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Sequential onset of permeability changes in mouse ascites cells induced by Sendai virus

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(1) The addition of haemolytic Sendai virus to cells induces membrane changes in the following sequence: (i) Increased permeability to ions, (ii) increased permeability to low molecular weight metabolites, (iii) increased permeability to proteins. (2) The consequences of an increased permeability to ions are: (a) alteration of membrane potential, (b) net changes in intracellular cations and (c) cell swelling, in that order. (3) Depending on virus:cell ratio, Ca^{2+} concentration and temperature, it is possible to observe ion leakage without metabolite or protein leakage, and ion and metabolite leakage without protein leakage. (4) A model for the induction of permeability changes is presented.

Introduction

Haemolytic paramyxoviruses such as Sendai virus and Newcastle Disease virus induce a change in the permeability properties of susceptible cells such that membrane potential collapses [1–4] and such that monovalent [5–8] and divalent [3,7–10] cations, and phosphorylated intermediates of metabolism [6,11,12], leak out. The change, which occurs in the absence of cell lysis [6,7,13], becomes repaired with time [3,14] and can be controlled by extracellular Ca^{2+} [3,6,15–17]. It underlies the ability of haemolytic paramyxoviruses to form stable cell syncytia [18,19], it provides a novel method for permeabilizing cells [20–22], and it has been used to probe the origin of membrane potential in Lettre cells [23,24]; it bears some resemblance to the permeability change caused by activated complement [25,26] and may have a clinical role with regard to excitable [27] and other [28] types of cell.

One of the characteristics of virally-mediated permeability changes is a temperature-dependent lag [6,25]. Since there is evidence that haemolytic Sendai virus possesses a permeable envelope [29], it is possible that the lag or induction period represents the accumulation of membrane damage, introduced into the cell plasma membrane by fusion with the viral envelope, to a critical level. Since cations such as Na^+ and K^+ are smaller than phosphorylated intermediates of metabolism such as phosphorylcholine, it is possible that an increased permeability to ions precedes the leakage of phosphorylcholine. The present experiments, which have been reported briefly earlier [23], were carried out in order to investigate this possibility.

Methods

Radiochemicals, Lettre cells and Sendai virus, which was of the '3-day' type, were obtained as

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described by Impraim et al. [3]. For most experiments, Lettre cells were removed from the peritoneal cavity of a Swiss white mouse one week after inoculation and were diluted in Hepes-buffered saline [4]. The suspension was spun at 700 $\times g$ for 3 min and the pellet suspended in Ham's F10 medium containing 3–10% foetal calf serum, at concentrations up to 40% v/v of cells (approx. $3 \cdot 10^8$/ml). Cells in this medium were used directly, or were pre-incubated with $^{86}Rb^+$ (Amersham International) or [^{3}H]choline for 20–60 min at 37°C. The radioactive cell suspension was generally not washed, but was added directly, at a dilution of 1 in 100, to various incubation media. Ouabain, furosemide, prenylamine and R24571 were dissolved in DMSO prior to addition; an equivalent amount of DMSO was added to control cells; up to 0.05% DMSO was without effect on leakage from Lettre cells. Samples (generally 0.1–0.2 ml) were removed at intervals and radioactivity, Na^+ and K^+ content analysed after spinning through oil as described by Bashford et al. [4].

Membrane potential was measured as previously described [4,24]. Water content in cell pellets after spinning through oil was measured as described by Bashford et al. [4] except that in this instance dilution into choline medium was omitted. The protein content of such pellets was assayed by the procedure of Lowry et al. [30]. Each experiment reported in this paper was carried out at least three times, with essentially similar results. The values quoted refer to a typical experiment in each case.

