ULTRASTRUCTURAL EVIDENCE FOR MYOSIN OF THE SMOOTH MUSCLE TYPE AT THE SURFACE OF TRYPSIN-DISASSOCIATED EMBRYONIC CHICK CELLS

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SUMMARY

Cells dissociated from embryonic chick muscle tissue using trypsin were rotated in the presence of globulin-enriched rabbit antisera against both smooth and striated muscle actomyosins originating from chicken gizzard (GAM) and pectoralis (PAM) muscles respectively. The presence of the rabbit antibodies was demonstrated using peroxidase-labelled sheep anti-rabbit y-globulins, the enzyme-antibody conjugate being located by electron-microscope histochemistry.

Anti-GAM γ-globulins reacted strongly with the plasma membrane. Judging from the complete absence of staining, γ-globulins from non-immunized rabbit serum did not interact with the membrane. When γ-globulins of sheep anti-rabbit IgG serum were applied alone, that is in the absence of pretreatment with rabbit γ-globulin, there was an observable reaction with the cell surface. Preincubation of anti-GAM with the heavy meromyosin fraction from smooth-muscle myosin inhibited the interaction of the antibodies with the membrane, as evidenced by the absence of staining. A weak positive reaction obtained with anti-PAM was due to components of the antibody preparation which were reactive with actin and not with PAM.

It was concluded that a smooth-muscle myosin-like protein is an integral part of the plasma membrane of embryonic chick muscle cells.

INTRODUCTION

Recent studies have shown that the aggregation of trypsin-dissociated embryonic chick muscle and liver cells is inhibited by the presence of antisera against actomyosin of the smooth-muscle type, believed to be present at the cell surface (Jones, Kemp & Gröschel-Stewart, 1970; Kemp, Jones & Gröschel-Stewart, 1971, 1973). Although it is considered that the major antigenic determinants for these antibodies are situated on the myosin molecule, proximal to the ATPase (Gröschel-Stewart, 1971; Kemp et al. 1973), the exact relationship of these determinants to the cell surface has not been fully established. Ultraviolet fluorescence microscopy using a fluorescein isothiocyanate (FITC)-antibody conjugate has been employed both for the direct detection at the cell periphery of rabbit anti-smooth-muscle actomyosin γ-globulins and for the location of bound rabbit antibodies by subsequent treatment of cells with labelled sheep anti-rabbit γ-globulins (Gröschel-Stewart, Jones & Kemp, 1970).
The evidence from the above quoted experimental work supports the theory that a surface-localized actomyosin-like protein may be involved in the regulation of cell adhesion (Jones, 1966, 1967; Jones & Kemp, 1970). A demonstration that this protein is an integral component of the plasma membrane, as defined by Robertson (1959), would strengthen the hypothesis. Owing to limited instrumental resolution, it is not possible using ultraviolet fluorescence microscopy, to locate an antigen-antibody complex within the membrane. However, the use of horseradish peroxidase as a label can enable the position of antibodies to be identified by electron microscopy (Nakane & Pierce, 1966; Avrameas & Bouteille, 1968). By conjugating the peroxidase to sheep anti-rabbit \( \gamma \)-globulins this method can be used to locate rabbit \( \gamma \)-globulin binding to the cell surface.

To summarize, the aim of the present investigation has been to detect the position of a myosin-like protein at the cell surface and to report evidence for its presence in the plasma membrane.

**MATERIALS AND METHODS**

**Dissociation**

Cells were obtained from the muscle tissue of 9-day-old chick embryos by treatment with 0.25 % (w/v) crude trypsin (Burroughs Wellcome, 1:300) for 10 min at 37 °C (Kemp, Jones, Cunningham & James, 1967). They were then suspended in Eagle's minimum essential medium (MEM) at a concentration of 2.0 \( \times \) 10\(^6\) cells/ml.

**Aggregation**

The cell suspension was placed in 25-ml Erlenmeyer flasks and the appropriate test solution was added to give a final concentration of 250 \( \mu \)g \( \gamma \)-globulin per 10\(^8\) cells. The flasks were rotated at 70 rev/min on a gyratory shaker (Moscona, 1961) for 4 h. The aggregates were then gently washed by centrifugation and resuspended in fresh Eagle's MEM for a further period of 1 h. Viability of the cells was tested by a dye exclusion method using lissamine green (Kemp et al. 1967).

