Permeability Changes Elicited by Influenza and Sendai Viruses: Separation of Fusion and Leakage by pH-jump Experiments

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SUMMARY

Permeability changes elicited in Lettre cells by influenza virus at pH 5.3 were maintained when the pH was shifted to 7.4. Permeability changes elicited by Sendai virus at pH 7.4 were maintained when the pH was shifted to 5.3. In each case permeability changes at the new pH were sensitive to inhibition by extracellular Ca^{2+}. The time at which the pH was shifted was critical: if the shift was made prior to the onset of permeability changes, no subsequent changes occurred. It is concluded that the pH-sensitive event, namely virus–cell fusion, is related to the induction of permeability changes through attainment of some type of 'threshold' level of membrane damage.

INTRODUCTION

Permeability changes induced by haemolytic paramyxoviruses underlie the toxin-like action of these viruses (Forda et al., 1982) as well as their ability to mediate cell–cell fusion (Knutton & Pasternak, 1979; Knutton & Bachi, 1980). The changes are restricted to those viruses [Sendai and Newcastle disease virus (Klemperer, 1960; Poste & Pasternak, 1978; Foster et al., 1980)] that fuse with susceptible cells at neutral pH. A preliminary report has shown that influenza virus, which fuses with erythrocytes and other cells at pH 5 to 6 (Maeda & Ohnishi, 1980; Huang et al., 1981; Lenard & Miller, 1981; Marlin et al., 1981; White et al., 1981), elicits permeability changes in Lettre cells maintained at pH 5.3; conversely, Sendai virus elicits permeability changes at pH 7.4 but not at pH 5.3 (Patel & Pasternak, 1983). The pH-sensitive step appears to be membrane fusion between the envelope of the virus and the plasma membrane of the recipient cell, catalysed by the activity of the HA glycoprotein in the case of influenza virus and the F glycoprotein in the case of Sendai virus; lipid-depleted preparations of HA glycoprotein alone are also able to cause haemolysis at pH 5 to 6 (Maeda et al., 1981; Sato et al., 1983).

Under physiological conditions, the pH-dependent step for influenza virus appears to be fusion between the viral envelope and the lysosomal membrane, following endocytotic uptake of the virion (Yoshimura et al., 1982). A low pH-requiring step leading to pore formation has also been shown to be involved in the interaction between diphtheria toxin and membranes (Kagan et al., 1981; Sandvig & Olsnes, 1981), and it is therefore of interest to examine in greater detail the relationship between pH sensitivity and an increased permeability. Since it is more satisfactory to measure permeability changes in the absence of cell lysis, we have analysed virus-induced changes in Lettre cells rather than in erythrocytes (Pasternak, 1984). A characteristic feature of Sendai virus-induced permeability changes is a lag, or induction period. Lag is prolonged by Ca^{2+} and is reduced by Ca^{2+} antagonists such as the calmodulin inhibitor R24571 (Van Belle, 1981); the lag period to leakage of ions is less than that to leakage of phosphorylated metabolites (Bashford et al., 1983a; Micklem et al., 1984, 1985a, b). We show here that influenza virus-mediated permeability changes at pH 5.3 have similar characteristics, and have attempted to separate the fusion event from subsequent leakage by pH-jump experiments.
METHODS

Radiochemicals, Lettre cells and Sendai virus, which was of the '3-day' type, were obtained as described by Impraim et al. (1980) and Bashford et al. (1983b). Influenza virus was the X47 strain (Baez et al., 1980) kindly donated by Dr J. S. Oxford.

Lettre cells, which contained <10% contaminating macrophages or red blood cells, were pre-incubated with [3H]choline and washed in order to remove free [3H]choline. Leakage of [3H] from such cells is predominantly leakage of phosphoryl [3H]choline (Impraim et al., 1980). Virus-treated and control cells remained impermeable to trypan blue throughout incubation at pH 7.4 or 5.3. Cells pre-incubated with 86Rb+ were not washed, but were diluted directly into incubation medium. 150 mM-NaCl, 5 mM-KCl, 1 mM-MgCl2 buffered with 5 mM-MES and 5 mM-HEPES to a final pH of 5.3 or 7.4. pH-jump experiments were carried out by adding 0.012 ml 1 M-HCl to 2.1 ml cell suspension in buffer at pH 7.4 (to give pH 5.3) or by adding 0.01 ml 1 M-NaOH to 2.1 ml cell suspension in buffer at pH 5.3 (to give pH 7.4).

