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SEPARATION OF PLASMA MEMBRANE MARKERS BY GLYCEROL-INDUCED BLISTERING OF MUSCLE CELLS

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Summary

Glycerol (50%, w/w) was found to cause blistering of chick primary myoblast and fibroblast plasma membranes and extensive blistering of 5—6-day-old-myotube plasma membranes in tissue culture. The tips of myoblasts and fibroblasts appeared to be the most sensitive portion of the plasma membrane to the blistering effect of glycerol. The glycerol-induced blistering of myotubes was reduced and delayed by brief EDTA pretreatment.

Glycerol treatment (50, 15 and 8% sequentially) of myotubes was used to remove plasma membrane blisters and a plasma membrane-enriched fraction was isolated from these blisters using a modified Dextran T500-polyethyleneglycol 6000 aqueous two-phase polymer system. This fraction was found to be enriched 4.1-fold for 5′-nucleotidase activity, but not for other putative plasma membrane markers, $(Na^+ + K^+)$-ATPase activity or α-[^{125}I]bungarotoxin binding material. Autoradiographs of α-[^{125}I]bungarotoxin, glycerol-treated (50%, w/w) myotubes showed the plasma membrane blisters to be devoid of reduced silver grains.

5′-Nucleotidase was shown to be an ectoenzyme on myoblasts and 5-day-old myotubes and the total cellular activity was present on the cell surface. During the period of myoblast fusion and myotube formation, cell surface activity decreased to a low level while total cellular activity was elevated.

Introduction

The plasma membrane of the multinucleated myofiber is a complex structure derived from the fusion of mononucleated myoblasts during differentia-

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tion; it is chemically, electrically and mechanically excitable, contains specialized motor endplate regions and is highly dependent on nervous innervation for its structure and activity [1,2]. It is a difficult membrane system to study due to the thick external lamina and collagen layers on its surface. After tissue homogenization additional difficulties are encountered due to the large intramembranous systems. For example, the T-tubule and sarcoplasmic reticulum membrane systems in adult frog sartorius muscle have been calculated to contain 150—160-times the surface area of the plasma membrane [3]. In addition, this ratio differs from one myofiber type to another (e.g. fast versus slow [4]), during differentiation [5] and following denervation [6], making interpretation of experimental analyses of such membrane preparations equivocal.

In tissue cultured muscle, the plasma membrane is accessible to analysis since it is directly exposed to the ambient medium. The acetylcholine receptor and the electrophysiological properties of muscle during differentiation have thus been successfully studied [7—9]. Glycerol treatment of adult muscle has been used for a number of years to study the mechanisms of contraction [10] and excitation-contraction coupling [11]. More recently, glycerol has been used to study the distribution of plasma membrane macromolecules [12,13]. It was found in this study that glycerol caused plasma membrane blistering of myoblasts, fibroblasts and 5—6-day-old myotubes in tissue culture. The effect of glycerol on the distribution of several plasma membrane markers of the intact myotube and in a plasma membrane-enriched fraction isolated without cell lysis, has been studied. Part of this work has appeared in abstract form (Northeast Regional Developmental Biology Conference, Boston, Mass. February 19—21, 1976).

Materials and Methods

Fertilized chicken eggs were obtained from G.F. Shaw, Inc., West Chester, Pa. Cultures were prepared from 11-day-old embryonic breast muscle. Embryos were rapidly dissected in Simms' balanced salt solution [14] and the breast muscle was reduced to small, 1-mm^3 pieces. The tissue was pelleted by centrifugation at 1550 rev./min for 3 min in a clinical centrifuge (Precision Scientific Co.), resuspended in 0.25% trypsin in calcium/magnesium free Simm's balanced salt solution and incubated at 37°C for 15 min. The cells were again pelleted by centrifugation, resuspended in 10 ml of growth medium and dissociated into single cells by repeated pipetting. The growth medium contained 80% basal medium (Eagle) with L-glutamine, 10% horse serum, 10% embryo extract and 45.5 units penicillin, 45.5 μg streptomycin and 22.8 μg fungizone per ml. The dispersed cells were passed through a Nitex 22 μm filter (Tetko, Inc., Elmsford, N.Y.), counted and plated on 100 mm Falcon tissue culture dishes (#3003) coated with 1 ml of rat tail collagen; $5.0—5.5 \times 10^6$ cells were added to each dish in 8 ml of growth medium. Collagen was prepared by the method of Ehrman and Gey [15] except that 1% acetic acid was used for extraction. The medium was changed at 24, 48 and 96 h. Cytosine arabinoside (Sigma), 1 μg per ml, was added to the medium from 48 to 96 h to kill most of the fibroblasts in the cultures. The cultures were maintained at 37°C in a humidified 5% CO_2/95% air incubator. Embryo extract was prepared by passage of 12-day-

old chick embryos through a 20 ml plastic syringe, diluted 1 : 1 with balanced salt solution, gently agitated at 23°C for 1 h and stored at −20°C. On the day of medium preparation, the embryo extract was thawed, centrifuged at 1600 × *g* for 30 min at 5°C and the supernatant removed for use. Fresh growth medium was prepared every 48 h. All tissue culture products were obtained from Grand Island Biological Co.

