Biochemical Pharmacology, Vol. 58, pp. 487–492, 1999.

ELSEVIER

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00118-5

9-[2-(Phosphonomethoxy)ethyl]adenine Diphosphate (PMEApp) as a Substrate toward Replicative DNA Polymerases α, δ, ε, and ε*

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ABSTRACT. The diphosphoryl derivative of the acyclic nucleotide phosphonate analog 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA), found previously to weakly inhibit DNA pol δ/proliferating cell nuclear antigen, was studied as a substrate for pol α, δ, ε, and ε*. A comparison of the V_{max} and K_m for this derivative (PMEApp) and dATP demonstrated that the relative efficiency of the incorporation of this analog into the DNA chain is decreasing in the following order: pol δ ≃ pol ε ≃ pol ε* > pol α. Under the reaction conditions, this incorporation amounted to 4.4 to 0.7% of dAMP molecules. Similar K_m values for PMEApp and dATP in pol ε and pol ε* catalyzed reactions revealed that proteolysis of the enzyme probably does not affect the dNTP binding site. The DNA polymerases tested were inhibited by the reaction product (PMEA terminated DNA chain) with similar K_i/K_m ratios (pol α 0.2; pol δ, 0.1; pol ε 0.05; and pol ε*, 0.06). The associated 3′-5′-exonuclease activity of pol δ, ε, and ε* was able to excise PMEA from the 3′-OH end of DNA with a rate one order of magnitude lower than that of the dAMP residue. BIOCHEM PHARMACOL **58**;3:487–492, 1999.

KEY WORDS. adefovir; PMEA insertion; PMEA excision; pol α; pol δ; pol ε

PMEA§ (Adefovir) is a promising antiviral drug that exhibits activity against DNA viruses [1], hepadnaviruses [2, 3], and retroviruses [4]. Its oral prodrug (bis-POM-PMEA, Adefovir Dipivoxil) is now in clinical trials for AIDS and hepatitis B treatment (phase II/III). Because the retroviral infections are accompanied very often by opportunistic herpesvirus infections, PMEA becomes still more attractive due to its dual antiviral potency [5].

PMEA, bearing a phosphonomethyl ether group in the molecule, is resistant against cellular catabolism; the mechanism of its cellular uptake depends on the cell type [6, 7]. It is phosphorylated by AMP kinase to PMEApp (a dATP analog) [8], which is a competitive inhibitor of HSV-1 DNA polymerase and retroviral reverse transcriptase, and terminates the nascent DNA chain [9, 11]. Kramata *et al.* [12] have shown recently that the cytostatic activity of PMEA and other acyclic nucleotide analogs correlates with their inhibitory potency toward replicative DNA polymerases α, δ, and the proteolyzed form of pol ε (pol ε*). The authors claim that DNA replication is one of the possible targets of this group of potential drugs; nonetheless, the ability of PMEApp to serve as a substrate of cellular replicative DNA polymerases has not been exhaustively investigated.

In this study, we have focused on the substrate activity of PMEApp toward pol α, δ, ε, and its proteolyzed form pol ε*. Moreover, we studied the relation between polymerases and PMEA-terminated DNA chain and the excision of PMEA from the 3′-OH end of DNA by the associated 3′-5′-exonuclease of pol δ, ε, and ε*.

MATERIALS AND METHODS

Chemicals

Nucleotides (dTTP, dATP, dCTP, dGTP, and ATP), MicroSpin G-25 columns, PD-10 columns, and poly dT were products of Pharmacia P-L Biochemicals (Sweden). Oligo dA_{18} was synthesized in the Institute of Organic Chemistry and Biochemistry (Prague, Czech Republic). Labeled deoxyribonucleotides [8-^{3}H]dATP (888 GBq mmol^{-1}), [γ-^{32}P]ATP (110 TBq mmol^{-1}), T4 polynucleotide kinase, and urea were products of Amersham International (GB). DEAE Sephacel, EDTA, BSA, terminal deoxynucleotidyl transferase (TdT) and DTT were purchased from Sigma (Czech Republic). Glycerol (99%, Riedel-deHaën) was redistilled in vacuum (glass apparatus). PMEA and its diphosphate were prepared according to procedures described previously [13, 14].

