptic domain in addition to the AChR. It may be hypothesized that these functions would result from the expression of a set of genes by particular nuclei located in the vicinity of the postsynaptic membrane already referred to as "fundamental" by Ranvier (32). Such localized and specialized synthesis of membrane and secreted proteins has received experimen-

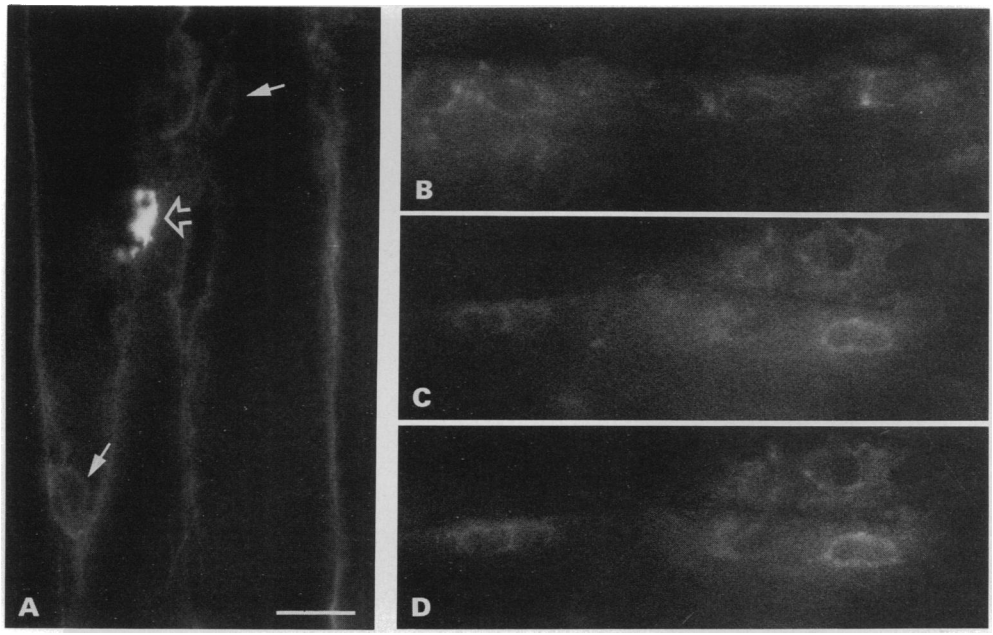

FIG. 4. AChR and GA distributions in denervated ALD muscle. (*A*) AChR labeling of the sarcolemma and of the perinuclear domain (small arrows). The large arrow points to a remaining postsynaptic AChR cluster. (*B*) Muscle fiber stained with CTR433 antibody. Note the numerous perinuclear GA. (*C* and *D*) Double-labeled field for AChR and GA, respectively. Note the perfect perinuclear codistribution. (Bar = 20 μm.)

tal support (10, 33, 34) and is consonant with the suggestion of Palade (35) of a common transport pathway for these proteins.

An important issue raised by our observations is the mechanism by which the motor innervation of the muscle fiber causes the observed reorganization of the GA. One current view (3, 29) is that different first messengers regulate distinct intracellular second messenger pathways in junctional versus nonjunctional areas, thereby leading to different patterns of genes expressed. The observation that AChR clusters immobilize nuclei probably by way of the cytoskeleton (36) is suggestive of the involvement of the subsynaptic cytoskeleton in this process. The present results further extend this model to the morphogenesis of intracellular organelles involved in the posttranslational processing and targeting of characteristic proteins of the postsynaptic domain.

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