JOURNAL OF VIROLOGY, Aug. 1993, p. 4688–4695
0022-538X/93/084688-08$02.00/0

Vol. 67, No. 8

Inhibition of Proteolytic Activity of Poliovirus and Rhinovirus 2A Proteinases by Elastase-Specific Inhibitors

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Received 24 February 1993/Accepted 6 May 1993

A polyprotein cleavage assay has been developed to assay the proteolytic activities in vitro of the 2A proteinases encoded by poliovirus and human rhinovirus 14, which are representative members of the *Enterovirus* and *Rhinovirus* genera of picornaviruses, respectively. The elastase-specific substrate-based inhibitors elastatinal and methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MPCMK) inhibited both 2A proteinases in vitro. The electrophoretic mobilities of both 2A proteinases were reduced upon incubation with elastatinal, whereas the mobility of a Cys-109→Ala poliovirus $2A^{pro}$ mutant was unchanged, an observation suggesting that this inhibitor may have formed a covalent bond with the active-site Cys-109 nucleophile. Iodoacetamide, calpain inhibitor 1, and antipain inhibited poliovirus $2A^{pro}$. MPCMK caused a reduction in the yields of the enteroviruses poliovirus type 1 and coxsackievirus A21 and of human rhinovirus 2 in infected HeLa cells but did not affect the growth of encephalomyocarditis virus, a picornavirus of the *Cardiovirus* genus. MPCMK abrogated the shutoff of host cell protein synthesis that is induced by enterovirus and rhinovirus infection and reduced the synthesis of virus-encoded polypeptides in infected cells. These results indicate that the determinants of substrate recognition by 2A proteinases resemble those of pancreatic and leukocyte elastases. These results may be relevant to the development of broad-range chemotherapeutic agents against entero- and rhinoviruses.

Many positive-sense RNA viruses encode proteinases which play important roles in viral replication (18, 29). These enzymes have discriminatory and unusual cleavage specificities, and much current interest in understanding their structures and mechanisms of action derives from the realization that they are therefore attractive targets for the rational development of selective and specific antiviral inhibitors (20, 28). One of the largest families of human pathogenic RNA viruses is the *Picornaviridae*, whose members include poliovirus and rhinovirus, the etiological agents of poliomyelitis and the common cold, respectively. Poliovirus, a member of the genus *Enterovirus*, is the prototypic picornavirus, and its structure and biological properties have been characterized in the greatest detail. In contrast to the effective control of infectious poliovirus, vaccination against human rhinoviruses (HRV) is impracticable, since over 100 different HRV serotypes have been described (14). The development of a group-specific inhibitor of viral replication is therefore an attractive alternative to immune prevention. This strategy is encouraged by the extensive similarities between the genomic nucleotide sequences of all members of the *Enterovirus* and *Rhinovirus* genera and, hence, between the polypeptides that they encode (44).

All poliovirus proteins are generated by proteolytic processing of a single 247-kDa polyprotein that is translated from the genomic RNA (24), following initiation by the novel mechanism of internal ribosomal entry (23). All but one of the processing steps are catalyzed by three virally encoded proteinases, $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$, which are themselves part of the polyprotein (16, 18). The initial event in the ordered proteolytic cascade is the primary cleavage by which the P1 structural protein precursor is separated from the nascent P2-P3 nonstructural precursor. This reaction is catalyzed by $2A^{pro}$, which cleaves a Tyr-Gly dipeptide at its own amino terminus (50). It must occur before the P1 precursor can be processed to yield capsid proteins (34). $2A^{pro}$ is also involved in the rapid shutoff of host cell protein synthesis that occurs upon infection with poliovirus and which is associated with proteolytic cleavage of the 220-kDa γ subunit (p220) of eukaryotic translation initiation factor eIF-4F (11, 27). $2A^{pro}$ probably activates a latent cellular proteinase which then cleaves p220 (17, 54, and references therein). Other possible functions of $2A^{pro}$ are the *trans* activation of picornavirus translation (13) and an unknown role in poliovirus genome replication (33a).

The activity of poliovirus $2A^{pro}$ is inhibited by the alkylating agents iodoacetamide and *N*-ethylmaleimide (25, 55), an observation indicating that the enzyme contains an active-site thiol group. This supported an earlier classification of $2A^{pro}$ as a cysteine proteinase (6). However, $2A^{pro}$ is not related to the papain superfamily, since it is not inhibited by the classical epoxide inhibitor E-64 (25, 55) and shows no sequence similarity with this family. More recently, it has been suggested that $2A^{pro}$ is structurally related to the small subclass of trypsin-like serine proteinases, such as alphalytic protease, and specifically, that His-20, Asp-38, and Cys-109 form the catalytic triad (3). The proposed substitution of a cysteine residue for a serine residue in the viral catalytic triad, which is a notable difference between the cellular trypsin-like and the viral trypsin-like proteinases, is supported by the results of recent experiments (17, 55). Other differences between corresponding residues in $2A^{pro}$ and cellular trypsin-like proteinases are likely to contribute to changes in the active-site environment and to substrate-binding interactions. $2A^{pro}$ cleaves only two of the 10 Tyr-Gly dipeptides in the poliovirus polyprotein (50) and is likely to recognize an extended substrate amino acid sequence, since residues flanking the scissile bond between P1 and 2A, for example, are strongly conserved in different

