Two Mechanisms in Cell Adhesion Revealed by Effects of Divalent Cations

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ABSTRACT. The effect of divalent cations on cell-to-cell and cell-to-substrate adhesions was studied using two different types of chicken embryonic cells (fibroblastic and neural retina cells). Mn^{2+} , Co^{2+} , Mg^{2+} and Ca^{2+} were effective in promoting the aggregation of fibroblastic cells, while Sr^{2+} and Ba^{2+} were not effective. Among these ions, Ca^{2+} showed some unique effects on cell aggregation. Aggregation was initiated immediately after the addition of Mn^{2+} , Co^{2+} or Mg^{2+} whereas with Ca^{2+} a lag period was always observed. When the medium contained both Ca^{2+} and Mg^{2+} , the aggregate size formed was bigger than that formed with either ion at an equimolar concentration. The divalent cation dependence of fibroblastic cell adhesion to non-cellular substrate was essentially the same as that of cell-to-cell adhesion. The aggregation of neural retina cells specifically depended on the presence of Ca^{2+} . For adhesion of neural retina cells to non-cellular substrate, however, Ca^{2+} was not effective, and only Mn^{2+} or Co^{2+} was effective. These results suggest two different mechanisms in cell adhesion.

Cells dispersed experimentally from animal tissues are known to reaggregate under appropriate conditions. The aggregation of cells generally requires the presence of divalent cations in the medium. Elucidation of the role of divalent cations in cell adhesion, therefore, is fundamental to a general understanding of the mechanism of cell adhesion (6). One difficulty in interpreting the results of published works on the effect of divalent cations is that the studies have been carried out in different experimental systems or used different types of cells. While the importance of Ca^{2+} has been repeatedly emphasized (2, 4, 15, 17, 24), recent studies have shown that Mg^{2+} or Mn^{2+} is more effective than Ca^{2+} in promoting cell adhesion (1, 3, 12, 14, 22, 26).

Most animal cells are able to adhere to non-cellular substrates and to each other. It has not been demonstrated, however, whether the mechanisms of these two types of adhesion are the same. In the present study, we compared the effectiveness of various divalent cations on both cell-to-cell and cell-to-substrate adhesion.

MATERIALS AND METHODS

Cells. Fibroblastic cells and neural retina cells of chick embryos were used. For obtaining fibroblastic cells, the head skin was removed from 11-or 12-day-old chick embryos and the dermis was separated from the epidermis in Ca^{2+} - and Mg^{2+} -free modified saline G (CMFS) (21). Fragments of the dermis were incubated in CMFS with 0.5% crude trypsin (Difco, Detroit, Mich., 1:250) and 0.01% crude collagenase (Worthington, Freehold) for 30 min at 37°C. The dissociated cells were cultured with Eagle's MEM (Nissui, Tokyo) supplemented

with 6% fetal calf serum (Gibco, Grand Island) and 2.2g/l NaHCO_3 in Falcon plastic petri dishes in a water-saturated atmosphere containing 5% CO_2 for 4-5 days until cultures reached a confluent state.

Neural retina cells were used for assay directly after dissociation of neural retinae from 8-day-old chick embryos, as described in the following section.

Preparation of cell suspensions. Fibroblastic cells in confluent state were washed with CMFS and incubated with 2 ml of dissociation medium composed of 0.25% crude trypsin and 0.01% crude collagenase in CMFS for 15 min at 37°C. After removing the medium by centrifugation ($160 \times g$, 4 min), the cells were washed once with 1 mM EDTA in CMFS, resuspended in cold CMFS and were gently pipetted. They were filtrated through four layers of gauze and two layers of silk cloth, and washed twice with cold CMFS by centrifugation. The cells were finally suspended in basal assay medium at a density of 3×10^6 cells/ml. This medium was Ca^{2+} - and Mg^{2+} -free modified Eagle's MEM (CMF-MEM), which was composed of 6.8 g NaCl, 0.4 g KCl, 1.0 g glucose, 0.86 g salt-free Eagle's MEM powder (Nissui, Tokyo) and 2.38 g HEPES (Dojin, Kumamoto) in 1 liter of triple-distilled water, and was adjusted to pH 7.4 with 1 N NaOH. Almost all cells in suspensions obtained by such procedures were singly dispersed. All solutions used for assays were filtrated through Millipore filter to render them strictly dust-free.

