Polyphosphoinositide metabolism in baby-hamster kidney cells infected with herpes simplex virus type 1

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The incorporation of [^{32}P]P_i and [^3H]inositol into the inositol lipids of baby-hamster kidney cells was studied in herpes-simplex-virus-type-1 (HSV-1)-infected and mock-infected cells. The infection was conducted during incorporation of, as well as after prelabelling with, the precursors. These methods were used in order to study both synthesis *de novo* of, and steady-state changes in, the phosphoinositides. Both with infection during labelling, and after prelabelling, we found increased [^{32}P]- and [^3H]-phosphatidylinositol 4,5-bisphosphate (PIP₂) and decreased [^{32}P]- and [^3H]-phosphatidylinositol 4-monophosphate in infected as compared with mock-infected cells, whereas no effect was observed on phosphatidylinositol. This altered inositol-lipid metabolism was (at least in the case of PIP₂) not present until 3–6 h after infection and remained stable, or increased slightly, throughout the infection period. Polyphosphoinositide metabolism constitutes an important step in signal processing in many forms of cellular stimulation, and the results obtained suggest that HSV-1 infection may induce such events in our cell system.

INTRODUCTION

Considerable interest has been focused during the last few years on the role of phosphoinositides in cellular signal processing. The role of phosphoinositides in general has been reviewed (Fisher *et al.*, 1984; Berridge, 1985), as well as aspects concerning hormone action (Farese, 1984) and cellular proliferation (Berridge, 1984). A role for the inositol lipids in virally induced transformation of cells has been proposed, and results could indicate that the increased inositol-lipid turnover was caused by increased PI kinase activity (Macara *et al.*, 1984; Sugimoto *et al.*, 1984). However, it has recently been suggested that this effect is not likely to be due to a PI kinase activity (Sugano & Hanafusa, 1985).

The effect on phospholipids of HSV (and PRV) infection has been studied to some extent (Asher *et al.*, 1969; Ben-Porat & Kaplan, 1971; Tucker *et al.*, 1974; Diringer *et al.*, 1978; Daniel *et al.*, 1981; Steinhart *et al.*, 1981; Suzuki & Blough, 1982; Steinhart *et al.*, 1984). Some authors report effects on sphingomyelin (Ben-Porat & Kaplan, 1971; Steinhart *et al.*, 1981; Steinhart *et al.*, 1984), others on cardiolipin (Tucker *et al.*, 1974). One group studied the incorporation of [^3H]inositol into PI and found no effect on the phospholipids during PRV infection, though they observed a reduction in the amounts of free cellular [^3H]inositol after PRV infection (Diringer *et al.*, 1978). PPIs, however, have not been isolated in any of these studies. Therefore, very little is known about phosphoinositide metabolism in HSV-infected cells, and the possibility that infection induces some kind of signal processing via the PPI cycle has not been investigated.

The present study has been aimed to reveal if signal processing via the PPI cycle occurs during HSV infection. To test this we have radiolabelled the phosphoinositides involved, using both [^{32}P]P_i and [^3H]inositol as precursors. In order to distinguish between events related to the steady-state levels and the synthesis *de novo* of the inositol lipids, we have used both prelabelling of the cells (before infection) and simultaneous labelling and infection. Our results show an altered turnover of PPIs during HSV-1 infection.

EXPERIMENTAL

Materials

The cells used in all experiments were BHK 21 clone 13 (Macpherson & Stoker, 1962). The virus used was HSV 1 strain 17syn⁺ (Brown *et al.*, 1973). [^{32}P]P_i (10 mCi/ml, carrier-free) and *myo*-[2- ^3H]inositol (1 mCi/ml) were purchased from Amersham International (Amersham, Bucks., U.K.). Lipid standards were purchased from Supelco (Bellafronte, PA, U.S.A.) and Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cell-culture media: EMEM and NBC serum were purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). T.l.c.: t.l.c. plates (Kieselgel 60) and chemicals (chromatography grade) were obtained from E. Merck (Darmstadt, Germany). Film for autoradiography was Fuji RX medical X-ray film (Fuji Photo Film Co., Tokyo, Japan). BSA was purchased from Sigma.