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isolates and serotypes (19). In this respect, $2A^{pro}$ resembles alpha-lytic protease (2), but it differs significantly from this enzyme with respect both to its cleavage specificity and to the primary determinants of substrate recognition (19, 30). HRV2 $2A^{pro}$ has been characterized in similar detail as poliovirus $2A^{pro}$ (26, 41, 43) and has very similar properties. Considerably less is known about $2A^{pro}$ moieties encoded by other rhinoviruses and enteroviruses, although sequence comparisons indicate that they are all closely related to poliovirus $2A^{pro}$. We have shown that substrate specificity of coxsackievirus A21 (CAV21) is very similar to that of poliovirus $2A^{pro}$ (19); HRV14 $2A^{pro}$ probably also catalyzes cleavage of a Tyr-Gly dipeptide at its amino terminus and induces cleavage of p220 (10). It should be noted that members of the *Cardiovirus*, *Aphthovirus*, and *Hepatovirus* genera of picornaviruses do not encode a 2A-related proteinase (16, 18).

The efficiency of substrate-derived proteinase inhibitors increases as they mimic the proteinase cleavage site more closely, and effective inhibitors can therefore be highly specific tools for dissecting the structures and mechanisms of action of proteinases. Here, we report the efficient inhibition of poliovirus and rhinovirus $2A^{pro}$ proteolytic activity in vitro by two such inhibitors, elastatinal and methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MPCMK). These inhibitors also reduced the titer of infectious virus particles produced in HeLa cells infected with poliovirus type 1 (Mahoney) [PV1(M)], HRV2, or CAV21. Their efficacies against these viral proteinases therefore indicate that the substrate-binding pocket of picornavirus 2A proteinases is likely to be structurally very similar to that of elastase and suggests a feasible approach to the design of specific substrate-based inhibitors directed against this group of viral proteinases.

MATERIALS AND METHODS

Genetic engineering of DNA. Restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs. *Taq* polymerase and reagents for the polymerase chain reaction (PCR) were purchased from Perkin-Elmer Cetus. The sequencing primer 5′ GATCAAGT TCTT 3′ [complementary to PV1(M) nucleotides (nt) 3956 to 3967 (24)], primers 5′-CCATACGTACATATGGATTCG GACAC-3′ and 5′-GGGAAGCTTCTATTGTTCCATGGCT TCTTC-3′ for PCR amplification of PV1(M) $2A^{pro}$ cDNA, and primers 5′-CCATGGCCAATGGTTTAGGACCTAGG TACGG-3′ and 5′-GGCCCATGGTGACTGTTCCTCTGC GATAGACTCC-3′ for PCR amplification of HRV14 $2A^{pro}$ cDNA were synthesized on an Applied Biosystems apparatus. DNA manipulations were done by standard procedures (40).

Plasmids. The vector pS32A3, which contains a cDNA segment encoding five amino acids of the L protein of encephalomyocarditis virus (EMCV), one amino acid of VP1, and the entire $2A^{pro}$ coding region of PV3 (Sabin) [PV3(S)] cloned downstream of a T7 promoter and nt 260 to 833 of the EMCV 5′ nontranslated region (5′NTR), has been described previously (22).

The vector pBS[VP1-Δ2AΔ2B(PV1M)] was constructed by ligating the smaller *Bst*EII-*Bst*EII fragment of pMN29 (34) with the larger *Bst*EII-*Bst*EII fragment of pBS^-(VP1-2ABΔ2C) (17) to allow transcription of mRNA encoding a PV1(M) polyprotein substrate containing a single $2A^{pro}$ cleavage site. This vector contained a cDNA fragment encoding all of VP1 and most of $2A^{pro}$ and 2B cloned downstream of a T7 promoter and nt 260 to 833 of the EMCV 5′NTR. $2A^{pro}$ was inactivated by deletion of nine amino acid residues near its carboxy terminus (50); an additional deletion at the carboxy terminus of 2B resulted in truncation of the potential open reading frame of the partial poliovirus polyprotein, so that processing by $2A^{pro}$ of this translation product yielded the cleavage products VP1 (ca. 33 kDa) and Δ2AΔ2B (ca. 26 kDa).

For transcription of mRNA encoding wild-type (wt) PV1(M) $2A^{pro}$ and a derivative thereof containing a Cys-109→Ala substitution, the vectors pBS[E2A] and pBS [E2A(C109A)] were constructed by ligation of the *Eco*RI-*Msc*I fragment of pS32A3 (22) and *Eco*RI-*Hin*dIII fragments of PCR products derived from either pT7XL or pBS^-[VP1-2ABΔ2C(C109A)] (17) as appropriate, between the *Eco*RI and *Hin*dIII sites of pBS^- (Stratagene, La Jolla, Calif.).

