

(Na⁺ + K⁺)-ATPase Correlated with a Major Group of Intramembrane Particles in Freeze-Fracture Replicas of Cultured Chick Myotubes

DAVID W. PUMPLIN and DOUGLAS M. FAMBROUGH

Department of Anatomy, University of Maryland School of Medicine, Baltimore, Maryland 21202; and the Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210

ABSTRACT Immunofluorescence microscopy with a fluorescein-labeled monoclonal antibody was used to map the distribution of sodium- and potassium-ion stimulated ATPase ([Na,K]-ATPase) on the surface of tissue-cultured chick skeletal muscle. At this level of resolution it appeared that the (Na,K)-ATPase molecules were distributed nearly uniformly over the plasma membrane. These molecules could be cross-linked by use of the monoclonal antibody followed by a second antibody directed against the monoclonal antibody; the resulting fluorescent pattern was a set of small dots (patches) on the muscle surface. This pattern was stable over several hours, and there was little evidence of interiorization or of coalescence of the patches. Myotubes labeled with immunofluorescence were fixed in glutaraldehyde, cryoprotected with glycerin, then fractured and replicated by standard methods. Replicas of the immunofluorescence-labeled myotubes revealed clusters of intramembrane particles (IMP) only when the immunofluorescent images indicated a patching of the (Na,K)-ATPase molecules. Double antibody cross-linking of antigenic sites on myotubes with each of three other monoclonal antibodies to plasma membrane antigens likewise resulted in patched patterns of immunofluorescence, but in none of these cases were clusters of intramembrane particles found in freeze-fracture replicas. In each case it was shown that the (Na,K)-ATPase molecules were not patched. Other control experiments showed that patching of (Na,K)-ATPase molecules did not cause co-patching of one of the other plasma membrane proteins defined by a monoclonal antibody and did not cause detectable co-clustering of acetylcholine receptors. Detailed mapping showed that there was a one-to-one correspondence between immunofluorescent patches related to the (Na,K)-ATPase and clusters of IMP in a freeze-fracture replica of the same cell. We conclude that the intramembrane particles patched by double antibody cross-linkage of the (Na,K)-ATPase are caused by (Na,K)-ATPase molecules in the fracture plane. Quantification of the IMP indicated that the (Na,K)-ATPase-related particles account for up to 50% of particles evident in the replicas, or up to about 400 particles/ μm^2 of plasma membrane. Particles related to the (Na,K)-ATPase were similar to the average particle size and were as heterodisperse in size as the total population of IMP. A hypothesis is advanced to account for the reported difference in particle density observed in freeze-fracture replicas of normal and dystrophic skeletal muscle.

When cells are freeze-fractured, the fracture plane preferentially passes between the lipid leaflets of the plasma membrane lipid bilayer. Replicas of the fracture surfaces contain irregularities termed particles and pits, and these are thought to originate when the fracture plane passes around a membrane protein embedded in the bilayer (24). Although this interpretation of particles and pits seems reasonable, only a few

integral membrane proteins are demonstrably related to intramembrane particles (IMP).¹ Several membrane proteins generate IMP when reconstituted into liposomes; these include

¹ *Abbreviations used in this paper:* IMP, intramembrane particles; TMRITC, tetramethylrhodamine isothiocyanate.

the Band 3 protein of erythrocytes (42), (Na,K)-ATPase (6, 34, 38), the Ca⁺⁺-dependent ATPase of sarcoplasmic reticulum (30), and rhodopsin of rod outer segments (14). In other cases, a tight clustering of IMP corresponds to a clustering of some functional element of the membrane; for gap junctions (11) and the purple membrane of *Halobacterium halobium* (2), these specialized areas of membrane have been isolated and shown to contain primarily a single protein. Similarly, membrane fragments derived from *Torpedo* electric organ, which are enriched in acetylcholine receptors, are also rich in IMP (3, 18). Direct labeling of IMP in the postsynaptic membrane indicates that these IMP are the receptors (27). Other clusters of IMP appear in appropriate concentrations at sites of known physiological function and have therefore been assigned to that function; these include the voltage-sensitive sodium channels at nodes of Ranvier (28) and the voltage-sensitive calcium channels of nerve terminals (13, 26). The unclustered IMP that occur in virtually all cell membranes have not been so easily identified, and heretofore the bulk of them have remained uncorrelated with membrane proteins of defined function. In this report we offer evidence that the (Na,K)-ATPase accounts for up to 50% of the IMP revealed by conventional freeze-fracture and replication of the plasma membrane of tissue-cultured chick skeletal muscle myotubes.

MATERIALS AND METHODS

Monoclonal Antibodies: The four monoclonal antibodies used in this study were purified from ascitic fluid of mice injected intraperitoneally with hybridoma cells. Ascitic fluid was fractionated by ammonium sulfate precipitation, and the immunoglobulin fraction was fractionated by gel filtration on Ultragel AcA-22 (LKB Instruments, Inc., Rockville, MD) or by ion exchange chromatography on DE-52 (Whatman Chemical Separation Inc., Clifton, NJ). Details of immunizations, fusion with Sp 2/0 myeloma cells, screening for anti-muscle membrane antibodies, and cloning in soft agar as well as descriptions of the specificity of the monoclonal antibodies 24 and C3/1 are published elsewhere (8, 39). Monoclonal antibodies 1 and 10 were derived from the same fusion that produced monoclonal antibody-24.

Cell Culture and Immunofluorescence Microscopy: Myogenic cultures were established from 11-d chick embryo leg muscle. The myoblasts were grown on collagen-coated coverslips in Eagle's Minimum Essential Medium supplemented with 10% horse serum and 1% chicken serum. For immunofluorescence microscopy the cultures were generally incubated for 30 min at room temperature in phosphate-buffered saline supplemented with 10% horse serum and with monoclonal antibody at 5 µg/ml and/or tetramethylrhodamine-labeled α-bungarotoxin (TMRIC-α-bungarotoxin) at ~0.2 µg/ml. Frequently, fluorescein (FITC)-conjugated monoclonal antibody was used. In some experiments goat anti-mouse IgG, conjugated with FITC or with rhodamine (N. L. Cappel Laboratories Inc., Cochranville, PA), was used as a second reagent. In other cases unlabeled goat anti-mouse IgG was used after labeling the cultures with fluorescent monoclonal antibody.

Freeze-Fracture: After mapping by fluorescence microscopy, myotubes were fixed in 5% glutaraldehyde in 0.12 M phosphate buffer, pH 7.4, and equilibrated over 5–10 min to 1:2 (vol/vol) glycerol-water. Prescored 4-mm

