STUDIES ON THE FACTORS AFFECTING CELLULAR SPREADING UPON CULTURE-SUBSTRATE

1. THE EFFECTS OF DIVALENT CATIONS AND CONDITIONED MEDIUM ON CELLULAR SPREADING

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SUMMARY

The effects of various divalent cations on cellular spreading of in vitro cultured fibroblasts of chick embryos upon substrates were investigated in serum-free Eagle's MEM. Fibroblasts spread on the surfaces of culture dishes in the presence of Mn2+, Mg2+, Ca2+ or Co2+, but not in the presence of Zn2+, Cd2+, Sr2+ or Cu2+. The minimum cation concentration for permitting cellular spreading at 37 °C is different for each cation. The values are $10^{-4}$, $5 \times 10^{-4}$, $10^{-3}$ and $10^{-2}$ M for Mn2+, Mg2+, Ca2+ and Co2+, respectively. Below these concentrations the cells remain round. Mn2+ is about 1000-fold more effective in causing spreading than Ca2+. The cells do not spread at low temperature, but they do at 22 °C in the presence of $10^{-4}$ M Mn2+.

In a medium conditioned with a mass culture of fibroblasts, cell spreading occurs at one tenth of the cation concentrations given above. If conditioned medium is heated at 65 °C for 30 min or treated with trypsin, its effect is abolished; this suggests that the effective factor(s) in the conditioned medium is a protein.

INTRODUCTION

When cells are inoculated into culture dishes, they are usually spherical immediately after attachment to the surfaces of dishes. In serum-free Eagle’s MEM, they flatten and spread to form pseudopodia after several hours. The addition of serum to the medium retards spreading (Easty, Easty & Ambrose, 1960; Nordling, 1967; Taylor, 1961; Witkowski & Brighton, 1972). The effects of Ca2+ and Mg2+ on the occurrence of cellular spreading in the cell-substrate adhesion have been analysed (Garvin, 1968; Rabinovitch & DeStefano, 1973; Takeichi & Okada, 1972) and it is clear that Mg2+ is more effective than Ca2+. In this paper, the effects of divalent cations other than Ca2+ and Mg2+ on cellular spreading were examined. One surprising result is the great effectiveness of Mn2+. The enhancing effect on spreading of medium conditioned with a culture of a large number of chick fibroblasts was also studied.
MATERIALS AND METHODS

Cells used for assay

Fibroblasts originated from the lungs of White Leghorn chick embryos at 11-12 days of incubation were used. Primary cultures of dissociated cells were obtained by trypsin treatment of small pieces of lung according to the procedure given by Takahashi & Okada (1970, 1971). When the cultures attained confluence after 3 days of incubation with Eagle's MEM supplemented with 10 % calf serum, the cells were harvested by treatment with 0.25 % trypsin solution in Ca	extsuperscript{2+}- and Mg	extsuperscript{2+}-free Hank's saline (CMF) at 37 °C for 30 min. After thorough washing by repeated centrifugations, the cells were resuspended in CMF to give a cell density of $1 \times 10^5$ per ml.

Preparation of conditioned medium

Primary cultures of dissociated cells of thigh muscle from 12-day-old chick embryos were used for obtaining the conditioned medium (Takahashi & Okada 1971). When the cells were grown to confluence, usually 3 days after inoculation, the culture medium consisting of Eagle's MEM and calf serum was removed and the dishes were well washed with Hank's saline. Then, 10 ml of fresh Eagle's MEM without calf serum were added. After 24 h the medium was discarded. Thereafter, the serum-free medium was conditioned with mass cultures every 24 h for 5 days. The harvested conditioned medium (CM) was pooled, centrifuged at 10000g for 30 min, and then the supernatant was filtered through a Millipore filter (pore size 0.45 μm) and stored at 4 °C. Before use, CM was concentrated about 50-fold by means of passive pressure ultrafiltration through a collodion bag (Carl Schleichen and Shull, Germany). The concentrated CM was dialysed thoroughly against CMF to remove divalent cations contained in the original sample. As will be shown below, all of the spreading-enhancing activity of CM remained in the undialysable portion.