The vector pT7XL is derived from pT7PV1-5 (52) and contains the cDNA of the complete PV1(M) genome cloned downstream of a T7 promoter.

For transcription of mRNA encoding wt HRV14 $2A^{pro}$, the vector pBS[E2A(HRV14)] was constructed by ligation of the *Eco*RI-*Msc*I fragment of pS32A3 and the *Msc*I-*Nco*I fragment of the product derived by PCR amplification from pWR2C,173 (31) between the *Eco*RI and *Nco*I sites of pBS^-.

The vector pBS[VP1-2ABΔ2C(HRV14)] was constructed by ligating the *Msc*I-*Nco*I fragment (nt 2721 to 4304 of HRV14) derived from pWR2C,173 between the *Msc*I and *Nco*I sites of pBS[E2A(HRV14)] to allow transcription of mRNA encoding an HRV14 polyprotein substrate containing a single $2A^{pro}$ cleavage site. This vector contained a cDNA fragment encoding most of VP1, all of 2A and 2B, and part of 2C of HRV14 cloned downstream of a T7 promoter and nt 260 to 833 of the EMCV 5′NTR.

Cleavage and inhibitor assays in vitro. Plasmids pS32A3, pBS[VP1-Δ2AΔ2B(PV1M)], pBS[E2A], pBS[E2A(C109A)], and pBS[E2A(HRV14)] were linearized by digestion with *Dra*I, and pBS[VP1-2ABΔ2C(HRV14)] was linearized by digestion with *Hin*cII; all were transcribed in vitro with T7 RNA polymerase (a kind gift of J. Dunn, Brookhaven National Laboratory, Upton, N.Y.). Synthetic mRNA transcripts (0.25 μg/25 μl) were translated in a rabbit reticulocyte lysate (RRL) (Promega Biotec, Madison, Wis.) for 60 min at 30°C, in the presence of [^{35}S]methionine when indicated. Cleavage assays using [^{35}S]methionine-labelled polypeptide substrates and unlabelled $2A^{pro}$ derived by translation in vitro were done essentially as described previously (27). Inhibitors were preincubated with unlabelled $2A^{pro}$ for 15 min at 30°C, and a 10-μl aliquot of this mixture was then incubated with a 2-μl aliquot of the substrate for 120 min at 30°C. mRNA transcripts derived from pT7XL were translated in a HeLa cell extract prepared as described previously (33, 36). The inhibitors used in this study were leupeptin, iodoacetamide, antipain, aprotinin, *o*-phenanthroline, diisopropylfluorophosphate, ε-aminocaproic acid, soybean trypsin inhibitor (purchased from Sigma, St. Louis, Mo.), calpain inhibitor 1 (purchased from Calbiochem, San Diego, Calif.), MPCMK (purchased from Enzyme Systems Products, Dublin, Calif.), and elastatinal (purchased from Sigma and from Boehringer Mannheim, Indianapolis, Ind.).

Inhibitor assays in monolayer cell culture. HeLa R19 cells were maintained as monolayers in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) and were infected with PV1(M), the PV1(M) variant W1-P1/E/P2,3-1, HRV2, CAV21, or EMCV (a cardiovirus) at a multiplicity of infection of 25 PFU per cell. The sources of these virus

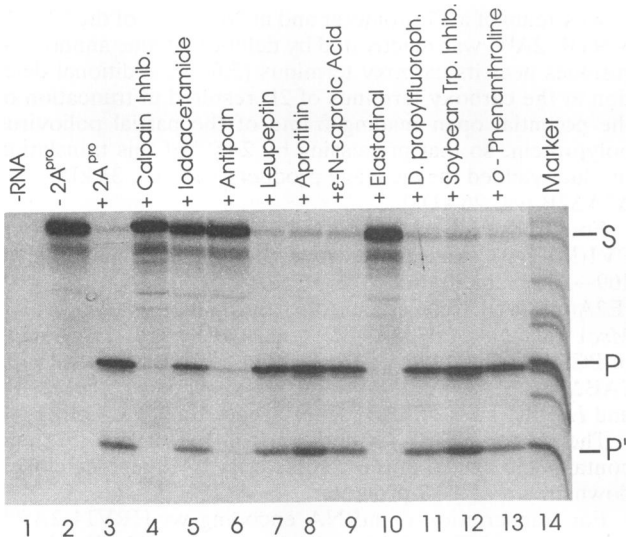


FIG. 1. Identification of inhibitors of the proteolytic activity of poliovirus $2A^{pro}$. mRNA transcripts derived from pBS$^-$[VP1-Δ2AΔ2B(PV1M)] that encode a partial poliovirus polyprotein were translated in vitro (lane 2) and incubated with unlabelled poliovirus $2A^{pro}$ in the absence (lane 3) or presence (lanes 4 to 13) of proteinase inhibitors as described in Materials and Methods. Lane 1 contains RRL incubated without exogenous mRNA, and the marker in lane 14 is a [^{35}S]methionine-labelled cell lysate prepared from HeLa cells infected with PV1(M). Proteinase inhibitors were added at the following final concentrations: calpain inhibitor 1, 250 μM; iodoacetamide, 2.5 mM; antipain, 500 μM; leupeptin, 500 μM; aprotinin, 5 U/ml; ε-aminocaproic (ε-N-caproic) acid, 5 mM; elastatinal, 250 μM; diisopropylfluorophosphate (D. propylfluoroph.), 500 μM; soybean trypsin (Trp.) inhibitor, 2 mM; *o*-phenanthroline, 1 mM. The primary translation product S (VP1-Δ2AB) and the two cleavage products P (VP1) and P′ (Δ2AB) are indicated on the right.