Cell cultures

BHK 21 cells were propagated in roller bottles and grown further in 32 mm-diameter culture dishes contain-

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PPI(s), polyphosphoinositide(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DG, diacylglycerol; IP₃, inositol 1,4,5-trisphosphate; HSV-1, herpes simplex virus type 1; PRV, pseudorabies virus; BHK cells, baby-hamster kidney cells; EMEM, Eagle minimum essential medium; NBC serum, newborn-calf serum; BSA, bovine serum albumin; p.f.u., plaque-forming unit(s).

ing EMEM supplemented with 10% (v/v) NBC serum. Cells were incubated at 37 °C until confluence, at which time experiments were performed.

Infection of cells

Virus stocks in 5 mM-Tris/HCl (pH 7.5)/0.1% BSA were kept at -80 °C. Portions were thawed shortly before use and aggregates dissolved in a sonication bath. Unless otherwise stated, cells were infected with 20 p.f.u. of HSV/cell. At the time of infection the medium was replaced with a fresh one containing 2% (v/v) NBC serum. Mock infection was performed with the Tris/HCl/BSA solution used for virus stock preparations.

Labelling of cells with [32 P]P_i and [3 H]inositol

When prelabelling the cells, 2 μ Ci of [32 P]P_i and/or 5 μ Ci of [3 H]inositol was added/ml of medium 18 h before infection. The medium was removed immediately before infection, the cells washed with non-radioactive medium, and infection carried out in the absence of radioactive isotope; the EMEM used contained unlabelled P_i and inositol.

When labelling from the time of infection, 5 μ Ci of [32 P]P_i was added/ml of medium immediately before the virus and remained present throughout the experiment.

Extraction of lipids

A modification of the extraction procedure described by Bligh & Dyer (1959) was used. All extraction procedures were performed on ice. Medium was removed, and methanol/chloroform/conc. HCl (40:20:1, by vol.) was added to the cells, which were scraped off the cell-culture dishes, transferred to test tubes and shaken briefly before further addition of chloroform/water (1:1, v/v). Tubes were shaken again before the water and chloroform phases were separated by a 10 min centrifugation at 200 g. The chloroform phase was collected and evaporated to dryness in an N₂ jetstream. The lipid residues were kept at -20 °C until further analysis.

T.l.c. of phospholipids

The lipid residues were dissolved in 20 μ l of chloroform and 10 μ l samples were applied on the t.l.c. plates. The inositol lipids were separated in a chloroform/methanol/40% (v/v) methylamine/water (60:36:5:5, by vol.) solvent system as described by de Chaffoy de Courcelles *et al.* (1984). The remaining phospholipids were separated by two-dimensional chromatography as follows: direction 1, chloroform/methanol/25% NH₃/water (70:45:4:6, by vol.); direction 2, chloroform/acetic acid/methanol/water (81:45:10:4, by vol.). Plates were dried under N₂ for 1 h between the systems. Lipid standards were revealed by spraying the plates with 10% molybdo-phosphoric acid in ethanol. Radiolabelled compounds were detected by autoradiography. Detected lipids were scraped off the plates, transferred to scintillation vials, and 4 ml of scintillation liquid (Opti-Scint, from Packard-Becker B.V. Chemical Operations, Groningen, The Netherlands) was added to each sample. Radioactivity was measured in a Packard Tri-Carb 460 CD liquid-scintillation counter.

Data presentation

The [32 P]P_i incorporation into the various lipids of the infected cells relative to that in the mock-infected cells

showed the same pattern in different experiments. The exact time after infection, however, at which a certain change of incorporation took place varied slightly between experiments. Time courses are therefore not directly comparable. Because of this, statistical evaluation of the data is not directly applicable. Therefore, the results of a typical experiment are shown rather than the mean of the six to ten experiments performed with [32 P]P_i or [3 H]inositol labelling. Within-experiment variability of data was determined to be 6.0% for PIP₂, 8.0% for PIP, 5.5% for PI, and 6.2% for PC (six parallels).

