

Nitric oxide inhibits the adenovirus proteinase in vitro and viral infectivity in vivo

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ABSTRACT

Nitric oxide (NO) is an antiviral effector of the innate immune system, but few of the viral targets of NO have been identified. We now show that NO inhibits adenovirus replication by targeting the adenovirus proteinase (AVP). NO generated from diethylamine NONOate (DEA-NONOate) or spermine NONOate (Sp-NONOate) inhibited the AVP. Inhibition was reversible with dithiothreitol. The equilibrium dissociation constant for reversible binding to the AVP by Sp-NONOate, or K_i , was 0.47 mM, and the first-order rate constant for irreversible inhibition of the AVP by Sp-NONOate, or k_i , was 0.0036 s^{-1} . Two hallmarks of a successful adenovirus infection were abolished by the NO donors: the appearance of E1A protein and the cleavage of cytokeratin 18 by AVP. Treatment of infectious virus by DEA-NONOate dramatically decreased viral infectivity. These data suggest that NO may be a useful antiviral agent against viruses encoding a cysteine proteinase and in particular may be an antiadenovirus agent.

Key words: S-nitrosylation • antiviral agent • proteinase inhibitor • cysteine proteinase • diethylamine NONOate

The adenovirus proteinase (AVP) is a potential target for antiviral therapy. Adenovirus with a temperature-sensitive mutation in the proteinase is not infectious when grown at the nonpermissive temperature (1). One function of AVP is to cleave virion precursor proteins after virion assembly. Only then does the virus become infectious (2). A second function is to destroy the cytoskeleton late in infection to facilitate cell lysis (3–5).

AVP is unusual in that it requires cofactors for maximal enzyme activity. One cofactor is pVIc, the 11-amino acid residue peptide from the C terminus of adenovirus precursor protein pVI (6–8). The penultimate amino acid residue in pVIc forms a disulfide bond with Cys104 of AVP (9, 10). A second cofactor is the viral DNA (6, 11). A third, nonviral, cytoplasmic cofactor is actin (5). In vitro, actin stimulates AVP as does pVIc. AVP alone cannot cleave cytokeratin 18, but in the presence of actin, AVP can cleave cytokeratin 18. Furthermore, actin itself is cleaved by AVP. Thus, actin can be a cofactor for its own destruction. Because the three cofactors act at

different times and places in infected cells, administration of antiviral agents at these specific times and places will have different effects.

AVP is the first member of a new class of cysteine proteinases (12). Comparison of the crystal structure of papain (13–15) with that of an AVP-pVIc complex (9) revealed that the crucial amino acid residues involved in catalysis are in identical positions; the amino acid residues in papain are Cys25, His159, Asn175, and Gln19 and those in AVP are Cys122, His54, Glu71, and Gln115.

In this study, we explored nitric oxide (NO) as a potential antiviral agent. NO is an effector of the innate immune system that inhibits the replication of various viruses (16–23). NO inhibits coxsackievirus B3 replication in part by nitrosylating the nucleophilic cysteine residue in the active site of the viral proteinase (24). Here, NO was shown to inhibit AVP both in vitro and in vivo. In vitro, the equilibrium dissociation constant for the reversible binding of spermine NONOate (Sp-NONOate) to AVP and the first-order rate constant for irreversible inhibition of AVP by Sp-NONOate were measured. In vivo, treatment of infectious virus by diethylamine NONOate (DEA-NONOate) or Sp-NONOate dramatically decreased viral infectivity.

MATERIALS AND METHODS

pVIc was purchased from Research Genetics (Huntsville, AL). DEA-NONOate and Sp-NONOate were purchased from Cayman Chemical (Ann Arbor, MI). Stock solutions were freshly prepared in 0.01 N NaOH. The fluorogenic substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine was synthesized and purified as described previously (6, 25). AVP was purified as described previously (6, 26). The computer program Table Curve was from Jandel (Chicago, IL).

Formation of AVP-pVIc complexes

The covalent complex AVP-pVIc, in which a disulfide bond is formed between Cys104 of AVP and Cys10' of pVIc, was prepared by incubating 100 μM AVP with 150 μM pVIc for 16 h at 4°C in 20 mM Tris-HCl (pH 8.0), 5 mM NaCl, 0.1 mM EDTA, and 1 mM octylglucoside.

Assay for proteinase activity

Assays, in 1 ml, contained 10 mM Tris (7.5), 2 mM octylglucoside, 100 mM NaCl, 50 nM AVP-pVIc complex, 5 μM (Leu-Arg-Gly-Gly-NH)₂-rhodamine, and indicated concentrations of spermine, Sp-NONOate, diethylamine, or DEA-NONOate. Assay mixtures containing diethylamine or DEA-NONOate were preincubated for 10 min at 37°C, and assay mixtures containing spermine or Sp-NONOate were preincubated for 30 min at 37°C, before the addition of substrate. The preincubation allowed for the binding equilibrium to be reached and for the maximal release of NO. The increase in fluorescence was monitored as a function of time in a PTI spectrofluorometer (Photon Technology International, London, Ontario, Canada). The excitation wavelength was 492 nm and the emission wavelength 523 nm, with a bandpass of 8 nm.

Protein concentration

Protein concentration was determined by using the BCA protein assay kit from Pierce Chemical (Rockford, IL). The concentration of AVP was also determined by using a calculated molar absorbance coefficient at 280 nm of $26,510 \text{ M}^{-1}\text{cm}^{-1}$ (27).

