0026-895X/92/030537-08$03.00/0

MOLECULAR PHARMACOLOGY, 42:537-544

Cell-Protecting Effect against Herpes Simplex Virus-1 and Cellular Metabolism of 9-(2-Phosphonylmethoxyethyl)adenine in HeLa S3 Cells

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Received July 31, 1991; Accepted May 27, 1992

SUMMARY

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) is a selective and potent inhibitor of retrovirus and herpesvirus replication *in vitro* and *in vivo*. In cell culture studies, pretreatment of HeLa S3 cells with PMEA before infection enhanced its antiviral potency by almost 10-fold, compared with treatment of the cells only after viral infection. To elucidate the basis for this observation, the uptake, metabolism, and retention of PMEA metabolites were examined in uninfected and herpes simplex virus type 1-infected cells, by using [2,8-^{3}H]PMEA. Uptake of the drug into both acid-soluble and acid-insoluble fractions was slow and did not begin to plateau until close to 24 hr. High performance liquid chromatographic analysis of acid-soluble extracts revealed at least four metabolites in addition to PMEA itself, designated as X, Y, DP, and TP. Metabolites X and Y, which were distinct from PMEA and its mono- and diphosphoryl derivatives, represented almost 90% of the radioactivity associated with the cells after 24 hr of incubation. Dephosphorylation of acid-soluble metabolites resulted in accumulation of radioactivity in the peaks associated with PMEA and X. Most of the radioactivity in the acid-insoluble fraction was associated with DNA. Enzymatic digestion of [^{3}H]PMEA-labeled DNA from either infected or uninfected cells yielded both metabolite X and PMEA itself. The role of newly discovered PMEA metabolites in its antiviral activity and cytotoxicity is not clear.

Among the phosphonylmethoxyalkyl derivatives of purine and pyrimidine bases that have been recently synthesized, several exhibit considerable antiviral activity against DNA viruses and/or retroviruses (1). Fig. 1 shows the structure of one of these compounds, PMEA. PMEA is active against several herpesviruses, including HSV-1, HSV-2, and Epstein-Barr virus (1–3). In addition, PMEA is active against several retroviruses, including HIV, Moloney murine sarcoma virus, and the LP-BM5 retrovirus in a murine acquired immunodeficiency syndrome model (1, 4–6). Therefore, PMEA has potential for use as an antiviral agent against HSV, Epstein-Barr virus, or HIV.

As anti-HSV compounds, PMEA and its congeners that contain an acyclic phosphonylmethoxyalkyl moiety, in place of the phosphorylated sugar, bypass the initial phosphorylation by viral thymidine kinase and are converted into their diphosphate forms by cellular enzymes (7–9). This is in contrast to compounds, such as acyclovir, that require initial phosphorylation by viral thymidine kinase in order to be further phosphorylated to the triphosphate form by cellular enzymes (10–12). The phosphorylation of PMEA to its mono- and diphosphate forms in an L1210 cell crude extract has been reported, but the specific enzyme activities responsible were not identified (8). It has also been proposed that 5-phosphoribosyl-1-pyrophosphate synthetase plays an important role in the metabolism of PMEA, by direct conversion of PMEA to PMEApp (9).

The mechanism of the anti-HSV-1 action of PMEA involves inhibition of the viral DNA polymerase, which results in an inhibition of viral DNA synthesis (13). The diphosphate of PMEA is an inhibitor of HSV DNA polymerase and can be incorporated into DNA by the enzyme *in vitro* (8, 13).

Because PMEA has potential for use as an antiviral agent and is representative of a new class of nucleotide analogs, the cellular metabolism of PMEA is of great interest. A metabolic study of PMEA in a T lymphoblastic cell line, with emphasis on its implications for anti-HIV activity, was recently published (9). In that study, which used the T lymphoblastic cell line MT-4, the only metabolites of PMEA detected were PMEAp and PMEApp, in addition to PMEA itself.

This work was supported by National Cancer Institute Grant CA-44358.

ABBREVIATIONS: PMEA, 9-(2-phosphonylmethoxyethyl)adenine; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; HIV, human immunodeficiency virus; acyclovir, 9-(2-hydroxyethoxymethyl)guanine; DHPG, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine; HPLC, high performance liquid chromatography; PMEAp, 9-(2-phosphonylmethoxyethyl)adenine monophosphate; PMEApp, 9-(2-phosphonylmethoxyethyl)adenine diphosphate.

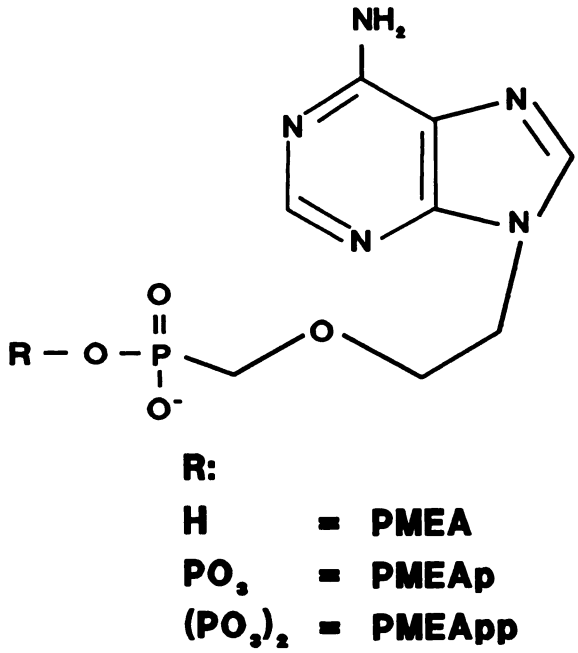

Fig. 1. Structure of PMEA and phosphorylated derivatives.