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§ *Abbreviations:* PMEA, 9-[2-(Phosphonomethoxy)ethyl]adenine; PMEApp, 9-[2-(Phosphonomethoxy)ethyl]adenine diphosphate; bis-POM, bis(pivaloyloxymethyl); PCNA, proliferating cell nuclear antigen; and DTT, dithiothreitol.

Received 22 June 1998; Accepted 4 December 1998.

Preparation of Template-Primer Poly dT-oligo dA_{18}PMEA

Oligo dA_{18} (5 nmol) was incubated for 4 hr at 37°C in a reaction mixture (125 μL) that contained 200 mM potassium cacodylate, pH 7.2, 0.5 mM DTT, 2.5 mM $MnCl_2$, 200 μM PMEApp, and 250 U TdT. The reaction was stopped by thermal denaturation for 25 min at 75°, and unreacted monomer was separated on a MicroSpin G-25 column. Purified oligomer (yield > 98%) then was annealed with poly dT in the base ratio 1:50 (TE buffer, pH 7.5) and used for inhibition of DNA polymerases.

Oligo dA_{18}PMEA (1500 pmol) was labeled on the 5′-end with ^{32}P using T4 polynucleotide kinase (Amersham–Tested User Friendly™, Polynucleotide Kinase 5′-End Labeling Protocol, USB™), and purified on 20% denaturing PAGE (1800 V, 3 hr). After autoradiographic detection, the band containing [5′-^{32}P]oligo dA_{18}PMEA was cut out and homogenized, and the gel was loaded on 300 μL of DEAE Sephacel equilibrated in sterile water. Then the column was washed exhaustively with water (overnight), and oligomer was eluted in 1 M LiCl, desalted on a PD-10 column, and concentrated in a Savant vacuum evaporator. Purified [5′-^{32}P]oligomer was annealed with poly dT in the base ratio 1:50 (TE buffer, pH 7.5) and used for 3′-5′-exonuclease experiments. Purified oligo dA_{18} for the control experiments was labeled on the 5′-end with ^{32}P by the same method as oligo dA_{18} PMEA.

DNA Polymerases and PCNA

DNA polymerases α, δ, ε, and ε* were isolated from Sprague–Dawley rat compact transplantable lymphomas with the purification procedure described in our previous papers [15, 16] except for the last step (glycerol gradient). One unit (U) of the DNA polymerase activity is defined as the amount of enzyme that catalyzes an incorporation of 1nmol of dATP into acid-insoluble precipitate after 30 min under the published conditions [12]. PCNA was purified to homogeneity according to the published method [17] [from *Escherichia coli* strain BL 1/DE 3 harboring a plasmid encoding the human PCNA cDNA sequence; this bacterial strain was provided by Dr. B. Stillman, Cold Spring Harbor Laboratory].

DNA Polymerase Assay

Activity of DNA polymerases α, δ, ε, and ε* on template-primer poly dT-oligo dA_{18} was determined according to Kramata *et al.* [12]; activity of both pol ε and pol ε* was measured under the same conditions. The estimation of K_m for the 3′-OH end of poly dT-oligo dA_{18} was carried out at 37° in the presence of 50 μM [^3H]dATP (40 μCi/mL) and an appropriate amount of substrate (template-primer in base ratio 50:1; time interval: 5, 10, and 15 min) in a reaction volume of 25 μL. The inhibitory effect of poly dT-oligo dA_{18}PMEA toward the DNA pol α, δ, ε, and ε* (in a reaction volume of 25 μL) on the template-primer poly dT-oligo dA_{18} was measured after 10 min of incubation at 37°C. It should be pointed out that DNA polymerase δ also requires the replication factor RFC under more physiological conditions, but it can be replaced in in vitro experiments on linear templates with PCNA in high concentrations [18, 19]. For the pol δ assay used in this study, this concentration was found previously to be 18 μg/mL [15].

