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RAPID PHOSPHOLIPASE A_2 STIMULATION AND DIACYLGLYCEROL CHOLINEPHOSPHOTRANSFERASE INHIBITION IN BABY HAMSTER KIDNEY CELLS DURING INITIATION OF DENGUE VIRUS INFECTION

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SUMMARY

Infection of baby hamster kidney (BHK-21) cells by dengue type 2 (DEN-2) virus is accompanied by a rapid but transient stimulation of phospholipase A_2 activity which reaches a maximum within 40 min. Thereafter, the phospholipase A_2 activity, that is associated with the microsomal fraction, returns to normal levels at an equally rapid pace. In contrast, microsomal phospholipase A_1, lysophosphatidylcholine acyltransferase, or lysophospholipase are not altered early in the infection. Microsomal diacylglycerol cholinephosphotransferase was inhibited in phase with phospholipase A_2 stimulation. The data obtained on the DEN-2/BHK-21 system suggest that virus-triggered phospholipase A_2 activation and ensuing lysophospholipid production may facilitate initial interaction of the virion with the cell plasma membrane and may aid viral penetration.

Viral infection is initiated by adsorption of the virion to the host cell surface followed by viral penetration of the plasma membrane. Virus adsorption can involve interaction between the virion and specific receptor sites, whereas mechanisms of virus penetration are thought to include fusion of the viral envelope with the plasma membrane and pinocytosis (viropexis) (1-3). The biochemical processes that govern these early events of virus infection are of current interest.

In the present study we show that initiation of infection of baby hamster kidney (BHK-21) cells with dengue (DEN-2) virus is accompanied by a rapid but transient induction of phospholipase A_2 activity and simultaneous inhibition of diacylglycerol cholinephosphotransferase.

Abbreviations: BHK-21, baby hamster kidney cells; DEN-2, dengue virus type 2 (New guinea C strain).

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MATERIALS AND METHODS

Cells and media. BHK-21 cells were grown in shaker culture in serum-free Waymouth 752/1 medium (4,5). Cells were used in their logarithmic phase of growth within 72 hrs after serial passage. Maintenance medium was prepared as described previously (5). Spent medium was prepared after growing BHK-21 cells for 48 hrs. The cells were sedimented at 500 x g for 10 min at 22°C, and the supernatant was separated from residual debris by centrifugation at 17,000 x g for 30 min. Bovine serum albumin (0.2%) was added to the spent medium, and the pH was adjusted to 7.8.

Virus. Dengue type 2 (New Guinea C strain) virus was grown in BHK-21 cells in serum-free shaker culture (6), and a partially purified virus was isolated after the third virus passage in BHK-21 cells. For this purpose, 5 x 10^7 BHK-21 cells were infected with DEN-2 at a multiplicity of infection of 0.05 plaque forming units per cell (PFU/cell) and the cells were incubated in shaker culture at 37°C for 60 min (6). Unadsorbed virus was removed, the cells were resuspended in 50 ml of growth medium and reincubated at 37°C for 42 hrs to obtain maximum virus titer (6). The virus suspension was purified at 4°C by first sedimenting the cells at 500 x g for 5 min, then the debris at 17,000 x g for 30 min; 0.2% bovine serum albumin was added to the supernatant (pH 7.8). Partially purified virus samples were quick-frozen and stored at -70°C. The virus titer was determined by plaque assay (7) and was 4.1 x 10^8 PFU/ml.

Cell infection. BHK-21 cells (5 x 10^7 cells) in their logarithmic phase of growth (48 hrs) were washed twice with 50 ml of maintenance medium (5), and were resuspended in 5 ml of serum-free growth medium (5,6). A 0.5 ml aliquot of this suspension ("time zero sample") was diluted with 5 ml of maintenance medium and kept at 0°C for cell fractionation (see below). The remaining 4.5 ml portion of resuspended cells was diluted with 40 ml of purified DEN-2 and was incubated at 37°C. At designated time intervals, 5 ml aliquots were removed, quickly chilled by mixing with 5 ml of frozen maintenance medium, and the sample was immediately used for cell fractionation.

Control experiments were done on uninfected cells. For this purpose, BHK-21 cells were incubated in spent medium (see above) instead of DEN-2 suspension prior to cell fractionation.

The percentage of cells infected during incubation with virus was determined by plaque assay (7) after washing the cells twice with 150 ml of growth medium to rinse off adhering virus.