TABLE 1

Effect on anti-HSV-1 activity of the time of exposure of HeLa S3 cells to various concentrations of PMEA and comparison with ACG and DHPG

Schedule of drug exposure[a]		Virus yield		
Before infection	After infection	10 μM	50 μM	100 μM PMEA
hr		*% of control*		
	0 to 24	82	36	11
−24 to −1	0 to 24	12	6	2
−9 to −1	0 to 24	16	8	5
−5 to −1	0 to 24	ND[b]	ND	7
−3 to −1	0 to 24	ND	ND	10
−24 to −1		86	56	38
−9 to −1		ND	ND	50
−5 to −1		ND	ND	64
−3 to −1		ND	ND	57
−1 to 0		98	103	102
	0 to 24	98[c]	99[c]	96[c]
−24 to −1	0 to 24	85[c]	69[c]	44[c]
Schedule of drug exposure		Virus yield		
Before infection	After infection	1.5 μM	7.5 μM	15 μM ACG[d]
hr		*% of control*		
	0 to 24	87	38	9
−24 to −1	0 to 24	85	40	8
Schedule of drug exposure		Virus yield		
Before infection	After infection	1 μM	5 μM	10 μM DHPG
hr		*% of control*		
	0 to 24	75	6	1
−24 to −1	0 to 24	46	4	2

[a] Time 0 is time of infection.
[b] ND, not determined.
[c] PMEA^r −1; others are strain KOS.
[d] ACG, acyclovir.

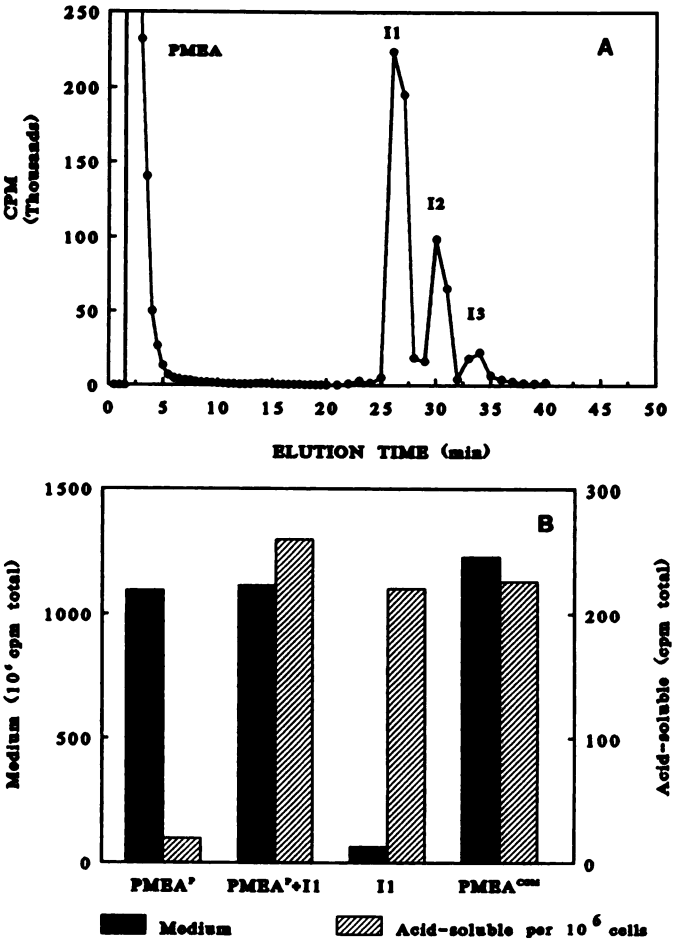

Fig. 2. Comparison of uptake of commercial and purified [^{3}H]PMEA. A, Purification scheme for commercial [^{3}H]PMEA, by using system 3. Fractions (0.5 ml) containing PMEA and I1 were taken, concentrated under vacuum, dissolved in 100 μl of water, and used immediately in the uptake study. B, HeLa S3 cells were treated with 10 μM unlabeled PMEA containing the following: purified [^{3}H]PMEA (90 mCi/mmol) (*PMEA^P*), a mixture of purified [^{3}H]PMEA (90 mCi/mmol) and I1 (2.5 mCi/mmol of PMEA) (*PMEA^P + I1*), I1 (2.5 mCi/mmol of PMEA) (*I1*), or commercial [^{3}H]PMEA (100 mCi/mmol) (*PMEA^{COM}*). Cells were harvested by scraping after 2 hr, and acid-soluble radioactivity was determined as described in Materials and Methods.

In this study, we focused on elucidating the relationships between the anti-HSV-1 effect of PMEA and the presence of its metabolites within the cells. Cell culture studies revealed that preincubation of uninfected cells with PMEA before viral infection dramatically enhanced its anti-HSV activity. Thus, the uptake, metabolism, and retention of PMEA metabolites were examined in both uninfected and HSV-1-infected HeLa S3 cells, by using [2,8-^{3}H]PMEA, to explain this observation.