TABLE 1. 3′-5′-Exonuclease activity of DNA pol δ, ε, and ε* toward template-primers poly dT-oligo dA_{18} and poly dT-oligo dA_{18}PMEA*

DNA polymerase	±dATP (50 μM)	$^{dA_{18}}V$† (fmol/μg · min)	^{PMEA}V† (fmol/μg · min)	Δ‡
Pol δ	−	4.6 ± 0.8	0.36 ± 0.05	13
	+	21.7 ± 4.0§	0.40 ± 0.07	*n*‖
Pol ε	−	103.3 ± 10	9.60 ± 1.15	11
	+	69.7 ± 8.7§	1.88 ± 0.28	*n*
Pol ε*	−	146 ± 13	20.80 ± 2.29	7
	+	51.7 ± 6.2§	3.47 ± 0.45	*n*

*V values are means ± SD of at least three separate experiments. For experimental details, see Materials and Methods.

†V is defined as a decrease of oligomer (dA_{18} or dA_{18}PMEA) concentration at a given time.

‡Δ represents the $^{dA18}V/^{PMEA}V$ ratio. For details, see Results and Discussion.

§Value represents elongation step only.

‖*n* In this case Δ is not relevant.

3′-5′-Exonuclease Activity

Exonuclease activity of DNA polymerases δ, ε, and ε* was determined in a reaction mixture (25 μL) that contained 37.5 nM substrate (poly dT-oligo dA_{18}PMEA or poly dT-oligo dA_{18} in a base ratio of 50:1), 40 mM Tris–HCl (pH 7.6), 0.3 mM $MgCl_2$, 200 μg/mL of BSA, 10% glycerol, and 1 mM DTT at four different time intervals in the presence or absence of 50 μM dATP. After incubation the reaction was terminated by addition of an equivalent amount of 98% deionized formamide containing 1 mM EDTA, 0.2% Bromophenol Blue, and 0.2% Xylene Cyanol FF. Samples then were heated to 60° (5 min), cooled to 4°, and loaded on 15% PAGE gels containing 89 mM Tris-borate (pH 8.3), 2 mM EDTA, and 7 M urea. After electrophoresis (1800 V, 4 hr) the gel was dried and quantified on a PhosphorImager. The rate of reaction was defined as the decrease of oligo dA_{18}PMEA and/or of oligo dA_{18} concentration at a given time.

Primer Extension Assay

Synthetic oligonucleotides were purchased from Lambda Bio-Med (Prague, Czech Republic) and purified by electrophoresis on 20% PAGE gels. The primer 5′GAGATCTC-CTTGGGG-3′ was labeled on the 5′-end with ^{32}P using T4 polynucleotide kinase and annealed with the template 5′-CTAATGCTTAAGCTCTATCCATTTTCCCCAA-