5-day-old myotube cultures were treated with α-[^{125}I]bungarotoxin (gift of Dr. D. Fambrough) according to the procedure of Hartzell and Fambrough [16] to label surface acetylcholine receptors. For autoradiography, the cultures were fixed in 2% glutaraldehyde in balanced salt solution (pH 7.4) at 4°C overnight, rinsed 3 times with water and covered with Kodak NBT-2 emulsion (diluted 1 : 1 with water) at 55°C. The autoradiographs were kept at 4°C for 4—14 days and developed with D-19 at 20°C for 2.5 min, fixed, rinsed 3 times with water, dehydrated through 70%, 95% and 100% ethanol, air-dried and covered with immersion oil.

Myoblast and myotube cultures were rinsed 3 times with balanced salt solution, pH 7.4 at 2—4°C and treated at 4°C with 5 ml of 50% glycerol in balanced salt solution (w/w), pH 7.4 for 45 min. The plasma membrane formed blisters during this period. The myotube cultures were then swirled on a rotary shaker at 5°C for 10 min. The supernatant, containing released membrane blisters, was removed. Additional membrane blisters could be released by several glycerol extractions. Therefore, 5 ml of 15% glycerol (w/w), pH 7.4 was added to the cultures, swirled for 90 s and the supernatant again removed. 5 ml of 8% glycerol (w/w), pH 7.4 was added, swirled for 90 s and the supernatant removed, leaving what will be referred to as the extracted plate. The glycerol extractions were done at 5°C. The majority of the myotubes on the extracted plate contained structurally intact plasma membranes; thus, if a hypotonic solution was added to the extracted monolayer, the plasma membrane of the myotubes could be seen by phase microscopy to swell and then collapse on greater than 98% of them.

To further study the mechanism of membrane blistering, myotube cultures were pretreated with several reagents which are known to affect membrane structure, before glycerol treatment. Trypsin treatment was performed by the method of Hynes [17] (10 μg trypsin per ml balanced salt solution for 10 min at 23°C), Triton X-100 treatment by the method of Fambrough [7] (0.01% Triton X-100 in growth medium for 1 h at 37°C), and ethylenediamine tetraacetic acid (EDTA) treatment by the method of Bischoff and Lowe [18] (0.5 mM EDTA in calcium/magnesium free balanced salt solution for 10 min at 23°C). The cultures were also treated with cytochalasin B (5 μg/ml, 0.2% bovine serum albumin in balanced salt solution) for 15 min at 37°C or with colchicine (10^{-4} M in 0.2% bovine serum albumin in balanced salt solution) for 1 h at 37°C prior to glycerol treatment. The plasma membrane remained intact for all of these procedures, as evidenced by myotube shrinkage when placed in the hypertonic glycerol solution.

The supernatants from the glycerol treatment, containing released plasma membrane blisters, were combined and used for subsequent plasma membrane isolation. A modification of the aqueous two-phase polymer system described by Brunette and Till [19] was used. To 20 ml of combined supernatant, 10 ml

of 20% Dextran T500 (Pharmacia Fine) in balanced salt solution (w/w), was added, shaken well, passed through a Nitex 36 μm filter and centrifuged at 15 000 × *g* (SS34 rotor, Servall), 20 min. The supernatant was decanted and to 30 ml, 10 ml of 14% polyethyleneglycol 6000 (Matherson, Coleman and Bell Co.) in balanced salt solution (w/w), pH 7.4 was added, shaken well and centrifuged at 15 000 × *g* for 20 min. All the above steps were performed at 5°C. After centrifugation, any precipitate formed at the interface was removed and discarded. The test tubes containing the two phases were stored overnight (18 h) at 0°C for complete separation of the two phases; during this time a flocculent precipitate formed at the interface. This precipitate was removed, diluted in three volumes of 5 mM Tris · HCl, pH 7.5 and centrifuged for 2 h at 50 000 × *g* (Sw 25.1 rotor, Beckman Model L). The precipitate was gently rinsed 3 times with 5 mM Tris · HCl, pH 7.5 and then resuspended by sonication in 200 μl of 5 mM Tris · HCl, pH 7.5 for subsequent assays. Control and extracted cultures of the same age were scraped into 5 ml of 5 mM Tris · HCl, pH 7.5, homogenized for complete cellular disruption, diluted to 30 ml and centrifuged at 50 000 × *g* for 2 h. The precipitate was gently rinsed three times, resuspended by sonication in 5 ml of 5 mM Tris · HCl, pH 7.5 and used for subsequent assays.

