Effects of Calcium Ions and the Bivalent Cation Ionophore A23187 on the Agglutination and Fusion of Chicken Erythrocytes by Sendai Virus

By C. ANTHONY HART, DEREK FISHER, TERENCE HALLINAN and JACK A. LUCY

*Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine,
University of London, 8 Hunter Street, London WC1N 1BP, U.K.*

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1. Ca^{2+} (0.4–16 mM) had no detectable action on the agglutination of hen erythrocytes by Sendai virus. 2. Pretreatment of the cells with Ca^{2+} (0.1–8 mM) in the presence of the bivalent cation ionophore A23187 led, however, to a significant decrease in the subsequent agglutination of the cells by the virus. 3. It thus appears that the entry of Ca^{2+} into the interior of these cells decreases cellular agglutination by Sendai virus; possible interpretations of this phenomenon are discussed in terms of the movement of intramembranous particles. 4. With a small number of virions, maximum cell fusion by Sendai virus occurred in the presence of EGTA [ethanedioxybis(ethylamine)tetra-acetate]. 5. Virus-induced cell fusion was significantly decreased by Ca^{2+} , even at a concentration of 0.2 mM; it is suggested that this may result from diminished interactions between virus particles and erythrocyte membranes.

The involvement of Ca^{2+} in the fusion of biological membranes seems to be an almost universal phenomenon. For example, Ca^{2+} is necessary in myoblast fusion (Shainberg *et al.*, 1969), in cell fusion induced by liposomes (Papahadjopoulos *et al.*, 1973) and in the fusion of nascent membranes of the protozoan *Echinospaerium nucleofilum* (Vollett & Roth, 1974).

Okada & Murayama (1966) showed that the concentration of Ca^{2+} required for the fusion of Ehrlich ascites cells by Sendai virus depends on the number of virus particles. Later, Toister & Loyter (1970, 1973) found that the fusion of hen erythrocytes by this virus increased with an increasing concentration of Ca^{2+} . The chemically induced fusion of chicken erythrocytes by glycerol mono-oleate is inhibited by EDTA (Ahkong *et al.*, 1973), and fusion of these cells can be induced by the bivalent ionophores X537A and A23187 (Ahkong *et al.*, 1975), which are known to facilitate the movement of Ca^{2+} through membranes. These observations indicated that Ca^{2+} is required for cell fusion induced both by Sendai virus and by chemical agents.

However, Peretz *et al.* (1974) reported that human erythrocytes treated with Sendai virus will fuse in the absence of bivalent-metal ions, or in the presence of EDTA, provided that low concentrations of virus are used to minimize haemolysis. Since this observation indicated that the mechanisms of Sendai-virus-induced and chemically induced fusion may be different, we have further investigated the fusion of hen erythrocytes by Sendai virus, small numbers of virus particles being used to minimize haemolysis. The effects of Ca^{2+} on the agglutination of hen erythrocytes by the virus have also been studied.

Materials and Methods

All reagent chemicals used were commercially obtained and were of analytical grade. The bivalent cation ionophore A23187 was a gift from Eli Lilly (Windlesham, Surrey GU20 6PH, U.K.). A solution of the ionophore in ethanol (1 mg/ml; 5 μ l) was added to 0.5 ml of cell suspension and the mixture was incubated with Ca^{2+} or EGTA [ethanedioxybis(ethylamine)tetra-acetate] as described below for the assessment of cell fusion. Valinomycin, which was from Sigma (London) Chemical Co. (London S.W.6, U.K.), was used in the same way as ionophore A23187.

Sendai virus was grown in 10-day chicken embryos (Watkins, 1971) from seed stock given by the Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, U.K. The Salk pattern method (Salk, 1944) was used to determine the number of virus particles from their haemagglutination titre, 0.5 ml of virus suspension per well and 60 μ l of a 4% (v/v) suspension of hen erythrocytes in the standard buffer being used (see below).

Blood was taken by venepuncture from the brachial vein of hens into a citrate anticoagulant solution (10 ml) (De Gowin *et al.*, 1949). It was washed three times in *N*-tris(hydroxymethyl)methylglycine buffer (20 mM, adjusted to pH 7.8 with KOH) containing KCl (140 mM), NaCl (5.4 mM) and MgSO_4 (0.8 mM) (Toister & Loyter, 1970). Erythrocytes were then resuspended in this buffer and used on the day that they were prepared.