Neural retinae isolated from embryos were incubated in 1 mM EDTA for 10 min at room temperature. After additional incubation in 0.5% crude trypsin in CMFS for 40 min at 37°C, the tissues were dissociated by flushing with a pipette. Dispersed cells were treated with 50 $\mu\text{g}/\text{ml}$ DNase (Sigma, St. Louis, 1 x crystallized) in CMFS containing 1 mM MgCl_2 for 10 min at 37°C to remove the DNA released from lysed cells, and washed once with 1 mM EDTA and twice with CMFS. The cells were finally suspended in CMF-MEM to a density of 2×10^7 cells/ml.

Culture dish. For studying cell-to-cell adhesion (cell aggregation), the condition of excluding cell adhesion to the culture dish was essential. The aggregation dishes used were polyvinyl chloride cylinder (inner diameter 28 mm) firmly attached to the bottom of plastic petri dishes (Jintan-Termo, Tokyo). Into each aggregation dish 5 ml of 0.5% bovine serum albumin (Armour, Chicago, fraction V) and 0.8% NaCl dissolved in water was added and left overnight in a refrigerator. After removing the solution the dishes were repeatedly rinsed with distilled water. In such albumin-coated dishes, cell adhesion to the substrate was effectively prevented. All test tubes and pipettes used for assaying cell adhesion were also previously coated with albumin as described above.

For studying cell-to-substrate adhesion, Falcon plastic petri dishes (6 cm in diameter) were coated with 5 ml of 0.5% gelatin (Merck) and 0.8% NaCl in double distilled water in the same manner as in coating with albumin.

Measurement of cell adhesion. For assaying cell-to-cell adhesion, 0.3 ml of cell suspension was added into each aggregation dish with 2.7 ml of CMF-MEM prewarmed to 37°C. The final cell density in each dish was 3×10^5 cells/ml for fibroblastic cells and 2×10^6 cells/ml for neural retina cells. The following method was used for measurement of the rate of cell-to-cell adhesion, unless otherwise noted. The aggregation dishes with cells were placed on a Gyrotory Shaker (model G-25, New Brunswick Scientific Co.) and rotated at 80 rpm at 37°C. After about 30 min, the cells concentrated in the center of each cylinder. A given amount of divalent cation(s) was then added to the cylinder from the stock solution, which determined the zero period of aggregation. Dishes were removed from the Gyrotory Shaker at appropriate intervals, and cells were immediately fixed by adding 2 ml of 5% glutaraldehyde in CMFS at pH 8, under constant medium agitation. This fixation did not cause artificial aggregation or dissociation of cells if the measurement was conducted within a few hours. The method for measuring aggregation is described below.

For assaying cell-to-substrate adhesion, 3 ml of cell suspension was inoculated in each

gelatin-coated Falcon plastic dish at the final cell density of 3×10^5 cell/ml. After adding the necessary quantity of divalent cation(s), the dishes were incubated in a water-saturated atmosphere at 37°C without agitation. Cells were fixed, following varying incubation intervals, and the number of non-adhering cell counted. Neither further attachment of still non-adhering cells nor detachment of once attached cells occurred after fixation.

Fixed cell suspensions were diluted with 10 ml of CMFS, and the total particle number which included the number of single cells plus the number of aggregates was counted with a Coulter counter (model Z_B, Coulter Electronics, Hialeah, Fla.) equipped with a $100\ \mu$ aperture.

The degree of aggregation was represented by the index N_t/N_0 , where N_0 is the initial particle number applied into each dish and N_t is the total particle number at the indicated time (7, 10). The degree of cell attachment to the substrate was represented as the percentage of unattached cells.

Stock solutions. All divalent cations were prepared as chloride salts. One hundred-fold concentrated stock solutions were prepared in H_2O . Crystallized trypsin (Sigma, type I) and trypsin inhibitor (Sigma, Type I-S) were dissolved in CMF-MEM in 10-fold concentration.

RESULTS

Effects of Mg^{2+} and Ca^{2+} on cell-to-cell adhesion of fibroblastic cells. Fibroblastic cells which hardly aggregated in CMF-MEM started aggregation immediately after addition of 1 mM Mg^{2+} , and the aggregation continued for about 3 hours (Fig. 1). The degree of aggregation was dependent upon the concentration of Mg^{2+} added, as judged from the N_t/N_0 ($t=60$ min) value in different concentrations of Mg^{2+} in the range between 0.01 and 20 mM (Fig. 2).