The 5′-nucleotidase assay was performed essentially as described by Trams and Lauter [20]. Cultures were rinsed twice with 5 ml of a phosphate-free buffer solution at 4°C (100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2 \cdot 6\ H_2O$, 2 mM $CaCl_2$, 10 mM $NaHCO_3$, 5 mM glucose, 15 mM Tris · HCl, pH 7.4) and then incubated at 2—4°C for 1 h in 5 ml of the buffer solution. The cultures were again rinsed with buffer at 23°C, 5 ml of fresh buffer containing 2.5 mM 5′-AMP (Sigma, Type III) added and incubated at 37°C on a rotary shaker (60 rev./min). Aliquots (100 μl) were removed at 15-min intervals, 100 μl of ice cold 10% trichloroacetic acid added, and inorganic phosphate determined on 100 μl of the centrifuged supernatant by the method of Baginski et al. [21]. After 45 min, the tissue was scraped from the plate in 5 ml of fresh buffer solution and sonicated with a Sonifier Cell Disruptor (model W185) (output, 25 watts) until the tissue was totally disrupted (approx. 60 s), as observed by phase microscopy. The total homogenate was preincubated for 10 min at 37°C, 2.5 mM 5′-AMP added and the release of inorganic phosphate followed for an additional 45 min. The 5′-nucleotidase activity was found to be linear within this time period. The activity present on the monolayer was taken to represent the activity present on the cell surface and the activity of the homogenate as the total cellular activity. Even after extensive washing of the monolayer, inorganic phosphate was released from the cultures to which no 5′-AMP had been added, sometimes in a near linear fashion. For this reason, time-matched cultures were always run without 5′-AMP for both the monolayer and homogenate and this amount subtracted from the activity in the presence of 5′-AMP. To determine if enzymatic activity was released into the medium, cultures were incubated as described above for 45 min in the absence of 5′-AMP and the buffer solution removed. To this solution, 2.5 mM of 5′-AMP was added and 5′-nucleotidase activity determined as described. The permeability of the cells to the 5′-AMP substrate was determined by incubating monolayer cultures as described for the 5′-nucleotidase assay, using 2.5 mM [^{3}H]AMP (New England

Nuclear, specific activity 4.3×10^5 dpm/micromole). After the 45-min incubation period, the cultures were rinsed 5 times at 4°C with incubation buffer, containing 1 mM unlabelled 5′-AMP. The cells were scraped from the plate, sonicated for complete cellular disruption and the radioactivity counted with an Intertechnique SL40 Scintillation Counter, using external standard ratios.

Protein was determined by the method of Lowry et al. [22] with bovine serum albumin as the standard. The $(Na^+ + K^+)$-ATPase activity was assayed on 5—20 μg of protein in 100 μl of Tris buffered reaction mixture (100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.5 mm EGTA, 3 mM ATP in 50 mM Tris · HCl, pH 7.5) for 45 min at 37°C. The reaction was stopped by the addition of 100 μl of 10% trichloroacetic acid, centrifuged and 100 μl removed for the determination of inorganic phosphate [21]. The $(Na^+ + K^+)$-ATPase activity was calculated as the difference in activity in the presence and absence of 1 mM ouabain. The activity of 5′-nucleotidase was measured on 10—30 μg protein in 100 μl of reaction buffer (10 mM KCl, 5 mM $MgCl_2$, 2.5 mM 5′-AMP in 75 mM Tris · HCl, pH 8.7) for 60 min at 37°C and released inorganic phosphate determined [21]. NADPH-cytochrome *c* reductase, an endoplasmic reticulum enzyme marker, and succinate-cytochrome *c* reductase, a mitochondrial enzyme marker, were assayed according to the method of Scottocasa et al. [23]. Total lipid phosphate was determined by the method of Baginski et al. [21] and multiplied by 25 to calculate the amount of phospholipid [24]. The amount of α-[^{125}I]bungarotoxin binding material in the various fractions was determined by countin in a Packard Gamma Counter. Ribonucleic acid was determined by incubation of the cultures for 48 h with 10 μCi [^{3}H]uridine (specific activity 46 Ci/mmol, New England Nuclear) and counting the level of activity in the various fractions with a Packard Tri-Carb, Model 574, Liquid Scintillation Counter.