Materials and Methods

Cells and viruses. HeLa S3, Vero, and MOLT-4F cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum and 100 μg/ml kanamycin, in a 37° incubator with 5% CO_2. Cells were tested periodically for *Mycoplasma* contamination by using a radioactive *Mycoplasma* detection system (Gen-Probe, San Diego, CA). The viruses used included HSV-1 strains KOS and PMEA^r-1 (13, 14). Stocks of viruses were prepared by low multiplicity passage in Vero cells, as previously described (15). Unless otherwise stated, viral infec-

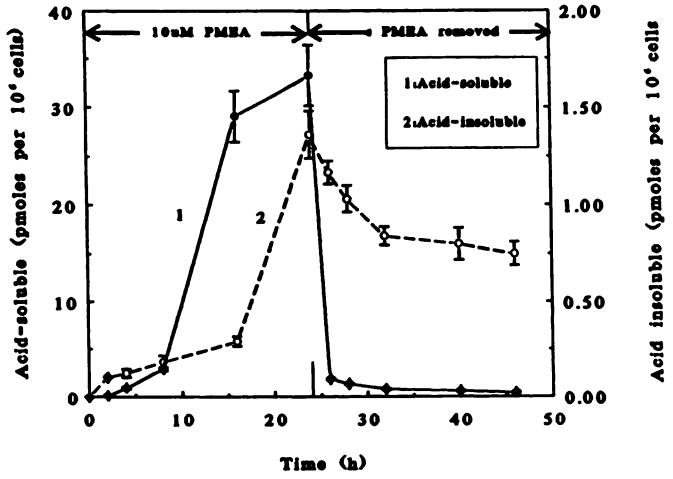

Fig. 3. Time-dependent uptake of [^{3}H]PMEA. HeLa S3 cells (5×10^6) were incubated with 10 μM [^{3}H]PMEA (100 mCi/mmol) and harvested at 2, 4, 8, 16, or 24 hr. At 24 hr, the medium was exchanged for medium without PMEA, and the cells were incubated for an additional 2, 4, 8, 16, or 22 hr. Acid-soluble and acid-insoluble radioactivity was determined as described in Materials and Methods.

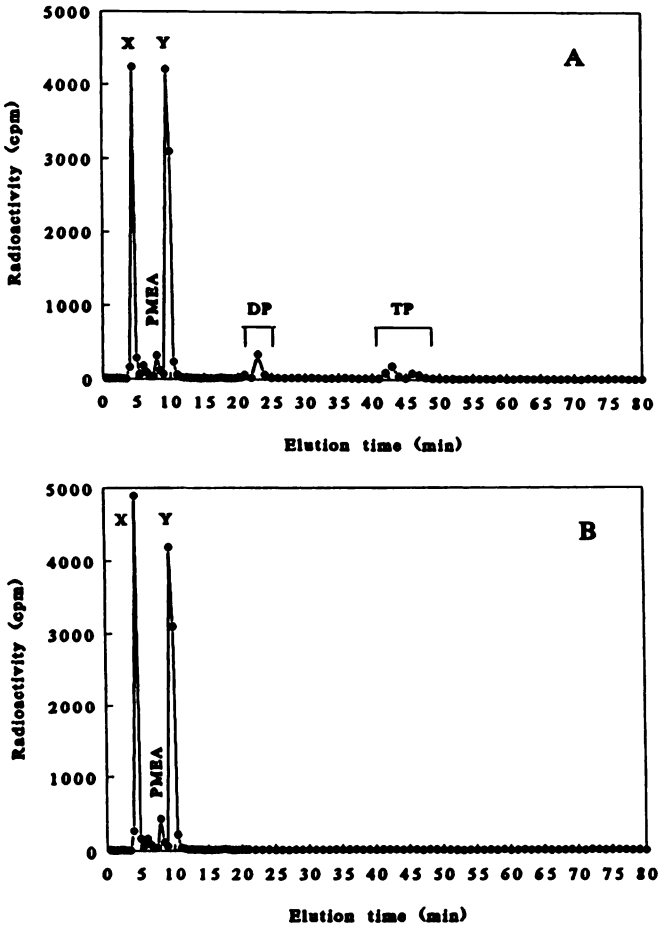

Fig. 4. Characterization of phosphorylated [^{3}H]PMEA metabolites in HeLa S3 cells. A, An aliquot of acid-soluble extract from 5×10^6 HeLa S3 cells treated for 24 hr with 10 μM [^{3}H]PMEA (100 mCi/mmol) was analyzed by anion exchange HPLC, using system 1. B, Identical analysis of an aliquot after treatment with snake venom phosphodiesterase I and alkaline phosphatase.

TABLE 2

Analysis of acid-soluble fractions of HeLa S3 cells

Acid-soluble extracts from cells treated with 10 μM [^{3}H]PMEA (100 mCi/mmol) were analyzed by anion exchange HPLC on a Partisil-10 SAX column. Cells were collected at the indicated times and extracted with 1.5 M perchloric acid.

Time	Amount				
	PMEA	X	Y	DP	TP
hr	*pmol/10⁶ cells*				
2	0.17	*a*	*a*	*a*	*a*
4	0.82	*a*	*a*	*a*	*a*
8	1.85	0.16	0.64	0.18	0.14
16	1.20	1.09	25.7	0.43	0.28
24	0.90	10.7	20.3	0.85	0.40

[a] Below the limit of detection.