Samples (0.19 ml) were removed at intervals and radioactivity and Na+ and K+ content analysed after spinning through oil as described by Bashford et al. (1983b). Experiments were carried out at least three times with essentially similar results; the figures present data for a typical experiment in each case.

RESULTS AND DISCUSSION

Sequential onset of influenza virus-mediated permeability changes

Fig. 1 shows that if Lettre cells that had been pre-incubated with [3H]choline were treated with low amounts of influenza virus, a lag to onset of 3H-labelled metabolite leakage was discernible (Fig. 1a). Ionic changes, on the other hand, occurred without a lag (Fig. 1b). If the temperature as well as the virus dose were reduced, a lag to onset of ionic changes became apparent (Fig. 2); in this instance, leakage of 86Rb+ from cells pre-incubated with this K+ analogue was measured, as well as ionic changes. Ca2+ increases the length of the lag period (Patel & Pasternak, 1983) and prevents the leakage of ions and metabolites through pores established in its absence (Fig. 3, 4). Thus, influenza virus-treated Lettre cells at pH 5.3 behave exactly like Sendai virus-treated Lettre cells at pH 7.4 (Bashford et al., 1983a; Micklem et al., 1985a, b).

Effect of R24571 on influenza virus-mediated permeability changes

Certain membrane-active drugs that are calmodulin antagonists, such as trifluoperazine and R24571 (calmidazolium), typically shorten the lag to onset of permeability changes and increase the subsequent extent of leakage (Micklem et al., 1984). Fig. 2 shows that the same was true of influenza virus-treated Lettre cells. Whether R24571 affects membrane fusion or some other part of the process leading to the onset of permeability changes is not yet clear, though it may be noted that trifluoperazine inhibits myoblast fusion (Bar-Sagi & Prives, 1983).

pH-jump experiments with influenza and Sendai viruses

The object of these experiments was to see whether permeability changes, once initiated, are pH-sensitive. Lettre cells pre-incubated with [3H]choline were exposed to influenza virus at pH 5.3 for a few minutes at 37 °C. The pH was then brought to 7.4 by addition of NaOH and incubation continued. In order to minimize recovery, that is the restoration of non-leakiness (Pasternak et al., 1976; Impraim et al., 1980; Micklem et al., 1985a), during this time, the temperature was reduced to 3 °C. At this temperature, no new pores are initiated. Fig. 3 shows that leakage of 3H-labelled metabolites (Fig. 3a) and ionic changes (Fig. 3b) continued at pH 7.4 at the same rate and to the same extent as at pH 5.3; in this instance the changes were not of a large magnitude, and they were therefore plotted on a linear axis. Since it has been shown that influenza virus does not induce permeability changes at pH 7.4 (Patel & Pasternak, 1983), the effect of Ca2+ on the leakage of metabolites and ions per se, free of any effect on the induction of such leakage (Micklem, et al., 1985a), can be tested.

The results of Fig. 3 show that Ca2+ inhibited leakage of 3H-labelled metabolites (Fig. 3a) and ions (Fig. 3b); this confirms that the leakage observed at 3 °C is indeed due to the effects of virus initiated at 37°C, since endogenous leakage of ions or metabolites is unaffected by Ca2+ (e.g. Patel & Pasternak, 1983).
Virus-induced permeability changes and pH

Fig. 1. Effect of Ca²⁺ on influenza virus-induced leakage from Lettre cells. Lettre cells pre-incubated with [³H]choline were washed, re-incubated (1-6 x 10⁶ cells/ml) with influenza virus (5 x 10⁵ HAU/ml) at 37 °C or 0 °C with (●) or without (○) 5 mM-CaCl₂ at pH 5-3, and ³H and cation content of cell pellets measured as described in Methods. (a) Radioactivity remaining in pellets expressed as a percentage of that present at zero time; (b) cation content expressed as a percentage of the K⁺/Na⁺ ratio at zero time.