Membrane pellets were fixed overnight (18 h) with 2% glutaraldehyde in balanced salt solution, pH 7.4 at 0°C. They were postfixed for 30 min at 0°C with 2% osmium tetroxide, dehydrated and embedded in Araldite and then sectioned for EM. The electron micrographs were taken by Dr. Sumner Zacks, Pennsylvania Hospital.

Results

Fig. 1 shows blister formation on myoblasts and fibroblasts after glycerol treatment. In most cases, blisters formed initially at the tips of the spindle- and stellate-shaped cells i.e. at the most active, mobile portions of the plasma membrane. With time, blisters appeared elsewhere on these cells, but rarely more than 4—6 blisters appeared per cell during the 60 min in 50% glycerol.

Fig. 2 shows the extensive blistering of 5-day-old myotube plasma membranes following treatment with glycerol. Myotubes blistered more extensively than myoblasts or fibroblasts, and myotube blisters were released spontaneously into the medium. The myotubes were evenly covered with blisters along their entire length. Sequential treatment with 50, 15 and 8% glycerol increased the size of the blisters and increased their release into the medium.

Trypsin treatment, known to remove the large external trypsin-sensitive protein of myoblasts and myotubes [25], or Triton X-100 treatment which affects

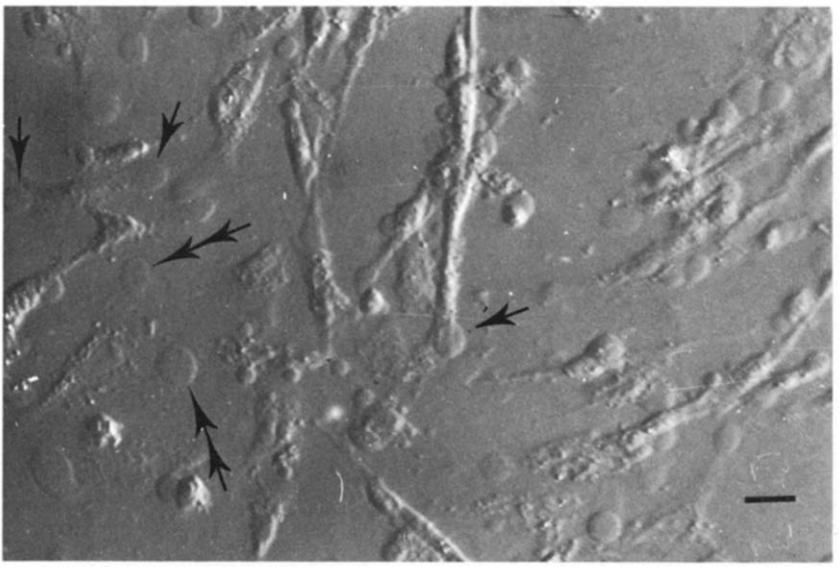

Fig. 1. Normarski interference phase micrograph of a 24-h culture treated with 50% glycerol as described in the text. The presence of membrane blisters at the tips of the myoblasts (arrows) and fibroblasts (double arrows) is evident. Bar represents 10 μm. × 625.

the mobility of acetylcholine receptor on myotubes [7], did not affect the glycerol-induced blistering phenomena. Pretreatment of myotubes with either cytochalasin B or colchicine did not inhibit glycerol-induced blistering of the plasma membrane; in some instances the drugs appeared to enhance blistering. Pretreatment of myotubes with 2.5% glutaraldehyde for 5 min at 5°C completely inhibited the blistering effect. EDTA pretreatment,used by Bischoff and Lowe [18] to remove plasma membrane proteins from intact cells, followed by 50% glycerol treatment, was found to significantly inhibit plasma membrane blistering of the myotubes. Whereas blisters appeared normally after 10—15 min in 50% glycerol, blisters did not appear in EDTA/glycerol treated myotubes for 2—3 h.

The released blisters were fractionated with the two-phase polymer system described. The interface material was observed by electron microscopy and found to consist mainly of trilaminar membranous fragments with some unidentified homogenous precipitate and ribosome-like particles approximately 250 Å in diameter. The results of the chemical and enzymatic assays performed on this plasma membrane-enriched fraction are shown in Table I. One interesting result of these determinations was that whereas two plasma membrane markers, $(Na^+ + K^+)$-ATPase and α-bungarotoxin binding material, were not enriched in the plasma membrane fraction, 5′-nucleotidase activity was enriched 4.1-fold over the particulate homogenate. The absence of enhanced α-[^{125}I]-bungarotoxin binding material in the plasma membrane-enriched fraction could not be explained by the dissociation of α-[^{125}I]bungarotoxin from the acetylcholine receptor during the isolation procedure since in control experiments, all of the label remained in the precipitable fraction (data not shown). A reduction in NADPH-cytochrome *c* reductase and succinate-cytochrome *c* reductase activ-