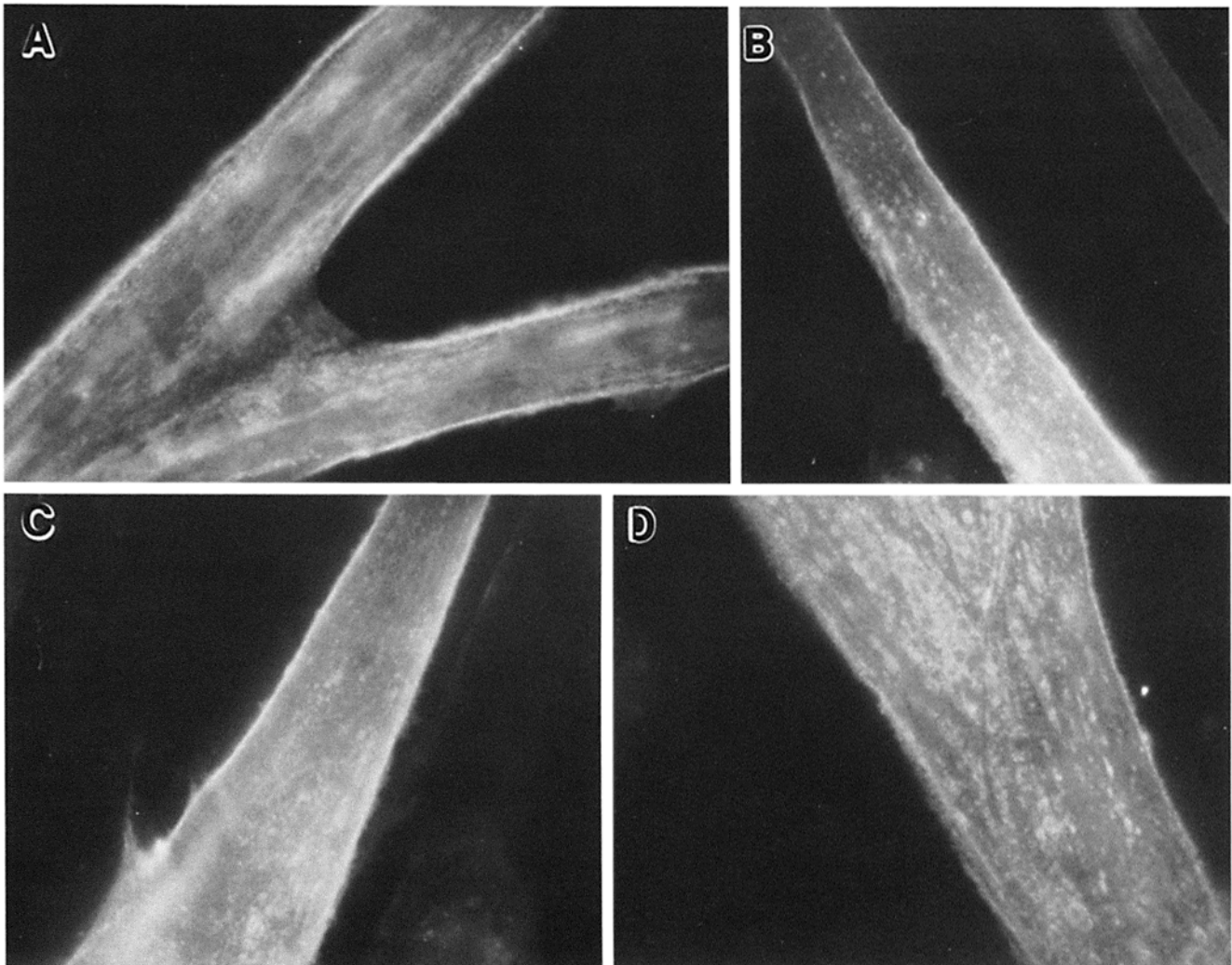


FIGURE 1 Immunofluorescent staining of living myotubes with FITC-conjugated monoclonal antibody to the skeletal muscle (Na,K)-ATPase. $\times 610$.

circles containing photographed areas were broken free of the coverslips and used to form a sandwich with a Balzers specimen carrier (Balzers, Hudson, NH) and a drop of polyvinyl alcohol solution (5, 23, 41). The sandwich was frozen in barely melted Freon, fractured at -119°C and 10^{-6} torr with the Balzers complementary replica device, and replicated within 3 s with sufficient platinum-carbon from an electron beam gun to give a 200-Hz shift on the quartz crystal monitor. After an additional deposition of carbon from a second beam gun, replicas were removed and cleaned by floatation on household bleach followed by dichromate-sulfuric acid cleaning solution. The replicas were picked up on slot grids and examined at $\times 12,000$ – $30,000$. Particle sizes were determined on micrographs enlarged to $\times 180,000$, while particle counts were obtained from micrographs enlarged to $\times 60,000$ – $75,000$. Membrane areas and particle sizes were measured with a Zeiss MOP-30 digitizer.

RESULTS

Monoclonal antibody-24 binds to an antigenic determinant of the sodium- and potassium-ion stimulated ATPase, which is exposed on the outside of living myogenic cells (8). This antibody, directly labeled with fluorescein isothiocyanate, was used to determine the distribution of (Na,K)-ATPase molecules on myotubes in tissue culture. The fluorescence micrographs in Fig. 1 illustrate the pattern of labeling by fluorescent antibody. With rare exceptions, all portions of the myotube

surface were labeled, and the fluorescence intensity varied moderately. Some of the variation was apparently due to the inability of the antibody to penetrate some areas of close apposition between cells or between cell and substratum.

The rather even distribution of fluorescence labeling of the myotubes with FITC-conjugated antibody-24, is better appreciated in comparison with the pattern of fluorescence of companion muscle cultures labeled with FITC-conjugated antibody-24 followed by goat anti-mouse IgG second antibody (Fig. 2, *a* and *b*). Cross-linking of the myotube-bound monoclonal antibody with second antibody resulted in rearrangement of the fluorescence pattern into a set of small (usual diameter $\leq 1 \mu\text{m}$) dots or patches on the myotubes. This patched pattern was stable for several hours at 37°C (Fig. 2 *b*), and there was no apparent tendency for the patches to coalesce or to be interiorized by the myotubes. This patching of the fluorescence pattern by second antibody could be blocked completely by prefixation of the myotubes with paraformaldehyde fixative (20) (Fig. 2, *c* and *d*).

Muscle cultures used for immunofluorescence analysis as described above were subsequently fixed with glutaraldehyde,