When 1 mM Ca^{2+} was added, aggregation started with a 30 min lag. After 60 to 90 min of incubation, N_t/N_0 value of Ca^{2+} became almost identical to that in Mg^{2+} , and sometimes even exceeded the Mg^{2+} value after prolonged incubation (Fig. 1). When

Fig. 1. Aggregation kinetics of fibroblastic cells in 1 mM Ca^{2+} , 1 mM Mg^{2+} or in the absence of both ions (CMF). The experiments were repeated five times, and similar kinetic patterns were obtained.

Fig. 2. Comparison of aggregation index N_t/N_0 of fibroblastic cells in various concentrations of six different cations, where t was 60 min. The figure represents the result of one of three experiments with essentially the same result.

the concentration of Ca^{2+} was tested in a range 0.01 to 20 mM, the maximum effect was obtained at 0.5 and 1 mM, and in higher concentrations, the aggregation was somewhat inhibited (Fig. 2).

When 1 mM Mg^{2+} was added to cells which were previously incubated in CMF-MEM for 180 min at 37°C , aggregation started immediately and continued at the nearly same rate as in cells without the previous incubation. This result ruled out the possibility that the failure of aggregation in CMF was due to cell death in such a special environment. Aggregation was completely inhibited at 4°C in any ionic condition (data not shown). When 0.05% crystallized trypsin was added in the aggregation medium, the aggregation was completely inhibited in the presence of Ca^{2+} or Mg^{2+} . Such trypsin effects were abolished with 0.1% soybean trypsin inhibitor.

Effect of Mn^{2+} , Co^{2+} , Ba^{2+} and Sr^{2+} on cell-to-cell adhesion. The effect of Mn^{2+} , Co^{2+} , Ba^{2+} and Sr^{2+} on aggregation of fibroblastic cells was compared with the effect of Mg^{2+} and Ca^{2+} by obtaining the N_t/N_0 value, where t was 60 min (Fig. 2). Mn^{2+} was the most effective ion in comparisons of the lowest effective concentration. The effectiveness of Co^{2+} was almost the same as that of Mg^{2+} . These three ions were not inhibitory at high concentrations within the tested range. It was also common to these cations that they initiated aggregation without a lag (Fig. 3). In Sr^{2+} and Ba^{2+} fibroblastic cells did not aggregate.

Effect of Ca^{2+} and Mg^{2+} simultaneously added to medium. The aggregation of fibroblastic cells was carried out in medium containing both Ca^{2+} and Mg^{2+} (Fig. 4). The rapid aggregation observed in medium with Mg^{2+} was inhibited by the coexistence of Ca^{2+} for 30 min which corresponded to the lag period of the aggregation in the medium with Ca^{2+} alone. After 60 min, the N_t/N_0 value was intermediate between the two control values with Ca^{2+} or Mg^{2+} alone. Then, the N_t/N_0 value of Mg^{2+} plus Ca^{2+} decreased below the control values, suggesting the additive effect of these ions on the later stage of aggregation.

Fig. 3. Aggregation kinetics of fibroblastic cells in six different cations (Mn^{2+} , 0.1 mM; others, 1 mM). The figure represents one of two experiments with similar results.

The inhibitory effect of Ca^{2+} on cell aggregation in the initial stage was shown in another way. When Ca^{2+} was added to an aggregating cell suspension 30 min after the addition of Mg^{2+} , the N_t/N_o value immediately increased and the value remained constant for about 20 min, before a sharp drop occurred (Fig. 5). This result strongly suggests that Ca^{2+} caused a partial dissociation of aggregates previously formed in Mg^{2+} and temporarily inhibited aggregation. Perhaps the appearance of an initial lag in aggregation by Ca^{2+} was due to such a Ca^{2+} inhibitory effect.

Fig. 4. Aggregation kinetics of fibroblastic cells in the medium with both 1 mM Mg^{2+} and 1 mM Ca^{2+} . Controls of 1 mM Mg^{2+} alone or 1 mM Ca^{2+} alone were conducted using the same cell suspension as the experiment. Essentially the same result was obtained in another assay.