**Actomyosin preparation**

Striated-muscle and smooth-muscle actomyosins were prepared from chicken pectoralis muscle (PAM) and gizzard (GAM) using a Weber-Edsall solution (Gröschel-Stewart, 1971) and purified by standard procedures (Gröschel-Stewart & Turba, 1963; Gergely, Martonosi & Gouvea, 1959). Myosin was isolated from GAM by fractional precipitation (Tsao, 1953) in the cold with neutral ammonium sulphate in the presence of 0.2 % (w/v) adenosine triphosphate, 10\(^{-5}\) M magnesium chloride and 5 \( \times \) 10\(^{-9}\) M cysteine (Huriaux, Hamoir & Oppenheimer, 1967). After dialysis against distilled water, myosin was digested with 0.01 % (w/v) crystalline trypsin (Worthington) for 10 min (Szent-Györgi, 1953) and heavy meromyosin (HMM) extracted as previously described (Kemp et al. 1973). The purity of the preparations was tested by electrophoresis on 7 % w/v polyacrylamide gels and they were found to contain HMM and a small proportion of myosin, but not actin. Actin was prepared as described by Gröschel-Stewart (1971).

**Antisera against actomyosins**

Anti-actomyosin sera were prepared by a standard course of injections, and the \( \gamma \)-globulins were precipitated with ammonium sulphate (Campbell, Garvey, Cremer & Susford, 1964). The non-specific \( \gamma \)-globulin antibodies were then removed by absorbing the preparation with
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liver powder (Garnett, Gröschel-Stewart, Jones & Kemp, 1973) and passing it through a column of resealed red blood cell ghosts adsorbed to glass beads. The presence of requisite antibodies and the absence from the anti-actomyosin γ-globulins of antibodies against the ground substance of muscle tissue were determined by immunodiffusion as described by Kemp et al. (1971).

The γ-globulins of anti-GAM and anti-PAM dissolved in Dulbecco phosphate-buffered saline (PBS-AB) were mixed with GAM-HMM, GAM-actin, or PAM powder in a weight ratio of 1:2, incubated for 1 h at 37 °C and stored for 16 h at 0 °C (Kemp et al. 1973). Insoluble material was removed by centrifugation at 10^4 g for 10 min at 4 °C. The supernatant was tested for residual antibody by immunodiffusion against HMM.

Preparation of sheep anti-rabbit globulin-peroxidase conjugate

Freeze-dried anti-rabbit IgG (SAR), 5 mg, obtained from sheep serum by precipitation with ammonium sulphate, and 10 mg of peroxidase (grade 1, Taab Laboratories, Reading) were dissolved in 1 ml of 0.05 M sodium phosphate buffer (pH 6.8). An 0.05-ml aliquot of a 1% (v/v) aqueous solution of glutaraldehyde (EM grade) was added, dropwise, to the mixture with stirring at 20 °C. The reaction mixture was then dialysed against Dulbecco PBS-A and the resultant precipitate was removed by centrifugation (Avrameas & Bouteille, 1968). The solution was diluted with 20 ml of 0.1 M sodium phosphate buffer (pH 6.8) before it was used.

The immunological activity of the peroxidase-conjugated SAR γ-globulins was tested against rabbit anti-actomyosin γ-globulins by immunodiffusion in agar. The γ-globulins were diluted to 0.1 mg/ml in Dulbecco PBS-A, the SAR γ-globulins being placed in the groove and the anti-actomyosin γ-globulins in the wells. In a further experiment, anti-GAM γ-globulins were placed in the groove and conjugated and unconjugated sheep anti-rabbit γ-globulins together with the γ-globulin fraction of sheep NIS were introduced into the wells. In both cases, the slides were incubated for 24 h at room temperature. They were then washed in Dulbecco PBS-A for 48 h, dried and stained (Gröschel-Stewart, 1968, 1971).

Exposure of treated aggregates to peroxidase-globulin conjugates

The aggregates were fixed in a mixture of 1% (w/v) formaldehyde and 1% (v/v) glutaraldehyde in o.01 M cacodylate buffer (pH 7.0) for 15 min. The formaldehyde solution was freshly prepared from paraformaldehyde by dissolving the polymer in water, while simultaneously heating the liquid and adding 1 N sodium hydroxide to give a pH of 7.0. After rinsing the aggregates in several changes of buffer for 15 h at 4 °C, the cells were exposed to SAR-peroxidase conjugate in the cold for 24 h. They were subsequently washed 3 times with cacodylate buffer over a period of 15 min at 4 °C and then further fixed in the formaldehyde-glutaraldehyde mixture for 15 min. After rinsing in 3 changes of 0.01 M cacodylate buffer over a period of 30 min, the material was transferred to the peroxidase substrate for 10 min (Graham & Karnovsky, 1966). This substrate was prepared by dissolving 5 mg of 3,3'-diaminobenzidine tetrahydrochloride in 10 ml of 0.05 M Tris-maleate buffer, pH 7.5, and adding 1 μl of 9% (v/v) hydrogen peroxide solution. The aggregates were washed in distilled water and then postfixed for 3 h in 1% (w/v) osmium tetroxide in 0.01 M cacodylate buffer. Following dehydration in ethanol, the aggregates were embedded in Taab resin (Taab Laboratories, Reading). Ultrathin sections, obtained using an LKB Ultrotome, were picked up on collodion-coated copper grids and examined using an AEI EM6B electron microscope.