The method of Toister & Loyter (1970) for the assessment of cell fusion was modified as follows. A

5% (v/v) suspension of hen erythrocytes (0.5 ml) was incubated at 37°C for 30 min. The above buffer (0.5 ml), containing the virus [usually 250–500 HA units (Salk, 1944) unless otherwise stated] and either an appropriate concentration of Ca^{2+} or 4 mM-EGTA, was then added to the cells. The mixture was placed in an ice bath for 20 min with intermittent shaking. For the investigation of agglutination, the cells were fixed by the addition of 3% (v/v) glutaraldehyde (2 ml) in the standard buffer for 1 h at 0°C. Alternatively, for experiments on cell fusion, the treated cells were incubated at 37°C, without shaking, allowing the cells to form a clump. After 30 min at 37°C, the cells were briefly shaken and placed in an ice bath. Samples were then taken for cell counting, by using a Vickers M26 inverted phase-contrast microscope, and approx. 500 cells were counted per sample. In the control samples (without virus) no cell fusion was detected. The fusion index was calculated from the formula:

$$\text{Fusion index} = \left(\frac{\text{no. of intracellular nuclei in treated cells}}{\text{no. of treated cells}} \right) - \left(\frac{\text{no. of intracellular nuclei in control cells}}{\text{no. of control cells}} \right)$$

Agglutination produced by the addition of virus was estimated by the following four methods, after fixation as described above.

1. Clumps of cells in the test tubes were scored visually on a scale + to ++++++. Absence of agglutination was scored as zero.

2. The time taken for the cells to settle as a clump at 0°C (settling time) was measured.

3. The E_{410} of the cell suspension was measured in a 1 cm-light-path cuvette with a Unicam SP.800 spectrophotometer. Absorbance decreased with increasing agglutination, but Sendai-virus-induced haemolysis of the erythrocytes prevented the values of absorbance obtained from providing a quantitative measure of agglutination.

4. After fixation with glutaraldehyde and dilution with a modified Eagle's solution containing 0.1% NaN_3 (Isoton; Coulter Electronics Ltd., Harpenden, Herts., U.K.), the total number of cells was counted with a Coulter Counter Model F_N (Coulter Electronics Ltd.) fitted with a 100 μm aperture. Triplicate samples (0.5 ml), each containing 10000–40000 cells, were counted. The cell numbers obtained were reproducible within $\pm 5\%$ of the mean value. Mean values were corrected for coincidence from the tables provided by the manufacturers. The Coulter Counter has been used to estimate agglutination of sea-urchin embryo and mouse teratoma cells by concanavalin A (Oppenheimer & Odencrantz, 1972) and of ery-

throcytes by antisera (Gibbs *et al.*, 1965; Bowdler & Swisher, 1964). For accurate work, as in earlier studies on the measurement of small changes in agglutination by different antisera (Gibbs, 1965), it is necessary to use both high and low thresholds, and this procedure was adopted in the present studies.

Results and Discussion

Ca^{2+} and cell agglutination

Incubation of hen erythrocytes with Ca^{2+} (0.4–16.0 mM) had no detectable effect on the agglutination produced by Sendai virus. A significant decrease in the agglutination of erythrocytes by the virus was observed, however, when the cells were preincubated with the bivalent cation ionophore A23187 and Ca^{2+} before the addition of the virus (Table 1). This pre-treatment led to a decrease in the macroscopic size of the clumps of erythrocytes, an increase in the time taken for clumps to settle out at 0°C, and an increase in the E_{410} , as well as a decrease in the number of cells agglutinated (Table 2). When cells that had been incubated with ionophore A23187 and Ca^{2+} and then agglutinated by Sendai virus were examined by phase-contrast microscopy, a marked decrease in the

Table 1. *Effect of exogenous Ca^{2+} on the agglutination of hen erythrocytes by Sendai virus in the presence of ionophore A23187*