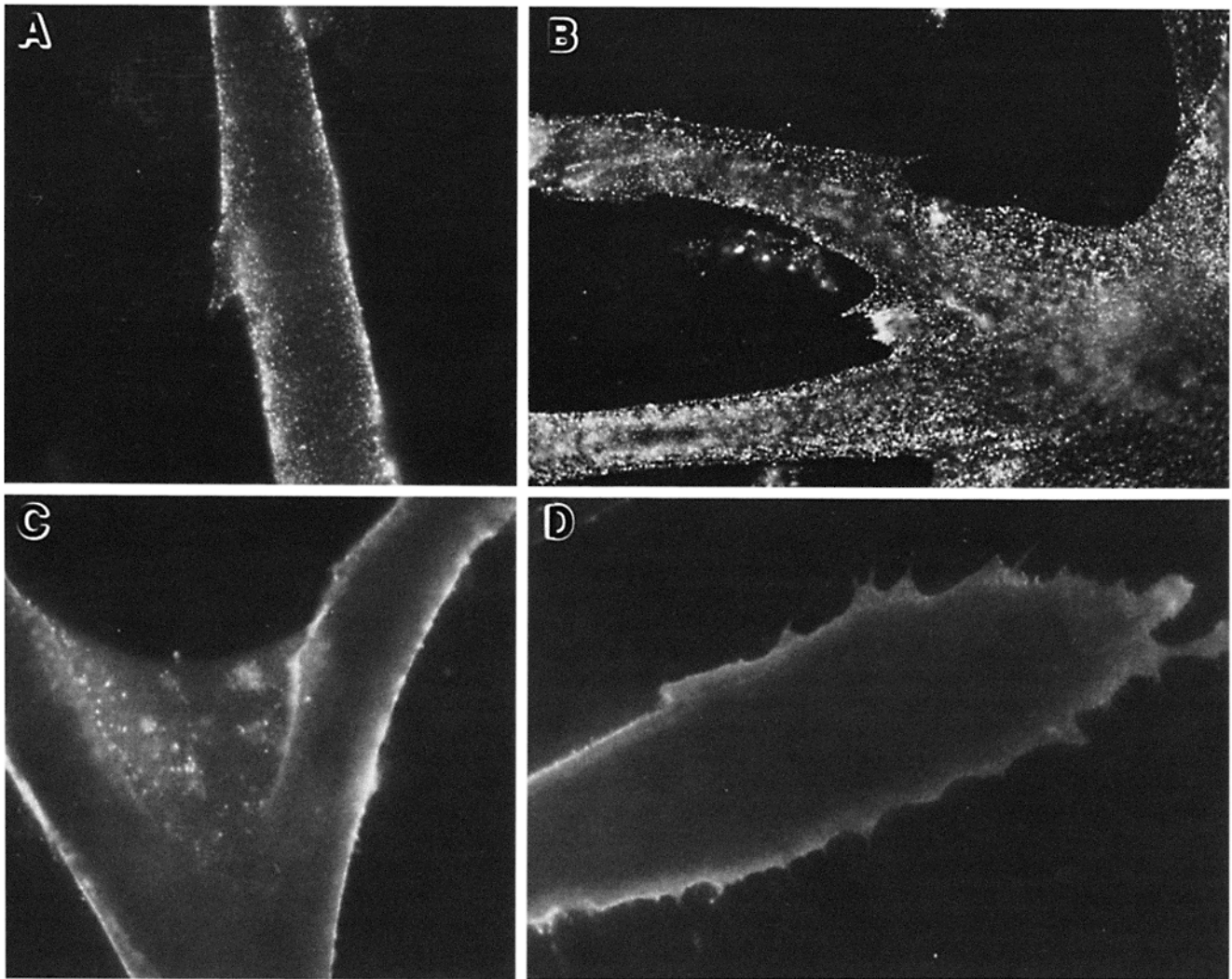


FIGURE 2 Effects of prefixation and of double antibody staining on the pattern of immunofluorescence of myotubes. (A and B) Myotubes were labeled with FITC-conjugated monoclonal antibody to the (Na,K)-ATPase followed by incubation with anti-mouse IgG for 30 min at 22°C (A), or 3 h at 37°C (B). Either treatment resulted in patching of fluorescence. (C and D) Myotubes prefixed with paraformaldehyde fixative and then labeled with FITC-conjugated monoclonal antibody followed by 1-h incubation with anti-mouse IgG. $\times 610$.

frozen, fractured, and replicated. Replicas of myotube plasma membrane from cultures treated only with FITC-conjugated antibody-24 and from cultures prefixed in paraformaldehyde fixative before immunofluorescent labeling showed a dispersed distribution of IMP indistinguishable from the distribution seen in replicas of untreated myotubes (Fig. 3*a*). However, replicas from myotubes exhibiting a patched distribution of fluorescence due to double antibody labeling showed clustering of a large fraction of the IMP (Fig. 3*b*).

We examined the correlation between patched immunofluorescence and IMP clustering more closely to determine whether there was a one-for-one match between immunofluorescent patches and particle clusters. This was accomplished by mapping the immunofluorescent patches on selected myotubes and later examining freeze-fracture replicas of the same areas of myotube surface. Since it was known that the fracture plane generally passes between the lipid leaflets of the plasma membrane apposed to the culture dish (5), high resolution fluorescence micrographs were made of the appropriate myotube surfaces after double antibody labeling. Lower magnification fluorescence and phase pictures were taken to facilitate relocation of the same myotube surfaces in the freeze-fracture replicas. Replicas were generated and the mapped P and E face areas examined. Figs. 4 and 5 illustrate a particularly complex set of fluorescent patches and the corresponding set of IMP clusters. Although the fluorescence images are near the limit of resolution of the fluorescence microscope, it is clear that there is a close correspondence in the positions and shapes of fluorescent patches and IMP clusters.

To control for the possibility that double antibody fluorescent labeling of myotubes leads to a clustering of IMPs independent of the specificity of the antibodies, we used three other monoclonal antibodies that bind to different antigenic determinants on the myotubes in experiments parallel to those involving antibody-24. Each of these other monoclonal antibodies (Fig. 6*a-c*), together with FITC-conjugated second antibody, yielded a patched pattern of fluorescence on the myotubes, virtually indistinguishable from that observed with antibody-24 and second antibody. The overall fluorescence patterns could be recognized because of different relative intensities of immunofluorescent staining of different cell types in the cultures (fibroblasts, myoblasts, myotubes). There was no obvious clustering of IMP in freeze-fracture replicas of myotubes with patched fluorescent labeling due to any of these three monoclonal antibodies together with second antibody (Fig. 7). As a further control, myotubes were labeled with each of the three monoclonal antibodies followed by TMRITC-conjugated goat anti-mouse antibody, producing a pattern of red fluorescent patches. Any residual anti-mouse activity was quenched with an irrelevant mouse IgG and the cultures were postlabeled with FITC-conjugated antibody-24. On these doubly labeled myotubes the rhodamine fluorescence remained patched, but the distribution of bound FITC-conjugated antibody-24 resembled the nonpatched distribution seen on myotubes labeled with FITC-conjugated antibody-24 only (Fig. 8*a* and *b*; compare with Fig. 1).

Although the control experiments described above prove that patching antigenic determinants other than the (Na,K)-ATPase does not lead to a copatching of the (Na,K)-ATPase and does not result in formation of IMP clusters in freeze-fracture replicas, it remained possible that patching of (Na,K)-ATPase molecules might sweep other antigenic determinants into the patch areas, and that the IMP clusters reflected the

clustering of molecules other than the (Na,K)-ATPase. While we could not test every surface antigen, we did determine that patching of (Na,K)-ATPase did not result in copatching of antigen C3/1 defined by an FITC-conjugated monoclonal antibody (Fig. 9), or acetylcholine receptors, defined by TMRITC-conjugated α -bungarotoxin.

It is well known that large IMP are associated with acetylcholine receptors (5, 27) and that acetylcholine receptor clusters occur spontaneously on cultured myotubes. We hypothesized that if a tight cluster of acetylcholine receptors occurred on a myotube, such an area might lack (Na,K)-ATPase molecules and would thus appear as a "black hole" in the fluorescence pattern of myotubes labeled with FITC-conjugated antibody-24. ~100 acetylcholine receptor clusters labeled with TMRITC- α -bungarotoxin were examined on myotubes labeled also with FITC-conjugated antibody-24 with or without second antibody. Acetylcholine receptor clusters did not totally exclude (Na,K)-ATPase, although there was a tendency for much lower fluorescein fluorescence to occur in the receptor cluster regions (Fig. 10). A technical difficulty was encountered in the search for "black holes." Acetylcholine receptor clusters usually occurred at points of contact of thin myotube extensions or flanges and the substratum. At such points the myotubes were so thin that in immunofluorescence microscopy there was appreciable fluorescence emanating from both upper and lower myotube surfaces, regardless of the plane of focus.

Freeze-fracture replicas of areas including acetylcholine receptor clusters revealed that each cluster consisted of a constellation of smaller patches of relatively large IMP, interspersed with regions containing dispersed IMP. In replicas of myotubes with patched (Na,K)-ATPase, we found areas containing clusters of IMP characteristic of those previously related to acetylcholine receptors interspersed with IMP clusters resembling those correlating with (Na,K)-ATPase (Fig. 11). Thus it seems unlikely that there is a significant exclusion of (Na,K)-ATPase molecules from regions of acetylcholine receptor clusters except for the space actually occupied by the receptors themselves.