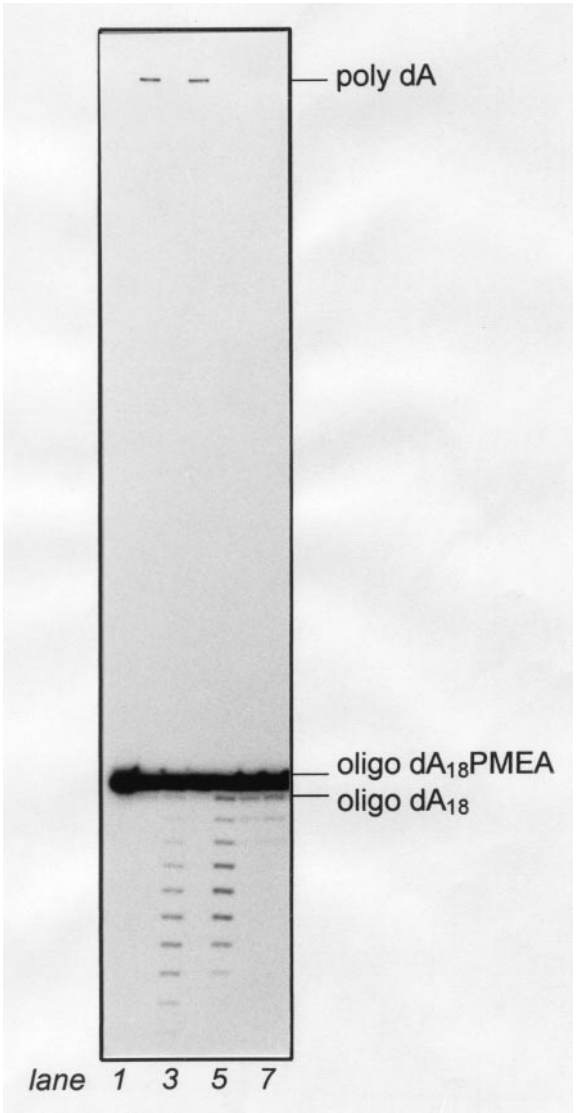


FIG. 1. Excision of incorporated PMEA from template-primer poly dT-oligo dA_{18}PMEA catalyzed by the associated 3′-5′-exonuclease of DNA polymerases δ, ε, and ε∗. Pol ε (2 U/mL), ε∗ (2 U/mL), and δ (2 U/mL, without PCNA) were incubated with 37.5 nM poly dT-oligo dA_{18}PMEA in the presence and/or absence of 50 μM dATP (see Materials and Methods for details) for 30 min at 37°. The lanes contained (from left to right): *1*, control without enzyme; *2*, pol ε∗ + dATP; *3*, pol ε∗ − dATP; *4*, pol ε + dATP; *5*, pol ε − dATP; *6*, pol δ (−PCNA) + dATP; and *7*, pol δ (−PCNA) − dATP.

GGAGATCTC-3′ in the molar ratio 1.5:1 (template-primer, pX_{40}-oY_{15}). The reaction (25 μL) was carried out at saturation with the 3′-OH end of the ^{32}P-labeled primer (2 μmol/L) in the presence of 50 μM dCTP, 50 μM dGTP, 50 μM dTTP, and an appropriate concentration of PMEApp or dATP, respectively [in 40 mM HEPES K^+ (pH 7.0), which contained 10 mM $MgCl_2$, 200 μg/mL of BSA, 10% glycerol, and 1 mM DTT], at three different time intervals under different ionic strength as follows: (i) pol α, 50 mM KCl; (ii) pol δ, PCNA (18 μg/mL), 25 mM KCl; and (iii) pol ε and pol ε∗, 100 mM KCl. After incubation, the reaction was analyzed on 20% PAGE gels by the procedure mentioned above. The kinetic constants of dATP incorporation were determined from the sum of full-length product and all elongation intermediates.

TABLE 2. Inhibitory potency of poly dT-oligo dA_{18}PMEA towards DNA pol α, δ, ε, and ε∗

DNA polymerase	K_m(nmol/L) poly dT-oligo dA_{18}	K_m(nmol/L) poly dT-oligo dA_{18}PMEA	K_i/K_m
Pol α	1.8 ± 0.2	0.36 ± 0.06	0.20
Pol δ/PCNA	9.7 ± 1.0	1.21 ± 0.15	0.12
Pol ε	2.8 ± 0.5	0.14 ± 0.02	0.05
Pol ε∗	9.3 ± 1.0	0.52 ± 0.1	0.06

Kinetic constants are the averages of at least three separate experiments; K_i and K_m values are means ± SD. For experimental details, see Materials and Methods.

RESULTS AND DISCUSSION

The data in Table 1 and Fig. 1 (Δ, ratios of the rate of decrease of oligo dA_{18} and/or oligo dA_{18}PMEA concentration) demonstrate that the ability of pol δ (without PCNA), pol ε, and pol ε∗ to excise PMEA from the 3′-OH end in the absence of dATP was comparable, i.e. 7–13