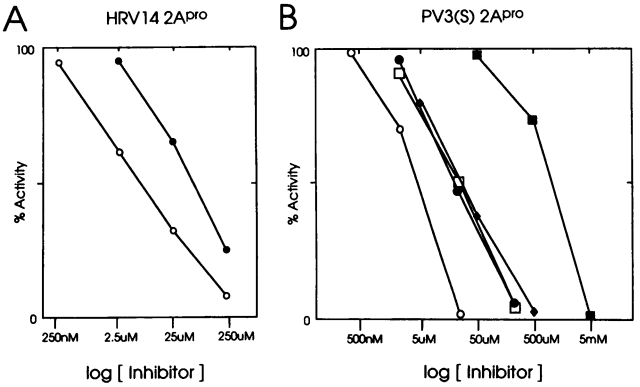


FIG. 2. Concentration-dependent inhibition of PV3(S) $2A^{pro}$ and HRV14 $2A^{pro}$ proteolytic activities by iodoacetamide, calpain inhibitor 1, antipain, elastatinal, and MPCMK. mRNA transcripts encoding a partial HRV14 polyprotein (ΔVP1-Δ2A) were translated in vitro and incubated with unlabelled HRV14 $2A^{pro}$ for an hour at 30°C (A), and those encoding a partial PV1(M) polyprotein (VP1-Δ2AΔ2B) were translated in vitro and incubated with unlabelled PV3(S) $2A^{pro}$ for an hour at 30°C (B) in the presence of iodoacetamide (■), calpain inhibitor 1 (□), antipain (◆), elastatinal (○), or MPCMK (●) at the indicated concentrations. The extents of cleavage were determined by scanning laser densitometry of autoradiographs and are shown as percentages of cleavage in the absence of inhibitor.

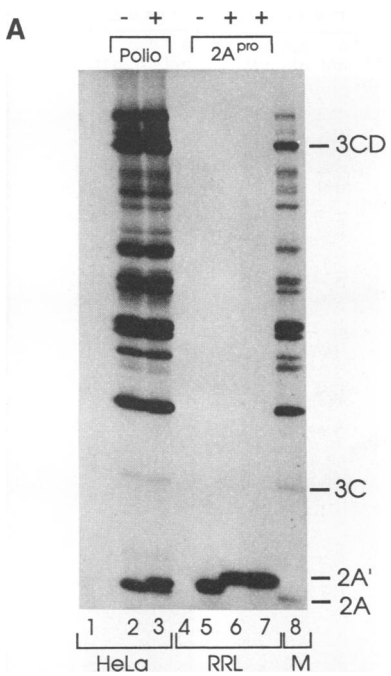
isolates have been described previously (9, 32). The culture medium was removed from cells by aspiration and was replaced with a 200-μl suspension of virus in phosphate-buffered saline (PBS) containing inhibitor and the appropriate solvent (MPCMK in a final concentration of 5% dimethyl sulfoxide [DMSO] or elastatinal in a final concentration of 3.3% ethanol). Cell monolayers were rocked gently on an oscillating shaker for 30 min at room temperature, the virus suspension was removed by aspiration, and the cell monolayers were washed twice with PBS before addition of 2.4 ml of culture medium (Dulbecco's modified Eagle's medium and 5% fetal calf serum) containing the appropriate concentrations of solvent and inhibitor. Cells infected with PV1(M), W1-P1/E/P2,3-1, or EMCV were incubated for 7 h at 37°C, and cells infected with HRV2 or CAV21 were incubated for 9 h at 35°C. Cells were then scraped from culture dishes and removed with culture medium, and virus was released by three cycles of freeze-thawing. Cell debris was removed by low-speed centrifugation, and the supernatant was stored as viral stock at −80°C. Virus titers were determined by plaque assay in 35-mm petri dishes under semisolid medium (0.9% agarose). Cells were incubated at 35 or 37°C, as appropriate, for 48 h before being stained with crystal violet (21). Protein synthesis in the presence of MPCMK was examined by labelling virus-infected HeLa R19 cells with a mixture of [^{35}S]cysteine and [^{35}S]methionine (TransLabel; ICN Biomedicals) essentially as described previously (30), except that actinomycin D was not included in the culture medium. MPCMK was present at concentrations as indicated above in a final concentration of 5% DMSO.