Fig. 5. The effect of 1 mM Ca^{2+} added to samples of fibroblastic cells under aggregation in 1 mM Mg^{2+} . Note the immediate increase of the aggregation index N_t/N_o after addition of Ca^{2+} (arrow). Similar results were obtained in two other experiments.

Difference in size-distribution between aggregates formed in Mg^{2+} and Ca^{2+} . Microscopically, the maximum aggregate size formed in the medium with a physiological concentration of Ca^{2+} was always larger than in the medium with an equimolar concentration of Mg^{2+} , even when the Nt/No value was almost identical (Fig. 6). Such an

Fig. 6. Photographs of aggregates of fibroblastic cells formed after 120 min of gyration in 1 mM Mg^{2+} (A) and 1 mM Ca^{2+} (B). The Nt/No value was 0.47 in Mg^{2+} and 0.48 in Ca^{2+} . $\times 50$.

observation was confirmed quantitatively by determining the number of different-sized aggregates with the Coulter counter. The window was set between 15 and 60. In this range, more than 80% of particles in "single" cell suspensions were counted at $1/\text{amplification}=4$, and $1/\text{aperture current}=1$. The remaining particles were detected at a window setting higher than 60. By increasing the value of the settings four-fold, $(1/\text{amp.} \times 1/\text{a.c.})$ particles four-fold larger in volume were counted. This process was repeated until all size particles were counted.

Table 1 shows the distribution of the number of aggregates with 4-, 16-, 64- or 256-fold volume relative to a "single" cell volume after 120 min of incubation in Mg^{2+} and Ca^{2+} media, when the Nt/No was almost equal in 1 mM Mg^{2+} and 1 mM Ca^{2+} .

TABLE 1. SIZE DISTRIBUTION OF AGGREGATES IN Mg^{2+} AND Ca^{2+} MEDIA

Particle size ^a	No. particles/ml medium $\times 10^{-1}$					
	Before aggregation	1 mM Mg^{2+}	1 mM Ca^{2+}	2 mM Mg^{2+}	2 mM Ca^{2+}	1 mM Mg^{2+} + 1 mM Ca^{2+}
1 $\times$	25718	15386	16204	13138	18077	12919
4 $\times$	5957	2590	1859	1906	1820	1501
16 $\times$	17	470	68	415	41	50
64 $\times$	0	90	148	152	137	76
256 $\times$	0	0	5.3	2.7	5.3	38.7
Total	31692.0	18536.0	18288.3	15613.7	20080.3	14584.7
Nt/No	1.00	0.58	0.58	0.49	0.63	0.46

^a Relative value to the average volume of single cell.

Cells after 120 min of gyration were assayed,

The number of the largest aggregates was greater in Ca^{2+} than in Mg^{2+} . The number of aggregates in Mg^{2+} was successively decreased with an increase in size, while the size distribution of the aggregates in Ca^{2+} showed a peak at the 64-fold aggregate. In 2 mM cation concentration, the maximum size of aggregates was 256-fold in both Mg^{2+} and Ca^{2+} . The size distribution of the aggregates, however, was still different in these two media as observed in assays using 1 mM media.

In the medium with both Ca^{2+} and Mg^{2+} , larger aggregates were formed than those in medium with each ion alone at an equimolar concentration (Table 1). The higher aggregability of cells in medium with both cations, therefore, is not due to the simple increase of divalent cation concentration, but due to the additive action of the two ions on cell adhesion.

Effect of divalent cations on cell-to-substrate adhesion. Our previous paper revealed that Mg^{2+} was more effective than Ca^{2+} in promoting adhesion of chick fibroblastic cells to plastic substrate coated with acidic proteins (26). In the present study, the effect of various divalent cations was re-examined on cell adhesion of fibroblastic cells to Falcon plastic dishes coated with gelatin. The kinetics of cell adhesion in Mg^{2+} or Ca^{2+} are shown in Fig. 7. As in the case of cell-to-cell adhesion, there was a lag in Ca^{2+}

Fig. 7. Kinetics of adhesion of fibroblastic cells to gelatin coated Falcon plastic dishes in 1 mM Ca^{2+} or 1 mM Mg^{2+} .