Fig. 2. Effect of R24571 on influenza virus-induced leakage from Lettre cells. Lettre cells pre-incubated with ⁶²⁵RbCl were diluted in Ca²⁺-free medium (2.3 x 10⁶ cells/ml) at pH 5-3 at 19 °C with (●, ■) or without (○, □) influenza virus (500 HAU/ml) in the presence (●, ●) or absence (□, □) of 10⁻⁶ M-R24571, and ⁶²⁵Rb and cation content of cell pellets measured as described in Methods. Radioactivity (a) and cation ratios (b) are expressed as in Fig. 1.

A similar experiment was carried out with Sendai virus. That is, Lettre cells pre-incubated with [³H]choline were exposed to Sendai virus for a few minutes at pH 7-4 at 37 °C and then shifted to pH 5-3 at 3 °C. Fig. 4(a) shows that the subsequent leakage of ³H-labelled metabolites was as extensive at pH 5-3 as at pH 7-4; indeed, it was somewhat higher at pH 5-3, but this can be attributed to the endogenous leakage of ³H-labelled metabolites at low pH (K. Patel & C. A. Pasternak, unpublished observations). Fig. 4(b) depicts the ionic change in this experiment. Note that there was only a marginal effect of Ca²⁺; this may be because the cells were well past the threshold for ion changes and it is known that the longer cells are exposed to virus, the more difficult it is to reverse the increased permeability by removal of Ca²⁺ (Micklem, et al., 1985a). In other words, Ca²⁺ is much more effective at preventing the onset of permeability changes than at closing existing pores.

Fig. 4(c, d) illustrates the results of a similar experiment in which the initial incubation was in the presence of Ca²⁺, and EGTA was added at the time of the temperature and pH shift. Fig. 4(c) confirms that leakage subsequent to the temperature shift is independent of pH; in this instance leakage in the presence of Ca²⁺ at 37 °C was marginally greater than leakage in the absence of Ca²⁺ at 3 °C; maintaining Ca²⁺ in the incubation medium at 3 °C abolished further leakage. When the ionic changes in this experiment were examined, cells at 3 °C treated with EGTA again showed leakage compared with cells kept in the presence of Ca²⁺ (Fig. 4d). In this instance, cells kept at 37 °C in the presence of Ca²⁺ showed no change whatsoever in Na⁺/K⁺ ratio. This surprising result may be attributed to the effective operation of the Na⁺ pump acting to reverse any permeability changes that are undoubtedly occurring (Fig. 4c); moreover, cells
Fig. 3. Effect of pH jump on influenza virus-induced leakage from Lettre cells. Lettre cells pre-incubated with [3H]choline were washed, re-incubated (1.6 x 10^6 cells/ml) with influenza virus (5 x 10^3 HAU/ml) in Ca^{2+}-free medium at pH 5.3 at 37 °C (Δ), and ^3H and cation content of supernatants and cell pellets measured as described in Methods. At the time indicated by the arrow, portions of the incubation mixture were chilled to 3 °C; the pH was either maintained (□, ■) or shifted to 7.4 (○, ●); the cells were either maintained in Ca^{2+}-free medium (○, □) or exposed to 5 mM-Ca^{2+} (●, ■). (a) Radioactivity released into the medium, expressed as a percentage of that in the cells at zero time; (b) Na^+/K^+ ratio in cell pellets.

kept at pH 5.3 (at 37 °C or 3 °C) shrink (unless they become permeabilized by virus, in which case they swell) due to concomitant loss of K^+ and Na^+, and this effect complicates the interpretation of Na^+/K^+ data. An experiment similar to the one shown in Fig. 3(b) was carried out with influenza virus. That is, cells were incubated at pH 5.3 with influenza virus in the presence of Ca^{2+} and then switched to pH 7.4 at 3 °C +/− EGTA. The results (not shown) were essentially similar to those obtained with Sendai virus. It may be noted that Ca^{2+} is less effective at inhibiting permeability changes at pH 5.3 than at pH 7.4, irrespective of the virus used to initiate the changes. Since the ionization of Ca^{2+} is little affected by decreasing the pH from 7.4 to 5.3 (pK values for Ca^{2+} are > 7), it is likely that it is the receptor to which Ca^{2+} binds that is the pH-sensitive component.