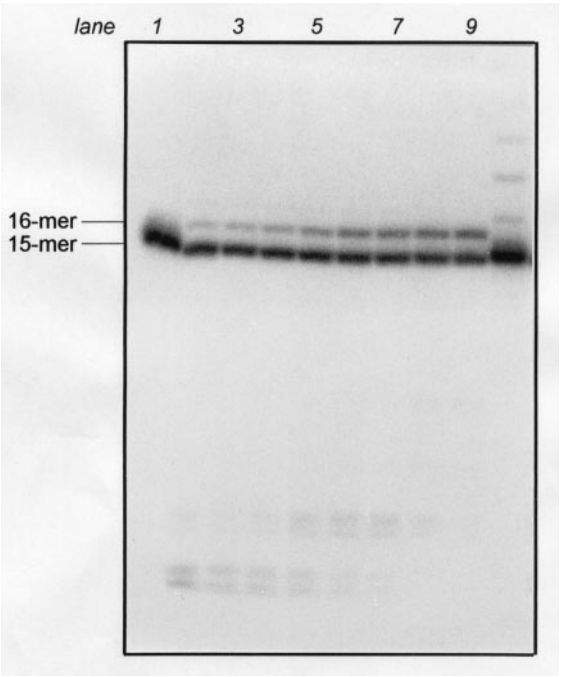


FIG. 2. Kinetics of PMEA incorporation catalyzed by DNA pol ε∗. A reaction mixture containing the enzyme (170 U/mL) and PMEApp at various concentrations (1.2, 1.8, 2.5, 4, 8, 10, 20, and 40 μM) in the presence of synthetic template-primer pX_{40}-oY_{15} was incubated for 30 min at 37° (lanes *2–9*; see Materials and Methods for details). The increasing amount of PMEA-terminated oligomer (oY_{15}PMEA) possesses slightly higher mobility than oY_{15}dA (lane *10*). The minor component with the lowest mobility represents an elongated mismatch product of primer oY_{15}. The influence of the associated 3′-5′-exonuclease is also visible at lower concentrations of PMEApp. Lane *1*: control, non-elongated oligomer without enzyme; lane *10*: elongation intermediates of primer oY_{15}.

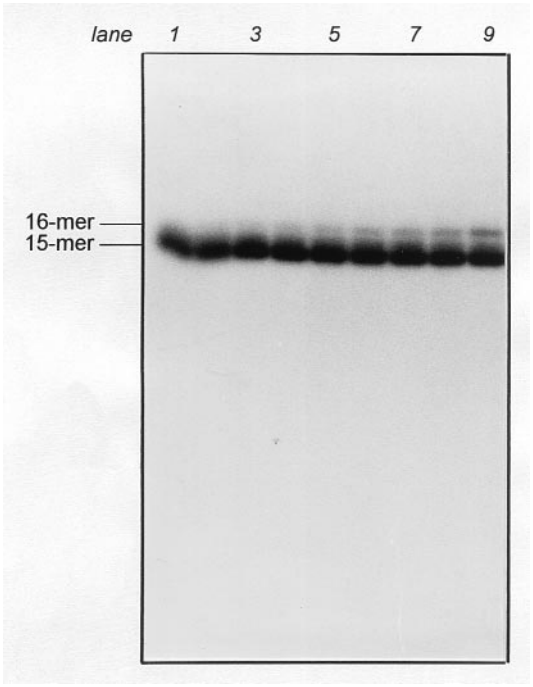


FIG. 3. Kinetics of PMEA incorporation catalyzed by DNA pol α. A reaction mixture containing the enzyme (30 U/mL) and PMEApp at various concentrations (1.2, 1.8, 2.5, 4, 8, 10, 20, and 40 μM) in the presence of synthetic template-primer pX_{40}-oY_{15} was incubated for 30 min at 37° (lanes *2–9*; see Materials and Methods for details). Lane *1*: control, non-elongated oligomer without enzyme.

times slower than the excision of dAMP from dA_{18}. The presence of dATP in the reaction catalyzed by pol δ (without PCNA) did not affect the liberation of PMEA from poly dT-oligo dA_{18}PMEA. On the other hand, in pol ε and pol ε* catalyzed reactions, dATP addition strongly inhibited the excision of PMEA (the rate of decrease of oligo dA_{18}PMEA was 5–6 times slower than in the absence of dATP). PCNA did not influence the rate of PMEA excision catalyzed by the 3′-5′-exonuclease activity of DNA pol δ (data not shown).

The inhibitory effect of poly dT-oligo dA_{18}PMEA toward DNA polymerases α, δ, ε, and ε* was measured on template-primer poly dT-oligo dA_{18}. The results presented in Table 2 show that the PMEA-terminated template-primer is a comparatively potent competitive inhibitor of poly dT-oligo dA_{18} elongation. K_i/K_m ratios demonstrated similar inhibition of all the DNA polymerases studied (Table 2). In the control experiments, poly dT did not inhibit DNA polymerase-catalyzed reactions (reactions were carried out in the same concentration interval of poly dT as for poly dT-oligo dA_{18}PMEA; data not shown).