Gel electrophoresis of protein samples. Samples were prepared and electrophoresis was done by using 12.5% polyacrylamide–sodium dodecyl sulfate (SDS) gels as described previously (34). Gels containing products of translation in vitro were treated with En^{3}Hance (New England Nuclear Corp.). Kodak XAR-5 film was exposed to dried gels for 18 h. The extent of cleavage was quantitated by scanning X-ray films with an LKB laser densitometer.

RESULTS

Characterization of the proteolytic activity of poliovirus $2A^{pro}$. A number of proteinase inhibitors inhibited translation in RRL and could therefore not be used to investigate the intramolecular (*cis*) cleavage activity of $2A^{pro}$ in vitro (data not shown). An alternative approach was therefore developed to investigate intermolecular (*trans*) cleavage of the VP1-2A scissile bond (34): mRNA transcribed from the vector pBS$^-$[VP1-Δ2AΔ2B(PV1M)] was translated to yield a fragment of the poliovirus polyprotein (Fig. 1, lane 2). This substrate was efficiently cleaved at the VP1-2A site by exogenous $2A^{pro}$ to yield two products of the expected sizes (Fig. 1, lane 3).

The effects of 10 different proteinase inhibitors on the *trans* cleavage activity of $2A^{pro}$ were assayed (Fig. 1, lanes 4 to 13), resulting in the identification of four inhibitors of $2A^{pro}$. These were calpain inhibitor 1 (lane 4), iodoacetamide (lane 5), antipain (lane 6), and elastatinal (lane 10). The metalloproteinase inhibitor *o*-phenanthroline (lane 13) and the serine and thiol proteinase inhibitors leupeptin (lane 7), aprotinin (lane 8), ε-aminocaproic acid (lane 9), diisopropylfluorophosphate (lane 11), and soybean trypsin inhibitor (lane 12) had no effect on the activity of $2A^{pro}$. Inhibition of $2A^{pro}$ by *o*-phenanthroline and leupeptin has been reported elsewhere (25, 55), and we have no explanation for the lack



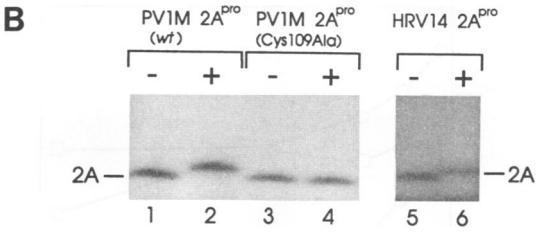


FIG. 3. Modification of poliovirus and HRV14 $2A^{pro}$ following incubation with elastatinal. (A) Elastatinal (250 μM) was added before (lane 6) or after (lanes 3 and 7) translation of mRNA transcripts derived from pT7XL translated in a HeLa cell lysate (lanes 2 and 3) or derived from pS32A3 and translated in RRL (lanes 5 to 7). Exogenous mRNA was not added to the HeLa cell lysate (lane 1) or to the RRL in lane 4. Elastatinal was not added to the translation mixtures in lanes 2 and 5. The marker (M) in lane 8 is a [^{35}S]methionine-labelled cell lysate prepared from HeLa cells infected with PV1(M). (B) mRNA transcripts encoding wt poliovirus $2A^{pro}$ (lanes 1 and 2), mutant (Cys-109→Ala) poliovirus $2A^{pro}$ (lanes 3 and 4), and wt HRV14 $2A^{pro}$ (lanes 5 and 6) were translated in RRL. Elastatinal (250 μM) was added to the translation mixtures shown in lanes 2, 4, and 6 after translation and was not included in the mixtures shown in lanes 1, 3, and 5.

of inhibition by these compounds in the experiments described here.

Titration of the effect of the four inhibitors calpain inhibitor 1, iodoacetamide, antipain, and elastatinal on the *trans* cleavage activity of poliovirus $2A^{pro}$ (Fig. 2B) indicated that of these, elastatinal was the most efficient inhibitor (50% inhibitory concentration [IC_{50}] = 4 μM), that antipain (IC_{50} = 23 μM) and calpain inhibitor 1 (IC_{50} = 28 μM) were less efficient, and that iodoacetamide (IC_{50} = 1.2 mM) was the least efficient inhibitor. Antipain strongly inhibits a range of different proteinases, including trypsin, papain, cathepsins A and B, and to a lesser extent, thrombokinase (1), while iodoacetamide is a general inhibitor of cysteine proteinases. Elastatinal is a 513-Da substrate mimetic with the structure *N*-(1-carboxyisopentyl)carbamoyl-α-(2-iminohexahydro-4-pyrimidyl)glycyl-glutaminyl-alinal. Inhibition of cellular proteinases by this compound is restricted to porcine pancreatic elastase (1). The efficient inhibition of poliovirus $2A^{pro}$ by elastatinal shown in Fig. 1 suggests that elastase and poliovirus $2A^{pro}$ have similar determinants of substrate binding. To investigate this relationship further, the effect of MPCMK (a highly specific peptide chloromethylketone inhibitor of human leukocyte elastase [38]) on the proteolytic activity of $2A^{pro}$ was assayed. MPCMK (IC_{50} = 20 μM) was a less efficient inhibitor than elastatinal of the proteolytic activity of poliovirus $2A^{pro}$ (Fig. 2B).