Cell culture and virus infection

HeLa cells were cultured in modified Eagle's medium (MEM) with 10% heat-inactivated FBS, penicillin, and streptomycin. The cells were plated in six-well plates 1 day before the experiment. They were infected with virus after 80–90% confluence had been reached. Wild-type adenovirus (Ad5) was treated with various amounts of DEA-NONOate for 10 min or with Sp-NONOate for 30 min, or with vehicle (0.01 N NaOH) at 37°C. The cells were infected with virus at a multiplicity of infection (MOI) of 100 in MEM with 0.2% FBS for 3 h, and they were then washed and fed with MEM and 10% FBS.

Assay of virus titers

HeLa cells were infected at an MOI of 10 with wild-type adenovirus (Ad5) that was pretreated with various amounts of DEA-NONOate, as described above. Forty-eight hours after infection, Ad5 infectivity was measured with the AdenoX Rapid titer kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Plaques were counted from at least three fields from each well.

NO inhibition of AVP cleavage of cytokeratin 18

HeLa cells were infected with Ad5 as described above and harvested 16 h after infection. Cell lysates were prepared and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was immunoblotted with antibody to adenovirus E1A protein or to cytokeratin 18 (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

NO inhibited AVP-pVIc

To determine whether AVP-pVIc is inhibited by DEA-NONOate and Sp-NONOate, 50 nM AVP-pVIc was incubated with three different concentrations of DEA-NONOate for 10 min or three different concentrations of Sp-NONOate for 30 min. The fluorogenic substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine was then added, and the rate of substrate hydrolysis was measured ([Fig. 1](#)). Both compounds inhibited AVP-pVIc in proportion to their concentrations. DEA-NONOate inhibited AVP-pVIc by 3% at a concentration of 0.1 μM, by 75% at 10 μM, and by 94% at 10 mM. Sp-NONOate inhibited AVP-pVIc by 7% at a concentration of 0.1 μM, by 83% at 10 μM, and by 91% at 10 mM.

Dithiothreitol (DTT) reversed NO inhibition of AVP-pVIc

If NO inhibits AVP-pVIc by S-nitrosylation, presumably of the active-site nucleophile Cys122, then the presence of DTT should reverse the covalent modification. To test this hypothesis, 50

nM AVP-pVIc was incubated with or without 0.01 mM DEA-NONOate for 10 min. Then, (Leu-Arg-Gly-Gly-NH)₂-rhodamine was added, and the increase in fluorescence was recorded as a function of time. After 5 min, 1 mM DTT was added and monitoring of the rate of substrate hydrolysis resumed. The results showed that DTT could indeed reverse the inhibition of AVP-pVIc by DEA-NONOate (Fig. 2). Compared with the control reaction with no DEA-NONOate, the rate of the reaction incubated with DEA-NONOate was much lower. When DTT was added to the reaction incubated with DEA-NONOate, the reaction rate increased to approach the slope of the control reaction.

Inhibition of AVP-pVIc was caused by NO, not by vehicle

The inhibitory effect on AVP-pVIc produced by the NO generators DEA-NONOate and Sp-NONOate was due to NO generation and not the presence of diethylamine or spermine. This was shown by incubating 50 nM AVP-pVIc with substrate and monitoring the rate of substrate hydrolysis for 5 min, adding 0.01 mM diethylamine or spermine, and then resuming the monitoring of the rate of substrate hydrolysis. No change in the rate of substrate hydrolysis was observed by the presence of either diethylamine or spermine (data not shown).

Inhibition of AVP-pVIc activity by NO was not an artifact caused by an alteration of substrate or product by NO

Can an NO donor alter the fluorescence that is generated after substrate cleavage? To answer this question, 5 nM AVP-pVIc was incubated with (Leu-Arg-Gly-Gly-NH)₂-rhodamine for 30 min. Then, 5 mM iodoacetamide was added to inhibit the enzyme. Addition of 1 mM DEA-NONOate or Sp-NONOate caused no change in the fluorescence of the Leu-Arg-Gly-Gly-NH-rhodamine that had been generated (data not shown).

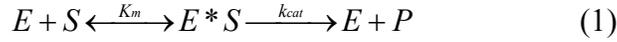
Can an NO donor alter the fluorogenic substrate such that it cannot be cleaved by the enzyme? When 5 μM (Leu-Arg-Gly-Gly-NH)₂-rhodamine was incubated with 10 mM DEA-NONOate for 30 min followed by addition of 1 mM DTT and 50 nM AVP-pVIc, the rate of substrate hydrolysis was unaffected by preincubation of the substrate with DEA-NONOate (data not shown). Thus, DEA-NONOate did not alter the ability of the fluorogenic substrate to be cleaved by AVP-pVIc.

Quantitative characterization of the inhibition of AVP-pVIc by Sp-NONOate

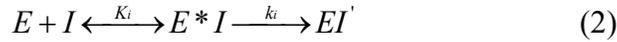
Sp-NONOate was predicted to inhibit AVP-pVIc by S-nitrosylating the nucleophilic sulfur atom of Cys122. The data presented thus far are consistent with this hypothesis. A competition experiment was performed to show unequivocally that the inhibition of AVP-pVIc by Sp-NONOate is irreversible. The competition experiment allows one to measure the equilibrium dissociation constant for the reversible binding of Sp-NONOate to AVP as well as the first-order rate constant for irreversible inhibition of AVP-pVIc by Sp-NONOate. At zero time, 50 nM AVP-pVIc and 0.2, 0.4, 0.6, or 3 mM Sp-NONOate were added simultaneously to 5 μM substrate, and the amount of substrate hydrolyzed as a function of time was measured (Fig. 3). If competitive reversible inhibition had occurred, the rate of substrate hydrolysis would be constant and proportional to the Sp-NONOate concentration. It was not. Instead, the rate of substrate hydrolysis decreased as a function of time, eventually reaching zero. The amounts of substrate

hydrolyzed when the rate of substrate hydrolysis was zero were proportional to the concentrations of Sp-NONOate (Fig. 3). These data are hallmarks of competitive, irreversible inhibition.