Analysis of IMP Related to the (Na,K)-ATPase

Freeze-fracture replicas were analyzed to determine whether patching of (Na,K)-ATPase molecules resulted in a change in the total number of IMP per unit area of myotube surface and to estimate the ratio of patched to total IMP (Table I). The data confirmed our impression that total IMP per unit area was not significantly affected, and, on average, ~20% of the IMP were clustered in these randomly chosen samples. In myotubes that were most affected by antibody treatment, such as that illustrated in Fig. 4, ~50% of the IMP were clustered. There seemed to be about as much variation in total IMP per unit area from myotube to myotube within a single culture as from one culture to another in a set or from one culture set to another, although we did not analyze this matter rigorously.

Measurements of particle diameter were made on 239 particles in antibody-induced IMP clusters and on 227 unclustered particles in adjacent membrane. The mean particle diameter (9.1 ± 3.3 nm, mean \pm SD vs. 9.5 ± 3.6) and the variance in diameter did not differ ($P > 0.1$). The two distributions of particle size did not differ significantly ($P > 0.05$, Kolmogorov-Smirnov test) and were approximately Gaussian, though slightly skewed toward larger diameters (Fig. 12).

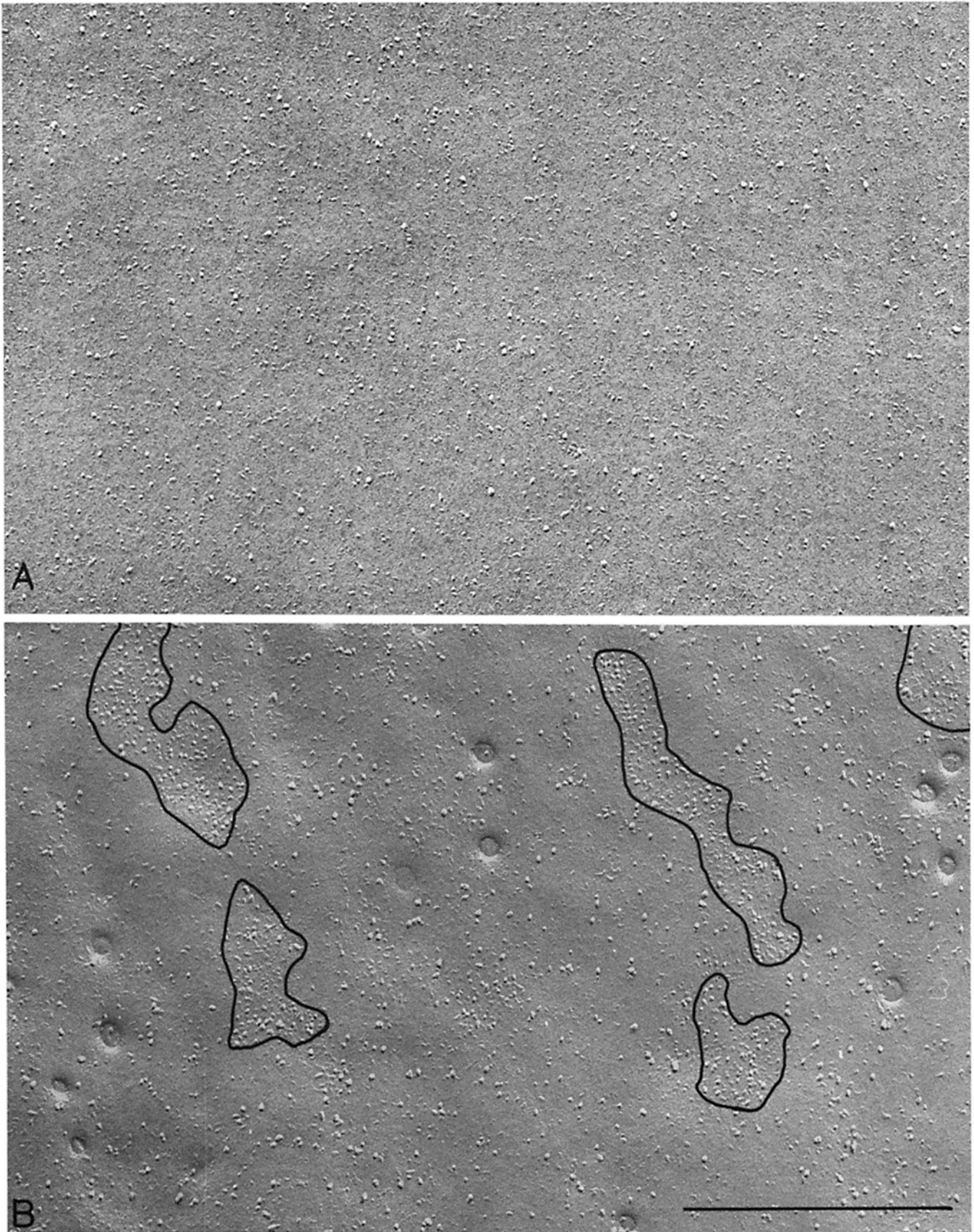


FIGURE 3 Freeze-fracture replicas of chick myotubes. (A) Portion of a chick myotube treated only with monoclonal antibody to the (Na,K)-ATPase. Intramembrane particles are not clustered. (B) Replica with clustered particles from a myotube incubated first with monoclonal antibody to the (Na,K)-ATPase and then with anti-mouse IgG. P face. Bar, 0.5 μm . $\times 96,000$.



FIGURE 4 Freeze-fracture replica of myotube treated with monoclonal antibody to the (Na,K)-ATPase and then with FITC-labeled anti-mouse IgG. The same area is presented in both A and B, with boundaries of particle clusters indicated in B. Bar, 0.5 μm . $\times 48,000$.

In other words, the clustered IMP were not morphologically distinct from the total population of IMP. (However, the diameter of 345 particles in clusters of AChR [recognizable by their characteristic arrangement] was $14.1 \pm 4.1 \text{ nm}$,

significantly larger than that of the remaining population [$P < 0.01$, Student's t test].) The mean particle diameter measured for IMP in antibody-induced clusters is identical to the mean diameter reported for IMP in vesicles reconstituted

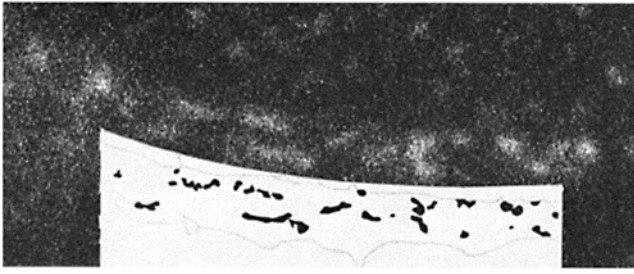


FIGURE 5 Correlation of intramembrane particle clusters and fluorescent patches on myotube labeled with anti-(Na,K)-ATPase antibody followed by FITC-conjugated anti-mouse IgG. The map of particle clusters (from an area including that shown in Fig. 4) is superimposable on the corresponding fluorescence pattern from that area. Particle clusters are indicated by black regions within the outlined myotube. $\times 6,500$.

from purified lipids and (Na,K)-ATPase (34). The size distribution in our study was slightly broader than that reported for reconstituted (Na,K)-ATPase. Although most of the clustered IMP occurred on the P face replica, there were also IMP in cluster regions on the complementary E face replica (Fig. 13). The ratio of P to E face particles was 4.4 ± 1.1 (mean \pm SD) for nine particle clusters on the myotube shown in Figs. 4 and 5.