Inhibition of HRV14 $2A^{pro}$ by elastatinal and MPCMK. The sensitivity of poliovirus $2A^{pro}$ to inhibition by elastatinal and MPCMK was unexpected. Intermolecular cleavage by HRV14 $2A^{pro}$ was therefore also assayed to investigate whether this sensitivity to inhibition was restricted to poliovirus. Appropriate cloned cDNA segments encoding HRV14 $2A^{pro}$ and ΔVP1-2ABΔ2C were transcribed into mRNA and translated in RRL to yield unlabelled $2A^{pro}$ and the [^{35}S]methionine-labelled substrate ΔVP1-Δ2A, respectively (data not shown). Both elastatinal and MPCMK were effective inhibitors of the proteolytic activity of HRV14 $2A^{pro}$ in *trans* cleavage assays of the type described above (Fig. 2A). Elastatinal (IC_{50} = 7 μM) was a more efficient inhibitor than MPCMK (IC_{50} = 65 μM), and the effects of both on HRV14 $2A^{pro}$ activity were comparable to their effects on poliovirus $2A^{pro}$.

Modification of poliovirus and rhinovirus 2A proteinases by elastatinal and MPCMK. Incubation with elastatinal consistently resulted in a small decrease in the electrophoretic mobility of poliovirus $2A^{pro}$ (Fig. 3A, lane 6). This modification was not due to the failure of $2A^{pro}$ to cleave itself from the six amino acid residues that are present at its amino terminus upon translation of pS32A3 mRNA, since it also occurred when elastatinal was added to the RRL after translation had ceased (Fig. 3A, lane 7). This observation shows that the inhibitor must have interacted with the synthesized protein. The modification was also not restricted to serotype 3 of poliovirus, from which this $2A^{pro}$ species originated, since a similar modification of $2A^{pro}$ was apparent upon translation of genomic-length PV1(M) mRNA transcripts with subsequent addition of the inhibitor (Fig. 3A, lane 3). Altered electrophoretic mobility due to elastatinal was specific to $2A^{pro}$ and was not observed for $3C^{pro}$, the second proteinase encoded by poliovirus (Fig. 3A, lane 3). The electrophoretic mobility of HRV14 $2A^{pro}$ was modified by the addition of elastatinal after translation in a manner identical to that observed for the PV1(M) and PV3(S) $2A^{pro}$ moieties (Fig. 3B, lane 6).

The carboxy-terminal moiety of elastatinal is alinal (1), and by analogy with other aldehyde analogs of peptide substrates (47, 53) it is likely that it forms a covalent bond with the active site Cys-109 residue of poliovirus $2A^{pro}$. The small decrease in the electrophoretic mobility of $2A^{pro}$ after incubation with the inhibitor is consistent with covalent linkage to a small peptide moiety. To eliminate the possibility that elastatinal was linked to a residue other than Cys-109, RNAs encoding wt PV1(M) $2A^{pro}$ and $2A^{pro}$ containing a Cys-109→Ala substitution RNA (17) were translated in RRL in the presence or absence of elastatinal (Fig. 3B). Elastatinal caused a decrease in the electrophoretic mobility of wt $2A^{pro}$ (Fig. 3B, lane 2) but not of the Cys-109→Ala mutant (lane 4).

Inhibitory effects of MPCMK on the growth of enteroviruses

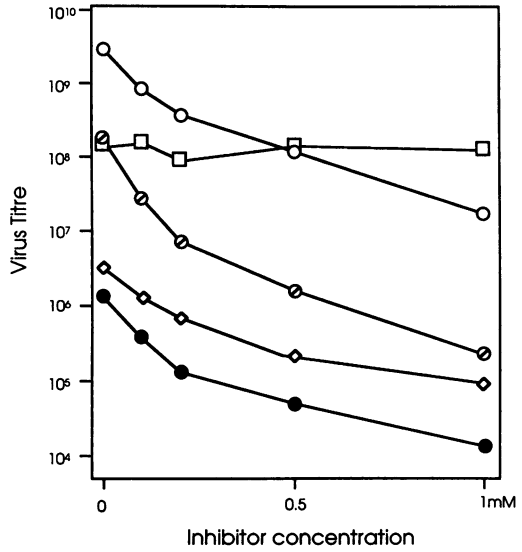


FIG. 4. Effect of MPCMK on virus yield. HeLa R19 cells were infected with EMCV (□), CAV21 (◇), HRV2 (●), PV1(M) (○), and a dicistronic derivative W1-P1/E/P2,3-1 (ϕ) at a multiplicity of infection of 25 PFU per cell for 30 min in the presence of 5% DMSO and the indicated concentration of MPCMK. Cell monolayers were washed twice with PBS and were then incubated for 7 h at 37°C [EMCV, PV1(M), and W1-P1/E/P2,3-1] or for 9 h at 35°C (HRV2 and CAV21) in culture medium containing 5% DMSO and the indicated concentration of MPCMK. Viral titers in the combined cell and culture medium fractions were determined by plaque assay.

