Inhibition of Agonist-induced Activation of Phospholipase C following Poxvirus Infection*

(Received for publication, February 24, 1992)

Kerry G. Oliver§§, R. Mark L. Buller¶, Philip J. Hughes¶¶, James W. Putney, Jr.‡, and Gregory J. Palumbo¶¶

From the §Laboratory of Cellular and Molecular Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709 and the ¶Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Recent studies indicate that viruses may influence polyphosphoinositide levels. This study has examined the effects of vaccinia virus infection on phospholipase C activity. Infection of BS-C-1 cells, an African Green Monkey kidney cell line, or A431 cells, a human carcinoma cell line, with vaccinia virus inhibits receptor-mediated phospholipase C activation. As a consequence, agonist-mediated Ca²⁺ mobilization in BS-C-1 cells also was inhibited by vaccinia virus infection. Alleviation of the inhibition of phospholipase C activation was observed in vaccinia virus-infected cells treated with cycloheximide without influencing uninfected cells. Treatment of infected cells with α-amanitin, an inhibitor of host mRNA synthesis but not virus mRNA synthesis, failed to alleviate the inhibition of phospholipase C activation. Together these results suggest that a virus-encoded gene product mediates the inhibition of phospholipase C activation without the need of a virus-induced host factor.

Analysis of the processes involved in the formation of inositol (1,4,5)-trisphosphate and mobilization of intracellular Ca²⁺ indicate that the vaccinia virus gene product exerts its inhibitory effects at the level of phospholipase C activity. This may occur by either directly reducing the amount of phospholipase C, reducing the specific activity of phospholipase C, or by inhibiting the association of phospholipase C with its substrate, phosphatidylinositol 4,5-bisphosphate.

Poxviruses are large complex double-stranded DNA viruses that replicate in the cytoplasm of the infected cell in a highly regulated manner (1). Following virus entry into a cell and uncoating of the virion, virus enzymes packaged in the virion initiate early transcription which results in expression of virus-encoded proteins and enzymes and initiates a progression through the virus replication cycle (2). It has been estimated that approximately 75% of the poxvirus genome is required for the production of virus progeny, with the remaining 25% of the genome nonessential for virus replication in tissue culture cells (3). Although the functions of many of the nonessential genes are unknown, they may determine pathogenicity and be vital for virus replication in vivo.

Within several hours post-infection, poxvirus-encoded gene products initiate extreme changes in the physiology of the host cell which include inhibition of host protein (4), RNA (5), and DNA synthesis (6-8) as well as radical alterations in the internal architecture of the cell as virus gene products accumulate and form a viroplasm or virus factory (2). The effect these drastic changes have on the ability of infected cells to respond to external stimuli such as hormones or growth factors is unknown. The association of some growth factors or hormones with their specific receptors can induce protein, RNA, and DNA synthesis, and thus the ability of poxvirus gene products to alter these signaling events may be beneficial to poxvirus replication. One such signaling network that can induce protein, RNA, and DNA synthesis occurs through the activation of phospholipase C (PLC) (9). Phospholipase C cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into two second messengers, inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol, which initiate a cascade of events including increases in intracellular free Ca²⁺ and activation of protein kinase C, respectively (10). Alternatively, PLC activation has been associated with the secretion of inflammatory mediators (11, 12), and thus the ability of poxviruses to inhibit PLC activity could be beneficial to poxvirus replication and spread.

Indications that poxvirus infection and replication may affect the inositol phosphate signaling pathway have arisen from the discovery of two open reading frames in poxviruses with homology to mammalian gene products that either up-regulate or down-regulate PLC activity. Vaccinia growth factor (VGF) (13-15), a homologue of epidermal growth factor (EGF), acts as a functional ligand for the EGF receptor (16-18). Since the association of EGF with its receptor stimulates PLC (19), it is likely that VGF also stimulates PLC. In addition, poxviruses recently have been shown to encode a homologue of the mammalian actin binding protein, profilin (20, 21). Using artificial lipid vesicles, mammalian profilin has been demonstrated to inhibit the association of PLC with its substrate, PIP2 (22, 23). Thus, these products suggest that