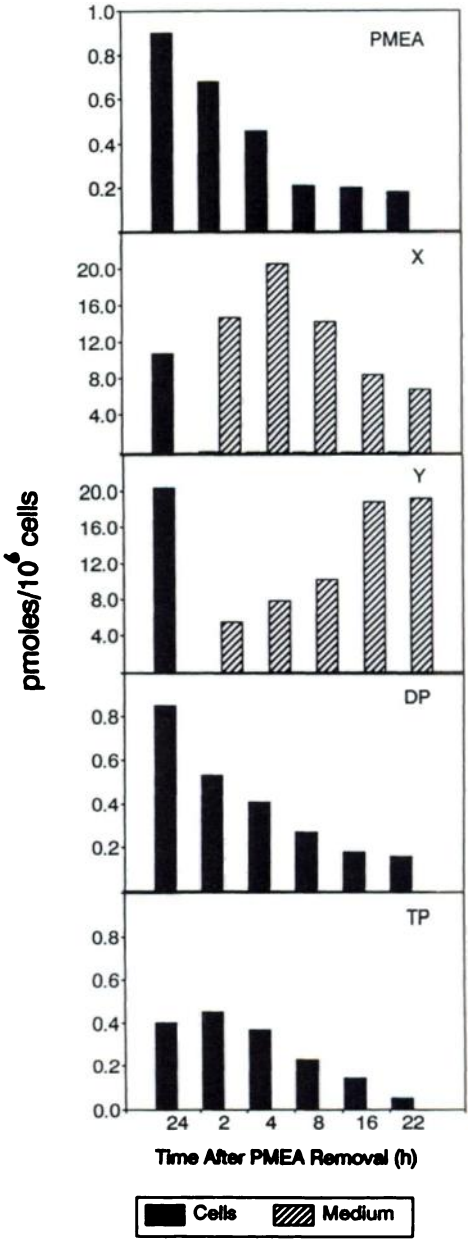

Fig. 5. Retention and efflux of [^{3}H]PMEA metabolites. HeLa S3 cells were treated for 24 hr with 10 μM PMEA (100 mCi/mmol). At various times after drug removal, acid-soluble fractions and corresponding methanol-extracted media were prepared and analyzed by anion exchange HPLC, using system 1, and reverse phase HPLC, using system 4.

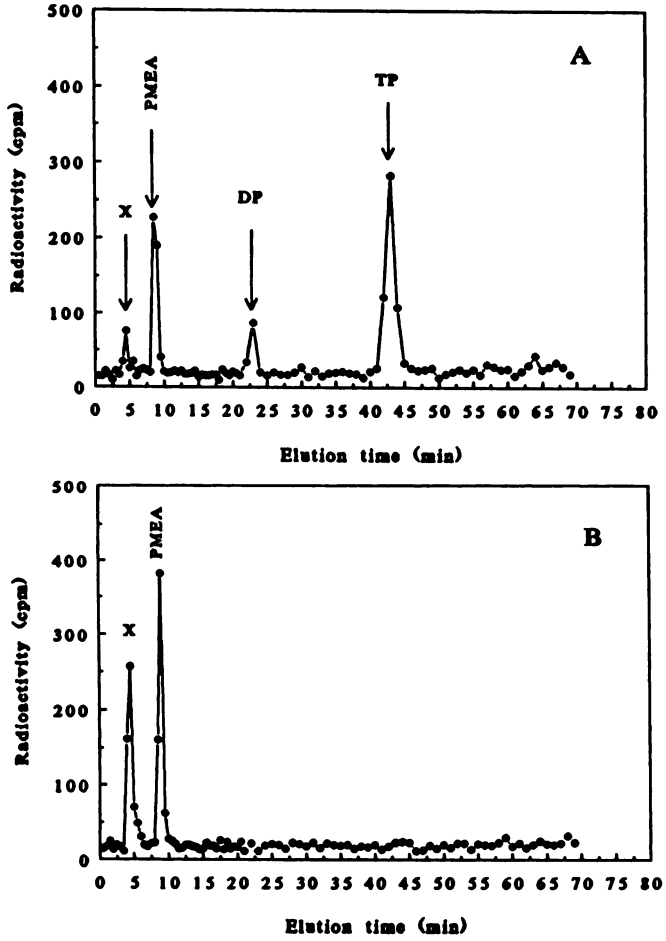

Fig. 6. Characterization of phosphorylated [^{3}H]PMEA metabolites in MOLT-4F cells. A, An aliquot of acid-soluble extract from 5 × 10^6 MOLT-4F cells treated for 24 hr with 10 μM [^{3}H]PMEA (120 mCi/mmol) was analyzed by anion exchange HPLC. B, Identical analysis of an aliquot after treatment with snake venom phosphodiesterase I and alkaline phosphatase.

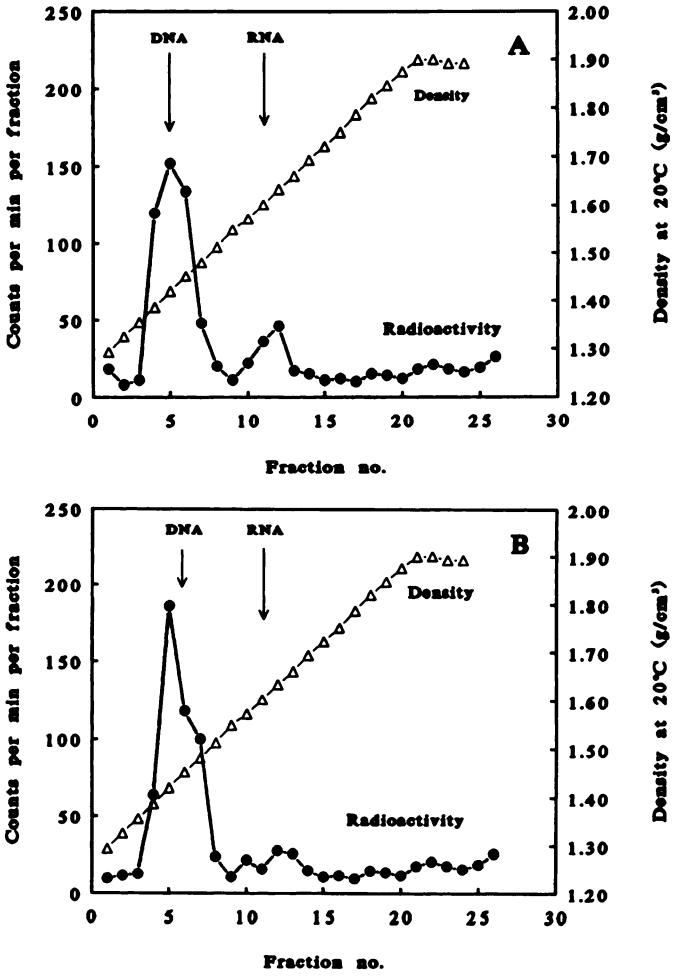

Fig. 7. Cesium sulfate density gradient centrifugation of [^{3}H]PMEA-labeled nucleic acids from mock- and HSV-1-infected HeLa S3 cells. Purification of nucleic acids from 5 × 10^7 mock-infected (A) or HSV-1 infected (B) HeLa S3 cells treated for 20 hr with 0.5 μM [^{3}H]PMEA (5 Ci/mmol) and centrifuged in a $CsSO_4$ gradient was performed as described in Materials and Methods.

tions were performed at 10 plaque-forming units/cell. Virus yield assays were performed essentially as described (16).