Results

Membrane potential and ionic changes

The first demonstrable change following the addition of Sendai virus to cells is a decrease in the membrane potential. That this precedes a change in intracellular cation concentration (Fig. 1) is hardly surprising, since the number of ions which must cross the membrane in order to evoke a change in potential is many-fold less than that needed to cause an observable change in intracell-

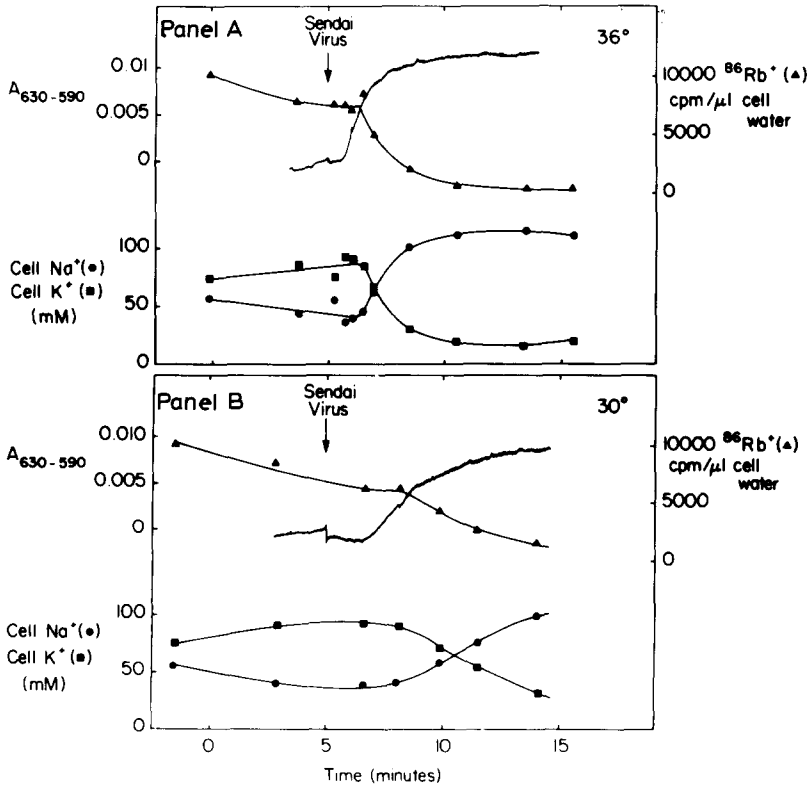

Fig. 1. Membrane potential and cation leakage of Lettre cells. Lettre cells preincubated with $^{86}RbCl$ were diluted into medium ($5 \cdot 10^6$ cells/ml) containing 150 mM NaCl, 5 mM KCl, 5 mM Hepes, 1 mM $MgCl_2$, 1 mM glucose, 2 μM oxonol V, pH 7.4, at 36°C (upper panel) or 30°C (lower panel) and membrane potential (continuous trace) and the content of $^{86}Rb^+$, Na^+ and K^+ in cell pellets measured as described in Materials and Methods. Sendai virus (10 HAU/ml) was added at the time indicated by the arrow. The cellular content of Na^+ (●———●) and K^+ (■———■) is expressed as mM; the cellular content of $^{86}Rb^+$ (▲———▲) is expressed as cpm/μl of cell water, calculated from the sum of cellular $Na^+ + K^+$ on the assumption that 130 nmol of $Na^+ + K^+$ is equivalent to 1 μl of cell water [4]. Membrane depolarization is indicated by an increase in $A_{630-590}$ [4,24].

ular cation concentration. In order to resolve the change in potential from that of ion content, the efflux of Rb^+ from cells pre-incubated with $^{86}Rb^+$ was measured, together with changes in potential and in concentration of Na^+ and K^+. Fig. 1 indicates that $^{86}Rb^+$ leakage and net cation changes begin simultaneously. Note that there is a short, but significant, lag to onset of membrane depolarization, just as there is to onset of ionic changes, which is increased as the temperature is reduced (compare panel A with panel B of Fig. 1).

An additional reason why membrane potential is collapsed by Sendai virus before the onset of ionic changes might be postulated to be related to the activity of the viral neuraminidase. For if surface potential, a considerable proportion of which is due to neuraminic acid-containing glycoproteins and glycolipids at the cell surface [31], were to contribute to potential of lettre cells as it does to that of cultured neuroblastoid cells [32], then a loss of neuraminic acid residues would depolarize Lettre cells; certainly viral neuraminidase acts on Lettre cells without lag [33]. However, (a) viral neuraminidase is unaffected by Ca^{2+}, whereas the decrease in membrane potential is prevented by Ca^{2+} [3], and (b) 'early harvest' virus, which has full neuraminidase activity [34] does not depolarize Lettre cells [3].