A formal analysis has been derived to show how data from the competition experiment (Fig. 3) can be used to calculate the equilibrium dissociation constant for reversible binding and the first-order rate constant for irreversible inhibition (28). The analysis is based on two competing reactions:



where E is the enzyme; S is the substrate; $E * S$ is the adsorptive enzyme-substrate complex; P is the product; K_m is the Michaelis constant; and k_{cat} is a first-order rate constant. The interaction between AVP-pVIc and Sp-NONOate may be characterized by



where I is an irreversible inhibitor; $E * I$ is an adsorptive enzyme-inhibitor complex; EI' is a stable enzyme-inhibitor complex; K_i is an equilibrium dissociation constant; and k_i is a first-order rate constant.

Analysis of the competition defined by equations 1 and 2 indicated that the amount of product formed as a function of time should obey the following equation:

$$P = \frac{k_{cat}[E]_0}{qb} (1 - e^{-bt}) \quad (3)$$

where q and b are defined as

$$q = (K_m/[S]_0 + (K_m[I]_0/K_i[S]_0) + 1 \quad (4)$$

$$b = k_i/r \quad (5)$$

and r is

$$r = (K_i[I]_0 + (K_i[S]_0/K_i[I]_0) + 1 \quad (6)$$

with K_i being the equilibrium dissociation constant for reversible binding, k_i being the first-order rate constant for irreversible inhibition, and $[E]_0$, $[I]_0$, and $[S]_0$ being the initial concentrations of enzyme, inhibitor, and substrate, respectively.

Before the individual kinetic constants K_i and k_i could be calculated, values for the pseudo-first-order rate constant b in equation 3 had to be determined. After importing the data (Fig. 3) into the program Table Curve, a plot of the instantaneous velocity vs. time was made (Fig. 4A). Taking the natural logarithm of both sides of equation 3 yields

$$\ln\left(\frac{dP}{dt}\right) = -bt + \ln\left(\frac{k_{cat}[E]_0}{q}\right) \quad (7)$$

A plot of the natural logarithm of the instantaneous velocity, $\ln(\text{rate of substrate hydrolysis})$, vs. t (seconds) is shown ([Fig. 4B](#)). This generated a series of straight lines with slopes of $-b$.

The individual values for k_i and K_i were obtained by using the following equation:

$$b = \left(\frac{k_i[I]_0}{K_i \left(1 + \left(\frac{[S]_0}{K_m} \right) + [I]_0 \right)} \right) \quad (8)$$

which predicts that a plot of b vs. $[I]_0$ will yield a rectangular hyperbola that is similar in form to the Michaelis-Menten equation ([Fig. 5](#)). The K_m used was $3.7 \mu\text{M}$ (data not shown). These data show that Sp-NONOate inhibited AVP-pVIc with a K_i of $0.47 \pm 0.083 \text{ mM}$, and a k_i of $0.0036 \pm 0.0002 \text{ s}^{-1}$.

NO inhibited AVP activity in infected cells

Although NO clearly inhibited AVP-pVIc in vitro, does NO incubated with adenovirus before infection of HeLa cells affect AVP activity in vivo, within infected cells? One indication of an active AVP within adenovirus-infected HeLa cells is cleavage of cytokeratin 18 (3, 5). Accordingly, adenovirus, pretreated with 10 mM DEA-NONOate for 10 min or with 10 mM Sp-NONOate for 30 min, was used to infect HeLa cells at an MOI of 100. After 16 h, the cells were lysed, the lysates were fractionated by SDS-PAGE, and the gel was immunoblotted with an antibody to cytokeratin 18.

The results showed that incubation of adenovirus with NO before infection of HeLa cells resulted in inhibition of AVP activity within the infected cells ([Fig. 6](#)). As expected, cytokeratin 18 was cleaved in cells after infection with untreated virus or with virus treated with vehicle alone ([Fig. 6](#)). Cytokeratin 18 was not cleaved in uninfected cells ([Fig. 6](#)). In contrast, AVP cleavage of cytokeratin 18 was blocked by pretreatment of adenovirus with NO ([Fig. 6](#)).

NO inhibited adenovirus infectivity

Although treatment of adenovirus with NO before infection of HeLa cells clearly inhibited the cleavage of cytokeratin 18 by AVP, does such treatment by NO also alter the expression of adenovirus proteins in infected HeLa cells? One of the major viral proteins to appear early in an adenovirus infection is the E1A protein. Its presence can be used as a hallmark of a successful early infection of HeLa cells by adenovirus. In uninfected HeLa cells, E1A was absent in the immunoblot ([Fig. 7](#), lane 1). In cells infected by adenovirus ([Fig. 7](#), lane 2) or in cells infected by adenovirus treated with 0.01 N NaOH ([Fig. 7](#), lane 3), the E1A protein was clearly present. However, E1A was not expressed in cells infected by virus pretreated with DEA-NONOate or Sp-NONOate ([Fig. 7](#), lanes 4 and 5).