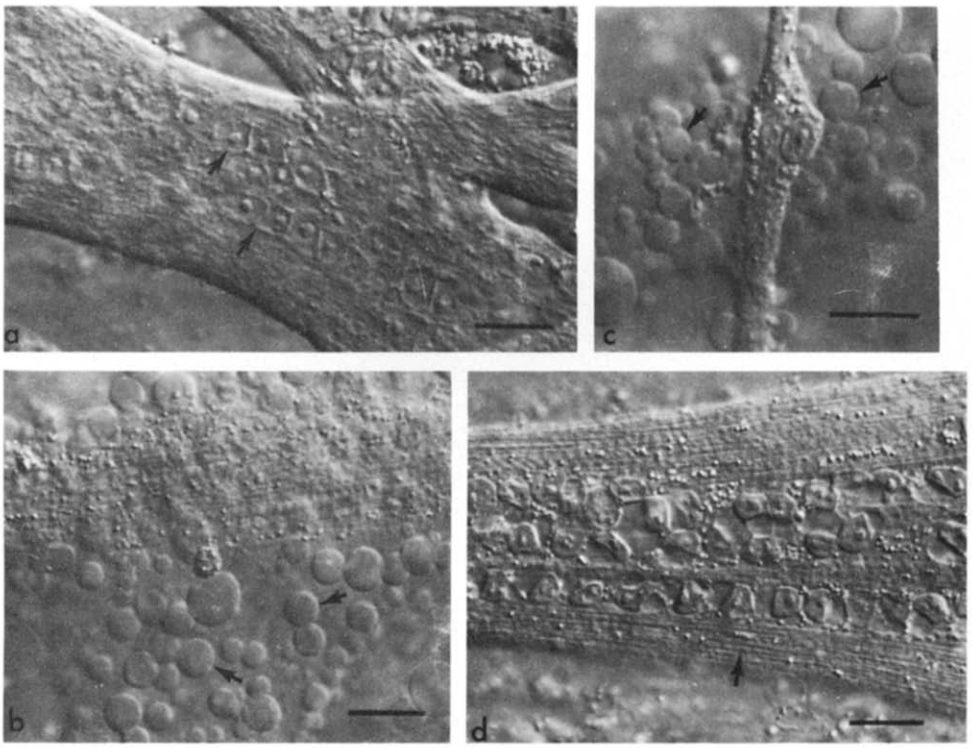

Fig. 2. Normarski interference phase micrographs of glycerol-treated myotubes. (a) Control myotube. The branched myotubes are striated and contain rows of nuclei (arrows) with prominent nucleoli. × 1020. (b) Myotube treated with 50 and 15% glycerol as described. Part of the myotube is in focus through the center of the cell, showing myofibrils. Other parts are in focus on the cell surface, showing numerous membrane blisters (arrows) of various sizes. × 1020. (c) Myotubes treated with 50, 15 and 8% glycerol. The thin, vertical myotube with a hypolemmal nucleus, is in focus through the center of the cell, while the larger myotube underneath is in focus near the cell surface, showing membrane blisters (arrows). × 1200. (d) Myotube treated with 50, 15 and 8% glycerol as described. The myotube is in focus through the center of the cell, showing myofibrils (arrow) and nuclei. The myotube appears dehydrated; the surface (not shown) is still covered with membrane blisters. × 990. The bar represents 10 μm in each photograph.

ities, and in RNA content was also found in the plasma membrane-enriched fraction. Phospholipids were enriched 11.23-fold and a very high lipid to protein ratio (2.54, w/w) was found, similar to the high lipid to protein ratio found by Kent et al. [26] (2.3—2.8, w/w) for their plasma membrane-enriched fraction from tissue culture myotubes.

Although 5′-nucleotidase has been shown to be an ectoenzyme on a number of cell types[20,27,28], it has not been studied in tissue cultured muscle. Therefore, the 5′-nucleotidase activity of the muscle cultures was measured. It was found that intact myoblasts are able to hydrolyze the 5′-AMP substrate and that no additional activity was detected on disruption of the cells (Fig. 3). During the period of myoblast fusion and myotube formation, the surface activity of 5′-nucleotidase decreased to a low level while total cellular activity was elevated. As the myotubes differentiated further, becoming striated and contractile, the surface activity increased and total cellular activity decreased, so that by 4—5 days, the total cellular activity was again present on the cell sur-

TABLE I

CHEMICAL AND ENZYMATIC DETERMINATIONS OF THE PLASMA MEMBRANE FRACTIONATION

4—5-day-old myotubes were treated with glycerol and fractionated as described in the text. Enzymatic specific activities are in nmol/mg protein per min. % recovery is the % of recovery of the activity in the plasma membrane-enriched fraction of the initial activity in the particulate homogenate. Total activity is the total activity present in the plasma membrane-enriched fraction. Relative specific activity is the specific activity of the plasma membrane-enriched fraction/specific activity of the particulate homogenate. Each value represents the mean of at least three determinations.