1 The abbreviations used are: PLC, phospholipase C; inositol phosphates are abbreviated according to the "Chilton Convention" (83) as, for example, (1,4,5)IP3 for D-myo-inositol (1,4,5)-trisphosphate; VGF, vaccinia growth factor; EGF, epidermal growth factor; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; GPIP, glycerophosphatidylinositol 4-phosphate; GPIP2, glycerophosphatidylinositol 4,5-bisphosphate; DMEM, Dulbecco's modified Eagles medium; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
vaccinia virus infection can alter host cell responses to extracellular signals. Therefore, this study has examined the effects of poxvirus infection on PLC activity. To distinguish the effects of VGF from other potential regulators of PLC, a mutant virus, vSC20 (24), that lacks a functional VGF gene was used to infect the African Green Monkey kidney cell line BS-C-1. A comparison of PLC activity in infected and uninfected cells should indicate whether poxvirus encoded gene products can regulate basal or agonist-stimulated PLC activity.

**EXPERIMENTAL PROCEDURES**

Chemicals—Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, Puck's saline A, penicillin/streptomycin, and trypsin/EDTA were purchased from GIBCO. myo-[3H]inositol was purchased from American Radiolabeled Chemicals, St. Louis, MO. Cycloheximide, ATP, phosphocreatine, creatine phosphokinase, and 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma. ATP for receptor stimulation was purchased from Boehringer Mannheim. Receptor-grade EGF was purchased from Collaborative Research Inc. (Bedford, MA) and bradykinin from Peninsula Laboratories (Belmont, CA). Fura-2/AM was purchased from Molecular Probes (Eugene, OR). Thapsigargin was purchased from LC Services, Woburn, MA. α-Amanitin was purchased from Fluka Chemical Corp., Ronkonkoma, NY. Adsorbosphere SAX HPLC columns were purchased from Alttech Associates, Deerfield, IL.

Cell Culture—BS-C-1 cells were grown in DMEM containing high glucose (4.5 g/liter), 10% FBS, L-glutamine (2 mM), penicillin-streptomycin (500 units/ml and 500 μg/ml), and 1 mM HEPES buffer. BS-C-1 cells were labeled with [3H]myoinositol for 3 days prior to the direct addition of poxvirus (50 plaque-forming units/ml) into the medium. The effective virus input was 5–15 plaque-forming units/ml during the 18-h infection period. Cell viability was measured by MTT (25). A543 human epidermoid carcinoma cells were generously provided by Dr. Graham Carpenter, Vanderbilt University and were cultured as described previously (26). A431 cells were labeled as described above for BS-C-1 cells; however, for the final 18 h the medium was aspirated, and DMEM in the absence of FBS with myo-[3H]inositol (50 μCi/ml) was added.

[3H]Insitol Phosphate Measurements—Following culture of BS-C-1 cells with [3H]myoinositol for 3 days, the cells were infected with virus for 18 h, unless otherwise specified, in the continued presence of myo-[3H]inositol (50 μCi/ml). After infection the medium was aspirated, washed with fresh medium (containing 1% FBS in the case of BS-C-1 cells) or no FBS for A431 cells either with or without agonist was added to the cells for various times. Cells were quenched by the rapid aspiration of the medium followed by the addition of 6% perchloric acid to 250 μg/sample of phytic acid. The plates were incubated at 4 °C for 20 min after which time the supernatants were removed and neutralized by the free/triethylamine method of Downes et al. (27) as modified by Shears et al. (28). The [3H]inositol phosphates were eluted from an anion exchange HPLC column (Adsorbosphere SAX, 5-μm particle size) using a linear ammonium phosphate gradient (0–1.0 M, pH 3.35) and radioactivity was measured on-line as described previously (29, 30).