RESULTS

Incorporation of [32 P]P_i and [3 H]inositol into the inositol lipids

Two different approaches to labelling the cellular phospholipids were used. In the first approach the cells were incubated with [32 P]P_i and/or [3 H]inositol for 18 h before infection. Before addition of virus the medium with the radioisotopes was replaced by a fresh one containing non-radioactive P_i and inositol. These experiments are referred to as 'prelabelling' experiments and are, in principle, pulse-chase experiments. In mock-infected cells, the radioactivity of the phospholipids gradually decreased during the observation period, probably because of exchange between the radioactive moieties and their non-radioactive counterparts in the medium. In the virus-infected cells, however, [3 H]- or [32 P]-PIP₂ decreased less than in the mock-infected cells; thus, at any time after the start of infection, the PIP₂ radioactivity was increased relative to that in control cells and will be referred to below as 'increased' radioactivity. In the second approach, both virus and [32 P]P_i were added to the cells at the same time; this is referred to as 'simultaneous incorporation and infection'. Steady-state levels of the phospholipid labelling are not reached within the infection periods observed in these experiments. Other experiments showed that steady-state levels of [32 P]P_i incorporation into PIP and PIP₂ were reached after about 15 h of incubation; more than 25 h were required to reach steady states in PI, PC, PE and PS (results not shown).

Prelabelling experiments

Fig. 1 shows the results of a typical prelabelling experiment. Relative to control cells, the [32 P]PIP₂ increases in infected cells. This relative [32 P]PIP₂ increase, which will hereafter be referred to as the 'PIP₂ response', started 3-6 h after infection (the exact time varied between experiments), and remained unchanged, or became gradually greater, during the infection period. The maximum relative increase in [32 P]PIP₂ and [3 H]PIP₂ (separate experiments, not simultaneous incorporation of the precursors) in infected cells averaged 70% (eight experiments, range 20-170%) and 60% (three experiments, range 41-88%), respectively (results not shown). Fig. 1 also shows that [32 P]PIP in infected cells decreases more than in control cells. (This is called the 'PIP response' below. The PIP₂ and PIP responses will collectively be called the 'PPI responses'.) The maximum relative decrease in [32 P]PIP averaged 19% for [32 P]PIP and 25% for [3 H]PIP. Fig. 1 also shows that the decrease in [32 P]PIP started very soon after infection, in contrast with the increase in [32 P]PIP₂, which had a delay of 3-6 h. PI, PC (Fig. 1), PE and PS (results not shown) did not

Fig. 1. Effect of HSV on the incorporation of $[^{32}\text{P}]\text{P}_i$ into different phospholipids in prelabelled BHK cells

Confluent BHK cells were incubated in EMEM containing $2 \mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i/\text{ml}$ for 18 h. The medium was then removed, and fresh medium added before the experiment started. Cells were then infected with (●) or without (○) HSV at a multiplicity of 20 p.f.u./cell and incubated at 37°C . Phospholipids were extracted for analysis at the times indicated. For further details, see the Experimental section.

Fig. 2. Simultaneous $[^{32}\text{P}]\text{P}_i$ incorporation into phospholipids and HSV infection of BHK cells

The experimental procedures and symbols are as described in the legend to Fig. 1. However, $[^{32}\text{P}]\text{P}_i$ ($5 \mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i/\text{ml}$ of EMEM) was here added at the time of infection and remained in the medium throughout the experiment.

alter their ^{32}P content during infection, relative to mock-infected cells.

Simultaneous $[^{32}\text{P}]\text{P}_i$ incorporation and infection

Fig. 2 shows the results of a typical experiment with simultaneous $[^{32}\text{P}]\text{P}_i$ incorporation and infection. The

incorporation of ^{32}P into PIP₂ increased more in infected than in mock-infected cells. As in the prelabelling experiments, the time after infection at which this accelerated increase in $[^{32}\text{P}]\text{PIP}_2$ started varied from 3 to 6 h between experiments. Incorporation of ^{32}P into PIP was slower in infected than in mock-infected cells and

Fig. 3. Effect of HSV infection on BHK cells prelabelled with both $[^3\text{H}]$ inositol and $[^{32}\text{P}]\text{P}_i$ simultaneously