Determination of the degree of cellular spreading

Cells suspended in Ca	extsuperscript{2+}- and Mg	extsuperscript{2+}-free Eagle's MEM were inoculated into Falcon plastic dishes (3-3 cm diameter). It is necessary to distinguish the initial attachment of cells to the substrate from the subsequent spreading of the attached spherical cells. Therefore, to eliminate the effects of divalent cations and CM on the attachment of cells to the substrate, the spherical cells adhering to the surfaces of the dishes were used for an assay of cellular spreading. After incubation at 37 °C for 15-20 min, almost all of the cells were attached to the substrate. Then the fluid was replaced with 1-5 ml of one of the media to be tested. Ca	extsuperscript{2+}- and Mg	extsuperscript{2+}-free MEM was a control medium and the cation to be tested and/or CM was added to it for assay by micropipette. All the divalent cations tested were used as chlorides. Cell number per dish was adjusted to be about $5 \times 10^5$. The 3 stages of cellular spreading identified in human conjunctiva strain cells upon culture substrates (Taylor, 1961) were similarly observed in the present case. At stage I attached cells were spherical, having no flattened process. At stage II cells had some flattened processes. Stage III was identified by the presence of pseudopodia from sufficiently spreading cell bodies with very flattened nuclei (see Fig. 4). The degree of spreading was expressed as a percentage of the number of cells at stage III to the total attached cell number after 6 h of incubation. At least 1000 cells were scored in several representative areas of culture dishes observed directly under a phase-contrast microscope (Nikon, Tokyo) or from photographs. Experiments were performed 3 times independently. Viability tests of cells by dye exclusion using Trypan Blue indicated that about 60 % of cells at stage I, 50 % at stage II and 100 % at stage III were alive.

Assay for testing the activity of CM

Freshly prepared conditioned medium before concentration usually contained about 20 μg of protein per ml, measured using the Lowry procedure (Lowry, Rosebrough, Farr & Randall, 1951). The relationship between the protein concentration of CM and % of spreading cells...
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at 10^{-4} M Ca^{2+} is presented in Fig. 1. The number of spreading cells increased linearly up to a protein concentration of 10 \mu g per ml of medium, where a plateau was observed. In all subsequent assays, the concentration of CM was fixed at 20 \mu g protein per ml of medium being tested.

![Graph showing effect of concentration of protein contained in CM on cellular spreading.](image)

**Fig. 1.** Effect of concentration of protein contained in CM on cellular spreading. Cells were cultured for 6 h in CMF-MEM containing 10^{-4} M Ca^{2+} and various concentrations of CM.

**Enzyme treatment of CM**

Enzyme treatments of CM with trypsin (Recrystallized, Type 1, Sigma Chemical Co., St Louis), pronase (B grade, Calbiochem, Los Angeles, Calif.), RNase (Sigma) and DNase (Worthington Biochemical Corp., Freehold, N.J.) were carried out at neutral pH and 37 °C for various lengths of time. At the completion of the treatment, if necessary, the enzymes were inhibited using specific inhibitors, Trypsin Inhibitor (Sigma Type 1-S, from soybean) and Albumin (Merck, Darmstadt, Germany). The enzyme inhibitors added had little effect on cellular spreading.

**RESULTS**

**Rate of spreading**

Kinetics of spreading of cells at 10^{-3} M Mg^{2+} in the absence or presence of CM are shown in Fig. 2. The number of spreading cells increased linearly up to a maximum value of about 60% by 6 h. After 24 h of incubation the plateau value was slightly decreased. By adding CM to the medium, the increase of spreading cells was promoted in the initial phase. The maximum value was about 1.5-fold greater than in the absence of CM. Since the percentage of spreading cells reached a plateau by 6 h in any media tested, measurements of the degree of spreading were made at 6 h in the following tests. In control medium, CMF-MEM, little cellular spreading occurred. No cells spread if the contaminating level of Ca^{2+} or Mg^{2+} was removed by addition of a chelating reagent, such as 0.1 mM EDTA, to the control medium.