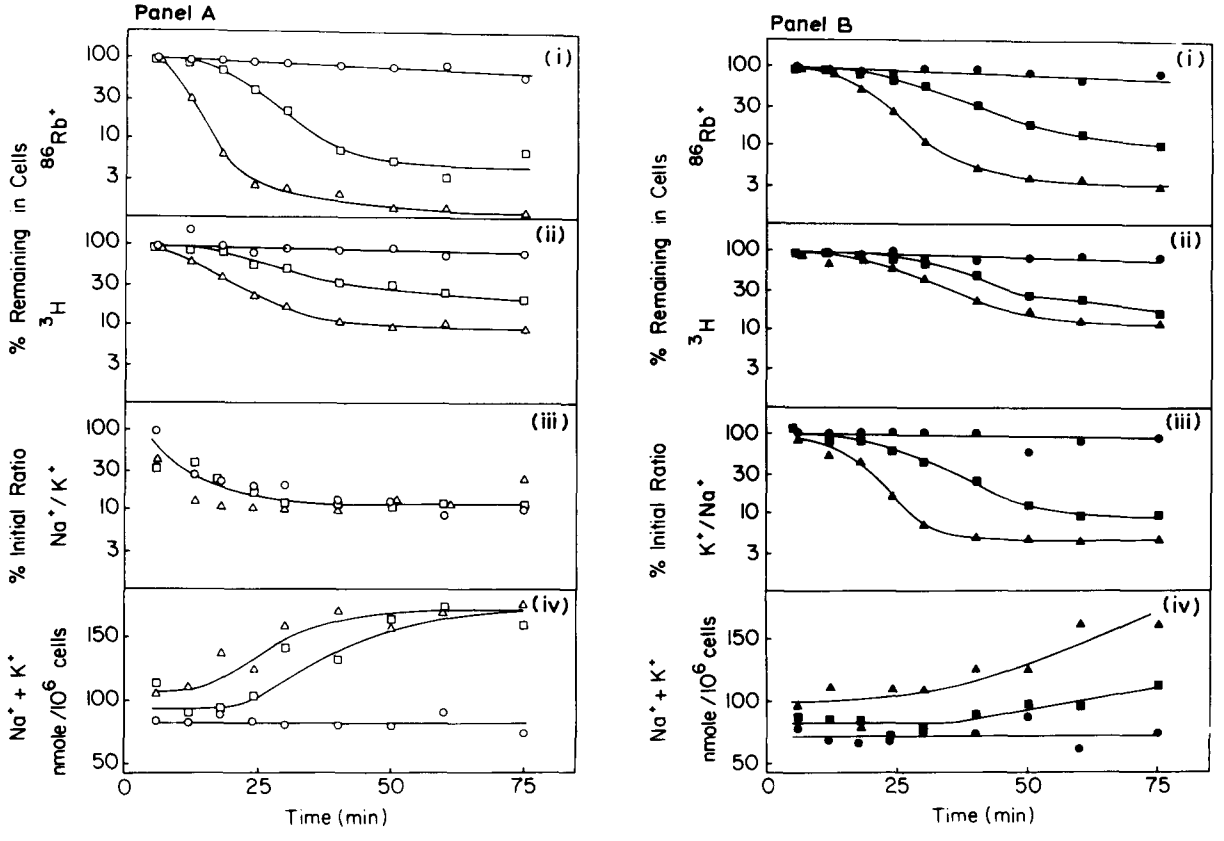

Fig. 2. Effect of R24571 on leakage of ions and metabolites from Lettre cells in high and low [K^+] medium. Lettre cells, preincubated either with $^{86}RbCl$ or with [^{3}H]choline, were diluted into medium ($3 \cdot 10^6$ cells/ml) containing 5 mM Hepes, 1 mM $MgCl_2$, 1 mM ouabain, 1 mM furosemide (pH 7.4), and 150 mM KCl (panel A) or 150 mM NaCl (final K^+ concentration 0.5 mM (panel B), incubated at 19°C and the content of $^{86}Rb^+$, ^3H, Na^+ and K^+ in cell pellets measured as described in Materials and Methods. No additions, ○———○ (panel A) and ●———● (panel B); Sendai virus (10 HAU/ml) present throughout, □———□ and ■———■; Sendai virus (as above) and R24571 ($3 \cdot 10^{-7}$ M) present, △———△ and ▲———▲. Radioactivity remaining in pellets is expressed as a % of that present at zero time: $^{86}Rb^+$, (i); ^3H, (ii). The content of Na^+ and K^+ in pellets is expressed (iii) as a % of the K^+/Na^+ ratio at zero time and (iv) as the total $Na^+ + K^+$. R24571 in the absence of virus induced no leakage of ^{86}Rb or ^3H (data not shown; see also Fig. 3).