The experimental procedures and symbols are as described in the legend to Fig. 1, except that the cells were preincubated with both $2\ \mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i$ and $5\ \mu\text{Ci}$ of $[^3\text{H}]$ inositol/ml for 18 h before infection. This was removed, and fresh medium without additions was added before the experiment started.

never reached the incorporation level of control cells during the observed period of infection. As mentioned above, there were experiment-to-experiment variations in results. However, after 10 h of infection, both in prelabelling and simultaneous-labelling experiments, the level of $[^{32}\text{P}]$ - and $[^3\text{H}]\text{-PIP}_2$ was significantly ($P < 0.005$) increased compared with control cells, as was $[^{32}\text{P}]\text{PIP}$ and $[^3\text{H}]\text{PIP}$ significantly ($P < 0.01$) decreased.

Double-labelling experiments

Fig. 3 shows the results of an experiment where the cells had been prelabelled with both $[^3\text{H}]$ inositol and $[^{32}\text{P}]\text{P}_i$. The PIP_2 response appears at the same time during infection and is of approximately the same magnitude, both for $[^3\text{H}]\text{PIP}_2$ and $[^{32}\text{P}]\text{PIP}_2$. One difference between incorporation of $[^3\text{H}]$ inositol and $[^{32}\text{P}]\text{P}_i$ is easily seen in the control cells: there is a more rapid decrease in $[^{32}\text{P}]\text{PIP}_2$ than in $[^3\text{H}]\text{PIP}_2$.

Effect of varying the multiplicity of infection

To examine whether the PPI effects were dependent on the amount of virus added to the cells, HSV at different

Fig. 4. Effect of infecting BHK cells with increasing amounts of virus on the incorporation of labelled phosphate into PIP_2

The experimental procedures are as described in the legend to Fig. 1. Cells were infected with multiplicities of virus varying from 0 (control) to 60 p.f.u./cell. The infection period was 11 h. Results are means $\pm$ S.E.M. for three separate dishes.

multiplicities was used in experiments. Cells were prelabelled with ^{32}P , and then infected with various multiplicities of virus (Fig. 4). The infection period was 11 h. The results show that the PIP_2 response is dependent on the amount of virus added up to about 20 p.f.u./cell. Further increase in the amount of virus added does not significantly increase the PIP_2 response further. Results for PIP, on the other hand, show that the PIP response is maximal at lower multiplicities of infection. Less than 5 p.f.u./cell gives virtually full response. PI and PC labelling was not affected, even at 60 p.f.u./cell.

DISCUSSION

Signal processing via the PPI cycle (Scheme 1) has been revealed in many cell systems and kinds of cellular stimulation and response (Fisher *et al.*, 1984; Farese, 1984; Berridge, 1984). However, in HSV-infected cells, this has not been studied in detail. In earlier studies (Ben-Porat & Kaplan, 1971; Diringer *et al.*, 1978), neither separation of the individual inositol lipids nor necessary acid-extraction conditions were used. When isolated as one group of substances, PI predominates quantitatively, so that events concerning the PPIs will escape detection. Also, as stated by Ben-Porat & Kaplan (1971) and others (Rampini & Dubois, 1978), the physiological state of the cells may influence the experimental results.

By infecting the cells and labelling with $[^{32}\text{P}]\text{P}_i$ simultaneously, we aimed to detect any increase or decrease in the synthesis *de novo* of the inositol lipids. As shown in Fig. 2, HSV infection increases and decreases the incorporation of ^{32}P into PIP_2 and PIP respectively.

Scheme 1. The PI cycle

PI is phosphorylated to PIP, which is again phosphorylated to PIP_2 by PI kinase (PIK) and PIP kinase (PIPK). These reactions are reversed by phosphomonoesterase(s) (PME). All the phosphoinositides can also be converted to diacylglycerol and IP, IP_2 or IP_3 by the action of phospholipase C (PLC). DG can, in turn, be phosphorylated to phosphatidic acid (PA). The cycle is completed when phosphatidic acid incorporates inositol to form PI. Phospholipase A_2 (PLA) removes the fatty acid in position 2 from the phospholipid. Further abbreviations used: IP and IP_2 , inositol mono- and bis-phosphate; LPI, LPIP and LPIP_2 , lyso-PI, -PIP and - PIP_2 .