RESULTS

In general the ultrastructure of trypsin-dissociated embryonic chick muscle cells was not changed by treatment with either SAR-peroxidase conjugate or rabbit antisera. However, the plasma membrane did appear incomplete in places, although there was no evidence from the dye exclusion tests of a loss of cellular permeability.

The SAR-peroxidase conjugate alone did not bind with the plasma membrane of
the cells (Fig. 1), indicating the absence of non-specific labelling by this conjugate. Cells not treated with either sheep or rabbit antibodies but stained for peroxidase showed no evidence for this enzyme at their surface. It will be seen from Fig. 2 that the surfaces of cells treated with NIS were not stained by the conjugate, thus pointing to the absence of a non-specific reaction between rabbit γ-globulins and the cell membrane. The immunodiffusion experiments conducted in parallel with these electron-microscope studies demonstrated that the SAR-peroxidase conjugate had retained its binding activity to rabbit γ-globulins.

Table 1. Summary of the results of experiments involving pretreatment of anti-actomyosins with possible antigenic determinants, before applying the antisera to the cells

<table>
<thead>
<tr>
<th>Pretreatment of antibody</th>
<th>None</th>
<th>GAM</th>
<th>PAM</th>
<th>HMM</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GAM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-PAM</td>
<td>+</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NIS</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>No pretreatment</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sheep anti-rabbit peroxidase conjugate</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Peroxidase substrate</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

The presence or absence of cell surface staining is indicated by + and − respectively. Qualitative estimation of the intensity of staining is represented as follows: +, very weak staining; + +, weak staining; + + +, intense staining; −, no staining; N, not tested.

An intense staining reaction was obtained in experiments in which cells were incubated with anti-GAM (Figs. 5–8), which had been shown by immunodiffusion experiments to contain neither ground substance nor actin, but to react with GAM myosin and HMM. The majority of the cell surface was heavily stained (Fig. 5). However, the unstained regions were not restricted to areas of apparent membrane perforation (Fig. 6). Microvillous projections (Fig. 6) and areas of close membrane apposition (Fig. 8) were particularly intensely stained. Fig. 7 shows the staining at a higher magnification and indicates that the antigenic determinant was probably located close to the outer hydrophilic part of the unit membrane.

Immunodiffusion experiments showed that anti-PAM did not react with smooth-muscle actomyosin or actin. However, some staining of the cell surface was observed (Fig. 3) with this antiserum, which was largely abolished by pretreatment of the anti-PAM with actin (Fig. 4). The results of control experiments involving the pretreatment of the antibodies with possible antigenic determinants are summarized in Table 1. Pretreatment of anti-PAM with actin reduced the reaction of the antibody with the cell surface suggesting that most of the binding of the anti-PAM was due to the presence of antibodies against small quantities of actin not detectable by immunodiffusion experiments.

There was no observable staining reaction when cells were treated with anti-GAM previously incubated with GAM-HMM at a concentration known, by tests employing
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immunodiffusion, completely to neutralize anti-GAM activity. This finding confirmed that there is a similarity between the antigenic sites on the cell surface and HMM.

DISCUSSION

Highly purified and specifically tested antibodies against smooth-muscle actomyosins were found to bind to the cell surface, the reaction sites being localized in the plasma membrane, as defined by Robertson (1959). Following this definition, there can be little doubt that the active sites are closely associated with the outer hydrophilic region of the plasma membrane. The apparent discontinuity of the membrane may be an artifact of the peroxidase staining technique. In this context it is important to appreciate that areas lacking stain were not always coincident with areas of apparent membrane perforation. Further, it is evident that staining never occurred in these perforated regions. Damage which occurred in culture should have been detected by a loss of permeability and disruption of the cells; any suggestion that this is not so and that the peroxidase-positive material had escaped from within the cells, could be countered by the fact that the material forming the apparent cell boundary between the regions of osmium deposition should then have stained for peroxidase.

The results of the control experiments strongly suggested that there are no sites present on the dissociated embryonic chick muscle cell that reacted either with non-specific rabbit immunoglobulins, which are presumably removed with liver powder, or with sheep anti-rabbit sera. As a result of these experiments it is highly probable that the observed staining was due to the presence of antigenic determinants to specific rabbit antibodies and that these antibodies were directed against a smooth-muscle-type myosin. This reinforces previous evidence that the active factor in anti-GAM serum is not an impurity but is directed against the HMM fragment of smooth-muscle myosin (Kemp et al. 1971, 1973), in that the peroxidase staining reaction of anti-GAM was abolished by preincubation of the antibody with GAM-HMM, which itself did not react with the cell surface as shown by electrophoretic mobility studies (Kemp et al. 1973). One cannot completely exclude the possibility that the active antibody was directed against another membrane component common to gizzard smooth-muscle cells and embryonic chick fibroblasts, because an absorption test with membrane proteins is not feasible if myosin is itself one of these proteins. However, there is not a component against erythrocyte membrane proteins, because the antiserum was passed over resealed red blood cell ghosts, which do not contain myosin (Gröschel-Stewart, 1969), prior to treatment of the cells.