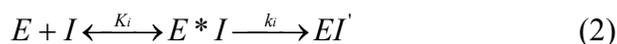
That E1A could not be observed in cells infected by adenovirus pretreated with DEA-NONOate or Sp-NONOate implied that these NO donors may be reducing the infectivity of the virus. Were other adenovirus proteins present in those cells? To answer this question, instead of using anti-E1A antibody for immunoblotting of the gel (Fig. 7, Immunoblot), the gel was stained with Coomassie blue (Fig. 7, Coomassie blue stain). The pattern of proteins observed also implied that in HeLa cells infected with adenovirus pretreated with NO, no expression of adenovirus-specific proteins could be observed. The results of this experiment plus those with cytokeratin 18 and E1A all implied that virus treated with NO lost its infectivity.

The effect of treating purified virus with NO on the infectivity of that virus was assessed directly. Purified adenovirus was incubated with 0.1 or 10 mM DEA-NONOate for 10 min. The infectivity of the virus was then measured in the plaque-forming assay. Pretreatment of adenovirus with NO decreased viral infectivity (Fig. 8). Treatment with 0.1 mM DEA-NONOate diminished the infectivity by 33%; treatment with 10 mM DEA-NONOate decreased the infectivity by 67%.

DISCUSSION

The major finding of this study is that NO inhibited the AVP. In addition, DTT reversed NO inhibition of AVP-pVIc. The equilibrium dissociation constant for the reversible binding of Sp-NONOate to AVP-pVIc and the first-order rate constant for irreversible inhibition of AVP-pVIc by Sp-NONOate were measured. Finally, treatment of infectious virus by DEA-NONOate or Sp-NONOate dramatically decreased viral infectivity. Consistent with this decrease in infectivity, two hallmarks of a successful adenovirus infection were abolished—the appearance of the E1A protein and the cleavage of cytokeratin 18 by AVP.

The equilibrium dissociation constant for the reversible binding of Sp-NONOate to AVP-pVIc and the first-order rate constant for irreversible inhibition of AVP-pVIc by Sp-NONOate were obtained from a competition experiment in which AVP-pVIc and Sp-NONOate were simultaneously added to a solution of substrate. If a substrate molecule had successfully competed with a Sp-NONOate molecule, a molecule of (Leu-Arg-Gly-Gly-NH)₂-rhodamine would have been cleaved to Leu-Arg-Gly-Gly-NH-rhodamine. If Sp-NONOate had successfully competed with a substrate molecule, the molecule of AVP-pVIc to which Sp-NONOate was bound would have become irreversibly inactivated. From an analysis of the data, the inhibition reaction mechanism was shown to be



If the inhibition reaction mechanism involved one step:



the data (Fig. 5) would conform to a straight line rather than an hyperbola (28).

Recently, with the computer docking program EUDOC (29), in silico screening of a chemical database for inhibitors of AVP identified 2,4,5,7-tetranitro-9-fluorenone (30). This compound inhibited AVP-pVIc selectively and irreversibly with a two-step reaction, reversible binding (K_i

= 3.09 μM) followed by irreversible inhibition ($k_i = 0.006 \text{ s}^{-1}$). The reversible binding is due to molecular complementarity between the inhibitor and the active site of AVP, which confers the selectivity of the inhibitor. The K_i for NO, 3.7 μM , is similar to that of 2,4,5,7-tetranitro-9-fluorenone, but the k_i for NO is 30-fold less than that for 2,4,5,7-tetranitro-9-fluorenone. Irreversible inhibition by 2,4,5,7-tetranitro-9-fluorenone is due to aromatic nucleophilic substitution of a nitro group of the inhibitor by the nearby Cys122 in the active site of AVP. The mechanism of inhibition is different compared with the S-nitrosylation of the sulfur atom of a cysteine residue by NO donors.

In AVP, there is some selectivity in S-nitrosylation over denitrosylation in that there appears to be a consensus S-nitrosylation motif (31). In hemoglobin, Cys β 93 can be nitrosylated; it is preceded by the basic amino acid histidine and followed by an acidic aspartate residue. In three-dimensional space, the sulfur in Cys β 93 is adjacent to the histidine in the R (relaxed) conformation and adjacent to the aspartate in the T (tense) conformation. The R conformation has the high affinity for oxygen and a conformation that facilitates base-catalyzed S-nitrosylation (31, 32). The T conformation has the low affinity for oxygen and a conformation that promotes denitrosylation. The structure of AVP-pVIc promotes S-nitrosylation, not denitrosylation, in that the sulfur of Cys122 is 3.87 Å from the N δ 1 atom of His54 (9). Glu71 is much farther away; the sulfur of Cys122 is 7.72 Å from the O ϵ 2 atom of Glu71.

Treatment of purified adenovirus with NO considerably diminished its capacity to successfully infect cells. The absence of E1A expression indicated that treatment with NO blocked an event early in virus infection. The data also implied that active AVP is required early in adenovirus infection, otherwise E1A would be expressed. There is evidence that AVP is required to degrade the capsid-stabilizing protein VI and that if this protein is not degraded early in infection the virus cannot uncoat at the nuclear membrane (33). The absence of cleavage of cytokeratin 18 in cells infected with NO-treated virus is consistent with an unsuccessful infection. Taken together, these two experiments implied that treatment of adenovirus with NO severely diminished the infectivity of the virus, and this was shown to be the case. After treatment with 10 mM Sp-NOOate, the titer of the virus decreased by more than 67%.

There is evidence that adenovirus can suppress production of NO in vivo by reducing expression of the inducible nitric oxide synthase (NOS2) (34). Adenoviral inhibition of NOS2 is mediated by the N terminus, CR1, and CR2 domains of the early adenovirus protein E1A. E1A inhibits NOS2 directly or indirectly by suppressing NOS2 transcription.