This may be caused by stimulation of PIP_2 synthesis while that of PIP is slowed. Alternatively, the reduced labelling of PIP may be a result of accelerated PIP_2 synthesis, and thus decreased absolute amounts of PIP, because of increased consumption of PIP through synthesis of PIP_2 without increased synthesis *de novo* from PI. The synthesis *de novo* of PIP needs therefore not be affected, despite the reduced labelling.

When the cells are prelabelled with $[^{32}\text{P}]\text{P}_i$ (or $[^3\text{H}]\text{-inositol}$), it is more likely that the $[^{32}\text{P}]\text{P}_i$ incorporation gives a picture of the turnover or degradation of the lipids. Also, an increase or decrease in the total amount of a particular phospholipid will influence the labelling. The results given in Fig. 1, with increased PIP_2 labelling and decreased PIP labelling, may indicate that HSV infection not only affects the synthesis *de novo* of PIP_2 (and PIP), but also the turnover and/or the total amounts of PIP and PIP_2 [as suggested in the interpretation of the PIP curves (Fig. 2) in the above paragraph]. The fact that the increase in $[^{32}\text{P}]\text{PIP}_2$ and decrease in $[^{32}\text{P}]\text{PIP}$ follow the same pattern both with prelabelling and simultaneous labelling and infection, makes it more likely that the steady-state levels of PIP_2 and PIP are altered by infection and that variations in ^{32}P incorporation reflect changes occurring in the total amounts of these phospholipids. Since PPI metabolism seems to be an important mediator of growth control in normal cells, it must be assumed that it is normally under strict control. It is therefore possible that this overall regulation mechanism is affected by HSV infection and that our results reflect this.

If the PIP_2 and PIP responses induced by infection result from increased PIP kinase or reduced PIP_2 phosphomonoesterase activity, or, also, altered overall regulation (see Scheme 1 for the enzymic pathways), one would expect that the PIP_2 response can be affected by

changing the number of viruses infecting each cell, thus changing the degree of stimulation/activation of the enzyme system. Fig. 4 shows that this is so. Increasing the multiplicity of virus up to 20 p.f.u./cell does increase the PIP_2 response. Even though multiplicities of infection of greater than 20 are quite possible [in receptor studies, as many as approx. 100–200 HSV-1 receptors on the surface of fibroblasts have been suggested (Addison *et al.*, 1984; Vahlne *et al.*, 1979)], our experiments with higher multiplicities have not led to further increase of the PIP_2 response. Thus there is apparently a 'saturation' or 'steady-state' level where maximum stimulation is achieved. It was surprising to observe that saturation of the PIP response occurred at a lower multiplicity of infection. It will require further investigation to determine the interrelationship between the PIP and PIP_2 responses at varying multiplicities of infection.

It follows from Scheme 1 that the PIP_2 level is regulated through at least four different enzymes: the PIP kinase, the phospholipase C (which splits PIP_2 into DG and IP_3), the phospholipase A_2 (which gives the lysophospholipid and non-esterified fatty acid from the 2-position on glycerol) and the phosphomonoesterase (which gives P_i and PIP). Stimulation/induction of the PIP kinase would increase the total amount of PIP_2 and reduce that of PIP, if this enzyme is considered alone. Even if increased synthesis of PIP_2 in turn leads to increased turnover of PIP_2 , and thus not increased total mass, this can be explained by our results. Any change in turnover of PIP would be expected to be reflected also in altered PI turnover. On the other hand, the PPIs are present in such small amounts that, for instance, a 20% decrease in PIP would only account for an approx. 2% decrease in PI, if both are reduced mol for mol, and this is not sufficient to cause significant changes under the experimental conditions we have used. (The exact

amounts of PPI have not been determined for BHK cells.)

In conclusion, our experiments suggest that HSV infection may induce, or otherwise affect, the PPI cycle in fibroblasts. One possible explanation is that HSV infection activates or induces the formation of PIP kinase or its activator. However, further studies are required to examine these possibilities.

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