Hen erythrocytes were incubated with the bivalent cation ionophore A23187 (5 μg) and the required concentration of Ca^{2+} at 37°C for 30 min as described in the Materials and Methods section. Sendai virus (250–750 HA units) was then added. The treated cells were placed in an ice bath for 20 min with intermittent shaking, and then fixed in the cold by the addition of 2 ml of a 3% (v/v) solution of glutaraldehyde in the standard buffer (pH 7.8). Agglutination was assessed as described in the text, by using the Coulter Counter. The number of un-agglutinated cells given is normalized with respect to the number found in the absence of exogenous Ca^{2+} , $\pm$ s.d. for the number of experiments in parentheses. In each experiment, the number of un-agglutinated cells was assessed in duplicate or triplicate: the values obtained were reproducible within $\pm 5\%$ of the mean value. *P* is the probability that the number of un-agglutinated cells is different from the number found in the absence of exogenous Ca^{2+} .

Concn. of Ca^{2+} (mM)	No. of un-agglutinated cells	<i>P</i>
0	1.0 (16)	—
0.1	4.61 $\pm$ 1.79 (4)	<0.02
0.2	5.51 $\pm$ 2.49 (4)	<0.05
0.4	4.31 $\pm$ 2.08 (4)	<0.05
1.0	5.61 $\pm$ 2.79 (3)	<0.1
1.6	7.39 $\pm$ 2.79 (6)	<0.01
4.0	4.78 $\pm$ 2.28 (16)	<0.001
8.0	4.39 $\pm$ 2.11 (4)	<0.05

Table 2. *Effect of ionophore A23187 and exogenous Ca²⁺ on the agglutination of hen erythrocytes by Sendai virus*

Hen erythrocytes were incubated at 37°C for 30 min with or without ionophore A23187 (5 µg), Ca²⁺ (4 mM) or EGTA (2 mM) and Sendai virus was then added as described in the text. Treated cells were then placed in an ice bath for 20 min with intermittent shaking. The cells were fixed at 0°C by the addition of 2 ml of a 3% (v/v) solution of glutaraldehyde in the standard buffer (pH 7.8). Agglutination was then assessed in duplicate, as described in the Materials and Methods section.

	Sendai virus (HA units)	Agglutination (visual assessment)	Settling time at 0°C (min)	E ₄₁₀	Percentage of un-agglutinated cells
Cells preincubated with Ca ²⁺ (4 mM)	128	+++	16	0.93	25.2
			16	0.81	17.0
	640	+++++	2.5	0.40	8.0
			2.5	0.42	9.8
	0	0	≥20	1.08	100
Cells preincubated with ionophore A23187 (5 µg) and Ca ²⁺ (4 mM)	128	+	>20	1.15	88.8
			>20	1.05	86.4
	640	+++	16	0.88	35.4
			16	0.84	35.8
	0	0	≥20	1.12	100
Cells preincubated with ionophore A23187 (5 µg) and EGTA	128	+++	16	0.86	18.6
			16	0.92	20.4
	640	+++++	2.5	0.52	7.6
			2.5	0.48	8.4
	0	0	≥20	1.16	100

number of cells per clump and an increase in the number of free cells was seen.

In these experiments it was necessary to fix the clumped cells with glutaraldehyde, since Sendai virus is rapidly eluted from erythrocytes at temperatures above 20°C. After fixation the agglutinated clumps remained stable for more than 48 h. Phase-contrast microscopy provided no evidence that glutaraldehyde itself induced the agglutination of the erythrocytes.

Shortening the incubation period with ionophore from 30 to 5 min (with 4 mM-Ca²⁺) still gave a decreased agglutination of the cells. Treatment with ionophore A23187 together with EGTA had no effect on the subsequent aggregation of cells by Sendai virus. Replacement of ionophore A23187 by the univalent cation ionophore valinomycin (in the presence of Ca²⁺) also had no detectable effect on cellular aggregation.

Erythrocytes that had been incubated with ionophore A23187 and Ca²⁺ were seen by phase-contrast microscopy to bud off small vesicles during a sequence of morphological changes similar to those observed by Allan & Michell (1975) for human erythrocytes in the presence of ionophore A23187 and Ca²⁺. However, in contrast with the behaviour of hen erythrocytes, we have not observed a decrease in the agglutination of human erythrocytes by Sendai virus on pre-treatment of the cells with Ca²⁺ and ionophore A23187.