Subcellular fractionations were carried out at 4°C. Cells were isolated by sedimentation at 500 x g for 5 min, resuspended in 5 ml of Tris-sucrose buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) and broken open in a Dounce homogenizer with an efficiency of 95% as judged by the trypan blue exclusion test. The homogenate was centrifuged for 5 min at 2,000 x g to remove unbroken cells and nuclei. The supernatant was then fractionated at 18,000 x g for 20 min; the pellet was resuspended in 2 ml of Tris-sucrose buffer and was used as the lysosomal fraction. The supernatant of the 18,000 x g fractionation was centrifuged at 100,000 x g for 60 min producing a pellet that was resuspended by homogenization in 2 ml Tris-sucrose buffer and used as the microsomal fraction. Subsequent assays were done immediately on the fresh samples as well as within 48 hrs after quick-freezing (-70°C); the data obtained on fresh and frozen samples were identical within normal experimental deviation.

Chemical and enzymatic assays. Protein was determined by the method of Lowry et al. (8). Intracellular potassium was measured by the atomic absorption technique as used previously (5). Radioactivity was measured as described previously (9).

Phospholipase A_2 [EC 3.1.1.4] activity was determined according to established procedures (10) based on labeled fatty acid release from 1-palmitoyl-2-[1'-^{14}C]linoleoyl-sn-glycerophosphocholine; substrate was prepared by enzymatic acylation (11) of 1-palmitoyl-sn-glycerophosphocholine (9) with [1-^{14}C]linoleic acid (Amersham Corp., Arlington Heights, IL). The assay mixture containing 150 mM Tris-HCl buffer (pH 8.5) 4 mM $CaCl_2$, 0.2 mM 1-palmitoyl-2-[1'-^{14}C]linoleoyl-sn-glycerophosphocholine (spec. act., 0.15 mCi/mmol) and 100 µg of the microsomal protein in a final volume of 400 µl was incubated for 15 min at 37°C. The reaction was terminated by addition of methanol. The mixture was extracted with chloroform, and released fatty acid was isolated by thin-layer chromatography and quantified by scintillation counting (9).

Phospholipase A_1 [EC 3.1.1.32] activity was determined under the conditions of the phospholipase A_2 assay (see above) using 1-[1'-^{14}C]palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (spec. act., 0.05 mCi/mmol) as substrate. The substrate was synthesized (12) by acylation of glycerophosphocholine ($CdCl_2$ complex; Sigma Chemical Co., St. Louis, MO) with [1-^{14}C]palmitic anhydride (prepared from [1-^{14}C]palmitic acid; New England Nuclear; see ref. 13), hydrolysis of the phosphatidylcholine with Ophiophagus hannah venom, and reacylation of 1-[1'-^{14}C]palmitoyl-sn-glycero-3-phosphocholine with linoleic anhydride. The substrate was shown to be unlabeled in the 2-acyl moiety as judged by snake venom hydrolysis. Phospholipase A_1 activity was measured by following labeled fatty acid release from the substrate.

1,2-Diacyl-sn-glycerol:CDPcholine cholinephosphotransferase [EC 2.7.8.2] activity was measured according to established procedures (14) as modified in our laboratory (9), using rat liver 1,2-diacyl-sn-glycerol as substrate.

Lysophospholipase [EC 3.1.1.5] activity was measured by following release of labeled glycerophosphocholine from 1-acyl-sn-glycero-3-[methyl-^{14}C]choline using an assay system containing 100 µmol of Tris-HCl buffer (pH 7.2), 50 µg of microsomal protein, and 200 nmol of the labeled substrate (spec. act., 300 dpm/nmol) in a total volume of 400 µl (15). Incubations were carried out at 37°C for 10 min, and the mixture was extracted (16) and water-soluble radioactivity was quantified. The substrate was prepared by Ophiophagus hannah venom catalyzed hydrolysis of [methyl-^{14}C]choline labeled phosphatidylcholine that was isolated from Novikoff hepatoma cells grown in the presence of [methyl-^{14}C]choline (New England Nuclear).

Acyl-CoA:lysolecithin acyltransferase [EC 2.3.1.23] activity was determined by the procedure worked out in our laboratory (17).

β-Glucuronidase [EC 3.2.1.3] activity and acid phosphatase [EC 3.1.3.2] activity were assayed according to established procedures (18).

RESULTS AND DISCUSSION

The effect of DEN-2 virus on baby hamster kidney cells was followed over the first two hours of virus-host cell interaction. We ascertained that the cells retained their viability (99%) throughout the experiment, and that

intracellular potassium content (28.3-32.0 μg K^+/mg protein) remained essentially unchanged (95-106% of controls) over the entire two hour period of DEN-2/BHK-21 infection. After 30 min, 67 ± 7% of the cell population had been infected (7), and the percentage of infected cells reached a maximum (82 ± 6.5%) essentially within one hr (84 ± 8.4% after 2 hrs).

