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Sendai virus causes a rise in intracellular free Ca^{2+} before cell fusion

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1. Sendai virus caused a large increase in the concentration of free Ca^{2+} within human erythrocyte ghosts detected by the Ca^{2+}-activated photoprotein obelin. 2. The increase in intracellular $[Ca^{2+}]$ preceded fusion. However, fusion could also be observed in the absence of a detectable rise in intracellular free $[Ca^{2+}]$. 3. It was concluded that the increase in intracellular free $[Ca^{2+}]$ was not an absolute requirement for cell fusion, but may be necessary to produce fusion at the maximum rate.

Cell fusion induced by Sendai virus has been exploited experimentally for more than 15 years (Harris & Watkins, 1965; Harris, 1970). However, the mechanism by which Sendai virus and other paramyxoviruses produce this phenomenon is still a source of considerable controversy (Knutton & Pasternak, 1979). In particular, the possibility that a rise in cytoplasmic free $[Ca^{2+}]$ is the trigger for cell fusion has been suggested by several workers (Okada & Murayama, 1966; Poste & Allison, 1973; Volsky & Loyter, 1978). The major problem in testing this hypothesis has been the inability to measure directly the cytoplasmic free $[Ca^{2+}]$ concentration during cell fusion. We have used the Ca^{2+}-activated photoprotein obelin, entrapped within red cell ghosts, to monitor changes in free $[Ca^{2+}]$ in the ghosts (Campbell *et al.*, 1981). Sendai virus can induce fusion between red cell ghosts (Peretz *et al.*, 1974). The aim of our work was to show whether Sendai virus did increase the free $[Ca^{2+}]$ within these ghosts, and to correlate any increase with the onset and progress of cell fusion.

Experimental

Preparation of human erythrocyte ghosts containing obelin

Human erythrocyte ghosts containing obelin were prepared as previously described by using a modification of the 'pre-swell' method (Hallett & Campbell, 1982). The ghost preparations were able to maintain a low intracellular $[Ca^{2+}]$ (0.2–0.5 μM) monitored by obelin luminescence in the presence of external Ca^{2+} (1 mM).

Abbreviation used: Tes, 2-{[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

Sendai-virus preparation

Infective Sendai virus, kindly donated by Professor H. Harris (Department of Medicine, Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.), was grown in the allantoic fluid of embryonated eggs for 3 days. Virus particles were separated from large debris in the allantoic fluid by differential centrifugation, and the virus pellet, obtained by centrifugation at 100000 ***g*** for 1 h, was resuspended in 150 mM-NaCl (spectrophotometrically pure)/10 mM-Tes (chelex-treated) and inactivated (non-infective but still fusogenic) by u.v. light. Portions of virus were stored frozen at −70°C and thawed when required, since it is important to avoid repeated freeze–thawing of the virus.

Quantification of fusion

Fusion between human erythrocyte ghosts was estimated by phase-contrast microscopy. Unfused ghosts, dumb-bell-shaped ghosts and large multilobed ghosts were observed. The latter two were intrepreted as being the result of fusion between two and three or more ghosts respectively. The extent of fusion was calculated from the following equation:

$$P = F/(F + U)$$

where P is the proportion of fused units, whether resulting from fusion of two or more ghosts, in the population, F is the number of fused units in the field of view and U is the number of unfused ghosts in the field. It should be noted that P is therefore smaller than the proportion of individual ghosts that had fused.

Measurement of free $[Ca^{2+}]$

Luminescence from the Ca^{2+}-activated photoprotein obelin was monitored as previously des-

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cribed (Campbell *et al.*, 1979). At defined time intervals the rate constant for obelin luminescence was calculated and compared with a calibration curve to give an estimate of the free $[Ca^{2+}]$ (Campbell *et al.*, 1981).

Results

Sendai virus causes an increase in free $[Ca^{2+}]$ within erythrocyte ghosts

Addition of Sendai virus to human erythrocyte ghosts containing obelin in medium containing Ca^{2+} (1 mM) resulted in a large increase in luminescence.