	Particulate homogenate (spec. act.)	Extracted plate (spec. act.)	Plasma membrane-enriched fraction			Relative specific activity
			(spec. act.)	(% recovery)	Total activity	
Protein (% of total)	100	101	0.54			
$(Na^+ + K^+ + Mg^{+2})$-ATPase	99.24	75.0	41.13	0.56	4.57	0.41
$(Na^+ + K^+)$-ATPase	11.08	14.07	8.11	0.32	0.90	0.73
5′-Nucleotidase	5.22	1.87	21.40	2.5	2.19	4.10
α-[^{125}I] Bungarotoxin binding material (cpm/μg protein)	35.2	38.9	4.06	0.11	405.2	0.12
NADPH-cytochrome *c* reductase	5.42	4.26	3.48	0.25	0.32	0.64
Succinate-cytochrome *c* reductase	4.33	4.81	0.10	0.16	0.01	0.02
RNA (cpm/μg protein)	83.8	79.2	18.0	0.12	936.0	0.22
Phospholipids (μg/mg protein)	222.0	298.0	2540.0	6.3	132.0	11.44

face. Whether this changing activity of 5′-nucleotidase is unique for the myoblast fusion period, or is a general cell cycle phenomenon, as found in other cell types [29] is not known. No 5′-nucleotidase activity was found released into the medium during the 45-min incubation period. The 5′-AMP substrate was found to be very impermeable for both myoblasts and myotubes, with the myotubes slightly less permeable (0.37% versus 0.54% of the total label inside

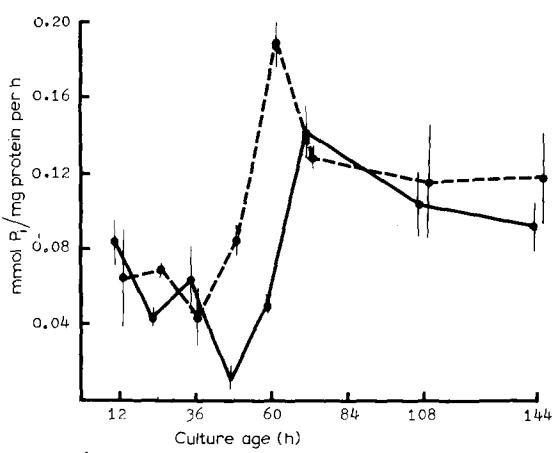

Fig. 3. 5′-Nucleotidase activity of muscle cultures. The enzymatic activity was measured as described in the text. ------, total cellular activity; ———, ectoenzyme activity. Each point is the mean of three determinations ± S.D.

the cells after the 45-min incubation). Thus, the total cellular activity of 5′-nucleotidase is present on the cell surface of 4—5-day-old myotubes, and this enzyme is therefore a good plasma membrane marker.

Autoradiographs of 5-day-old myotube cultures labelled with α-[^{125}I]-bungarotoxin showed reduced silver grains distributed over the entire myotube surface of many cells, with frequent clusters ('hot spots'), as described by others in older cultures [30—32] (Fig. 4a). A circular distribution of grains was seen on many of the heavily labelled myotubes (Fig. 4a, arrows). The cultures