Compounds. PMEA, PMEAp, PMEApp, and acyclovir were generously provided by A. Holy and I. Rosenberg from the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences (Prague, Czechoslovakia). DHPG was a gift from Syntex, Inc., (Palo Alto, CA). [2,8-^{3}H]PMEA was purchased from Moravek Biochemicals, Inc., and was further purified by reverse phase HPLC.

Preparation of cell extracts for metabolite analysis. HeLa S3 or MOLT-4F cells were grown in either 25- or 75-cm^2 flasks. Uninfected or HSV-1-infected HeLa S3 or MOLT-4F cells were incubated with various concentrations of [^{3}H]PMEA, in RPMI 1640 medium containing 2% dialyzed fetal bovine serum and 100 μg/ml kanamycin. At the indicated times, the cells were harvested by scraping and washed three times in cold PBS, and the cell pellets were either extracted with 1.5 M perchloric acid at 0° for 30 min and then neutralized (17) or extracted with ice-cold 60% methanol (18). Acid-soluble and methanol-soluble extracts were treated, where indicated, by venom phosphodiesterase I, bacterial alkaline phosphatase, or spleen phosphodiesterase (Sigma) (19).

HPLC analysis. Two types of columns were used; Partisil-10 SAX (250 × 4.6 mm; Whatman) and Lichrosorb RP-18 10-μm (250 × 4.6 mm; Alltech, Deerfield, IL). Four different systems were used, as outlined below.

In system 1, separation was on Partisil-10 SAX, using buffer A of 0.03 M potassium phosphate, pH 6.6, and buffer B of 0.3 M potassium phosphate, pH 6.6. The elution method ran isocratically for 10 min at 1 ml/min, using 100% buffer A. From 10 to 12 min, a linear gradient changed buffers to 50% buffer A/50% buffer B. From 50 to 52 min, the flow rate was increased to 2 ml/min. From 52 to 60 min, a linear gradient changed the solvent to 100% buffer B, which was then run isocratically from 60 to 80 min.

In system 2, separation was on Partisil-10 SAX, using buffer A of 50 mM NaH_2PO_4, pH 5.6, and buffer B of 50 mM NaH_2PO_4, pH 5.6, 1 M $(NH_4)_2SO_4$, 30% methanol. The elution method ran isocratically for 10 min, using buffer A, at 1 ml/min. From 10 to 40 min, a linear gradient changed the mobile phase to 100% buffer B. Buffer B then ran isocratically from 40 to 60 min.

In system 3, separation was on Lichrosorb RP-18. The elution method ran isocratically for 10 min, using water, at 1 ml/min. From 10 to 40 min, a linear gradient changed the solvent to 20% acetonitrile, which was then run isocratically from 40 to 60 min.

In system 4, separation was on Lichrosorb RP-18, using buffer A of 50 mM potassium phosphate, pH 6.6, and buffer B of 50 mM potassium buffer, pH 6.6, 50% methanol. The elution method ran isocratically for 10 min, using buffer A. From 10 to 50 min, a linear gradient changed buffers to 60% buffer A/40% buffer B. From 50 to 70 min, a linear gradient changed buffers to 100% buffer B. Buffer B was then run isocratically, at 100%, from 70 to 80 min.

The peaks were identified in three ways, 1) by retention times, 2) by addition of known standard substances, and, 3) when possible, by