The synthetic template-primer (pX_{40}–oY_{15}, see Materials and Methods) under saturation conditions, 50 μM dCTP, dGTP, dTTP, and different concentrations of PMEApp and/or dATP were used for quantitative evaluation of PMEA insertion into the DNA chain. dNTPs were added because of the high processivity of the DNA polymerases studied and also with the aim of suppressing 3′-5′-exonuclease activity of the enzymes mentioned [20]. The incorporation of PMEA was followed on 20% PAGE (Figs. 2–4; see Materials and Methods for details), and the kinetic data then were used for calculation of the relative efficiency of the PMEA incorporation [f_{INS} (%)] [21]. The results in Table 3 show that the relative efficiency of PMEA incorporation into the DNA chain by diverse DNA polymerases decreased in the following order: pol δ ≃ pol ε ≃ pol ε* > pol α.

According to Boosalis *et al.* [21], the kinetics of dNTP misincorporation is characterized by considerably higher K_m and lower V_{max}. We found that the rate of dNTP misincorporation (measured under the reaction conditions for dATP but in its absence; data not shown) was 100–300 times lower in comparison with V_{max} for dATP. On the other hand, the low V_{max} of PMEApp insertion into the DNA chain required a higher amount of enzyme in the reaction mixture and also a longer incubation period. Such conditions evidently facilitate misincorporation. Fortunately, PMEA-terminated chain could be distinguished from dNMP-mismatched product and from template-primer (see Figs. 2 and 4). In this case, the rate of misincorporation did not exceed 10% of V_{max} for PMEApp (measured in the presence of PMEApp; data not shown).

In this study we investigated the interaction of PMEApp (an analog of dNTP) with eucaryotic replicative DNA polymerases from SD lymphoma with the aim of evaluating

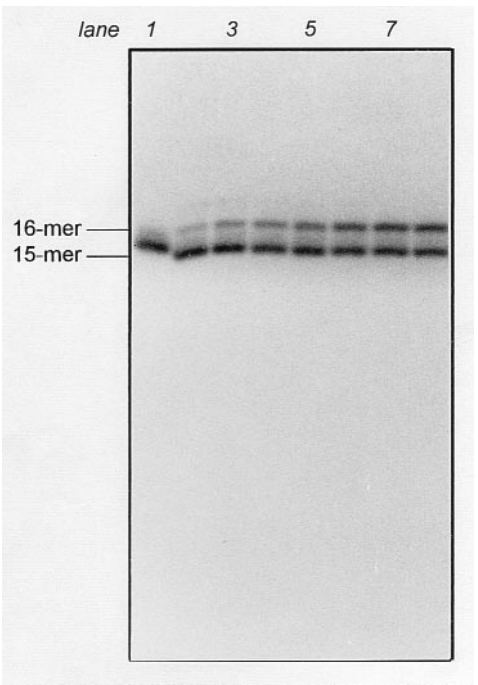


FIG. 4. Kinetics of PMEA incorporation catalyzed by DNA pol δ/PCNA. A reaction mixture containing the enzyme (9 U/mL) and PMEApp at various concentrations (0.8, 1.2, 1.8, 2.5, 4, 6, and 8 μM) in the presence of synthetic template-primer pX_{40}-oY_{15} was incubated for 30 min at 37° (lanes *2–8*; see Materials and Methods for details). The minor component with the lowest mobility represents an elongated mismatch product of primer oY_{15}. Lane *1*: control, non-elongated oligomer without enzyme.

TABLE 3. Insertion efficiency of PMEApp into synthetic template-primer $pX_{40}-oY_{15}$ catalyzed by DNA pol α, δ, ε, and ε*

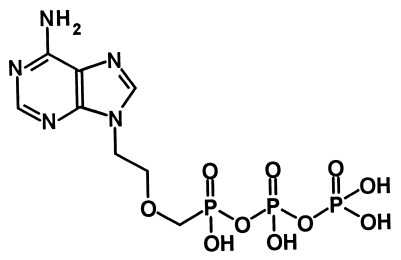
DNA polymerase	$^{PMEApp}K_m$ (μmol/L)	$^{PMEApp}V_{max}$ (pmol/μg · min)	$^{dATP}K_m$ (μmol/L)	$^{dATP}V_{max}$ (pmol/μg · min)	f_{INS}* (%)
Pol α	3.5 ± 0.7	0.560 ± 0.050	2.50 ± 0.15	59.6 ± 1.50	0.7
Pol δ/PCNA	1.1 ± 0.1	0.186 ± 0.030	0.45 ± 0.04	1.74 ± 0.09	4.4
Pol ε	3.1 ± 0.5	0.296 ± 0.020	9.10 ± 2.00	28.8 ± 1.40	3.0
Pol ε*	3.4 ± 0.3	0.166 ± 0.007	9.50 ± 1.70	23.0 ± 2.00	2.0