[3H]Insitol Lipid Measurements—Following removal of the acidic supernatants from the tissue culture plates, the remaining precipitates were scraped from the plates and the total [3H]inositol lipids were extracted according to the method of Schacht (31). Briefly, the lipids were extracted with chloroform/methanol/2.4 N HCl (2:5:2.3), and the chloroform fraction was counted by liquid scintillation spectroscopy. For analysis of individual [3H]inositol lipids, the lipids within the chloroform fraction were dried down under a stream of N2. Dried lipid samples were deacylated by the addition of monomethylamine reagent according to the method of Clarke and Dawson (32). Decacylated samples were analyzed by HPLC for the presence of glycerophosphoinositol (GPI), glycerophosphoinositol 4-phosphate (GPIP), and glycerophosphoinositol 4,5-bisphosphate (GPiP2) which are the deacylated products of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and PIP2, respectively (33).

Metabolism of Insitol Phosphates by BS-C-1 Cell Homogenates—[3H]Insitol was added to BS-C-1 cell homogenates (250 μg cellular protein/ml) and incubated at 37 °C in an intracellular-like medium with the following composition (mm): NaCl, 20.0; KCl, 100; MgSO4, 2.0; HEPES, 20.0; pH 7.2; EGTA, 1.0; and total Ca2+ to result in 150 nM free Ca2+. When indicated an ATP regenerating system with the following composition was added: 5 mM MgATP, 10 mM phosphocreatine, 0.1 mg/ml creatine phosphokinase. BS-C-1 cell homogenates were prepared by Dounce homogenization (10 strokes in the presence of 200 μg/ml saponin). Metabolism of [3H]Insitol phosphates was monitored by elution from an Adsorbosphere SAX column and on-line monitoring of the inositol phosphates (see above).

Determination of Intracellular Ca2+ in BS-C-1 Cells—BS-C-1 cells were grown on glass coverslips for 1–2 days. For Ca2+ measurements the coverslips were placed in a Teflon chamber, and the cells were incubated with 0.5 μM Fura-2/AM in DMEM + 10% FBS at 37 °C for 1 h. The cells were washed twice with phosphate-free nominally Ca2+-free HEPES-buffered Ringer's solution. Fluorescence emission of two to four cells was monitored at 515 nm following excitation at 350 and 380 nm with a photomultiplier-based spectrofluorimeter (Photon Technology International, Inc., Princeton, NJ) connected to a Nikon Diaphot microscope as described previously (28). Calcium ratios (350/380) were corrected for cell autofluorescence, determined by incubation with 10 μM ionomycin plus 4 mM MgCl2.

**RESULTS**

Inhibition of ATP-induced Activation of Phospholipase C by Vaccinia Virus Infection—Treatment of BS-C-1 cells with maximally effective concentrations of ATP (500 μM) induced a rapid increase in (1,4,5)IP3 and its immediate metabolites (Fig. 1), indicating the presence of functional P2 purinergic receptors. Infection of BS-C-1 cells with the VGF minus virus, vSC20, had no detectable effect on basal levels of (1,4,5)IP3 from 3–24 h post-infection (compare Fig. 1, A with B). This indicates that in the absence of VGF no vaccinia virus-derived gene products apparently activate PLC. In contrast, vSC20 infection dramatically inhibited the ATP-induced increase in (1,4,5)IP3 (compare Fig. 1, C with D). This inhibition was detectable as early as 9 h post-infection and was not a result of virus-induced decreases in cell viability through 24 h post-infection. In addition, the generation of two metabolic products of (1,4,5)IP3, (1,3,4,5)IP4, and (1,3,4)IP3, also were inhibited, suggesting that poxvirus infection decreased the ability of BS-C-1 cells to produce (1,4,5)IP3 rather than increased the metabolism of (1,4,5)IP3. Similar results were obtained following infection of BS-C-1 cells with other orthopoxviruses, including wild-type WR strain of vaccinia virus that possesses a functional VGF (16–18) and cowpox virus (strain

G. Palumbo, unpublished results.
PLC Activity in Poxvirus-infected Cells

Effect of vSC20 Infection on the P2 Purinergic Receptor—One potential mechanism for the inhibition of phospholipase C activation by poxvirus infection would be through an alteration in the affinity or expression of the agonist receptor. Generation of an ATP concentration response curve indicated that viral infection reduced the maximal response to ATP but did not substantially alter the apparent affinity of the receptor for ATP (Fig. 2A; average from three experiments $K_a = 10$ μM). In addition, circumvention of agonist-receptor interaction by direct activation of the G protein associated with PLC with aluminum fluoride (AlF$_3$) (35, 36) resulted in responses which also were inhibited in vSC20-infected cells (Fig. 2B). These results indicate that the major inhibiting action of poxvirus infection occurs at a site subsequent to receptor activation.