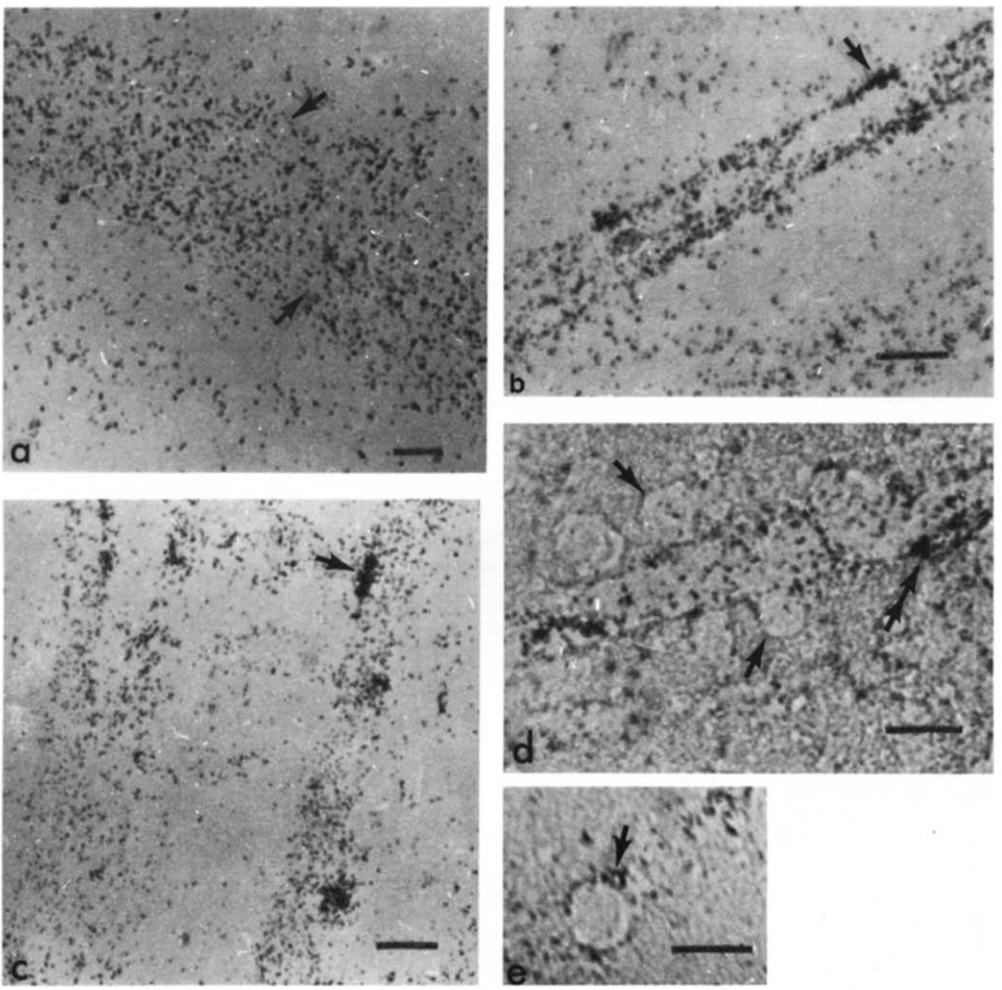

Fig. 4. Autoradiography of 5-day-old myotube cultures labelled with α-[^{125}I]bungarotoxin as described. (a) Control myotube covered with reduced silver grains. Myotubes containing many grains appeared to have grains arranged in circular patterns (arrows). × 800. (b) Myotubes treated with 50, 15 and 8% glycerol as described in the text. Areas of high grain clusters (arrows) are near areas devoid of grains. (c) Myotubes after treatment with 50, 15 and 8% glycerol. Grain clusters (arrow), common in older cultures, are not destroyed by glycerol treatment. × 775. (d) Myotube treated with 50% glycerol and fixed as described in the text. Membrane blisters are present (arrows) which are devoid of grains, along with grain clusters (double arrows) on the myotube. × 1000. (e) Myotube treated with 50% glycerol and fixed as described. A membrane blister centered over a myotube is devoid of grains, with several grain clusters (arrow) evident at its periphery. × 1075. Bar represents 10 μm in each figure.

also contained myotubes which had only a few scattered grains and some myotubes were not labelled. Following 50, 15 and 8% glycerol treatment, there was no noticeable decrease in the total density of grains (Figs. 4b, c), which agrees with the fact that all of the α-[^{125}I]bungarotoxin was recovered on the extracted plate (Table I). The glycerol-treated cultures had a grain distribution similar to the control cultures. Most of the membrane blisters which formed following glycerol treatment were not seen in the autoradiographs, probably due to their removal by the water washes necessary before the plates were covered with emulsion. Occasionally, blisters were seen after 50% glycerol treatment and fixation for 48 h in 2% glutaraldehyde/50% glycerol and these were always devoid of all but one or two grains (Figs. 4d, e). This agrees with the lack of enrichment for α-[^{125}I]bungarotoxin binding material in the plasma membrane-enriched fraction isolated from these blisters (Table I). When blisters were seen on myotubes after 50% glycerol treatment, there was usually a very uneven grain distribution associated with them, with clusters of grains present at the base or periphery of the blister (Figs. 4d and e, arrows). It is not evident whether blisters were devoid of grains because of glycerol-induced redistribution or due to blister formation at areas already devoid of grains, possibly corresponding with the circular patterns seen in control cultures (Fig. 4a, arrows). But, because of the large size of the blisters and the clusters of grains alongside them after 50% glycerol treatment, it is difficult to envision blister formation without some grain redistribution.