Fig. 8. The effect of six different divalent cations at different concentrations on the cell-to-substrate adhesion of fibroblastic cells. Percentages of unattached cells 60 min after incubation are plotted.

before the start of adhesion. The number of cells adhering to the substrate was always less in Ca^{2+} than in Mg^{2+} throughout the incubation period of 120 min, confirming our previous results. The cation effectiveness was $\text{Mn}^{2+} > \text{Co}^{2+} \approx \text{Mg}^{2+}$ judged from the lowest effective concentration that permitted cell adhesion to the substrate. Ca^{2+} displayed maximum effect at 1 mM (Fig. 8) but permitted adhesion of only about 40% of the inoculated population. Ba^{2+} and Sr^{2+} were hardly effective.

Effect of divalent cations on cell adhesion of neural retina. Kinetics of aggregation of neural retina cells was measured in the presence of 1 mM Mg^{2+} , 1 mM Ca^{2+} and CMF-MEM (Fig. 9). Aggregation of neural retina cells occurred more slowly than fibro-

Fig. 9. Aggregation kinetics of neural retina cells in three different media. Divalent cations were added at 1 mM.

blastic cells in Ca^{2+} , while the results in Mg^{2+} were essentially the same as in CMF-MEM during the incubation period. Other cations tested, Mn^{2+} , Co^{2+} , Ba^{2+} and Sr^{2+} , were similarly ineffective as Mg^{2+} , although Mn^{2+} in 10 mM concentration was slightly effective, as judged from the Nt/No value 120 min after inoculation (Table 2).

TABLE 2. EFFECT OF DIVALENT CATIONS ON AGGREGATION OF NEURAL RETINA CELLS

Divalent cations	Nt/No ^a	Ratio to*
Without divalent cations	0.83*	1.00
Mn 1 mM	0.79	0.95
10 mM	0.73	0.88
Co 1 mM	0.87	1.05
10 mM	0.81	0.98
Mg 1 mM	0.80	0.96
10 mM	0.79	0.95
Ca 1 mM	0.58	0.70
10 mM	0.51	0.61
Sr 1 mM	0.78	0.94
10 mM	0.80	0.96
Ba 1 mM	0.88	1.06
10 mM	0.87	1.05

^a No represents the initial particle number in the dish and Nt represents the total particle number after incubation.

Assays were performed after 120 min of incubation. Typical results are shown. The absolute values varied among the three experiments conducted, but the relative differences in cell adhesiveness among the different ionic conditions were reproducible.

A negligible adhesion of neural retina cells to substrate coated with gelatin was observed even after several hours of incubation in the presence of divalent cations effective for adhesion of fibroblasts. Adhesion of appreciable numbers of cells occurred

TABLE 3. EFFECT OF DIVALENT CATIONS ON ATTACHMENT OF NEURAL RETINA CELLS TO GELATIN-COATED PLASTIC^a

Divalent cations		Unattached cells (%) ^b
Without divalent cations		103
Mn	1 mM	100
	10 mM	5
Co	1 mM	96
	1 mM	91
Mg	1 mM	103
	10 mM	96
Ca	1 mM	96
	10 mM	101
Sr	1 mM	98
	10 mM	90
Ba	1 mM	100
	10 mM	92

^a Dishes were treated overnight with 0.005% gelatin.

^b The number of unattached cells was counted after 60 min of incubation. Percentages represent the mean of samples taken from three dishes initiated from a common inocula and incubated under the same conditions.

only when one hundred-fold diluted gelatin solution was used for coating. This, however, was observed only in the presence of 10 mM Mn^{2+} among cations tested (Table 3). When Falcon plastic dishes were coated with fetal calf serum, neural retina cells effectively adhered to the substrate in the presence of 10 mM Co^{2+} , as well as 10 mM Mn^{2+} , while the other cations were still ineffective.

DISCUSSION

The present study has demonstrated that divalent cation dependence of cell adhesion varied with either the cell type or the adhesion type. The four different cations (Mn^{2+} , Co^{2+} , Mg^{2+} and Ca^{2+}) tested were effective in promoting either the cell-to-cell adhesion or the cell-to-substrate adhesion of fibroblastic cells. For neural retina cells, however, Ca^{2+} was specifically effective among the above ions in promoting cell-to-cell adhesion, whereas Mn^{2+} or Co^{2+} was more effective than the others on cell-to-substrate adhesion as in the case of fibroblastic cell adhesion.