It is clear from these results (Fig. 3 and 4) that virus-mediated permeability changes, whether initiated by fusion with Sendai virus at pH 7.4 or with influenza virus at pH 5.3, are themselves insensitive to pH, have a low temperature coefficient (Q_10 of < 2 between 3 and 37 °C), yet are sensitive to inhibition by Ca^{2+}, or to reversal of Ca^{2+} inhibition by EGTA. Such conclusions allow one to investigate the lag to onset of permeability changes that is a characteristic of cells incubated at low temperatures, by pH-jump experiments as follows.

Lettre cells pre-incubated with ^86Rb^+ were incubated at pH 5.3 in the presence of influenza virus at 19 °C. The onset of permeability changes in this instance had a characteristic lag of some 15 to 20 min, as indicated in Fig. 2. Portions of such cells treated with influenza virus at pH 5.3 for 5, 10, 20 or 40 min then had their pH brought to 7.4; incubation was continued at the same
Fig. 4. Effect of pH jump on Sendai virus-induced leakage from Lettre cells. Lettre cells pre-incubated with [3H]choline were washed, re-incubated (2 x 10^6 cells/ml) with Sendai virus (80 HAU/ml) in Ca²⁺-free medium (Δ: a, b) or in medium containing 2.5 mM-CaCl₂ (▲: c, d) at pH 7.4 at 37 °C, and 3H and cation content of supernatants and cell pellets measured as described in Methods. At the times indicated by the arrow, portions of the incubation mixture were chilled to 3 °C; the pH was either maintained (O, •) or shifted to 5.3 (●, ○): the cells that were originally in Ca²⁺-free medium were either maintained in Ca²⁺-free medium (O, □: a, b) or exposed to 2.5 mM-CaCl₂ (●, ○: a, b); the cells that were originally in medium containing 2.5 mM-CaCl₂ were either maintained in medium containing 2.5 mM-CaCl₂ (●, □: c, d) or exposed to 5 mM-EGTA (O, □: c, d). Radioactivity released into the medium (a, c) and Na⁺/K⁺ ratios (b, d) are expressed as in Fig. 3.

Temperature for various periods of time and samples taken for ⁸⁶Rb⁺ and cation analysis. Fig. 5(a) shows that if cells were exposed to influenza virus for 5 or 10 min before the pH jump, little ⁸⁶Rb⁺ leakage subsequently took place; in contrast, cells exposed to influenza virus for 20 or 40 min showed immediate changes. Essentially the same results were obtained for measurement of ion ratios (Fig. 5b).

A similar experiment carried out by incubating prelabelled Lettre cells with Sendai virus at pH 7.4 for various periods of time before lowering the pH is shown in Fig. 6. Because cells tend to leak ions in the absence of virus at pH 5.3, control cells to which no virus was added were treated in the same way as cells with virus. The values of ⁸⁶Rb⁺ content (Fig. 6a) and ion ratio (Fig. 6b) in virus-treated cells were plotted as a percentage of the relative controls. As with influenza virus, a threshold action was clearly indicated: incubating at pH 7.4 for 4 or 8 min before the pH jump did not initiate subsequent leakage, but incubating for 19 or 30 min did; this is in good agreement with the observed lag to onset of leakage of around 20 min (Fig. 6a, b, upper figure).