Haywood (1974) has shown that gangliosides incorporated into liposomes act as receptors for Sendai virus. The major receptor for the closely

related influenza virus in human erythrocytes has, however, been shown to be associated with intramembranous protein particles, and the movement of intramembranous particles in human erythrocyte 'ghosts' causes a concomitant movement of the virus receptor (Tillack *et al.*, 1972). If the receptors for Sendai virus are similarly associated with intramembranous particles (approx. 8 nm in diameter), it seems possible that extensive clustering of these particles may effectively decrease the number of surface receptors available to the relatively bulky Sendai virus [120–250 nm in diam. (Watkins, 1971)]. This may perhaps be a mechanism by which treatment of hen erythrocytes with ionophore A23187 and Ca²⁺ decreases cellular agglutination by the virus. The proteins of human erythrocyte 'ghosts' are known to be relatively immobile (Peters *et al.*, 1974), and it is noteworthy that ionophore-treated human erythrocytes, unlike hen erythrocytes, do not show a decreased agglutination by Sendai virus.

Ca²⁺ and cell fusion

Virtually complete cell lysis occurred when a large number of virions (2560 HA units) were incubated with erythrocytes in the absence of exogenous Ca²⁺ or in the presence of EGTA (2 mM). Even with a low number of virions (250–500 HA units), haemolysis was still considerable in the absence of Ca²⁺, but many intact polykaryocytes were also formed. The addition of exogenous Ca²⁺ up to a concentration of 20 mM markedly decreased cell fusion, as shown in

Fig. 1. Effect of exogenous Ca^{2+} on the fusion of hen erythrocytes by Sendai virus

Hen erythrocytes were incubated at 37°C for 30 min with Ca^{2+} alone (○), or with Ca^{2+} and $5\mu\text{g}$ of ionophore A23187 (●). Sendai virus (256 HA units) was then added. The treated cells were maintained at 0°C for 20 min with intermittent shaking, and then incubated at 37°C for 30 min without shaking. The fusion index was measured as described in the text. The fusion index given is normalized with respect to that found in the absence of exogenous Ca^{2+} . The results of seven experiments are shown, in which the fusion index was determined in duplicate or triplicate for each concentration of Ca^{2+} with a reproducibility of $\pm 5\%$ of the mean value.

Fig. 1. The greatest decrease in fusion occurred on the initial addition of exogenous Ca^{2+} (0.2 mM). Statistical analysis of the results by Student's t test or by the Mann-Whitney U-test showed that Ca^{2+} at all concentrations investigated (0.2–20 mM) caused a highly significant ($P < 0.001$) decrease in cell fusion as compared with cells treated with the virus in the absence of Ca^{2+} .

The possibility that EGTA might directly increase fusion was investigated by washing the cells in buffer containing EGTA (4 mM) and resuspending them in buffer that was free of EGTA and of Ca^{2+} . Virtually no change in the fusion index was observed.

To see whether allowing ready access of Ca^{2+} to the interior of the cells modified the inhibitory effect of Ca^{2+} on cell fusion induced by Sendai virus, we incubated the erythrocytes (before treatment with the virus) in the presence of the bivalent cation ionophore A23187 and various concentrations of Ca^{2+} . Treatment of the cells with Ca^{2+} and ionophore A23187 had little effect on the subsequent virus-induced cell fusion (Fig. 1), despite the decrease in cellular agglutination. This indicates that the inhibitory action of Ca^{2+} on virus-induced cell fusion is mediated via the external surface of the erythrocyte, assuming that Ca^{2+} does not enter the erythrocytes on treatment of the cells with Sendai virus (cf. Pasternak & Micklem, 1974).

Fusion of erythrocytes by Sendai virus appears to involve three stages. First, the virus is adsorbed on the cell surface and this results in cross-linking and agglutination of the cells. Since we found that Ca^{2+} (0.4–16 mM) had no detectable action on the agglutination of hen erythrocytes by Sendai virus (in the absence of ionophore), it may be concluded that the inhibitory action of Ca^{2+} on cell fusion is not at the stage of cellular agglutination.