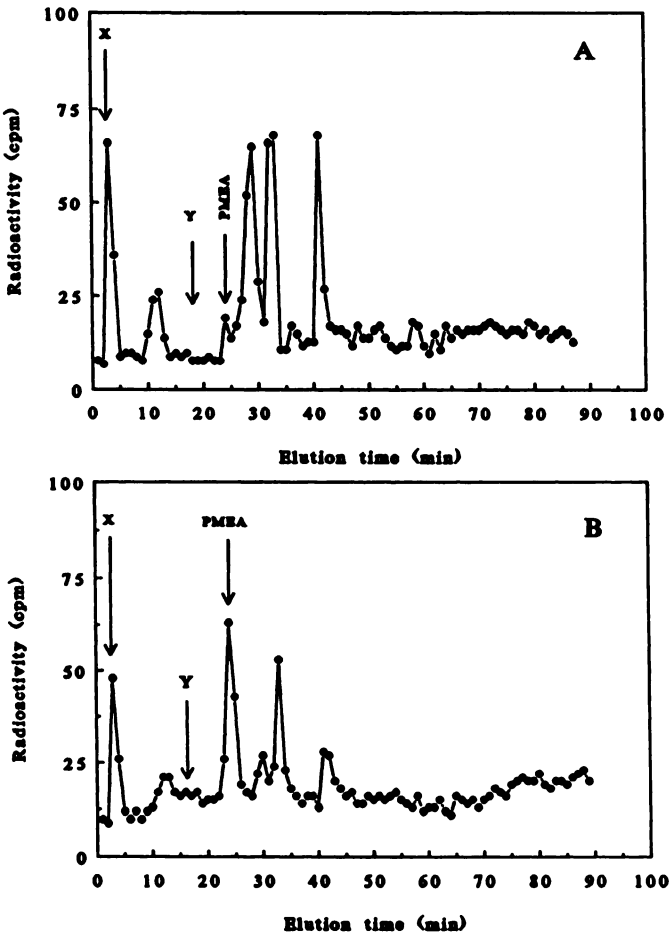

Fig. 8. HPLC analysis of digested [^{3}H]PMEA-labeled DNA from mock- and HSV-1-infected HeLa S3 cells. Purified DNA from mock-infected (A) or HSV-1-infected (B) HeLa S3 cells was treated sequentially with micrococcal nuclease, venom phosphodiesterase I, and alkaline phosphatase, followed by analysis by reverse phase HPLC, using system 4.

comparison of the ratios between absorbance at 254 nm and that at 280 nm.

Estimation of metabolites in culture medium. Media were taken and methanol was added to a final concentration of 60%. After 30 min at 0°, the precipitate was removed by centrifugation and the supernatant was lyophilized, redissolved in water, lyophilized again, and finally redissolved in 50 mM Tris·HCl buffer, pH 7.5. Aliquots were analyzed by HPLC.

Isopycnic centrifugation. Purified nucleic acids from mock-infected or HSV-1-infected HeLa S3 cells incubated with 0.5 μM [^{3}H] PMEA (5 Ci/mmol) for 20 hr were prepared as described (18). The cesium sulfate gradients were centrifuged at 40,000 rpm for 72 hr, in a Beckman SW 50.1 rotor, and were then fractionated from the top (17). The positions of DNA and RNA peaks were determined with purified HeLa S3 DNA and RNA and HSV-1 DNA, in parallel gradients.

Enzymatic digestion of radiolabeled DNA. The $CsSO_4$ gradient peak containing the labeled DNA was collected, dialyzed against 10 mM Tris·HCl, 1 mM EDTA, pH 8, precipitated with ethanol, and digested sequentially with micrococcal nuclease, venom phosphodiesterase, and bacterial alkaline phosphatase. After proteins were removed by precipitation with 1 M perchloric acid, the neutralized supernatants were analyzed by HPLC.

Results

Enhancement of the anti-HSV-1 effect of PMEA by preincubation of cells with the drug. Previous studies indicated that PMEA has a 90% inhibitory dose value of 80 μM for the inhibition of HSV-1 replication in HeLa S3 cells (13). This value was obtained by incubation of the cells with the drug for 24 hr after viral infection. In the present studies, HeLa S3 cells were preincubated with PMEA for various times before the viral infection and the drug treatment was continued for 24 hr after the viral infection. Table 1 shows that preincubation of cells with PMEA before viral infection resulted in a potentiation of its anti-HSV-1 activity, compared with treatment after viral infection alone. The maximal effect, within the time period tested, was observed when PMEA was present for the 23 hr before and the 24 hr after viral infection. Under these conditions, 10 μM PMEA inhibited HSV-1 by almost 90%. In contrast, PMEA had a 90% inhibitory dose value of 96 μM when the drug was present only for the 24 hr after the viral infection, in parallel assays. Because the enhancement of antiviral activity increased with increased preincubation time, these results suggest that uptake and/or conversion of PMEA to its active form were relatively slow or that prolonged exposure to PMEA induced an antiviral response in the cells.

When a PMEA-resistant strain of HSV-1, PMEA^r-1, was tested in the same experiment, the degree of resistance to PMEA was not altered substantially. Because PMEA^r-1 is resistant to PMEA by virtue of an altered HSV DNA polymerase (13), this result suggests that the enhanced antiviral activity of PMEA observed when the cells were preincubated with the drug is still related to the HSV DNA polymerase, rather than an alternate mechanism of PMEA action. Moreover, PMEA at 10 μM had little cytotoxicity to HeLa S3 cells, as estimated by several different assays (data not shown).

For comparison with PMEA, two other anti-HSV drugs, acyclovir and DHPG, that are activated by viral thymidine kinase (10, 16) were tested for enhanced antiviral action under the preincubation conditions. No significant enhancement of antiviral activity of acyclovir or DHPG was observed (Table 1).

Suitability of commercial [^{3}H]PMEA for metabolic studies. In an effort to understand the molecular basis of the enhancement of the antiviral action of PMEA after preincubation of the cells with PMEA, the metabolism of PMEA in HeLa S3 cells was explored. When commercially available [2,8-^{3}H]PMEA (Moravek Biochemicals, Inc.) was examined for this purpose, several impurities were found, representing 1–3% of the total cpm (Fig. 2A). One of these impurities, designated I1, was purified and found to be very metabolically active. This impurity had approximately 90-fold higher uptake than PMEA itself (Fig. 2B) and was rapidly converted into at least two metabolites, with retention times similar to those of PMEAp and PMEApp on anion exchange HPLC. The metabolites of the impurity were incorporated into DNA and RNA (data not shown). Although the identity of this impurity was not determined, adenine, adenosine, and deoxyadenosine were excluded as possibilities, by HPLC (data not shown). Uptake of purified and unpurified PMEA was also tested in MOLT-4F and peripheral blood mononuclear cells. The uptake pattern in each case was similar to that of HeLa S3 (data not shown). Hence, all further experiments were performed with purified [^{3}H] PMEA.