How did one-third of the virus escape inhibition of infectivity by NO? Possibly, the proteinase in those virions was oxidized and was therefore insensitive to NO. During virion assembly, maturation of the viral precursor proteins by AVP occurs in the reducing environment of the cell nucleus (33). However, after release of infectious virions from the cells, the oxidizing extracellular environment may cause AVP to become inactivated by the oxidation of Cys122. When reinfesting cells, the proteinase may become reactivated by being in the reducing environment of the endosome or cytosol.

The data presented here imply that NO can be an antiadenovirus agent. To be most effective, it should be synthesized in infected cells or macrophages adjacent to infected cells before virion assembly. If NO is produced on virion entry, it will prevent uncoating of the virus at the nuclear

membrane and abort the infection; if NO is produced 16 h after infection, it will prevent AVP from degrading the cytoskeleton, thereby preventing cell lysis and the release of nascent virus particles; and if NO is produced during virion assembly, it will prevent processing of the virion precursor proteins and therefore the synthesis of infectious virus.

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Fig. 1

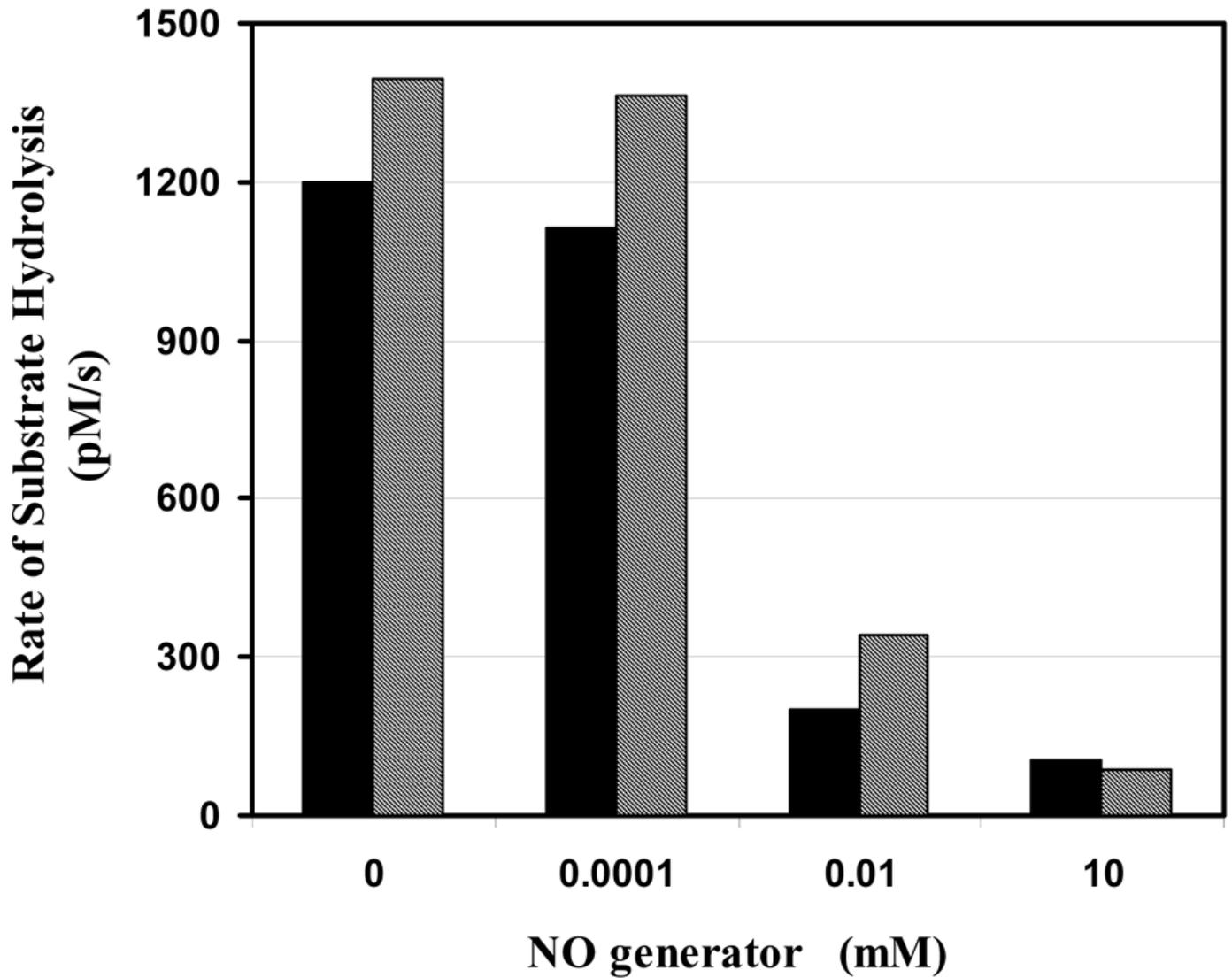


Figure 1. NO inhibition of AVP-pVIc. Recombinant AVP-pVIc, 50 nM, was incubated with the indicated concentrations of DEA-NONOate (striped bars) for 10 min or with Sp-NONOate (black bars) for 30 min. The fluorogenic substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine was then added to 5 μ M, and the rate of substrate hydrolysis was measured.