**Effect of Ca^{2+}**

The degree of cellular spreading was examined in CMF-MEM to which various concentrations of Ca^{2+} from 10^{-6} to 10^{-2} M were added in the absence or presence of
CM. As shown in Fig. 3A, Ca\(^{2+}\) was effective for permitting cell spreading. In the absence of CM, cells remained round at concentrations below \(10^{-4}\) M and cellular spreading occurred above \(5 \times 10^{-4}\) M Ca\(^{2+}\).

When CM was present, spreading of cells occurred at \(5 \times 10^{-8}\) M. The percentage of spreading cells increased between \(10^{-4}\) and \(10^{-3}\) M and remained constant above \(10^{-3}\) M. The maximum percentage of spreading cells in the presence of CM was about 1.5 times that in the absence of CM.

![Graph showing cellular spreading](image)

**Fig. 2. Rate of cellular spreading in medium containing \(10^{-3}\) M Mg\(^{2+}\) in the absence (\(\bullet\)-\(\bullet\)) or presence (\(O\)-\(O\)) of CM.**

**Effect of Mg\(^{2+}\)**

As shown in Fig. 3B, without CM cellular spreading occurred at concentrations above \(5 \times 10^{-4}\) M Mg\(^{2+}\). The percentage of spreading cells increased from \(5 \times 10^{-4}\) to \(10^{-3}\) M and remained constant over \(10^{-3}\) M. In the presence of CM, however, spreading of cells was observed at \(10^{-6}\) M. At the lower concentrations of Mg\(^{2+}\) the addition of CM was effective in increasing the percentage of spreading cells, though this effect disappeared in the presence of more than \(10^{-3}\) M of Mg\(^{2+}\).

**Effect of Mn\(^{2+}\)**

Fig. 3C shows that many cells spread well at \(10^{-5}\) M Mn\(^{2+}\) in the absence of CM and that cellular spreading occurred at \(10^{-7}\) M if CM was present. Mn\(^{2+}\) was found to be about 100-fold more effective than Ca\(^{2+}\) or Mg\(^{2+}\) for permitting cellular spreading with respect to the minimum effective concentration of ions. The percentage of spreading cells increased in proportion to the concentration and reached a plateau at \(10^{-5}\) M and \(10^{-4}\) M in the presence and absence of CM, respectively.

**Effects of other alkaline earth ions**

At \(10^{-3}\) M Co\(^{2+}\) about 20% of cells spread with or without CM, whereas ions of other alkaline earth metals such as Zn\(^{2+}\), Sr\(^{2+}\), Cd\(^{2+}\) and Ba\(^{2+}\) were not effective, as shown in Fig. 3D.
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Spreading of cells at various temperatures

Spreading of cells at various temperatures was tested at pH 7.0; pH of the media was maintained with 10 mM HEPES buffer. For the short-term cultures of the present experiments this buffer was not toxic to cells as tested by staining with trypan blue.

Results are shown in Table 1. With 10^{-8} M Mg^{2+} most cells remained round at 22 °C, whereas they spread at 37 °C. However, if CM was added, spreading of cells occurred at 10^{-8} M Mg^{2+} at 22 °C. On the other hand, at 22 °C cellular spreading occurred with 10^{-6}-10^{-3} M Mn^{2+} in the absence of CM. The addition of CM caused an increase in the percentage of spreading cells. At 10 or 4 °C, no spreading of cells occurred even at the highest concentrations of Mg^{2+} or Mn^{2+} tested with or without CM.