Analysis of Phospholipase C Activation in A431 Cells—Since direct G protein activation of phospholipase C by AlF$_3$ treatment was inhibited by vaccinia virus, it was conceivable that the mechanism of inhibition could be through either a reduction in the level of the G protein expressed or an inhibition of the association of the G protein with PLC. To address these possibilities, the effects of virus infection on PLC-γ activation, a G protein-independent isozyme of phospholipase C (37, 38), was compared with the effects of virus infection on PLC-7 activation, a G protein-independent tyrosine phosphorylation-dependent isozyme of phospholipase C (39, 40), but see (41). By utilizing A431 cells, which possess both a G protein-dependent receptor (bradykinin, 100 nM, (42)) and a G protein-independent receptor (EGF, 30 nM, (41)), activation of both PLC enzymes were examined in the same cell. Since EGF stimulates only minor changes in (1,4,5)IP$_3$ (26), the percent maximal formation of (1,3,4,5)IP$_3$ in response to EGF in infected or uninfected A431 cells was determined. Vaccinia virus infection inhibited both the G protein-dependent activation of PLC by bradykinin as well as the G protein-independent activation of PLC by EGF (Fig. 3). These data demonstrate that vaccinia virus infection can inhibit PLC activation in different cell lines as well as inhibit the activity of different isozymes of PLC whose activation occurs either through a G protein-dependent or G protein-independent mechanism. Therefore, if the mechanism of inhibition of the different isozymes of PLC is the same in the two cell lines, then these data suggest that vaccinia virus infection inhibits PLC activation subsequent to G protein association with PLC.

Kinetics of (1,4,5)IP$_3$ Metabolism by BS-C-1 Cells—Since the apparent inhibition of PLC activation occurred subsequent to receptor activation and G protein association with PLC, it was necessary to determine whether the decrease in (1,4,5)IP$_3$ levels was actually due to an altered metabolism of (1,4,5)IP$_3$. To directly address this possibility, the kinetics of exogenous [3H](1,4,5)IP$_3$ metabolism by infected or uninfected BS-C-1 cell homogenates was examined. To ensure that both pathways of (1,4,5)IP$_3$ metabolism (dephosphorylation to (1,4)IP$_2$ and phosphorylation to (1,3,4,5)IP$_3$) were examined, [3H](1,4,5)IP$_3$ was incubated with homogenates in the presence or absence of an ATP regenerating system. [3H]inositol phosphates were separated by HPLC. The percent catabolism of [3H](1,4,5)IP$_3$ is shown as the mean ± S.D. of duplicate determinations from one experiment of two.

Analysis of Inositol Lipids in BS-C-1 Cells—An additional mechanism for virus-mediated inhibition of (1,4,5)IP$_3$ formation could be through a reduction in availability of the PIP$_2$ substrate for PLC due to an overall decrease in total inositol lipids, a change in the ratio of the individual inositol lipids (PI to PIP to PIP$_2$), or a decrease in the accessibility of PLC to PIP$_2$. Following infection, there was...
The addition of ATP (20 μM) to uninfected cells resulted in a rapid and transient increase in [Ca²⁺], attributable to release of Ca²⁺ from internal stores (Fig. 6). Treatment of agonist-stimulated uninfected cells with thapsigargin (TG, 2 μM), which empties the microsomal Ca²⁺ stores by inhibiting the microsomal Ca²⁺-ATPase, resulted in the further release of only a small amount of Ca²⁺. Stimulation of vaccinia virus-infected cells with ATP resulted in a diminished Ca²⁺ response, and the subsequent addition of thapsigargin resulted in a much larger release of Ca²⁺ as compared with uninfected cells (Fig. 6). This indicates that agonist stimulation of vaccinia virus-infected cells only partially mobilizes the (1,4,5)IP₃-sensitive Ca²⁺ pool. These results demonstrate that vaccinia virus infection results in a reduced physiological Ca²⁺ response to agonist, consistent with the data demonstrating that vaccinia virus infection decreased agonist-induced production of (1,4,5)IP₃.