Figure 1 illustrates changes in the specific activities of five microsomal phospholipid enzymes of BHK-21 cells during the first 2 hrs of infection with DEN-2 virus. The microsomal enzymes followed were the phospholipases A_1 and A_2, lysophospholipase, acyl-CoA:lysolecithin acyltransferase and diacylglycerol:CDPcholine cholinephosphotransferase.

As is evident from Fig. 1, exposure of BHK-21 cells to DEN-2 virus immediately triggered activation of phospholipase A_2. In fact, as early as 5 min after initiation of the infection, phospholipase A_2 was stimulated by 50%, and maximum activity was reached within 40 min. Thereafter, phospholipase A_2 activity decreased at an equally rapid pace and returned essentially to control levels at 90 min. In contrast, phospholipase A_1 activity was not affected to any extent (<10%) throughout this initial phase of virus-host cell interaction.

Phospholipase A_2 activation can be expected to favor formation of 1-acyl-sn-glycero-3-phosphocholine and similar lysophospholipids provided that compensatory mechanisms such as lysolecithin reacylation (19) or lysolecithin catabolism (20) are not activated as well. However, as our data show (Fig. 1), neither acyl-CoA:lysolecithin acyltransferase nor lysophospholipase was affected at all during the first two hrs of infection.

On the other hand, DEN-2/BHK-21 cell interaction substantially inhibited 1,2-diacylglycerol:CDPcholine cholinephosphotransferase. The figure shows that cholinephosphotransferase inhibition progressed in phase with phospholipase A_2 stimulation and that the enzyme reached minimum activity (43% of controls) 40 min after initiation of infection. After 90 min, cholinephosphotransferase activity of infected cells again was essentially

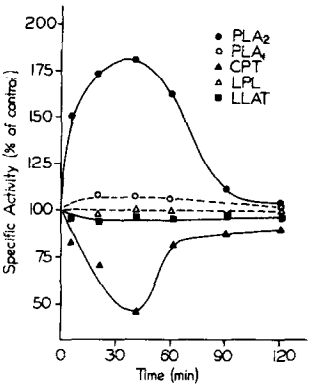

Figure 1. Effect of DEN-2 virus on the activities of several microsomal phospholipid enzymes of BHK-21 cells during initiation of infection. Changes in enzyme activities were followed over a two-hr period and are expressed in percent activity found in infected cells relative to that of uninfected control cells. Specific activities of controls at time zero were: phospholipase A_2 (PLA_2), 46.2 nmol/hr/mg protein; phospholipase A_1 (PLA_1), 49.1 nmol/hr/mg protein; diacylglycerol cholinephosphotransferase (CPT), 25 nmol/hr/mg protein; lysophospholipase (LPL), 28.6 nmol/hr/mg protein; lysolecithin acyltransferase (LLAT), 400 nmol/hr/mg protein. Data given are averages of at least three independent assays.

identical to that of uninfected control cells (Fig. 1). It is of interest in this context that diacylglycerol cholinephosphotransferase is known to be regulated by lysolecithin and that higher lysolecithin levels inhibit the cholinephosphotransferase system (9). Hence, observed inhibition of diacylglycerol cholinephosphotransferase, which coincides with phospholipase A_2 activation, may well reflect enhanced lysolecithin formation as a result of phospholipase A_2 stimulation early in the infection, although a direct virus-triggered cholinephosphotransferase inhibition can presently not be ruled out.

Viral RNA synthesis in the DEN-2/BHK-21 system commences at about 8 hrs after infection (21), and the entire replication cycle has been shown to take 20-24 hrs (6). Hence, stimulation of phospholipase A_2 within minutes after onset of the virus-cell interactions must clearly be attributed to very early events of the infection cycle. This would also agree with our observation that in the present 2-hr experiments consistently high β-glucuronidase (spec. act., 1.4 μmol/hr/mg protein) and acid phosphatase (spec. act., 2.3 μmol/hr/mg protein) activities were measured in the lysosomal fraction while these

activities were absent in the microsomal fraction. Evidently the structural integrity of the lysosomes was maintained throughout our experiments, and lysosomal phospholipase leakage (22) did not occur.

In summary, we have shown that phospholipase A_2 activity in BHK-21 cells is immediately stimulated after exposure to DEN-2 virus and that phospholipase A_2 activity returns to normal levels hours before virus replication takes place. Hence, virus-triggered phospholipase A_2 stimulation could be a controlling event in virus adsorption and/or virus penetration. Such a mechanism of initial virus-cell interaction may relate to the observation that certain viruses can cause structural (23-26) and dynamic (27,28) cell membrane changes similar to those brought about by lysolecithin (29-34).

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