A further reason why leakage of K^+ out of, and Na^+ into, cells occurs after membrane depolarization, is that leakage of these ions in normal media is counteracted by the operation of the Na^+ pump. Other compensating mechanisms may include electroneutral ion transport systems such as the furosemide-sensitive $Na^+ + K^+ + 2Cl^-$ cotransport described by Geck et al. [35] and other anion-dependent cation transporters [36]. A net change of ion content may only become apparent when the capacity of such mechanisms is exceeded by the extent of virally-mediated leaks. In an attempt to measure cation leakage uncomplicated by pumps and exchange mechanisms, Lettre cells were preincubated with $^{86}Rb^+$ as before and then placed in various media designed to limit cation pumping and cation exchanges. One medium chosen was 155 mM K^+ in place of 150 mM Na^+/5 mM K^+. It is possible to expose Lettre cells to high external K^+ without depolarizing them, because they have a surprisingly low K^+ conductance; indeed the membrane potential of Lettre cells is caused not by K^+ diffusion, but by electrogenic pumping [24,37]. Ouabain and furosemide were added to further limit compensatory ion movements. The flux of $^{86}Rb^+$, which in this medium is in the opposite direction to any net K^+ leakage that occurs, was then measured. Fig. 2, panel A (i) shows that $^{86}Rb^+$ leakage again has a lag, of approx. 10–15 min at this temperature. Because cells suspended in 150 mM K^+ rapidly accumulate K^+, whether virus is present or not (panel A (iii)),