Fig. 2

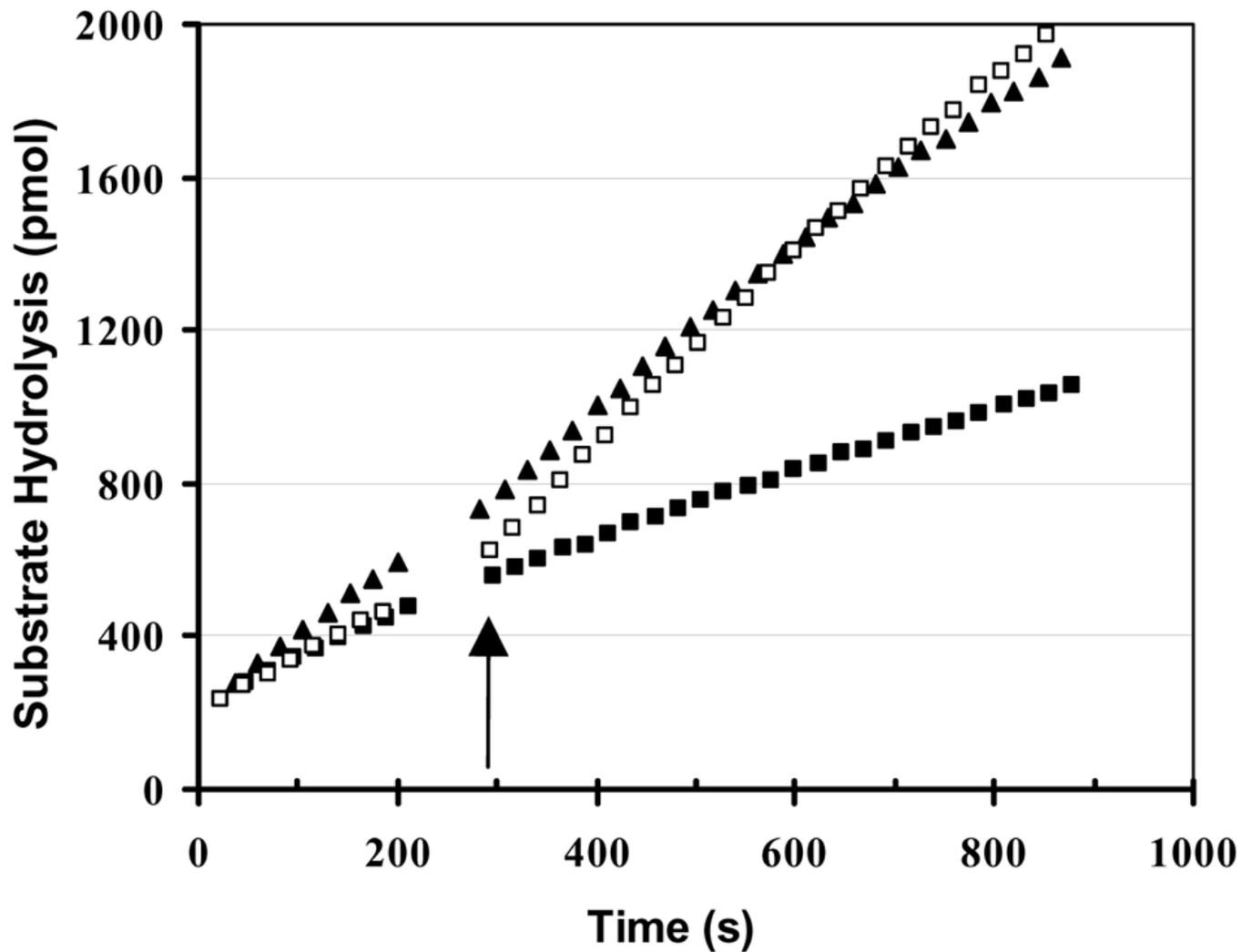


Figure 2. DTT reversal of inhibition of AVP-pVIc by NO. AVP-pVIc, 50 nM, was incubated with (□ and ■) or without (▲) 0.01 mM DEA-NONOate, and the increase in fluorescence with time was monitored continuously. After 5 min (↑), 1 mM DTT was added (▲ and □), and the continuous monitoring of the increase in fluorescence with time was resumed.