DISCUSSION

Monoclonal antibody-24 binds to (Na,K)-ATPase via an antigenic determinant which is exposed to the extracellular space (8). Thus FITC-conjugated antibody-24 could be used to determine the distribution of (Na,K)-ATPase molecules on tissue cultured myotubes. The distribution appeared to be relatively even, indicating that the monoclonal antibody by itself did not extensively cross-link (Na,K)-ATPase molecules into patches. This failure to patch antigenic sites is not unique to antibody-24. In fact, antibody C3/1 (39) also revealed a relatively uniform distribution of antigenic sites on myotubes. It is reasonable to propose that immunofluorescent staining of myotubes by antibody-24 is due to formation of discrete complexes in which an antibody molecule binds to two antigenic sites on a (Na,K)-ATPase molecule consisting of two alpha and two beta subunits (15); subsequent binding of polyclonal anti-mouse antibody to the complexes results in extensive cross-linking and generation of patches of antigen on the myotube surface.

The freeze-fracture study, particularly the mapping of IMP clusters relative to fluorescence patches, demonstrated a tight correlation between patching of (Na,K)-ATPase and formation of IMP clusters at the same loci. Control experiments showed that patching of other antigens did not produce IMP clusters nor cause patching of (Na,K)-ATPase molecules; patching of (Na,K)-ATPase did not cause copatching of antigen C3/1 (an integral membrane glycoprotein consisting of 38,000-dalton subunit(s) [39]) or of acetylcholine receptors. We conclude that the groups of IMP found at positions where (Na,K)-ATPase molecules are clustered result from the presence of the (Na,K)-ATPase in the plasma membrane. The patching of antigen did not alter the total number of IMP, but did result in clustering of up to 50% of the IMP on myotubes. It follows that the (Na,K)-ATPase is responsible for up to 50% of the IMP observed in freeze-fracture replicas from normal myotubes.

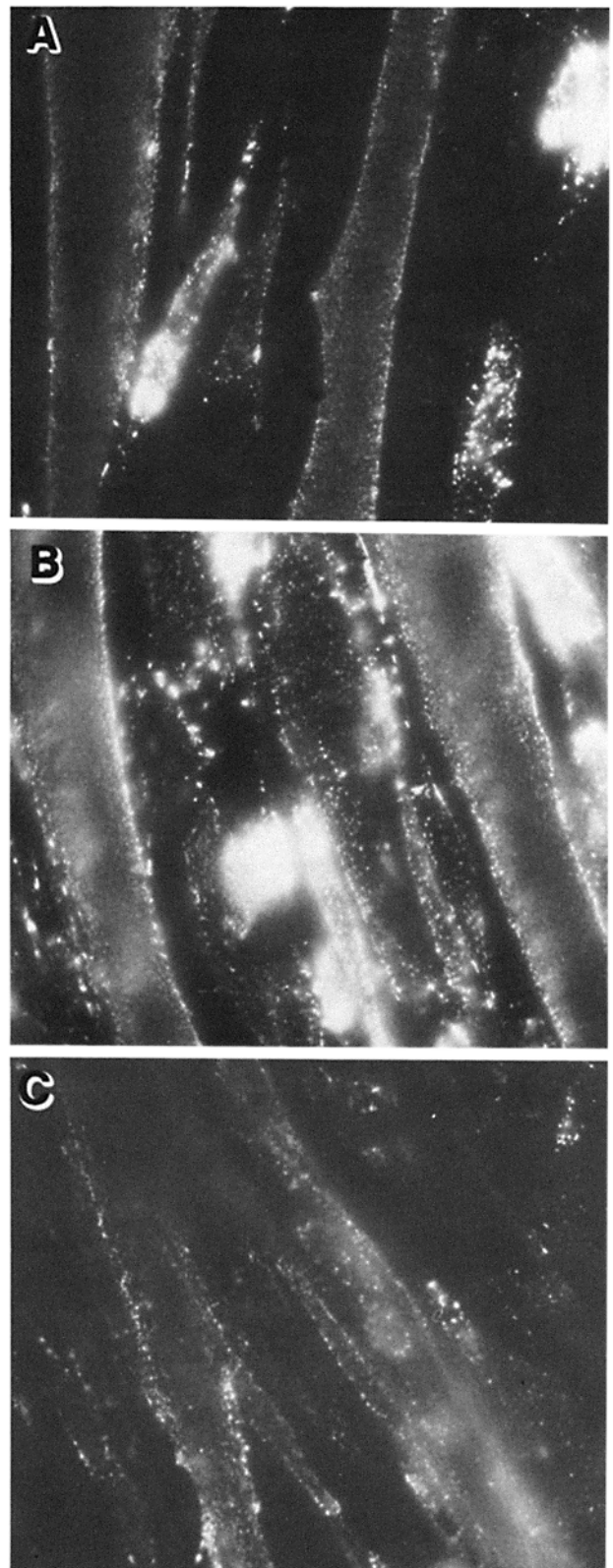


FIGURE 6 Immunofluorescence patterns from myogenic cell cultures labeled with monoclonal antibody 1 (A); monoclonal antibody C3/1 (B); and monoclonal antibody 10 (C). In each case FITC-conjugated anti-mouse immunoglobulin was used to detect bound monoclonal antibody. Myotubes have a patched pattern of immunofluorescence. $\times 610$.



FIGURE 7 Freeze-fracture replica of a myotube labeled with monoclonal antibody C3/1 followed by FITC-labeled anti-mouse IgG to patch the antigen. There is no apparent patching of intramembrane particles. P face: Bar, 0.5 μm . $\times 96,000$.

A number of problems arise in attempting to relate the number of IMP to the number of (Na,K)-ATPase molecules per unit area of myotube surface. Previously the number of ouabain binding sites and antibody-24 binding sites per myotube nucleus were estimated to be $\sim 5 \times 10^5$ (8). However, it was also demonstrated that the number of sites per myotube nucleus could be changed by culture conditions in a manner consistent with up-regulation of (Na,K)-ATPase under stress of low external potassium ion concentration. We have also observed (illustrated incidentally in Figs. 1*b* and 7*b*) that the density of antibody binding sites varies from one myotube to the next (as it does from one muscle fiber to the next in adult skeletal muscle [8]). Both this variability in density of surface sites and also the variability in surface area per nucleus in myotubes makes estimation of number of (Na,K)-ATPase molecules per unit area difficult. If we assume that there are between 500 and 1,000 μm^2 of surface per nucleus, then the number of (Na,K)-ATPase molecules (assuming two ouabain binding sites, per molecule) would be 250–500/ μm^2 . In the mapped myotube of Figs. 4 and 5, distributing the clustered particles (1,046 P face and 255 E face) evenly over the total area observed (2.77 μm^2) gave an average concentration of 470 particles/ μm^2 . The same procedure for the randomly chosen areas of myotube membrane of Table I (assuming a ratio of P/E face particles of 4.1) gave an average concentration of particles binding to antibody-24 of 108/ μm^2 (range 10–253). The average value is likely to be an underestimate, because the small fraction of IMP clustered in some micro-

graphs may be due to failure of the antibody to penetrate into regions of close approximation of myotube and substrate. In addition, low values may simply reflect low levels of (Na,K)-ATPase in some myotubes. At least we can conclude that there is not a huge discrepancy between the number of (Na,K)-ATPase molecules and the number of correlated IMP. In fact, recent reconstitution experiments suggest that an IMP results from a single (Na,K)-ATPase molecule (34). Our data are consistent with this finding, to the extent that quantification is possible.