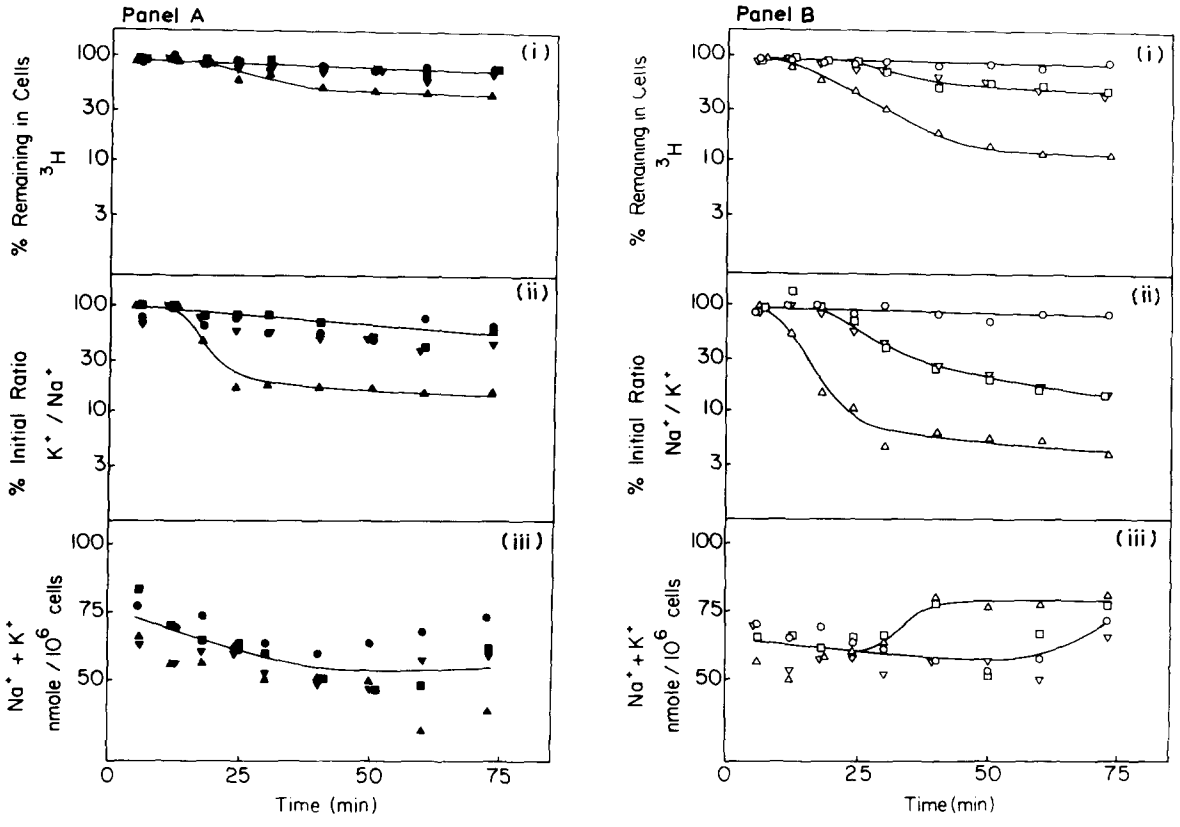

Fig. 3. Effect of virus dose and R24571 or leakage of ions and metabolites from Lettre cells. Lettre cells preincubated with [^{3}H]choline were diluted into medium ($3 \cdot 10^6$ cells/ml) containing 150 mM NaCl, 5 mM KCl, 5 mM Hepes, 1 mM $MgCl_2$ (pH 7.4), incubated at 24°C and the content of ^3H, Na^+ and K^+ in cell pellets measured as described in Materials and Methods. Closed symbols, no R24571 added; open symbols, in presence of 10^{-6} M R24571. Sendai virus was present at the following concentrations: no virus, ●——● and ○——○; 5 HAU/ml, ■——■ and □——□; 15 HAU/ml, ▼——▼ and ▽——▽; 50 HAU/ml, ▲——▲ and △——△. Radioactivity (i), K^+/Na^+ ratio (ii) and $K^+ + Na^+$ content of pellets (iii) are expressed as in Fig. 2.

a medium deficient in K^+ was next used. In such a medium, Na^+ pump activity is at a minimum [24], especially in the presence of ouabain; it is clear that ^{86}Rb leakage (panel B(i)) and cation movements (panel B (iii)) begin simultaneously.

'Ca^{2+} antagonists' like verapamil or prenylamine [38,39], and 'calmodulin antagonists' like R24571 [40], typically shorten the lag to onset of virally-mediated permeability changes [53]. In the presence of any of these compounds, the lag to onset of $^{86}Rb^+$ leakage, and that to onset of net cation changes, is shortened. This is illustrated with respect to R24571 in Figs. 2 and 3, and with respect to prenylamine in Fig. 4. It may be concluded that the lag to onset of net cation changes can be a reasonable measure of the lag to onset of increased cation flux.

Metabolite leakage

The leakage of phosphorylcholine and of other phosphorylated intermediates of metabolism is a characteristic response of virally-treated cells [3,6,25]. The onset of such leakage at low temperatures typically occurs after the onset of ionic changes (Figs. 2–4); addition of R24571 (Figs. 2 and 3) or prenylamine (Fig. 4) has a similar effect on metabolite and ion leakage. Under some conditions metabolite leakage is scarcely initiated, even though net ion changes are occurring (Figs. 3 and 4). Conversely, under other conditions, the lag to onset of metabolite leakage, $^{86}Rb^+$ leakage and net ion changes is similar, even though it can be as long as 15 min (Fig. 2). Note, however, that in this instance, the extent of ion leakage (measured as loss of $^{86}Rb^+$) is considerably greater than the extent of phosphorylcholine leakage; indeed, this is usually the case (Fig. 3 and 4). This observation is compatible with the proposal that the extent of leakage is a reflection of the proportion of cells affected by virus [33] and of the extent of damage induced by virus in those cells.

Cell swelling

Cell swelling is a characteristic response of Lettre cells to Sendai virus [13,41]. Under similar conditions red blood cells, which lack microvilli or other surface protuberances that enable cells to expand their volume under hydrostatic pressure, are lysed. This is one reason why Sendai virus is haemolytic but not lytic to Lettre cells [13].