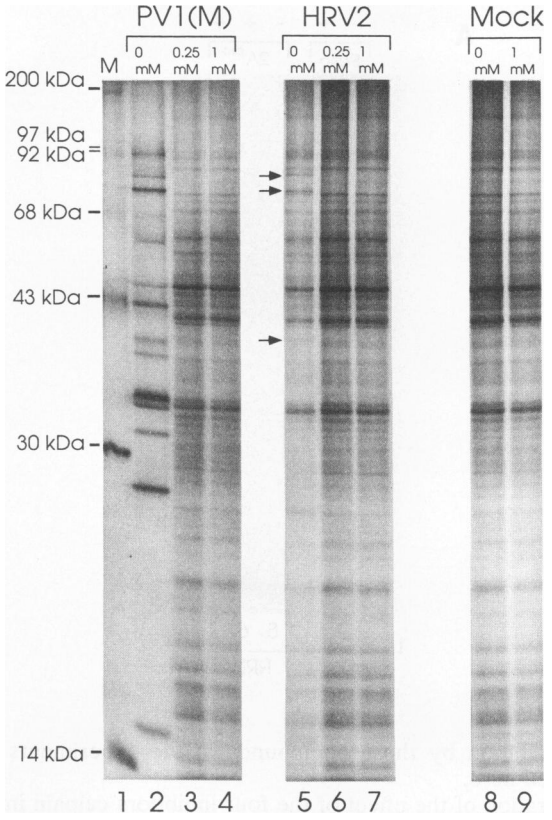


FIG. 5. Protein synthesis in uninfected and virus-infected HeLa R19 cells in the absence and presence of MPCMK. Mock-infected HeLa R19 cells (lanes 8 and 9) and HeLa R19 cells infected with PV1(M) (lanes 2 to 4) or HRV2 (lanes 5 to 7) were incubated in the presence of 5% DMSO and without MPCMK (lanes 2, 5, and 8), with 0.25 mM MPCMK (lanes 3 and 6), or with 1 mM MPCMK (lanes 4, 7, and 9) and were radiolabelled with TransLabel (ICN Biochemicals) 3 h postinfection. Proteins in cell extracts were resolved by electrophoresis in a 13% polyacrylamide–SDS gel, which was dried and exposed to X-ray film at −80°C for 18 h. The molecular sizes of marker (M) proteins (lane 1) are shown on the left, and HRV2-encoded polypeptides are indicated by arrowheads between lanes 4 and 5.

and rhinoviruses in HeLa R19 cells. Monolayers of HeLa R19 cells were infected with CAV21, HRV2, PV1(M), or a dicistronic derivative of PV1(M), W1-P1/E/P2,3-1, in the absence or presence of MPCMK. In W1-P1/E/P2,3-1, the poliovirus polyprotein is interrupted between the P1 and P2-P3 regions by insertion of the EMCV internal ribosomal entry site (32). Since the polyprotein is genetically interrupted between VP1 and 2A, the requirement for the primary role of 2A in separating the P1 capsid protein precursor from the nascent polypeptide has been abolished. Cells and medium were harvested at either 7 or 9 h postinfection, and virus yield was determined by plaque assay (see Materials and Methods). The yield of each of these viruses was unaffected by the presence of 5% DMSO but was progressively reduced by the additional inclusion of increasing concentrations of MPCMK in the culture medium (Fig. 4). The reduction in titer was between 100- and 1,000-fold in the presence of 1 mM MPCMK for each of these viruses as well as for PV2(S), PV3 (Leon), and HRV14 (data not shown). Growth of PV1(M) was reduced 10-fold in the presence of 0.5 mM elastatinal in the culture medium (data not shown). As a control, HeLa R19 cells were also infected with EMCV in the absence or presence of MPCMK and/or DMSO. The yield of EMCV was not affected by the presence of MPCMK in the culture medium (Fig. 4). EMCV is a picornavirus of the *Cardiovirus* genus and was used as a control because it encodes a 2A polypeptide that is wholly unrelated to the 2A proteinases of rhinoviruses and enteroviruses. The inhibitory effects of MPCMK on enterovirus and rhinovirus growth in HeLa cells thus are not due to impairment of cellular function but result directly from interference with one or more aspects of virus replication. As the proteolytic activity of $2A^{pro}$ was shown above to be inhibited by these compounds in vitro (Fig. 1), this polypeptide is therefore a likely target for inhibition in vivo.

The effects of MPCMK on the expression of PV1(M), CAV21, and HRV2 proteins in HeLa cells were investigated by SDS-polyacrylamide gel electrophoresis analysis of radioactively labelled proteins that had been synthesized in virus-infected and mock-infected cells. MPCMK did not affect the pattern of cellular protein synthesis at the concentrations tested (Fig. 5, lanes 8 and 9). The extent of host cell shutoff induced upon infection with HRV2 and CAV21 was less severe than that which resulted from poliovirus infection. The virus-induced shutoff of host cell protein synthesis in cells infected with PV1(M) and HRV2 (Fig. 5, lanes 2 to 4 and 5 to 7, respectively) was inhibited in the presence of 0.25 mM MPCMK, and synthesis of viral proteins was not apparent against the background of cellular proteins. Similar results were obtained for CAV21 and PV2(S) (data not shown). We have not been able to determine whether the failure to induce shutoff by these viruses in cell culture results from inhibition of $2A^{pro}$ or from direct inhibition of the induced activity that is responsible for cleavage of p220.