At first we were surprised by the high percentage of IMP related to the (Na,K)-ATPase. However, our failure to find IMP associated with any of three other surface antigens in this study and the failure of others to correlate capping of various surface antigens with aggregation of IMP (17, 19, 25) demonstrates that not all membrane proteins give rise to IMP upon freeze-fracture. Formation of IMP may be attributable to a special subclass of integral membrane proteins that not

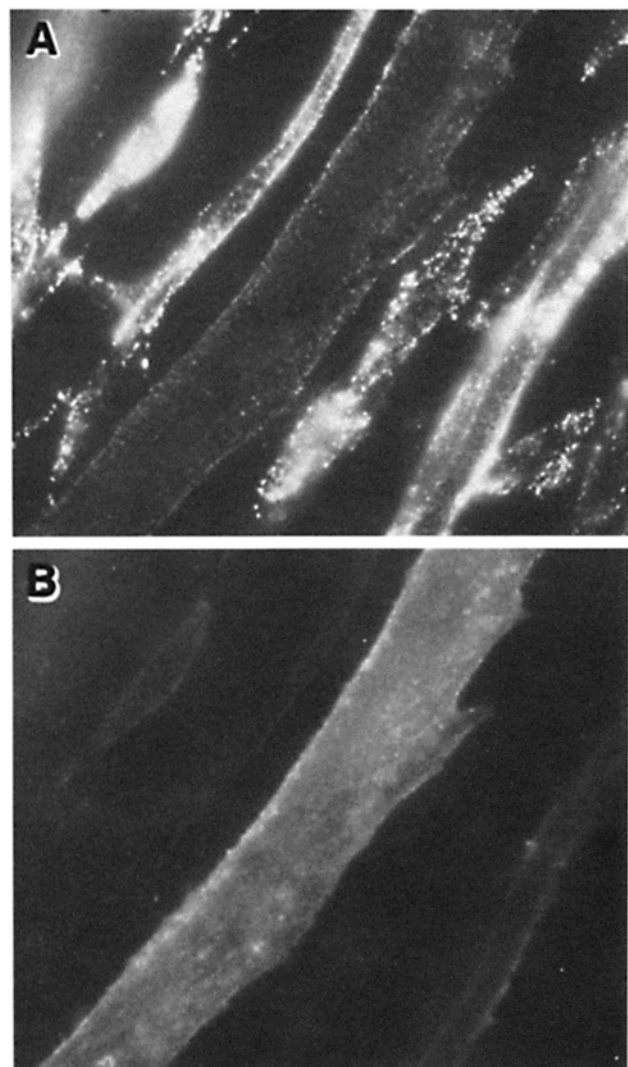


FIGURE 8 Immunofluorescence patterns on myotube showing that patching of antigen C3/1 does not cause patching of (Na,K)-ATPase. (A) Patched pattern to rhodamine fluorescence due to monoclonal antibody C3/1 followed by TMRITC-labeled anti-mouse antibody. (B) Dispersed pattern of fluorescein fluorescence on same myotube as in A, due to labeling with FITC-conjugated anti-(Na,K)-ATPase monoclonal antibody. $\times 610$.

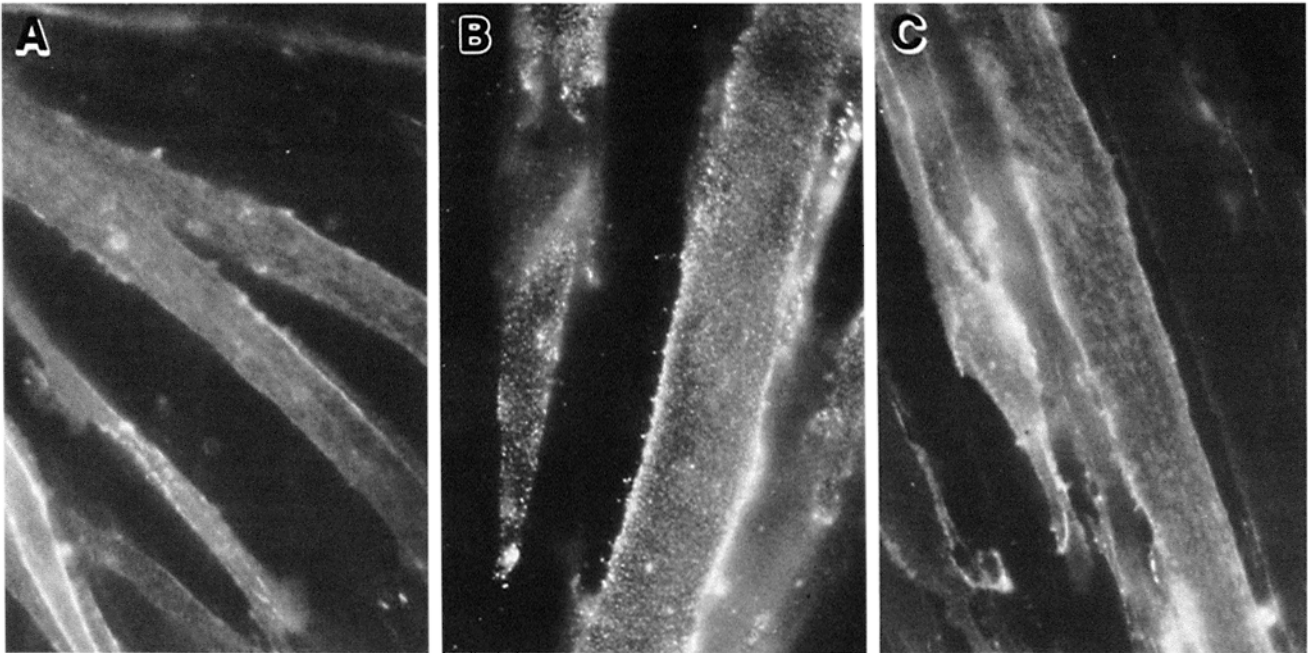


FIGURE 9 Immunofluorescence patterns on myotube showing that patching of (Na,K)-ATPase with monoclonal antibody followed by second antibody does not cause patching of antigen C3/1. (A) Myotube showing dispersed fluorescence due to labeling with FITC-conjugated monoclonal antibody C3/1. (B) Patched fluorescence pattern due to labeling with monoclonal antibody to the (Na,K)-ATPase followed by FITC-labeled anti-mouse IgG. (C) Dispersed fluorescence due to labeling with FITC-conjugated antibody C3/1 on myotubes with (Na,K)-ATPase clustered by monoclonal antibody-24 and second antibody. $\times 610$.