At the second stage there is fusion of the viral membrane with the plasma membrane of the cell, and thirdly, fusion of one cell with another. Apostolov & Poste (1972) have reported that concentrations of Ca^{2+} greater than 0.5 mM inhibit the fusion of Sendai virus with human erythrocytes. Thus it seems possible that Ca^{2+} may inhibit cell-to-cell fusion by inhibiting fusion of the virus membrane with the erythrocyte membrane in our experiments with small numbers of virus particles. Since Neurath *et al.* (1972) have shown that the irreversible binding of Sendai virus to hen erythrocytes is enhanced by inhibiting the viral neuraminidase with EDTA, it is also possible that the presence of Ca^{2+} shortens the time during which the virus is in contact with the erythrocyte membrane.

Our findings with small numbers of virions acting on hen erythrocytes are consistent with the observations of Peretz *et al.* (1974), who observed that fusion of human erythrocytes by Sendai virus can proceed in the absence of bivalent cations provided that low concentrations of virus are used. This is in contrast with the requirement for Ca^{2+} in the fusion of hen erythrocytes by chemical agents (Akhong *et al.*, 1973). Also, it would appear that the general hypothesis for the mechanism of membrane fusion proposed by Poste & Allison (1973), which involves the activity of a Ca^{2+} -dependent adenosine triphosphatase, does not apply to the fusion of these cells by Sendai virus.

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References

- Akhong, Q. F., Fisher, D., Tampion W. & Lucy, J. A. (1973) *Biochem. J.* **136**, 147–155
- Akhong, Q. F., Tampion W. & Lucy J. A. (1975) *Nature (London)* **256**, 208–209
- Allan, D. & Michell, R. H. (1975) *Nature (London)* **258**, 348–349
- Apostolov, K. & Poste, G. (1972) *Microbios* **6**, 247–261
- Bowdler A. J. & Swisher, S. N. (1964) *Transfusion* **4**, 153–168
- De Gowing, E. L., Hardin, R. C. & Alsever, J. B. (1949) *Blood Transfusion*, p. 330, Saunders, Philadelphia and London
- Gibbs, M. B. (1965) *J. Immunol.* **94**, 55–61

- Gibbs, M. B., Dreyfus, J. C. & Aguilu, L. A. (1965) *J. Immunol.* **94**, 62–66
- Haywood, A. M. (1974) *J. Mol. Biol.* **83**, 427–436
- Neurath, A. R., Vernon, S. K., Hartzell, R. W. & Rubin, B. A. (1972) *J. Gen. Virol.* **16**, 245–249
- Okada, Y. & Murayama, F. (1966) *Exp. Cell Res.* **44**, 527–551
- Oppenheimer, S. B. & Odencrantz, J. (1972) *Exp. Cell Res.* **73**, 475–480
- Papahadjopoulos, D., Poste, G. & Schaffer, B. E. (1973) *Biochim. Biophys. Acta* **323**, 23–42
- Pasternak, C. A. & Micklem, K. J. (1974) *Biochem. J.* **140**, 405–411
- Peretz, H., Toister, Z., Laster, Y. & Loyter, A. (1974) *J. Cell Biol.* **63**, 1–11
- Peters, R., Peters, J., Tews, K. H. & Bähr, W. (1974) *Biochim. Biophys. Acta*, **367**, 282–294
- Poste, G. & Allison, A. C. (1973) *Biochim. Biophys. Acta* **300**, 421–465
- Salk, J. E. (1944) *J. Immunol.* **49**, 87–98
- Shainberg, A., Yagil, G. & Yaffe, D. (1969) *Exp. Cell Res.* **58**, 163–167
- Tillack, T. W., Scott, R. E. & Marchesi, V. T. (1972) *J. Exp. Med.* **135**, 1209–1227
- Toister, Z. & Loyter, A. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1523–1530
- Toister, Z. & Loyter, A. (1973) *J. Biol. Chem.* **248**, 422–432
- Vollet, J. J. & Roth, L. E. (1974) *Cytobiologie* **9**, 249–262
- Watkins, J. F. (1971) *Methods Virol.* **5**, 1–32