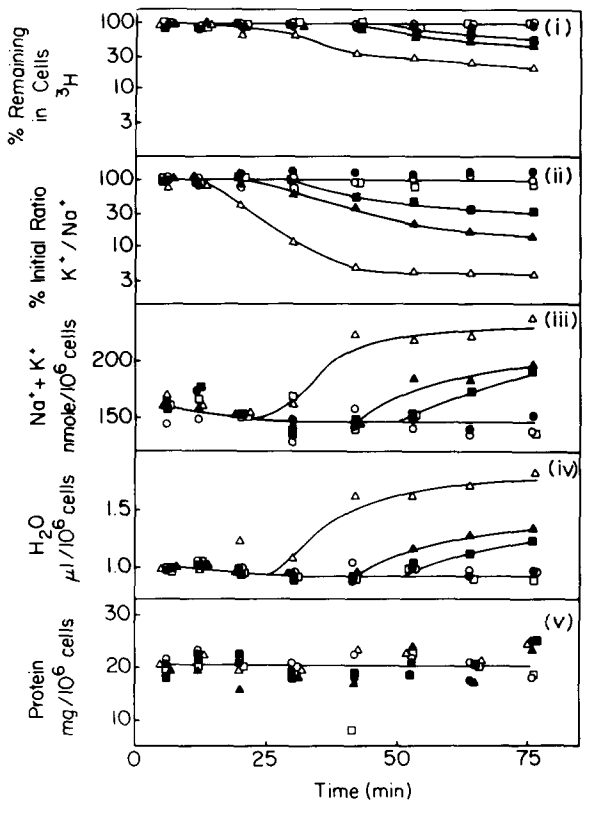

Fig. 4. Effect of prenylamine on water content and leakage of ions and metabolites from Lettre cells. Lettre cells preincubated with [^{3}H]choline were diluted into medium ($1.4 \cdot 10^7$ cells/ml) containing 150 mM NaCl, 5 mM KCl, 5 mM Hepes, 1 mM $MgCl_2$ (pH 7.4), incubated at 19°C and the content of ^3H, Na^+, K^+ and water in cell pellets measured by spinning samples (1 ml) without further dilution through oil as described in Materials and Methods. Sendai virus (10 HAU/ml), $CaCl_2$ (1 mM), prenylamine ($1.2 \cdot 10^{-5}$ M) and ouabain + furosemide (each 1 mM) were present as follows: prenylamine, ●——●; prenylamine + ouabain + furosemide, ○——○; virus, ■——■; virus + Ca^{2+}, □——□; virus + prenylamine, ▲——▲; virus + prenylamine + ouabain + furosemide, △——△. Radioactivity (i), K^+/Na^+ ratio (ii) and $K^+ + Na^+$ content of pellets (iii) are expressed as in Fig. 2. Water content and protein content of pellets are shown in (iv) and (v), respectively. Note that changes in $Na^+ + K^+$ content (iii) mirror changes in water content (iv); in cell pellets obtained as described above, a total $Na^+ + K^+$ content of 150 mM is equivalent to 1 μl of cell water.

In Lettre cells, total intracellular Na^+ and K^+ appears to be a good measure of intracellular water (cf. (iii) with (iv) of Fig. 4); this is because the sum of the concentrations of Na^+ and K^+ is

constant irrespective of cell volume. Note that increases in total Na^+ and K^+ or in cell water are not a consequence of cell aggregation, because total protein content remains the same under all conditions (Fig. 4 (v)). The value of total Na^+ and K^+ indicated by (iii) and (iv) of Fig. 4, namely 150 ± 12 mM is in reasonable agreement with the value of 130 ± 9 mM obtained by an independent method [4,24], bearing in mind that for the experiment depicted in Fig. 4, the cells were not diluted into choline medium.