Effect of Metabolic Inhibitors on the Generation of (1,4,5)IP₃ —To rule out the possibility that vaccinia virus-induced inhibition of the generation of second messengers was simply the result of the inhibition of host protein synthesis by virus (4), infected and noninfected cultures of BS-C-1 cells were treated with the protein synthesis inhibitor cycloheximide (100 ng/ml) 1 h prior to, and throughout, infection with vSC20. The inclusion of cycloheximide in noninfected cultures did not inhibit the ATP-induced generation of (1,4,5)IP₃ (Fig. 7) or its metabolites (data not shown). This suggests that inhibition of protein synthesis cannot account for the inhibition of (1,4,5)IP₃ generation observed in vSC20-infected cells. In contrast, cycloheximide treatment ablated the ability of vSC20 to inhibit agonist-induced increases in (1,4,5)IP₃ (Fig. 7). These data are consistent with a vaccinia virus-encoded gene product which either directly mediated or induced a host factor which mediated the inhibition of the (1,4,5)IP₃ generation.

To differentiate between these two possibilities BS-C-1 cells were treated with a-amanitin, a selective inhibitor of host RNA polymerase II but not the poxvirus encoded polypeptide (47). A reversal of the virus-mediated inhibition of (1,4,5)IP₃ generation would be consistent with a host-derived physiological interference with the production of a host-derived gene product.
FIG. 7. Effect of cycloheximide (CX), α-amanitin, and virus infection on agonist-stimulated increases in (1,4,5)IP$_3$. Uninfected (closed bars) or vSC20-infected (hatched bars) BS-C-1 cells were cultured in the presence or absence of either the protein synthesis inhibitor, cycloheximide (100 μg/ml; −1 to 18 h), or the RNA polymerase II inhibitor, α-amanitin (4 μg/ml; −1 to 18 h), and were stimulated with ATP (500 μM; 10 s). The stimulated increase in (1,4,5)IP$_3$ is presented as a mean and standard deviation of triplicate determinations and was calculated by subtracting the basal level of (1,4,5)IP$_3$ from the ATP-stimulated level of (1,4,5)IP$_3$; two other experiments gave similar results. * significantly different from uninfected; p < 0.05 (Student’s t test).

**DISCUSSION**

This is the first report that infection by a poxvirus can alter inositol phosphate metabolism in cell culture. Evidence presented in this study is consistent with a virus-encoded mediator being responsible for the observed inhibition in agonist-induced increases in (1,4,5)IP$_3$ formation which subsequently reduces mobilization of intracellular Ca$^{2+}$ (Figs. 1 and 6). Treatment of poxvirus-infected cells with cycloheximide, an inhibitor of protein synthesis, reversed the virus-induced inhibition of inositol phosphate formation (Fig. 7). In contrast, treatment of uninfected BS-C-1 cells with cycloheximide failed to affect agonist-induced increases in (1,4,5)IP$_3$, which suggests that a general virus-induced inhibition of host protein synthesis was not the mechanism of inhibition (Fig. 7). This latter result suggests that expression of a poxvirus gene is required to obtain inhibition of ligand-induced inositol phosphate formation. In addition, the failure of α-amanitin to reverse the inhibition in poxvirus-infected cells suggests that an induction of the synthesis of a host-derived gene product is not required.