Fig. 3. Effects of various concentrations of divalent cations. A, Ca^{2+} in the absence (●) or in the presence (○) of CM; B, Mg^{2+} in the absence (●) or in the presence (○) of CM; C, Mn^{2+} in the absence (●) or in the presence (○) of CM; D, Cd^{2+} in the absence (●) or in the presence (○) of CM, and the effects of Zn^{2+}, Sr^{2+}, Cd^{2+} and Ba^{2+} (○—○).
Table 1. Effect of temperature on percentage of spreading cells in the presence of various cations, without or with conditioned medium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10^{-4} M Mg^{2+}</th>
<th>10^{-3} M Mg^{2+}</th>
<th>10^{-4} M Mn^{2+}</th>
<th>10^{-4} M Mn^{4+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>- CM</td>
<td>+ CM</td>
<td>- CM</td>
<td>+ CM</td>
<td>- CM</td>
</tr>
<tr>
<td>37 °C</td>
<td>24.2</td>
<td>39.6</td>
<td>69.8</td>
<td>79.2</td>
</tr>
<tr>
<td>22 °C</td>
<td>24.4</td>
<td>37.7</td>
<td>53.4</td>
<td>60.9</td>
</tr>
<tr>
<td>0 °C</td>
<td>0.0</td>
<td>0.0</td>
<td>0.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The numbers of spreading cells were counted after 6 h in culture.

Table 2. Effect of CM, heated or boiled for various lengths of time, on the spreading of cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% spreading cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>65 °C, 10 min</td>
<td>7.3</td>
</tr>
<tr>
<td>65 °C, 30 min</td>
<td>4.1</td>
</tr>
<tr>
<td>65 °C, 60 min</td>
<td>1.3</td>
</tr>
<tr>
<td>100 °C, 10 min</td>
<td>1.0</td>
</tr>
<tr>
<td>100 °C, 30 min</td>
<td>1.0</td>
</tr>
<tr>
<td>Control (+ untreated CM)</td>
<td>41.3</td>
</tr>
<tr>
<td>(- CM)</td>
<td>13.3</td>
</tr>
</tbody>
</table>

Cells were cultured for 6 h in CMF-MEM containing 10^{-4} M Ca^{2+} and CM which had undergone various treatments as shown. For further details see text.

Table 3. Effect of CM treated with various enzymes on cellular spreading

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Concentration, µg/ml</th>
<th>Time, min</th>
<th>% spreading cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>1000</td>
<td>10</td>
<td>0.9</td>
</tr>
<tr>
<td>Trypsin</td>
<td>1000</td>
<td>30</td>
<td>0.0</td>
</tr>
<tr>
<td>Pronase</td>
<td>500</td>
<td>30</td>
<td>0.0</td>
</tr>
<tr>
<td>DNase</td>
<td>100</td>
<td>60</td>
<td>40.1</td>
</tr>
<tr>
<td>RNase</td>
<td>100</td>
<td>60</td>
<td>42.3</td>
</tr>
<tr>
<td>Trypsin</td>
<td>1000</td>
<td>0</td>
<td>35.4</td>
</tr>
<tr>
<td>Pronase</td>
<td>500</td>
<td>0</td>
<td>34.3</td>
</tr>
<tr>
<td>DNase</td>
<td>100</td>
<td>0</td>
<td>41.2</td>
</tr>
<tr>
<td>RNase</td>
<td>100</td>
<td>0</td>
<td>40.3</td>
</tr>
<tr>
<td>Control (+ CM)</td>
<td>43.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(- CM)</td>
<td>34.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

About 40-fold-concentrated CM was treated with various enzymes at 37 °C for the periods of time shown in the Table before using for assays of spreading. Fifty microlitres of treated CM and 1 mg of inhibitor (T.I. or Alb.) were added to 1.5 ml of CMF-MEM containing 10^{-4} M Ca^{2+}. Where the time is shown as zero, 50 µl of CM-enzyme mixture and 1 mg of inhibitor were added simultaneously to the test medium and then cells were cultured for 6 h.
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Characterization of the active factor(s) contained in CM