The aggregation of fibroblastic cells occurred rapidly in the presence of Mn^{2+} , Co^{2+} or Mg^{2+} . This result is consistent with the report by Armstrong (1) and Hornby (14) which showed a requirement for Mg^{2+} but not Ca^{2+} for aggregation of chick embryonic cells. The present study has demonstrated that Ca^{2+} is also effective although its effect could not be detected in the early stage of aggregation. The action of Ca^{2+} , however, was distinctive from the other ions in following aspects. First, Ca^{2+} seemed to have an inhibitory effect on cell adhesion, which is probably independent of its adhesion-promoting activity (25). Contraction of cells in Ca^{2+} may be relevant to such effects (16). Second, the cell adhesion-promoting activity of Ca^{2+} was less effective at high concentrations. Such property could be attributed to its inhibitory action on cell adhesion, as mentioned earlier. Thirdly, the size distribution pattern of aggregates in Ca^{2+} was found to be different from that in Mg^{2+} , in which the bigger aggregates

tended to be formed in Ca^{2+} than in Mg^{2+} , although the aggregation index Nt/No was not different in either ionic condition. This phenomenon suggests that cells undergo somewhat different aggregation procedures in each cation. The experiment using the medium with both ions suggested the presence of different sites for each ion. It should be emphasized here that the third point enumerated above complicates the comparison of cell adhesiveness in different environments. The measurement of cell adhesiveness by using only a single index, e. g., the decrease in total particle number (7), the decrease in single cell number (5, 19) or the comparison of the size of aggregates (18), is insufficient for a complete determination of the nature of cell adhesiveness.

The cell adhesion mechanism depending on Mn^{2+} , Co^{2+} or Mg^{2+} is designated here as the "Mg-mechanism" whereas the mechanism rather specifically depending on Ca^{2+} is designated as the "Ca-mechanism". There are several reasons to assume that the cell-to-substrate adhesion depends mostly on the "Mg-mechanism". The percentage of adhering fibroblastic cells always remained below 40% in Ca^{2+} , while it was usually higher than 70% in the Mg^{2+} -group cations at optimum concentrations. Neural retina cells adhered to the substrate only in the presence of Mn^{2+} or Co^{2+} , while Ca^{2+} was totally ineffective in promoting the cell-to-substrate adhesion. On the contrary, the "Ca-mechanism" seemed to be more important for cell-to-cell adhesion. The best example for this concept was that neural retina cells specifically required Ca^{2+} for cell-to-cell adhesion. Moreover, the distinctive effect of Ca^{2+} in promoting the adhesion of fibroblastic cells was observed particularly in the cell-to-cell adhesion system.

In summary, we suggest that there are two independent mechanisms, a "Mg-mechanism" and a "Ca-mechanism," in cell adhesion. Cell-to-cell adhesion of fibroblastic cells can be achieved with either of these mechanisms, while cell-to-substrate adhesion of these cells depends mostly on the "Mg-mechanism." In different types of cells the development of these two mechanisms would be different. In neural retina cells the "Mg-mechanism" may be relatively less developed, so that the "Ca-mechanism" could have been more easily detected.

It remains to be investigated which kind of cellular physiological activity is related to each hypothetical mechanism. It should be emphasized here that the order of effectiveness of various divalent cations for promoting cell-to-substrate adhesion, $\text{Mn}^{2+} > \text{Co}^{2+} \simeq \text{Mg}^{2+} > \text{Ca}^{2+}$, is parallel to the order for enhancing pseudopodia formation and spreading of cells (22, 26, 28). The importance of the tip of pseudopodia as a site of cell-to-substrate adhesion has often been pointed out (9, 13, 22, 23). It has also been theoretically suggested that pseudopodia formation is a necessary prerequisite for close contact of apposed cell surfaces (11, 20). Therefore, "Mg-mechanism" is likely to be associated with the motile activity of the cell surface.

The situation is still more speculative as to the nature of the "Ca-mechanism." The possibility that Ca^{2+} acts as an intercellular bridge (24) or that this ion is a necessary component for the function of intercellular macromolecules (8, 25, 27) to bind apposed cell surfaces should be taken into consideration in speculations on the nature of the "Ca-mechanism."

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