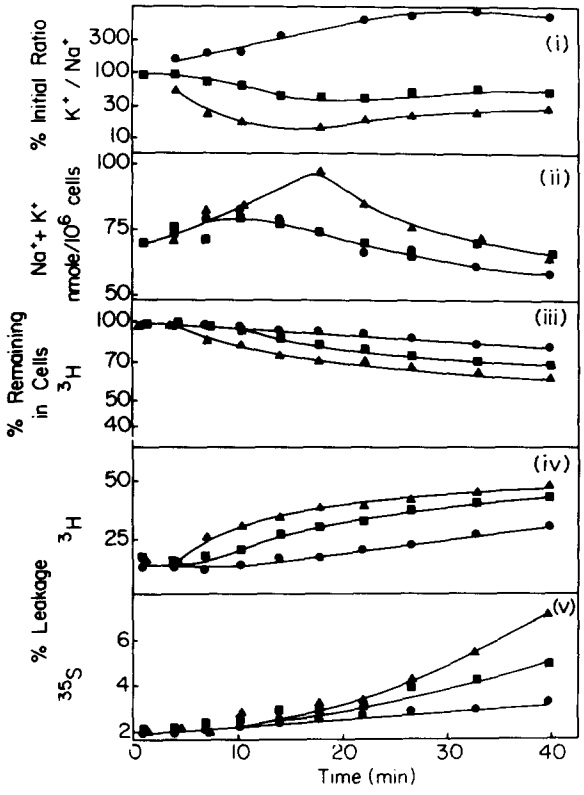

Fig. 5. Leakage of ions, metabolites and protein from Lettre cells. Lettre cells preincubated with 2-deoxy[^{3}H]glucose and [^{35}S]methionine were washed, reincubated ($2 \cdot 10^7$ cells/ml) in 150 mM NaCl, 5 mM KCl, 5 mM Hepes, 1 mM $MgCl_2$ (pH 7.4 at 37°C), and samples taken for assay of Na^+ and K^+ as described in Materials and Methods. Samples for assay of ^{3}H and ^{35}S were spun directly (i.e. not through oil) and ice-cold trichloroacetic acid added to supernatants and pellets (final concentration 5%). Radioactivity remaining in pellets (iii) is expressed as % of trichloroacetic acid-soluble ^{3}H present at zero time; is expressed as % of trichloroacetic acid-soluble ^{3}H (iv) or trichloroacetic acid-insoluble ^{35}S (v) that is in supernatants. Sendai virus was present at 0 (●———●), 30 (■———■), or 100 (▲———▲) HAU/ml throughout the experiment.

At low temperatures, swelling is a rather late event, and one that is separable from ion or metabolite leakage (e.g. panel B of Fig. 2). Virally-mediated changes in Na^+ and K^+ gradient alone are not necessarily sufficient to cause cell swelling (panel A of Fig. 3). Indeed Lettre cells treated in ways that induce ionic changes similar to those caused by Sendai virus, such as exposure to nigericin, do not swell (Bashford, C.L., unpublished data). While the exact mechanism responsible for virally-mediated cell swelling remains to be elucidated, the relevance of cell swelling to the formation of stable cell-cell syncytia, i.e. of giant cells, is clear [18,19,42].

Protein leakage

As mentioned above, the action of Sendai virus on Lettre cells is not cytolytic and soluble proteins do not generally leak out [7,11]. Nevertheless, under certain conditions (high virus: cell ratio, for example), cells can be made permeable to proteins [43,44]; cell lysis is unlikely to have occurred in these experiments, because the ability to introduce a toxic protein into cells capable of undergoing protein synthesis was assessed. When such protein leakage from Lettre cells does occur, it is a late event, clearly separable from the leakage of ions or metabolites (Fig. 5). The leakage of [^{3}H]2dGlc-6-*P* is plotted on a logarithmic axis (panel iii) for comparison with Figs. 2–4, and on a linear axis (panel iv), for comparison with leakage of ^{35}S-protein (panel v), which is too low to warrant plotting on a logarithmic axis. Note that in this experiment, carried out at 37°C, the K^+/Na^+ ratio of control cells increased during the incubation period, presumably because of the activity of the Na^+ pump. Fig. 5 shows that the extent of protein leakage is much less than that of ions or metabolites, suggesting that only a small proportion of cells is actually leaking protein. A similar relationship between protein (lactate dehydrogenase) and metabolite leakage has been observed in Sendai virus-treated mast cells [22].

Discussion

The experiments described in this paper show that virally-mediated permeability changes occur in a sequential manner; this is illustrated schemati-