Fig. 3

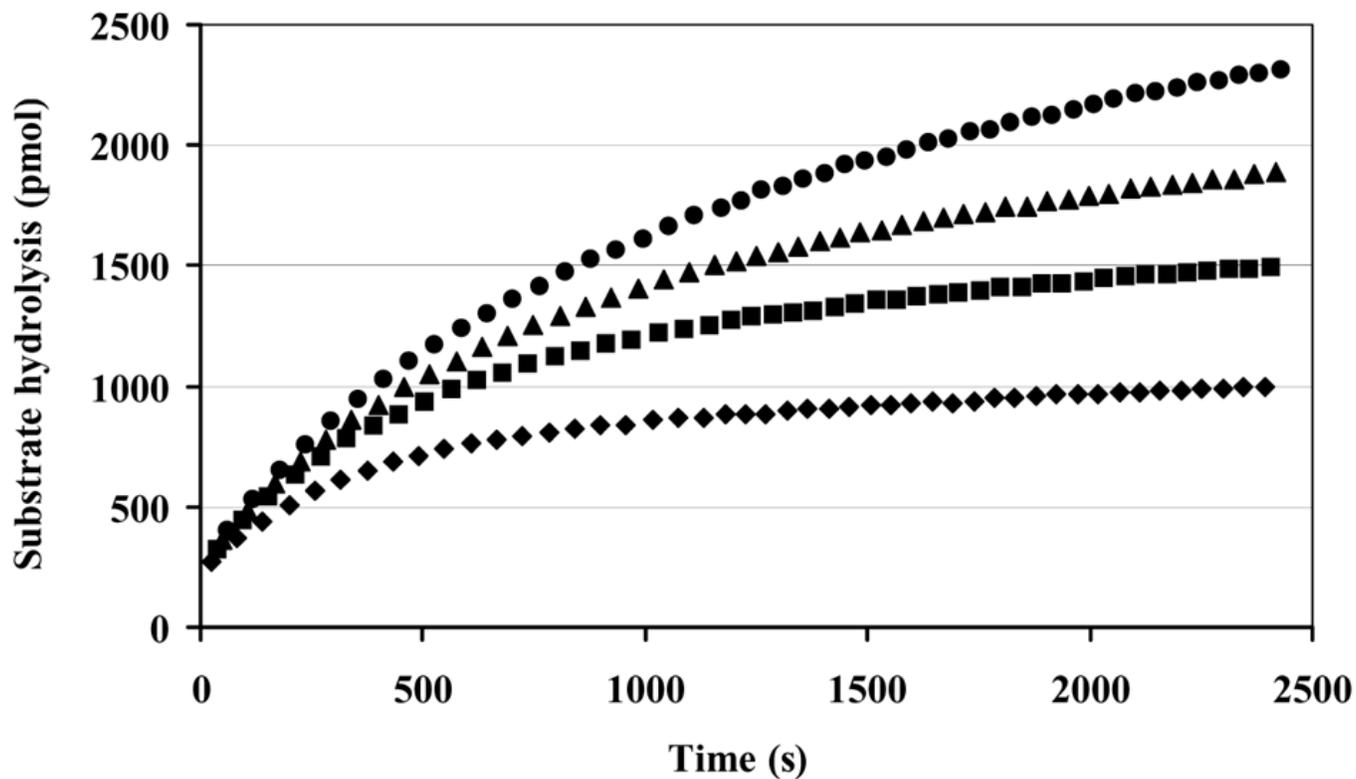


Figure 3. Competitive, irreversible inhibition of AVP-pVIc by Sp-NONOate. At time zero, 50 nM AVP-pVIc and 0.2 mM (●), 0.4 mM (▲), 0.6 mM (■), or 3 mM (◆) Sp-NONOate were added simultaneously to solutions containing 5 μ M (Leu-Arg-Gly-Gly-NH)₂-rhodamine. The increase in substrate hydrolysis was measured continuously as a function of time. The data can be described by **equation 3**.