The localization of smooth-muscle myosin in the plasma membrane should be related to reports of an inhibitory effect of anti-GAM γ-globulin on the aggregation of trypsin-dissociated embryonic chick cells (Kemp et al. 1971) and to the fact that this was lost after pretreatment of the antibody with HMM (Kemp et al. 1973). Earlier evidence had suggested that the effect of anti-GAM antibodies was surface-localized and Gröschel-Stewart et al. (1970) and Garnett et al. (1973) demonstrated the binding to cells of FITC-labelled antibodies directed against smooth-muscle
myosin. Furthermore, it was shown that a decrease in the negative electrophoretic mobility of chick fibroblasts resulted from treatment with anti-GAM γ-globulin, but not anti-PAM (Kemp et al. 1973). This indicated that the active site was within the electrophoretic plane of shear, that is within 1 nm of the plasma membrane. Thus the identification of smooth-muscle myosin as a component of the membrane, when associated with the inhibitory effect on aggregation of anti-smooth muscle antibodies can be taken to implicate this type of protein in the adhesive mechanism between cells, as has already been suggested (Jones, 1966, 1967; Jones & Kemp, 1970).

Several interpretations can be offered for the staining seen in areas of close membrane apposition. This observation suggests that the binding of the anti-GAM γ-globulins to the plasma membrane did not prevent adhesion between cells at these points. The absence of staining in control preparations confirms that the staining was not due to trapping of γ-globulin molecules but to the presence of antigenic determinants for anti-GAM. It is possible that the binding of antibody proximal to adhesive sites, limits only the overall strength of the initial adhesion, but does not prevent the formation of a certain number of adhesions. In this way, during the process of aggregation (Jones et al. 1970; Kemp et al. 1971, 1973), weakening of the adhesive strength by the binding of anti-GAM γ-globulins to myosin-controlling adhesive sites, would result in the observed overall inhibition of aggregation. This is because the cells would have less chance of making successful adhesions for a given number of collisions under the conditions of shear. Since there is an interchange of cells between the aggregate and the medium, any weakening of adhesive strength would also tend to increase the number of cells de-adhering and thus displace the equilibrium. While it is felt that adhesion is due to the discrete co-operation of the myosin-like protein with this initial adhesive mechanism, the possibility cannot be excluded that adhesive sites proximal to, but not co-operatively related to, this protein may be masked by the presence of the antibody.

The reaction of the anti-PAM preparation with the cell surface would appear to be due to a component which binds with actin. Anti-actin antibodies are not thought to be type-specific and in the past workers have experienced considerable difficulty in preparing them. Immunodiffusion experiments have consistently failed to reveal anti-actin antibodies in preparations used in previous studies (Kemp et al. 1971, 1973) and, in fact, it had been considered that the anti-GAM antibody is essentially an anti-myosin. It would appear that the sensitive peroxidase staining technique has revealed small quantities of an anti-actin antibody in the anti-PAM preparation though not in anti-GAM. This tentative conclusion reopens the possibility that the smooth-muscle type myosin at the cell surface may be associated with an actin-like protein.

Although the nature of the adhesive mechanism between cells is not known, the suggestion that a myosin-like protein is involved is further strengthened by the presence of this protein in the plasma membrane.

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REFERENCES


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Fig. 1. Cells treated with peroxidase-sheep anti-rabbit conjugate alone after fixation in aldehydes, and followed by staining for peroxidase prior to further processing, showing absence of staining reaction. × 35000.

Fig. 2. Aggregating cells treated with NIS, followed by peroxidase-labelled SAR. × 24450.
Fig. 3. Aggregating cells treated with anti-PAM, followed by peroxidase-labelled SAR. x 34,200.

Fig. 4. Aggregating cells treated with anti-PAM which had been pretreated with actin, followed by peroxidase-labelled SAR. x 31,000.
Figs. 5-8. Aggregating cells treated with anti-GAM, followed by peroxidase-labelled SAR.

Fig. 5. × 19,000.

Fig. 6. Note staining of the surface of the microvillus. × 24,000.

Fig. 7. High-power micrograph showing that the staining is closely associated with the plasma membrane. × 64,100.

Fig. 8. Staining of the plasma membrane in area of close membrane apposition (arrow). × 22,700.
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1.0 μm

0.25 μm