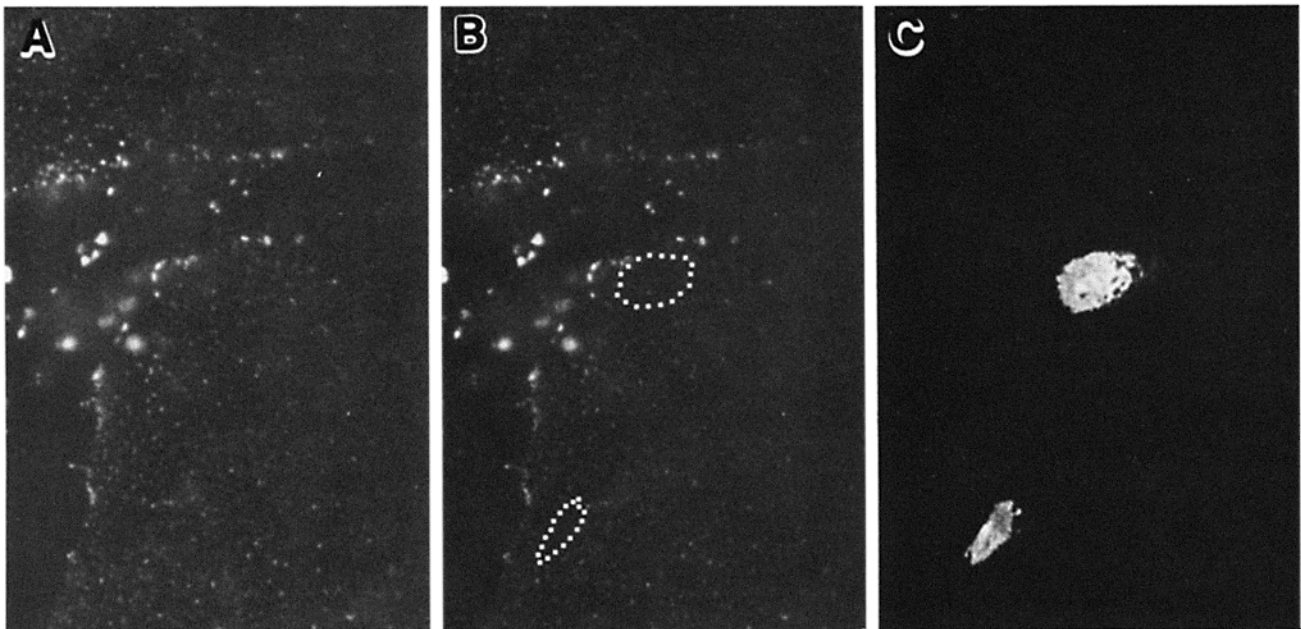


FIGURE 10 Immunofluorescence patterns on myotube labeled with TMRITC- α -bungarotoxin and with anti-(Na,K)-ATPase followed by FITC-conjugated anti-mouse IgG. Patched pattern of fluorescein fluorescence (A) contains small, relatively unoccupied areas that correspond to locations of acetylcholine receptor clusters (indicated by stippled perimeters in B and by rhodamine fluorescence in C). $\times 1,480$.

only span the bilayer, but also have extensive mass within the bilayer. To the extent that we know or can guess their structures, these features seem to be common to the membrane proteins so far correlated with IMP: rhodopsin (14), acetylcholine receptor (21, 35, 36, 40), Ca^{++} pump of sarcoplasmic reticulum (30), bacteriorhodopsin (12, 22), and connexin of gap junctions (4, 37). A structure recently proposed for the

(Na,K)-ATPase has each of the alpha subunits spanning the lipid bilayer six times (16). Two additional findings are related to the requirement for intramembrane mass. The antigen recognized by antibody C3/1, although it is an integral membrane protein, has a molecular weight of only 38,000, and may thus be too small to form a particle visible in the conventional procedures used. Although the bacteriorhodop-

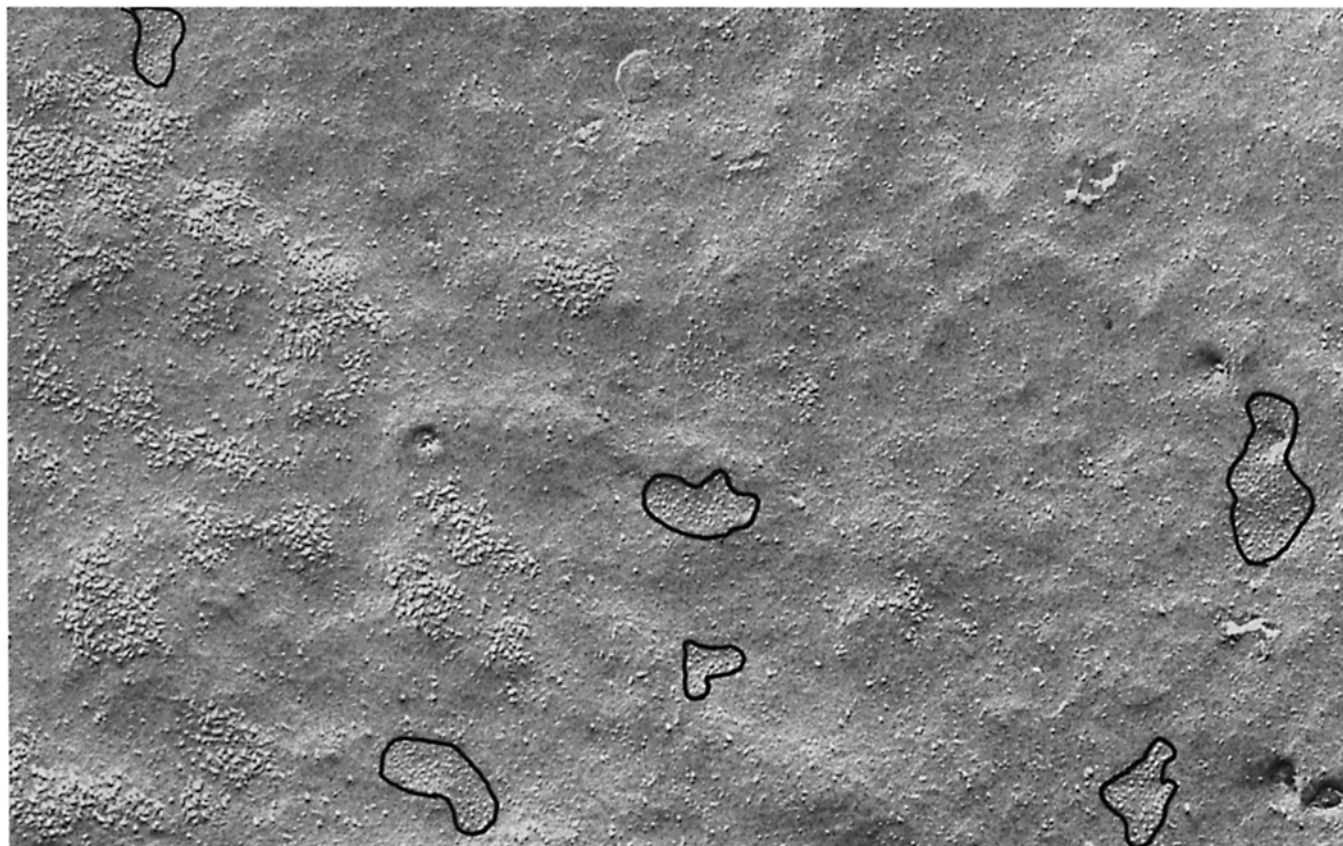


FIGURE 11 Freeze-fracture replica of chick myotube treated with anti-(Na,K)-ATPase monoclonal antibody followed by anti-mouse IgG. The left hand area includes part of an acetylcholine receptor cluster recognizable by its groups of tightly packed, large, angular IMPs. Clustered IMP (circled) related to the (Na,K)-ATPase are located adjacent to and within the field of particle clusters related to acetylcholine receptors. $\times 57,000$.

TABLE I
Comparison of Concentrations of Clustered and Nonclustered IMP

Treatment	% Particles clustered	Particles/ μm^2 (mean \pm SD)	Number of myotubes (cultures)
Control	0	468 ± 217	23 (2)
Antibody-24 + anti-mouse IgG	18 (range 3-37)	471 ± 100	12 (1)

Each area ($7.8 \mu\text{m}^2$) of smooth, flat P fact replica was chosen at random from a single myotube and enlarged $\times 75,000$. The concentration of particles was determined in regions containing clustered or nonclustered particles by counting 400 or more particles in a group of contiguous squares (40.3 nm^2 each) ruled on a transparent overlay to the micrograph. The total area containing clustered particles was then measured with a digitizer and subtracted from the total area of the micrograph. The two areas were multiplied by the concentration of particles determined for that micrograph. The total number of particles per unit area did not differ between treated and control myotubes. The greater variability in the concentrations of IMP in control cultures is apparently due to sampling. Myotubes with IMP concentrations differing widely from the mean were encountered in both control cultures.

sin molecule is also small (mol wt 26,000), some 10-12 individual molecules aggregate to form one particle (10).