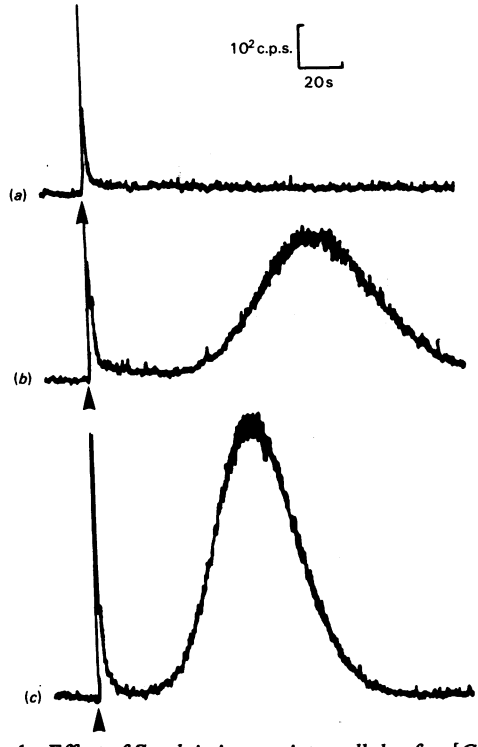

Fig. 1. *Effect of Sendai virus on intracellular free $[Ca^{2+}]$*
Human erythrocyte ghosts (5×10^7) were incubated in 500 μl of medium, containing 150 mM-NaCl, 0.25 mM-uranyl acetate, 10 mM-Tes and 1 mM-$CaCl_2$, pH 7.4 at 37°C. At the arrow-head 500 μl of a similar medium containing Sendai virus was added. The vertical axis shows luminescence and the horizontal axis time. (*a*) 600 haemagglutination units; (*b*) 3000 haemagglutination units; (*c*) 4500 haemagglutination units. At the end of the experiment 1% Triton X-100 was added to lyse the ghosts and stimulate remaining active obelin. In experiments (*b*) and (*c*), 99.9% of the obelin was consumed, showing that the decay in the luminescence was the result of obelin consumption and not a decrease in the intracellular free $[Ca^{2+}]$.

Under these conditions the concentration of ghosts was too low to allow aggregation and subsequent fusion to occur. The delay between the addition of virus and increase in luminescence ranged from 1 to 5 min and was dependent on the concentration of virus added. The increase in luminescence was dependent on external $[Ca^{2+}]$, being abolished by external EGTA (1 mM). It has been shown previously that Sendai virus also causes extensive lysis of human erythrocytes or erythrocyte ghosts (Peretz *et al.*, 1974). In our experiments too the virus caused lysis of the ghosts, releasing between 80% and 95% of the haemoglobin or [^{14}C]inulin entrapped within the ghosts within 10 min at the highest virus concentration used. It was therefore not possible to determine how much of the increase in obelin

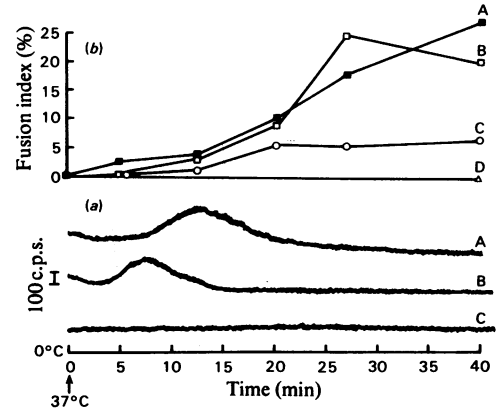

Fig. 2. *Effect of external $[Ca^{2+}]$ and internal [EGTA] on (a) intracellular $[Ca^{2+}]$ and (b) fusion*
(*a*) Human erythrocyte ghosts (5×10^7) were incubated in 100 μl of medium containing 150 mM-NaCl, 0.25 mM-uranyl acetate, 10 mM-Tes, pH 7.4 (trace C) or with $CaCl_2$ (1 mM) added (traces A and B). Sendai virus (1500 haemagglutination units) was added to the ghosts at 0°C for 10 min. At the arrow-head the ghosts were incubated at 37°C. Traces (B) and (C) show the obelin luminescence (vertical axis) from ghosts prepared containing only obelin, whereas trace (A) shows the response from ghosts prepared containing obelin and EGTA (5 mM). The horizontal axis represents time. (*b*) shows the percentage fusion, calculated as shown in the Experimental section, accompanying the intracellular Ca^{2+} changes, shown in (*a*). At various times during the experiments described in (*a*) samples (approx. 1 μl) were taken and immediately counted under a phase-contrast microscope to produce the percentage of fused cells present in the cell population. (A), (B) and (C) refer to the same cell populations and in the same conditions as shown in (*a*), namely (A) internal EGTA (5 mM), with external Ca^{2+}, (B) external Ca^{2+}, (C) no external Ca^{2+} and (D) no Sendai virus.

luminescence was due to its release into the external medium.