Stepwise analysis of the components involved in the inositol phosphate signaling pathway indicated that the inhibition in formation of (1,4,5)IP$_3$ in vaccinia virus-infected BS-C-1 cells occurred at a step subsequent to receptor activation (Fig. 2) or G protein association with phospholipase C (Fig. 3). Following poxvirus infection the metabolism of (1,4,5)IP$_3$ to either (1,4,5)IP$_2$ or (1,3,4,5)IP$_4$ (Figs. 4 and 5) also was unaffected. Analysis of the inositol lipids indicated that poxvirus infection had no inhibitory effect on either the total inositol lipid levels or the relative amounts of the individual inositol lipids PI, PIP, or PIP$_2$ (Table I). In addition, vaccinia virus infection inhibited the physiological mobilization of Ca$^{2+}$ mediated by (1,4,5)IP$_3$ (Fig. 6). Together, these results indicate that the mechanism of inhibition of (1,4,5)IP$_3$ formation in vaccinia virus-infected cells resides at the level of phospholipase C activity by either directly reducing the amount of phospholipase C, reducing the specific activity of phospholipase C, or by inhibiting the association of phospholipase C with its substrate phosphatidylinositol 4,5-bisphosphate.

One proposed mechanism by which the metabolism of PIP$_2$ by phospholipase C could be inhibited is through competitive binding of PIP$_2$ by one of the actin binding proteins such as profilin (22, 23). Thus, an increase in the amount of profilin within a cell would inhibit the ability of PLC to cleave PIP$_2$. The Copenhagen and WR strains of vaccinia virus both encode a gene with strong homology to mammalian profilin (20, 21). However, infection with a profilin homologue deletion mutant (21) (kindly provided by Drs. B. Moss and R. Blasco, Laboratory of Viral Diseases) resulted in comparable inhibition of PLC activity as was observed with vSC20 or WR vaccinia viruses.$^6$ This result does not rule out the possibility that the virus profilin homologue may play an important regulatory role in the activation of another form of PLC, but it does indicate that an additional vaccinia virus-encoded gene product inhibits PLC activity in the experimental protocol of this study.

In contrast to poxvirus-induced inhibition of inositol phosphate formation, several viruses have been found to enhance inositol phosphate levels either in the presence or absence of an exogenous agonist (48-51). Only one virus gene product, the middle T antigen of polyoma virus, has been identified and linked to changes in levels of polyphosphoinositides. In a previous manner to growth factor-induced signaling, the middle T antigen of polyoma virus associates with and activates a PI 3-kinase which produces a minor population of inositol lipids phosphorylated at the D-3 position of the inositol ring (51). In combination, these studies suggest that some viruses likely trigger activation or modification of host cell signaling pathways and thus possibly affect subsequent processes controlled by these pathways.

There are probably several genes encoded by poxviruses which are capable of influencing host cell pathways involved in the generation of second messengers. Evidence presented in this study suggests poxviruses encode at least one molecule which is capable of inhibiting PLC activity, although the identity of this virus-encoded gene product is presently unknown. Several systems have demonstrated that PLC stimulation can induce the secretion of inflammatory mediators (11, 12), and therefore, poxvirus inhibition of PLC activity may limit inflammation at the site of infection. Since an inflammatory response can limit virus infection (52), its down-regulation during infection may enhance virus survival in the host. In addition, it is likely that VGF will increase PLC activity, although it is not known if this can occur in infected cells and/or neighboring cells which could serve as targets for virus replication. Interestingly, the deletion of VGF from the parental WR strain of vaccinia virus decreases its virulence by approximately 5 orders of magnitude in the mouse intracranial LD$_{50}$ assay (24), which suggests that virus replication in vivo is enhanced by production of VGF. Studies are presently underway to identify poxvirus genes responsible for regulation of PLC so that the importance and function of these molecules in virus replication can be determined.

Acknowledgments—We thank Drs. A. J. Morris, V. M. Sanders, and P. Murphy for critical review of the manuscript; Drs. R. Blasco and B. Moss for their profilin minus mutant of vaccinia virus (strain WR); and B. R. Marshall for excellent editorial assistance.

**REFERENCES**

1. Moss, B. (1990) *Annu. Rev. Biochem.* 59, 681-698
2. Buller, R. M., and Palumbo, G. J. (1991) *Microbiol. Rev.* 55, 80-122
3. Perkus, M. E., Goebel, S. J., Davis, S. W., Johnson, G. P., Norton, E. K., and Paolotti, E. (1991) *Virology* 180, 406-410
4. K. Oliver, unpublished results.