Uptake of [^{3}H]PMEA into acid-soluble and acid-insoluble fractions of HeLa S3 cells. HeLa S3 cells were incubated with 10 μM [^{3}H]PMEA (100 mCi/mmol). Uptake of the radioactivity into acid-soluble and acid-insoluble fractions and

the retention of radioactivity in these fractions were analyzed. Uptake into both fractions is presented in Fig. 3. Upon exchange of the medium for drug-free medium, about 90% of acid-soluble radioactivity was lost from the cells within the first 2 hr, whereas >50% of the acid-insoluble radioactivity was retained after 22 hr of incubation in drug-free medium. When mock-infected and HSV-1-infected cells were compared, no significant difference in uptake of radioactivity was found after 24 hr of incubation with [^{3}H]PMEA (data not shown). Virtually the same results were obtained when methanol extraction was used instead of perchloric acid extraction (data not shown).

HPLC analysis of acid-soluble fractions. When the acid-soluble fractions were analyzed by anion exchange HPLC, at least four major metabolites, in addition to PMEA, were detected in HeLa S3 cells (Fig. 4A). The first two metabolites, designated X and Y, represented >90% of the radioactivity in the acid-soluble fraction at 24 hr of incubation and had retention times different from those of PMEA, PMEAp, and PMEApp. When retention times of these two metabolites were compared with those of possible metabolites generated from PMEA by the action of cellular nucleotidases, deaminases, phosphomonoesterases, or nucleosidases (PMEHx, adenine, adenosine, deoxyadenosine, inosine, deoxyinosine, or hypoxanthine), no close similarities were observed by using anion exchange and reverse phase HPLC (data not shown). The other two metabolites had retention times that were close to those of di- and triphosphate forms of various natural nucleosides on anion exchange HPLC and, thus, were denoted DP and TP. Because more than one peak of radioactivity was found within the regions designated DP and TP, the designations DP and TP each may include more than one species. None of the metabolites was detected in the absence of cells.

Table 2 shows the time course of appearance of the major PMEA metabolites in the acid-soluble fraction of HeLa S3 cells. After 4 hr of incubation with [^{3}H]PMEA, only PMEA itself was detected in the cell extracts. After 8 hr, the PMEA content reached its maximum and both X and Y were detected. After 24 hr, X and Y represented >90% of the radioactivity inside the cells.

After 24 hr, the medium was exchanged for drug-free medium and the retention and efflux of [^{3}H]PMEA metabolites were estimated (Fig. 5). PMEA and the peaks designated DP and TP were retained (half-lives of approximately 4 h and 12 h, respectively) in the cells and were not detected in the culture medium. In contrast, both X and Y were rapidly effluxed from the cells and were present in the culture medium within the first 2 hr of incubation in drug-free medium.

Characterization of phosphorylated [^{3}H]PMEA metabolites in acid-soluble fractions. In order to examine the origin of the radioactivity associated with DP and TP forms of PMEA and its metabolites, the acid-soluble fractions of HeLa S3 cells were subjected to enzymatic dephosphorylation by various enzymes. Fig. 4B shows an anion exchange HPLC profile of the products obtained by dephosphorylation with a mixture of alkaline phosphatase and venom phosphodiesterase I. Dephosphorylation with either alkaline phosphatase or venom phosphodiesterase I gave similar results (data not shown). In each case, the peaks designated DP and TP were lost and a corresponding increase in radioactivity was observed in peaks X and PMEA; about 80% of the radioactivity increase was associated with peak X and about 20% with PMEA. No increase in the radioactivity associated with peak Y was observed. These results indicate that the metabolites associated with the DP and TP peaks were phosphorylated forms of X and PMEA, but not Y. When unlabeled PMEAp and PMEApp were subjected to dephosphorylation under the same reaction conditions, only PMEA was recovered, indicating that the dephosphorylation reaction itself did not alter the basic PMEA structure (data not shown). These results suggest that metabolite X exists in phosphorylated form in HeLa S3 cells. Moreover, phosphonyl structure appears to be preserved during PMEA conversion to X, because there was no difference between the dephosphorylation pattern obtained with alkaline phosphatase and that obtained with venom phosphodiesterase I.

Because in HeLa S3 cells only a small amount of radioactivity was associated with higher phosphorylated products (DP and TP), we compared these results with data obtained by using cells having a higher ATP/ADP ratio than HeLa S3 cells. MOLT-4F cells had at least a 2-fold higher ATP/ADP ratio than did HeLa S3 cells under our conditions (data not shown). As can be seen in Fig. 6A, the profile of acid-soluble radioactivity in MOLT-4F cells treated with [^{3}H]PMEA differed from that of HeLa S3 cells. The relative amounts of the DP and TP material were higher in the MOLT-4F cells, compared with HeLa S3 cells, and peak Y was not detected in MOLT-4F cells. Similar to the results with HeLa S3 cells, dephosphorylation resulted in the elimination of the DP and TP peaks and an increase in peaks X and PMEA (Fig. 6B).

Analysis of acid-insoluble fractions of HeLa S3 cells treated with [^{3}H]PMEA. Because at least two metabolites in [^{3}H]PMEA-treated HeLa S3 cells appeared to be phosphorylated, the incorporation of the metabolites into DNA and RNA was examined. Purified nucleic acids from either mock- or HSV-1-infected HeLa S3 cells treated for 20 hr with 0.5 μM [^{3}H]PMEA (5 Ci/mmol) were subjected to ultracentrifugation in $CsSO_4$ gradients (Fig. 7). Most of the radioactivity was associated with DNA in both mock- and HSV-1-infected cells. DNA peaks were taken, dialyzed, and enzymatically digested, and acid-soluble materials were analyzed by reverse phase HPLC (Fig. 8). From both samples, metabolite X and PMEA itself were recovered. The origin of the other peaks of radioactivity is unknown, but they could be the result of incomplete digestion or they may represent other forms of metabolite X and/or PMEA.