Some physical and chemical properties of the spreading-enhancing activity (SEA) in CM were investigated. The factor(s) for SEA in CM did not pass through a dialysis membrane, indicating that the factor(s) is non-dialysable and thus probably a macromolecule; this hypothesis is supported by column chromatography (manuscript in preparation). When the concentrated CM was heated at 65 °C for 30 min or at 100 °C for 10 min, SEA was completely destroyed (see Table 2). Treatment of CM with trypsin or pronase for 30 min resulted in a complete loss of SEA as shown in Table 3. For this type of test the addition of trypsin inhibitor or albumin respectively to each assay medium was necessary, in order to abolish any effect of trypsin or pronase on cellular spreading. Therefore, control experiments with the medium containing CM, trypsin and trypsin inhibitor were carried out. In these media, the cells spread to the same extent as in medium with CM alone. Neither RNase nor DNase had any appreciable effect on SEA. These results indicate the protein nature of the effective factor(s) in CM.

DISCUSSION

Using electron microscopy (Eguchi & Okada, 1971; Lesseps, 1963) or a Stereoscan (Boyle, Weiss & Vesely, 1972; Witkowski & Brighton, 1971, 1972), cells are observed to adhere to the surface of the substrate by processes or microextensions on the cell surface, and cell contact is actually initiated at the crests of the undulating surfaces of the cells. The active spreading or protrusion of pseudopodia from cells can be assumed to play an important role in cell-to-substrate or cell-to-cell adhesion as well as in cellular locomotion. Studies on cellular spreading have disclosed that the medium constituents, such as sera and divalent cations, affect cellular spreading as well as cell-to-substrate and cell-to-cell adhesion. The reported effects of serum on this spreading, however, are not always consistent. Lieberman & Ove (1958) reported that cellular spreading required a fraction from bovine serum, and Fischer, Puck & Sato (1958) noted that the α-globulin-rich fraction of foetal calf serum, fetuin, was essential for spreading. On the other hand, Taylor (1961) and Witkowski & Brighton (1972) showed that the presence of serum retarded cellular spreading and that cells spread well in serum-free medium. As Nordling (1967) has pointed out, it is very difficult to make direct comparisons of the results obtained from experiments using different cells, different sera and different substrates. At any rate, the results of the present experiments, using minimal medium containing no serum protein, have revealed that cellular spreading occurs only in the presence of divalent cations, such as Mn²⁺, Ca²⁺ or Mg²⁺, the presence of serum proteins or other macromolecules being unessential.

Through different approaches from those mentioned above, several investigators have shown that conditioned medium contains a factor which enhances the growth of culture cells (Austin, McCulloch & Till, 1971; Rubin, 1966; Takahashi & Okada, 1970, 1971). Rubin assumed that the effective factor is a lipoprotein derived from cell
membranes. Some physical and chemical properties of the factor were discussed in the papers listed above. In the present study the presence of a factor(s) enhancing cellular spreading is demonstrated in the conditioned medium. Some similarities in chemical or physical properties of this factor(s) with Rubin's factor are also suggested. Therefore, the possibility exists that the factor(s) in CM for promoting cellular spreading is also derived from the cell membrane as assumed by Rubin. Of various divalent cations tested, Mn$^{2+}$ was most effective for cellular spreading (Rabinovitch & De-Stefano, 1973). It is well known that Mn$^{2+}$ acts as a cofactor for phosphotransferases and other enzymes in the membrane. Thus, it seems possible that the requirement of divalent cations for cellular spreading could be due to the metabolic activation of the protrusion of pseudopodia from cells which adhered to the substrate. The factor(s) in conditioned medium may encourage the protrusion of pseudopodia in collaboration with the necessary divalent cation, thus leading to enhanced cellular spreading. Further study on the physiological action of such factor(s) using a purified sample isolated from the conditioned medium should help to explain the mechanism of cellular spreading.

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REFERENCES


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Fig. 4. Phase-contrast photomicrographs of cells at various stages. Cells were cultured for 30 min in CMF–MEM containing $10^{-3} \text{M} \text{Mg}^{2+}$. a, b and c indicate cells at stages I, II and III, respectively. ×280.