These experiments therefore support the notion of a threshold level of membrane damage caused by membrane fusion (Micklem, et al., 1985a), below which permeability changes are not observed. Such a mechanism of threshold acquisition has also been demonstrated during the early stages of complement-mediated lysis (Edwards et al., 1983; Sims, 1983) and it should prove instructive to see in what other ways the onset of virus-mediated permeability changes resembles the onset of immunologically mediated changes leading to cell lysis (Bashford et al., 1984; Pasternak et al., 1985a, b).

The experiments described above, together with data presented elsewhere (Pasternak, 1981, 1984), clearly separate virus-mediated permeability changes following the initial binding of...
Fig. 5. Effect of pH jump at different times on influenza virus-induced leakage from Lettre cells. The same batch of Lettre cells used in Fig. 2 were incubated at 19 °C at pH 5.3 with influenza virus (500 HAU/ml) for 5 (1), 10 (2), 20 (3) or 40 (4) min as indicated by the arrows, the pH then adjusted to 7.4, and incubation continued at 19 °C. Radioactivity and cation content of cell pellets were measured as described in Methods. Radioactivity (a) and K⁺/Na⁺ ratio (b) each expressed as in Fig. 1, of batches of cells shifted to pH 7-4 at the four time points indicated in the upper curve are shown in the successive curves; the upper curve of (a, b) is reproduced from the respective curves (■) of Fig. 2.

virus to cells [stage (1)] into two further distinct stages: virus–cell fusion (or, in the case of lipid-depleted HA glycoproteins, membrane insertion), and the subsequent leakage of ions and metabolites (Fig. 7). Stage (2) is pH-specific, strongly temperature-dependent, and insensitive to the presence or absence of external Ca²⁺. Stage (3) is pH-insensitive, relatively temperature-independent, and sensitive to external Ca²⁺ at physiological concentration. The onset of the third stage is dependent on the acquisition of a threshold level of membrane damage; this may reflect (i) damage to an increasing number of cells and/or (ii) the accumulation of an increasing number, or an increasing size, of permeability 'pores' within any one cell. What is clear is that the threshold is lower for ions than for metabolites, and that below the threshold, leakage is not observed. A similar sequence of events probably occurs in the case of permeability changes elicited by bacterial toxins at low pH.

The significance of these results is twofold. First, it clarifies the type of permeability change that may occur when enveloped viruses fuse their way out of lysosomes during the initiation of an infectious cycle (e.g. Helenius & Marsh, 1982; Yoshimura et al., 1982). Second, it makes it unlikely that the chemiluminescence elicited by influenza virus when interacting at neutral pH with mouse spleen cells (Peterhans, 1980) or with human neutrophils (Mehta et al., 1985), which may be part of the mechanism whereby influenza virus impairs neutrophil function (Larson & Blades, 1976), is the result of a permeability change.
Fig. 6. Effect of pH jump at different times on Sendai virus-induced leakage from Lettre cells. Lettre cells pre-incubated with $^{86}$Rb were diluted (1.2 x 10^6 cells/ml) into medium at pH 7.4 and incubated with Sendai virus (13 HAU/ml) at 19 °C, and radioactivity (a) and K*/Na+ ratio (b) measured as described in Methods. The upper curve in each panel shows radioactivity and K*/Na+ ratio in cells maintained at pH 7.4. At the times indicated by the arrows, batches of cells were adjusted to pH 5.3, and incubation continued at 19 °C; radioactivity (a) and K*/Na+ ratios (b) of these cells are shown in the successive curves. The values of radioactivity and K*/Na+ ratio are calculated as a percentage of the values in cells kept at pH 5.3 without virus.

Fig. 7. Stages in the onset of virus-mediated permeability changes. Stage (1) is temperature-independent, pH-insensitive and unaffected by Ca2+. Stage (2) is highly temperature-dependent, pH-sensitive, and unaffected by Ca2+. Stage (3) is slightly temperature-dependent, pH-insensitive and inhibited by Ca2+. In the case of permeability changes elicited by lipid-depleted preparations of HA glycoproteins (Sato et al., 1983), stages (1) and (2) represent binding and insertion of HA respectively. Based on data presented in this and previous reports ((Pasternak, 1984; Micklem. et al., 1985a, b).
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REFERENCES


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