The addition of uranyl acetate (0.25 mM) reduced haemolysis, as determined by [^{14}C]inulin or haemoglobin release, to between 2% and 10% after 30 min incubation with virus. The increase in obelin luminescence induced by Sendai virus was still observed under these conditions (Fig. 1). The same results were obtained with virus preparations rendered non-haemolytic by a mild heat treatment (45°C for 30 min) (Fuchs & Giberman, 1973).

The rise in intracellular [Ca^{2+}] occurs before fusion

Fusion of ghosts was observed with a higher ghost concentration and after agglutination at 0°C. Absorption of virus particles to the surface of the ghosts at 0°C did not cause a rise in intracellular [Ca^{2+}] as detected by obelin luminescence. However, after absorption, when the cells were warmed to 37°C, a large rise in obelin luminescence was observed after a lag of approx. 3 min (Fig. 2*a*). The rise in intracellular free [Ca^{2+}] preceded fusion by approx. 5 min [Figs. 2*a* (B) and 2*b* (B)].

Is the increase in intracellular free [Ca^{2+}] required for fusion?

When internal EGTA was also entrapped within the ghosts the onset of the rise in intracellular free [Ca^{2+}] was delayed,but no delay in the rate of fusion was observed.

In the absence of external Ca^{2+} there was no detectable rise in intracellular free [Ca^{2+}]. However, fusion still occurred, although at a reduced rate (Fig. 2).

Discussion

These results show that interaction of Sendai virus with erythrocyte membranes results in a large rise in intracellular free [Ca^{2+}]. The effect only occurs at 37°C after absorption, and not at 0°C, and does not depend on cell–cell aggregation.

The rise in intracellular free [Ca^{2+}] was not a consequence of cell fusion, because with ghost concentrations too low to allow fusion (Fig. 1), the rise in intracellular free [Ca^{2+}] could still be detected.

In conditions where fusion occurred, the rise in intracellular free [Ca^{2+}] preceded fusion of the ghosts and also occurred before the reported time of onset of fusion for other cells (Pasternak & Micklem, 1973; Fuchs *et al.*, 1978). The time of onset of the rise in free [Ca^{2+}] was similar to the time at which there have been reported to be increases in the fluxes of other ions in other cells (Pasternak & Micklem, 1973; Fuchs *et al.*, 1978, 1980).

There appears to be no absolute requirement for a rise in intracellular free [Ca^{2+}] in ghost fusion, since fusion was observed when no rise in intracellular free [Ca^{2+}] was detectable, for example, in the absence of any external Ca^{2+}. However, the rate of fusion was reduced under these conditions, suggesting that either internal or external Ca^{2+} may therefore be required for the maximum rate of fusion. This could explain some of the discrepancies concerning the need for external Ca^{2+} that have been reported (Volsky & Loyter, 1978; Impraim *et al.*, 1979). Mammalian erythrocytes have no internal Ca^{2+} stores, but it is possible that in cells with internal stores, Ca^{2+} could be mobilized by Sendai virus. This is supported by the observation that Sendai virus can stimulate oxygen radical production from rat polymorphonuclear leucocytes in the absence of external Ca^{2+}, a process thought to be stimulated by a rise in intracellular free [Ca^{2+}] (Hallett *et al.*, 1981; Hallett & Campbell, 1982). Although some virus preparations contained up to 60 μM-Ca^{2+}, the possibility that viral Ca^{2+} was the source of the intracellular rise was excluded in the experiments reported here because the rise was shown to be dependent on extracellular [Ca^{2+}] (Fig. 2).

These studies emphasize that in order to clarify the role of Ca^{2+} in cell fusion, it is essential to measure directly the cytoplasmic free [Ca^{2+}] and to correlate this in the same cells with the extent and rate of fusion.

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