Kinetic constants are the averages of at least three separate experiments; V_{max} and K_m values are means ± SD. See Materials and Methods for details.
*$f_{INS}(\%) = 100 \times {}^{PMEApp}V_{max} \times {}^{dATP}K_m / {}^{dATP}V_{max} \times {}^{PMEApp}K_m$ [21].

its substrate activity. These enzymes (pol α, δ, ε, and ε*) were found to possess high processivity of dNTP incorporation [20]. The mechanism is characterized by a low rate of dissociation of the enzyme from template-primer compared with the rate of enzyme translocation and binding of the subsequent dNTP. Therefore, the rate-determining step is the catalytic step or a precatalytic conformational change of the substrate–enzyme complex. In those cases where the nucleotide can terminate the DNA chain, the reaction rate is determined strictly by the dissociation of the enzyme–template–primer complex [22].

The decreasing relative substrate specificity of PMEApp towards replicative polymerases (pol δ ≃ pol ε ≃ pol ε* > pol α) follows from the primer extension study (Table 3). The $^{PMEApp}V_{max}/^{dATP}V_{max}$ ratio shows that the relatively low efficiency of PMEA incorporation catalyzed by DNA polymerases α, ε, and ε* is most probably directed by slow rate-limiting dissociation of the enzyme from the terminated template-primer. Moreover, this ratio also should be influenced by the fact that the PMEA-terminated DNA-chain is a relatively potent inhibitor of all the polymerases studied (Table 2). Our data also show that the K_m for dATP and PMEApp and also the f_{INS} value of the proteolyzed and nonproteolyzed form of pol ε (pol ε and pol ε*) are practically the same. Thus, it is evident that the proteolysis most probably does not affect the binding site of pol ε [16]. All of these findings indicate that *in vitro* PMEApp inhibition of polymerases α, δ, ε, and ε* is a complex process affected by (i) competition for the active site between PMEApp and dATP [12], (ii) substrate properties of PMEApp, and (iii) PMEApp-terminated template-primer inhibitory potency.

The relative efficiency of PMEA insertion catalyzed by DNA pol α also was studied by Cihlář and Chen [23]. Their



PMEApp

K_m value for PMEApp (2.71 μM) is in good agreement with our data. However, their f_{INS} value for PMEApp is one order of magnitude higher than our results. This difference is most probably due to non-processive conditions of pol α, which they used for determination of dATP substrate activity in the assay. We have confirmed that lowering of the reaction processivity (dTTP was omitted) results in a small decrease of K_m for dATP (2 μM), whereas the relative efficiency of PMEApp (f_{INS}) incorporation increased from 0.7 to 2% (data not shown).

DNA polymerases δ, ε, and ε* contain an associated 3′-5′-exonuclease, which can excise the incorporated PMEA residues from the DNA chain (Fig. 1, Table 1). The relative rate of PMEA excision in the absence of dATP is one order of magnitude lower than that for dAMP and is comparable with the published value for AZT 5′-phosphate [24, 25]. It should be noted that dATP strongly inhibits the 3′-5′-exonuclease of pol ε and ε* (Table 1).

Our results clearly show that PMEApp is a weaker substrate of replicative DNA polymerases than dATP; its incorporation into DNA most probably depends on the intracellular PMEApp/dATP ratio. It is evident that the DNA damage by PMEA incorporation, which is manifested by the dose-dependent cytostatic effect [26, 27] and/or by certain chromosomal aberations [28], could be repaired by the action of pol δ- and pol ε-associated 3′-5′-exonuclease.

This work was supported by Grants 203/96/0284 and 203/96/K001 from the Grant Agency of the Czech Republic and by Gilead Sciences (Foster City, CA, U.S.A.).

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