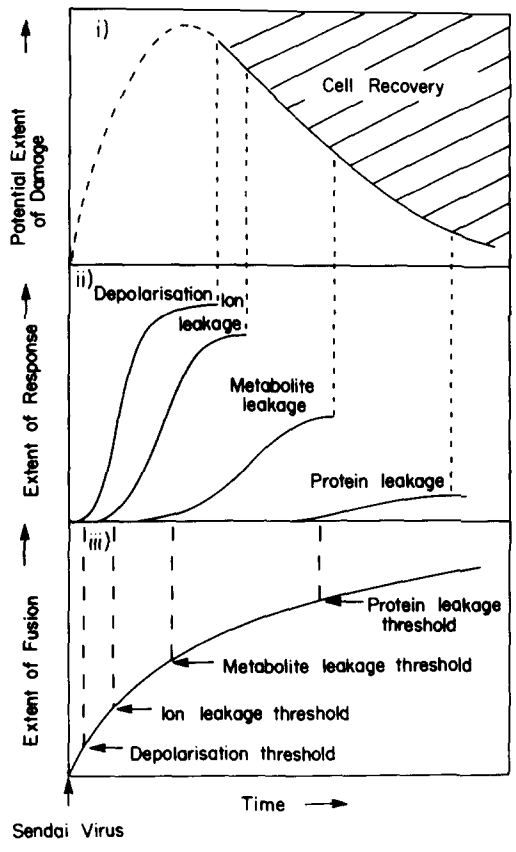

Fig. 6. Temporal relationship between depolarization, ion leakage, metabolite leakage and protein leakage. For details, see text.

cally in panel (ii) of Fig. 6. The shape of the curves in Fig. 6 (panel (ii) arises from the interplay of a number of factors [33] some of which are indicated in panels (i) and (iii). As virus-cell fusion proceeds (panel iii), the extent of damage to that particular cell increases and the cell is successively able to depolarize, to leak cations, to leak phosphorylated metabolites and to leak proteins. Ca^{2+} reduces the ability of virus to damage cells, effectively increasing the threshold extent of fusion needed to elicit an increase in permeability. At lower temperatures fusion is slower and the time taken to reach threshold is increased (see Ref. 33).

Opposing the ability of virus to damage the cell surface is the capacity of cells to recover; this is indicated in panel (i) of Fig. 6. When virus and cells are mixed, most cells bind virus, although the number of virus particles bound to any particular cell will vary. After binding has occurred, fusion between cell and viral envelope commences, and both damage and repair processes are initiated. The outcome of these opposing activities is that the extent of damage to the cell surface, which is detected by our measurements of membrane potential and cell contents in the entire cell population, depends on the proportion of cells in which the required threshold level of fusion/damage has been achieved.

Whether induction of permeability changes involves the accumulation of a 'threshold' number of pores that are of the same size, or whether induction involves the formation of pores of (successively) larger size, cannot be decided on the present experiments alone. The fact that the rate of virally-mediated leakage of $^{86}Rb^{+}$ is considerably greater than that of phosphoryl[^{3}H]choline (Fig. 2) is compatible with a pore size of around 1 nm in diameter [41].

As mentioned above, the continuous line in Fig. 6, panel (i) indicates the recovery process for each of the parameters listed (that is, the restoration of the original permeability barrier) [3]. If the initial membrane damage is too severe (high virus: cell ratio, absence of Ca^{2+}, or presence of drugs such as R24571) recovery is impaired; such cells begin to leak substantial amounts of protein and eventually lyse. In the case of red blood cells, which have little capacity for recovery and in which colloid osmotic swelling is more damaging than in Lettre cells [13], the latter situation is the normal outcome of viral action.

The demonstration that Sendai virus causes Lettre cells to become depolarized prior to a change in net cations or metabolite leakage, coupled with the fact that cells can recover from the effects of Sendai virus [3], provides an explanation for the dramatic effects of Sendai virus on excitable cells. Anterior pituitary cells, beating heart cells, and ganglionic neurones are all affected by virus in an entirely transient manner: complete restoration of activity is achieved within minutes [27]. It is therefore likely that the changes observed in these instances were elicited solely by membrane depolarization, and that subsequent changes, in net cation content or metabolite leakage, did not occur at all. Indeed, in the case of the anterior pituitary cells, it was subsequently shown that metabolite

leakage occurred only if the external Ca^{2+} concentration was lowered to below 0.5 mM [28].

There is evidence for a sequential onset of leakage of ions and other compounds across the plasma membrane of cells undergoing complement-mediated lysis [45–50] and a threshold of membrane damage has been postulated here also [51,52]. Moreover, membrane damage by complement, as well as by other pore-forming agents such as *Staphylococcus aureus* α-toxin and the bee venom melittin, is sensitive to extracellular Ca^{2+} [48–50]. It should prove instructive to see in what other ways the onset of virally mediated permeability changes resembles the onset of membrane changes initiated by complement, toxins and cytotoxic cells.

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