Discussion

The phenomena of plasma membrane blistering has been noted in a number of cell types following treatment with dimethylsulfoxide (L cells, 12), cytochalasin B (blastomeres, 33), local anesthetics (BALB/3T3 cells, 34), spectrin precipitation (erythrocytes, 35) and elevated intracellular Ca^{2+} (erythrocytes, 36). The mechanism(s) involved is not known, but membrane-associated microtubules and microfilaments have been implicated [33,34]. The various reagents may act by completely different mechanisms. Characterization of the plasma membrane of these blisters indicates that they are not representative of the cell surface, but appear to be enriched for certain components and not others. Thus, when blisters are formed by procedures known to precipitate spectrin [35], the plasma membrane of these blisters is almost totally devoid of protein, having a lipid to protein ratio of 20 : 1. When blisters are formed by increased intracellular Ca^{2+} [36], the plasma membrane of these blisters contains membrane protein band 3 and acetylcholineesterase, a superfical membrane enzyme, but are devoid of spectrin, actin and glycophorin. The plasma membrane of these blisters also contains a high lipid to protein ratio (2.8) and is enriched for diacylglycerol when compared to the total cell membrane. This selectivity of certain membrane components to the plasma membrane of blisters may be comparable to studies which showed that phagocytosis of particles by leukocytes internalized some plasma membrane components, but not others [37].

This separation of plasma membrane macromolecules may be due to differential response of these molecules to the reagent used to induce blistering.

Thus, treatment of L cells with glycerol or dimethylsulfoxide caused the aggregation of intramembraneous particles [12]. When membrane blisters were formed following dimethylsulfoxide treatment, the blisters were free of intramembraneous particles. Erythrocyte blisters formed following spectrin precipitation were also found to be devoid of intramembraneous particles [35]. In *Entamoeba histolotica*, glycerol also induced the aggregation of intramembraneous particles, but concanavalin A receptors still had a uniform distribution on the cell surface [13]. Others have shown that the fluidity of integral membrane components cannot be inferred from the demonstration of the mobility of surface components [38].

Glycerol was found in the present study to cause blistering of myoblasts, fibroblasts and myotubes. Plasma membranes in general appear to be elastic structures and the membrane blisters may be areas of the cell surface which for some structural reason, are weaker than other areas and therefore capable of expanding. The myotube surface contains filopodia [39,40], invaginations called caveolae and T-tubule connections [41,42] and the blisters may be the swelling or evagination of these structures. The plasma membrane-enriched fraction isolated from these blisters was found to be enriched 4—5 fold over the particulate homogenate for 5′-nucleotidase activity, but not for two other plasma membrane markers, $(Na^+ + K^+)$-ATPase and α-[^{125}I]bungarotoxin binding material. Schimmel et al. [43] also found a separation of these plasma membrane markers in membrane fractions isolated from 3-day-old chick myotubes using sucrose density gradients and more recently, Li and Hochstradt [44] found $(Na^+ + K^+)$-ATPase and 5′-nucleotidase activities enriched in different plasma membrane vesicle subfractions isolated from mouse fibroblasts. Since it was shown that 5′-nucleotidase is an ectoenzyme on these cells whose total cellular activity is present on the cell surface, this separation of labels represents either the morphological separation of these membrane proteins in the intact membrane or a differential response of membrane marcomolecules to reagents during the isolation procedure. 5′-Nucleotidase is a glycoprotein which binds concanavalin A [45,46], and is a plasma membrane ectoenzyme which probably does not penetrate deeply into the lipid bilayer. Its movement in the bilayer may therefore be controlled by a mechanism different from that for integral membrane macromolecules such as acetylcholine receptor and $(Na^+ + K^+)$-ATPase. Myofibers have been found to contain heterogenous populations of different sized intramembraneous particles [47] which probably represent integral membrane macromolecules and these may be aggregated away from the sites of glycerol-induced membrane blisters. On the other hand, it may represent a separation of these labels on the intact cell (e.g. 5′-nucleotidase may be associated with filopodia or caveolae) as found histochemically for other membrane macromolecules in other cell types [48].

Glycerol most readily induced blistering at the area of the myoblast plasma membrane most intimately involved in myoblast fusion (i.e. the tips, ref. 49). Since several recent studies looking for differences in prefused and postfused muscle cell membranes [26,43] have been done using the $(Na^+ + K^+)$-ATPase and α-bungarotoxin binding in membrane fraction, but not the 5′-nucleotidase fraction, it may be worth reexamining the 5′-nucleotidase fraction for membrane changes during cell fusion, especially since 5′-nucleotidase may be

associated with the lipid-rich, more fluid portion of the plasma membrane, which has been theorized by Lucy [50] to be involved in cell fusion.

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