Discussion

Unlike some of the anti-HSV nucleoside analogs, PMEA does not require phosphorylation by viral thymidine kinase and, thus, is not preferentially converted into its active form in virus-infected cells. Instead, this compound represents a nucleoside monophosphate analog. The phosphonyl group is believed to be stable against the action of the cellular phosphomonoesterase activities (20) and, thus, once the compound has been taken up by the cells, it can be directly phosphorylated into its mono- and diphosphoryl derivatives by cellular enzymes (8). However, due to the polar nature of PMEA, we anticipated that its uptake into cells would be rather slow. Considering that viral DNA synthesis under cell culture conditions starts approximately 4–8 hr after HSV-1 infection,[1] it was reasonable

[1] S. A. Foster, J. Cerny, and Y.-C. Cheng, unpublished observations.

to think that preincubation of the cells with PMEA before viral infection could lead to an accumulation of an active form of the drug before the onset of viral DNA synthesis and, thus, block virus replication more effectively than addition of the drug after viral infection.

Our results indicate that preincubation of the cells with PMEA before viral infection enhances its antiviral potency by almost 10-fold, compared with treatment of the cells after viral infection alone. This result has important implications for the estimation of antiviral activity of compounds that can be activated by cellular enzymes. The antiviral potency of such compounds could be underestimated when the result is based on the addition of the compound after the viral infection.

The enhancement of antiviral activity obtained by preincubating the cells with the drug does not appear to be limited to PMEA. We found that preincubation of cells with (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine resulted in a similar enhancement of anti-HSV activity.[2]

In an effort to explain the preincubation data in terms of uptake and metabolism of PMEA, we studied intracellular metabolism of PMEA by using [2,8-^{3}H]PMEA. Commercially available [^{3}H]PMEA was tested and found to contain an impurity that was only about 2% of the total cpm but was very metabolically active, being rapidly taken into the cells and converted into metabolites with retention times similar, but not identical, to those of PMEAp and PMEApp on Partisil-10 SAX HPLC (data not shown). Thus, further purification of this preparation was required before it could be used in metabolic studies. So far, two studies dealing with either uptake or metabolism of PMEA, in which labeled PMEA from the same commercial source was used, have been published (9, 21). In those reports, no mention was made of any impurities or further purification of the PMEA; thus, this impurity could have been specific to the preparation obtained by us or, alternatively, could have been overlooked.

When purified [^{3}H]PMEA was used, uptake of the drug was slow and did not begin to plateau until close to 24 hr. This very slow uptake (under our conditions) is different from the PMEA uptake observed by others, which reached a plateau by 2 hr (21). Therefore, these data support the idea that pretreatment of the cells with PMEA before viral infection enhances its antiviral effect by allowing more time for the intracellular accumulation of the drug.

HPLC analysis of the acid-soluble PMEA metabolites in HeLa S3 cells revealed at least four metabolites, in addition to PMEA itself. Two metabolites (or groups of metabolites) were designated DP and TP and probably represent mono- and diphosphoryl PMEA metabolites. In contrast, the metabolites designated X and Y did not appear to be phosphorylated forms of PMEA but may represent modifications of the basic PMEA structure. Metabolites X and Y were not detected until approximately 8 hr of incubation but formed the majority of the acid-soluble metabolites at 24 hr.

Analysis of the efflux of the individual metabolites after drug removal indicated that both X and Y crossed the plasma membrane more easily than PMEA or the DP and TP metabolites. This suggests that X and Y may be forms that are less charged than the parent PMEA. One possible explanation for this is that the phosphonyl group has been removed from PMEA. We feel that this is unlikely, because the phosphonyl group is thought to be resistant to phosphatases and our results with alkaline phosphatase and venom phosphodiesterase suggest that the phosphonyl structure is still intact. Alternatively, the addition of a charge-neutralizing group could be involved. Another possibility is that the X and Y metabolites could be still charged but recognized more efficiently by the membrane transport mechanism. The slow accumulation of intracellular acid-soluble radioactivity during [^{3}H]PMEA treatment seen in Fig. 3 is expected, based on the charged nature of PMEA, whereas the rapid loss of acid-soluble counts after drug removal is explained by the intracellular conversion of PMEA to X and Y, which more easily pass out of the cells.

The role of X and Y metabolites, if any, in the antiviral or cytotoxic effects of PMEA is unclear, although metabolite X does appear to exist in phosphorylated form(s). In addition, both PMEA and X appeared to be incorporated into DNA.

During the completion of this work, a study of PMEA metabolism was published, in which only "PMEA," "PMEAp," and "PMEApp" were detected in [^{3}H]PMEA-treated MT-4 cells (9). However, it is not clear how the metabolites were identified. The present study suggests that PMEA metabolism may be more complex in some cell types. Indeed, published ID_{50} values for PMEA against different cell lines vary widely (1, 22).[2] At least one explanation for this is different metabolic patterns in different cell types. Within our study, PMEA metabolism was different in HeLa S3 versus MOLT-4F cells, in that metabolite Y was not detected in MOLT-4F cells.

In summary, pretreatment of HeLa S3 cells with PMEA before viral infection greatly increased the antiviral potency of PMEA. This is probably the result of a slow accumulation of PMEA metabolites within the cells, along with the long retention of the phosphorylated metabolites. These results could also partly explain why PMEA has potent antiviral activity in animals when administered infrequently (23). At least two metabolites of PMEA that were distinct from PMEA itself, PMEAp and PMEApp, were detected in HeLa S3 cells; however, their role in antiviral activity and cytotoxicity is unclear. Attempts will be made to generate enough of the X and Y metabolites to pursue their further characterization.

[2] J. Cerny, S. A. Foster, and Y.-C. Cheng, unpublished observations.

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