These two proteolytic activities are both (independently) inhibited by elastatinal (data not shown) in in vitro assays of a type described elsewhere (27).

DISCUSSION

Poliovirus and rhinovirus 2A proteinases are thought to be structurally related to trypsin-like serine proteinases but to differ significantly from the cellular enzymes in having a cysteine residue in place of the serine component of the catalytic triad (3). This proposed substitution is supported by the results of mutational analysis (17, 43, 55) and is consistent with the inhibition of the proteolytic activity of the 2A proteinases by the alkylating agent iodoacetamide reported here and elsewhere (25, 55). In contrast to this general inhibitor of cysteine proteinases, elastatinal and MPCMK are highly specific inhibitors of mammalian elastases and inhibit other serine proteinases, such as trypsin, chymotrypsin, and cathepsin G, only weakly or not at all (1, 38, 39). The specificities of these two inhibitors derive from their close correspondence to the structure and conformation of elastase substrates and, consequently, their high affinities for the extended substrate-binding sites of these enzymes. Inhibition of the 2A proteinases encoded by PV1(M), PV3(S), and HRV14 by elastatinal and MPCMK was therefore unexpected. It suggests that the substrate-binding pockets of these viral enzymes closely resemble those of mammalian elastases. The interactions between the extended substrate-binding pocket and substrate residues have been determined at atomic resolution for several elastase-inhibitor complexes (7) and may be useful for deriving a model of the interaction between viral 2A proteinases and their substrates.

2A proteinases cleave only one or two sites within the ca. 250-kDa viral polyprotein (8, 15, 26, 30, 50), indicating that substrate recognition by these enzymes is highly discriminatory. Residues at positions P4, P2, P1, and P1′ of the VP1-2A cleavage site (nomenclature of Berger and Schechter [4]) are conserved in over 60 isolates of poliovirus (19), and a similar pattern is evident in all other entero- and rhinoviruses (17). The results of mutational analysis of residues at these positions suggest that 2A proteinases recognize an extended series of subsites that together define a cleavage site (19, 30, 41). The rate of hydrolysis by elastase is also highly dependent on substrate length (45, 46, 49) and parallels the length dependence of elastase inhibition by peptide chloromethylketones (38, 39, 48). The modes of productive binding of elastase with peptide substrates and with peptide chloromethylketone inhibitors are equivalent, and such inhibitors have therefore been used in the detailed characterization of the elastase substrate-binding site. The effective inhibition of PV1(M), PV3(S), and HRV14 2A proteinases by MPCMK suggests that a similar characterization of these and other 2A proteinases is now feasible, by using a panel of peptide chloromethylketone derivatives that differ in composition and length.

Synthetic neutrophil elastase inhibitors are potential therapeutic agents in the treatment of emphysema (12, 37, 42, 51), and we consider it possible that related inhibitors may find a role in the treatment of diseases caused by enteroviruses and rhinoviruses. We have shown that relatively high concentrations of MPCMK inhibited the growth of several members of these two picornavirus genera in tissue culture. In contrast, the inhibitor had no effect on the growth of the cardiovirus EMCV, which does not encode a serine protease-like $2A^{pro}$. Inhibition is thus selective and does not result from impairment of cellular functions required to support viral replication. The primary role of $2A^{pro}$ is the intramolecular cleavage of the P1 capsid protein precursor from the P2-P3 nonstructural protein precursor, but it plays several other roles that are advantageous to, and may be essential for, virus replication. These roles include mediating the shutoff of host cell protein synthesis (27), transactivation of viral translation (13), and an additional undefined role in viral growth (33a). The importance of these additional roles is emphasized by the inhibition of the growth of the poliovirus W1-P1/E/P2,3-1 by MPCMK. In this dicistronic virus, the requirement for the primary role of $2A^{pro}$ has been abolished following genetic separation of the P1 and P2-P3 coding regions. All of the functions of $2A^{pro}$ other than this cleavage may be inhibited by MPCMK, a possibility which could account for the observed inhibition of the shutoff and the reduction in viral protein synthesis in virus-infected cells. The availability of specific $2A^{pro}$ inhibitors and of genetically distinct 2A mutants (5, 33, 33a, 35) will facilitate dissection of these various functions of $2A^{pro}$.

ACKNOWLEDGMENTS

We thank J. Dunn for the T7 polymerase, S. K. Jang and R. Rueckert for the gifts of plasmids pS32A3 and pWR2C,173, respectively, P. Kissel for oligonucleotide synthesis, and C. Helmke for photography.

This work was supported in part by Public Health Service grants AI-15122 and CA-28146 to E.W. from the National Institutes of Health.

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