It is attractive to think that IMP size might be useful in classifying the particles and assigning them to particular membrane proteins. Some IMP associated with particular functional elements of the membrane (such as acetylcholine receptors) or with particular membrane structures (such as the active zones of nerve terminals) are distinctly larger than the

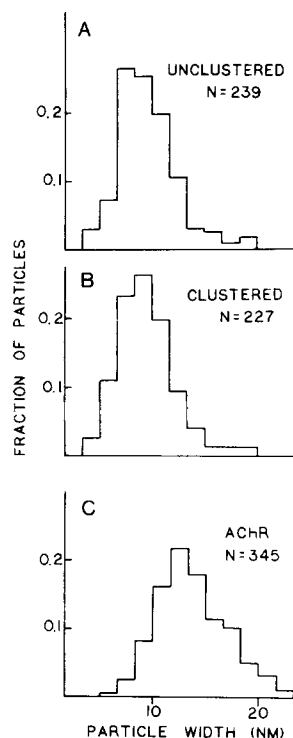


FIGURE 12 Histogram of size distributions of IMP: (A) not clustered by antibody-24 followed by anti-mouse IgG; (B) clustered by antibody-24 followed by anti-mouse IgG; (C) within an acetylcholine receptor cluster. A and B are from the same myotube, while C is from a different myotube replicated identically. The size distribution of non-AChR particles in C was similar to that shown in A and B.

remaining IMP. However, our data indicate that a single, well-defined membrane protein, the (Na,K)-ATPase, is responsible for IMP that are as heterogeneous in morphology

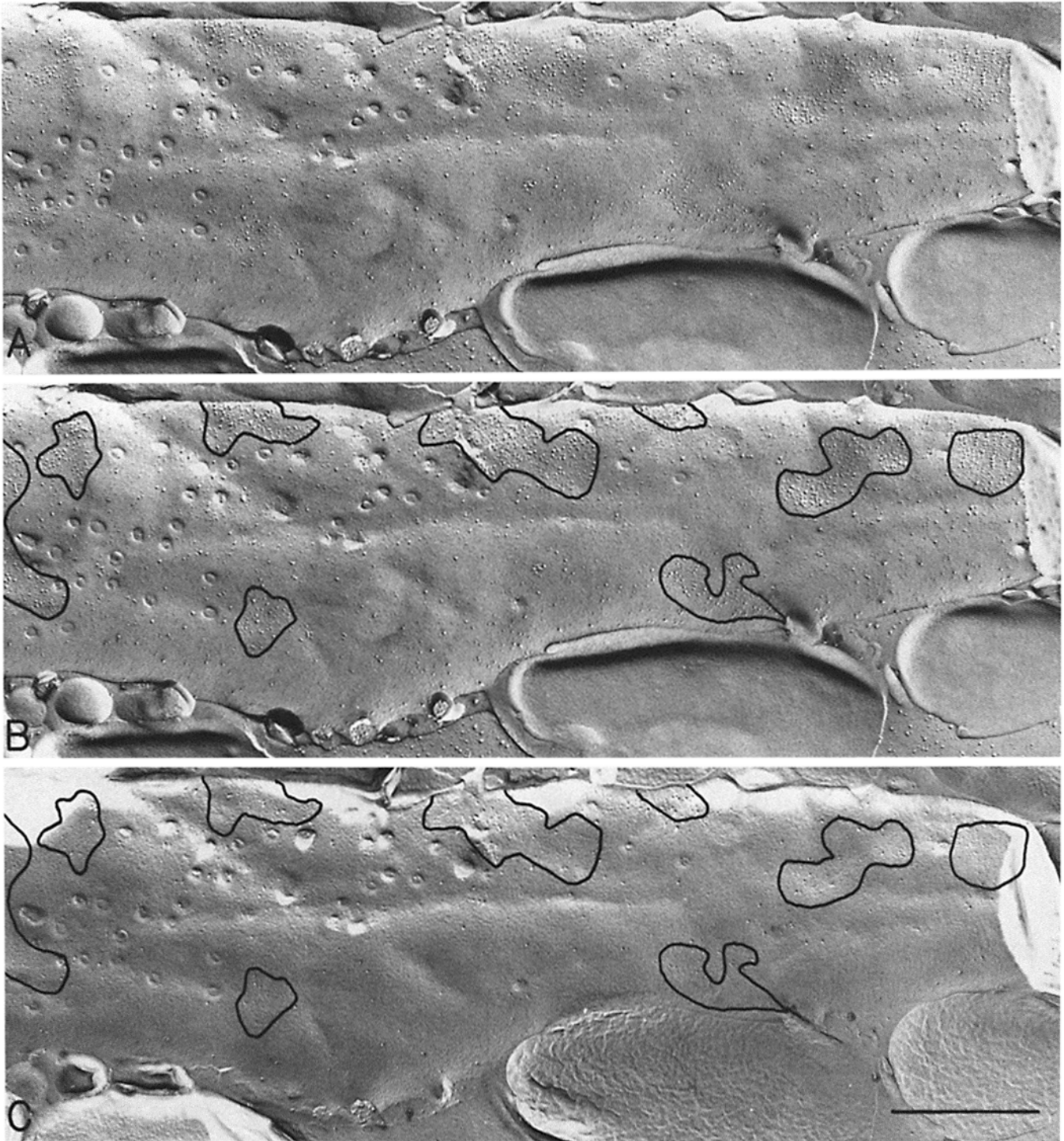


FIGURE 13 Freeze-fracture replicas of a chick myotube treated with anti (Na,K)-ATPase monoclonal antibody followed by anti-mouse IgG. (A and B) P-face replica. (C) E-face replica. Particle cluster areas outlined in B and C. $\times 48,500$.

as the total population of IMP in our replicas. Thus, the fracture plane may be perturbed in a variety of ways as it passes through an area occupied by a (Na,K)-ATPase molecule (6, 29). The fracture may cleave the molecule, leaving no trace in the replica, may rise over or dive under the molecule, producing P face and E face IMP, or may break through the molecule in an irregular manner, generating a smaller perturbation of the fracture plane. An earlier suggestion that intramembrane proteins of erythrocytes are broken in consistent places along the polypeptide chain during frac-

ture (7) recently has been disputed (9). Nonetheless, the good numerical correlation between (Na,K)-ATPase molecules and IMP, seen in vesicles reconstituted from purified lipids and (Na,K)-ATPase (34), suggests that relatively few fractures of these molecules result in a particle too small to be visible.

Finally, our conclusion that a large percentage of IMP seen in freeze-fracture replicas of myotubes are due to (Na,K)-ATPase may explain the reductions in IMP concentration seen in replicas of fixed, glycerinated fibers of tenotomized rat muscle (1) and human muscle from patients with muscular

dystrophy (31, 32). Although concentrations of IMP are sensitive to technical variations (cf. reference 33), these reductions were seen using conventional freeze-fracture methods similar to those used in this study. We note that a large fraction of the IMP may be related to (Na,K)-ATPase and that this molecule may be subject to rather large variations in abundance due to up- and down-regulation in response to variations in demand for sodium extrusion from muscle fibers. Therefore, we would predict that numbers of IMP per unit area should show nearly as great a variation, and the number of IMP might be significantly altered in replicas of otherwise homologous muscle fibers which have recently been subjected to very different levels of use. It is not unreasonable to expect that dystrophic, tenotomized, and normal muscles would be subjected to rather different levels of use, and thus might differ in density of (Na,K)-ATPase sites per unit area partly because of this difference.

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