Fig. 4

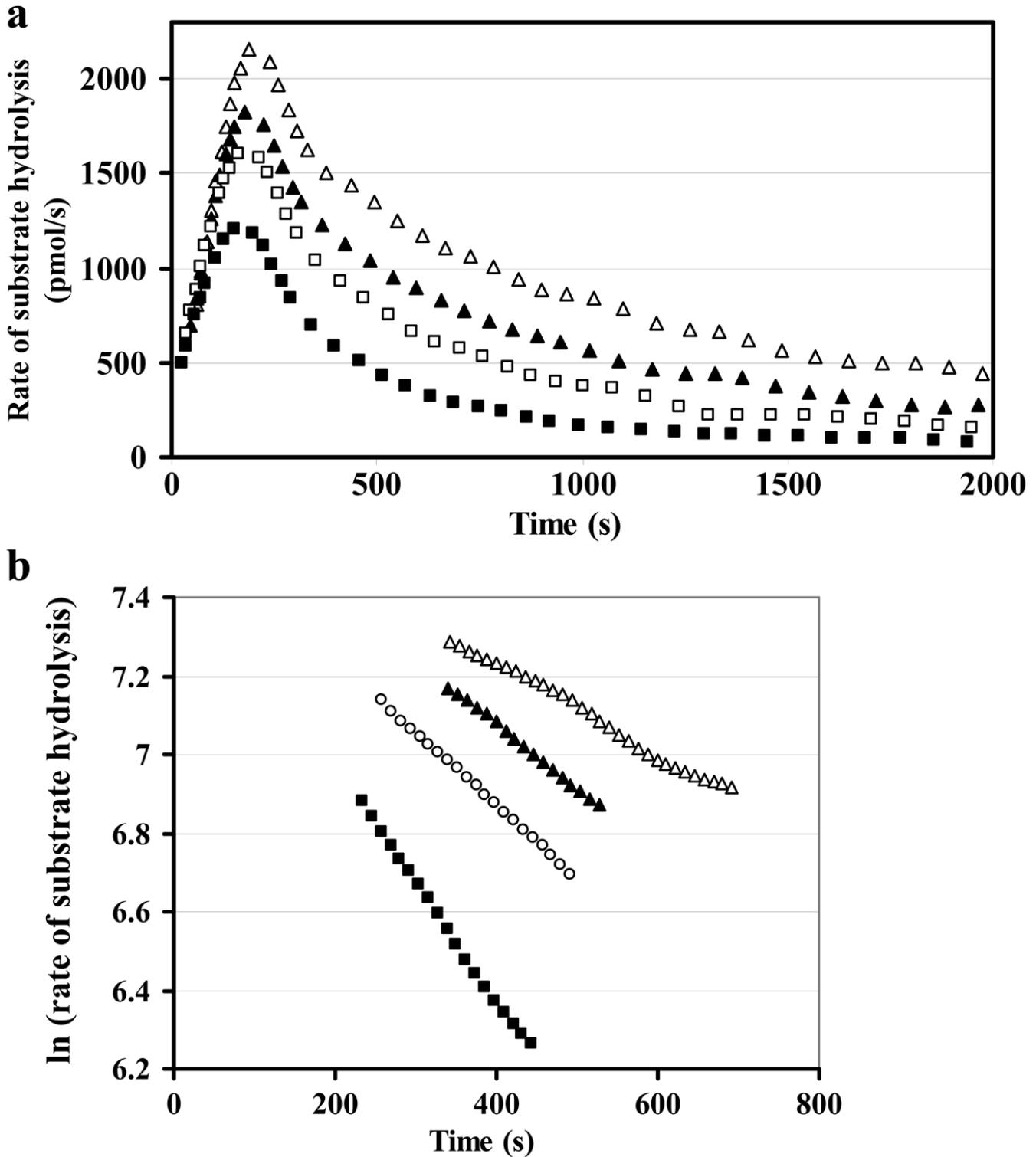


Figure 4. Determination of the apparent first-order rate constants b for the competitive, irreversible inhibition of AVP-pVic. A) The primary data in Fig. 3 were exported into the program Table Curve, and the program plotted the instantaneous velocity vs. time. The Sp-NONOate concentrations were 0.2 mM (\triangle), 0.4 mM (\blacktriangle), 0.6 mM (\square), and 3 mM (\blacksquare). B) The natural logarithm of the slope (rate of substrate hydrolysis) was plotted vs. time, using the data in A. The data can be described by equation 7. The Sp-NONOate concentrations were 0.2 mM (\triangle), 0.4 mM (\blacktriangle), 0.6 mM (\circ), and 3 mM (\blacklozenge).

Fig. 5

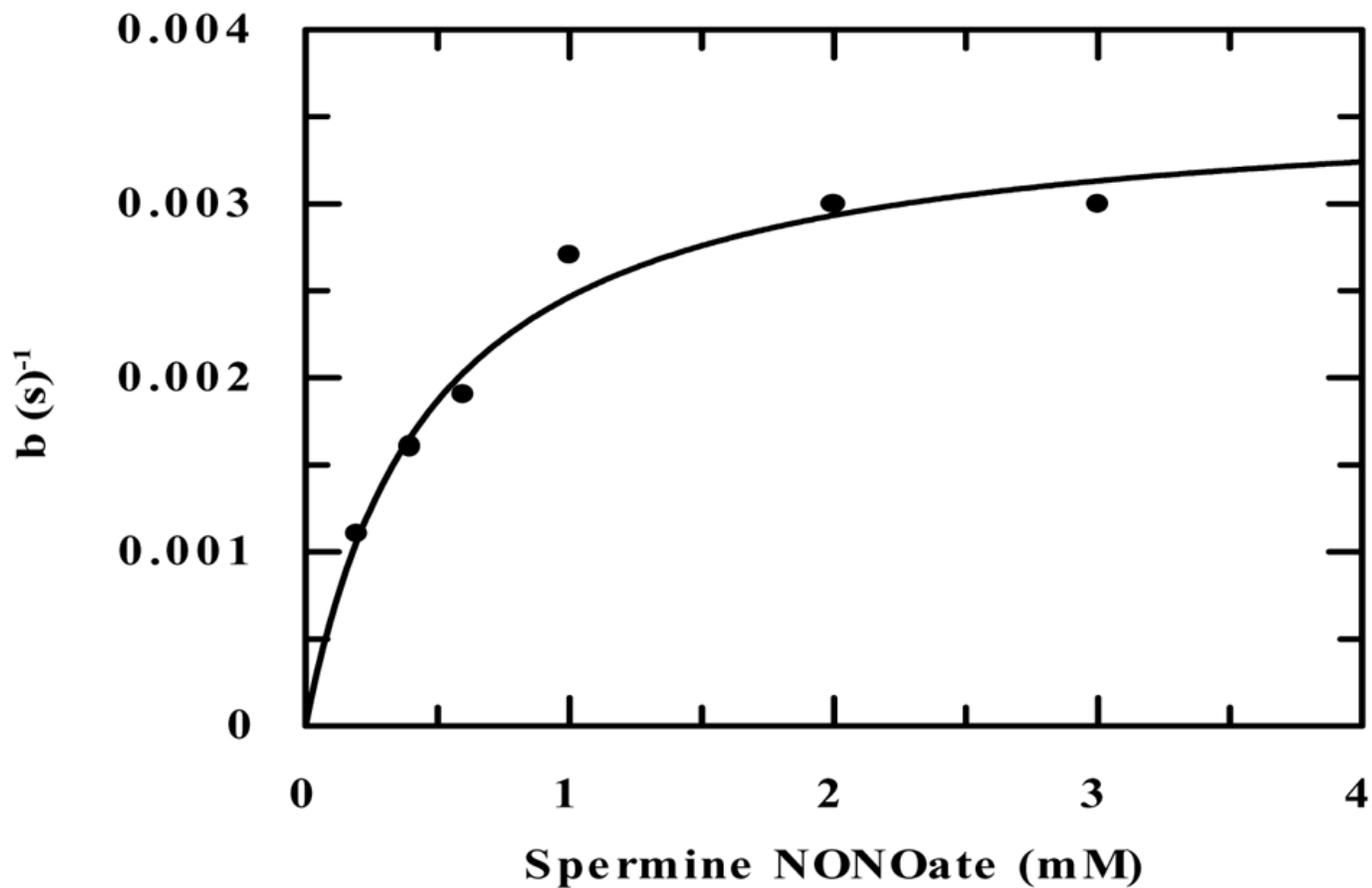


Figure 5. Determination of K_i and k_i for the irreversible inhibition of AVP-pVIc by Sp-NONOate. The apparent first-order rate constants b , obtained from the slopes of the lines in **Fig. 4B**, were plotted vs. the initial Sp-NONOate concentrations. The line drawn through the points conforms to the rectangular hyperbola described by **equation 8**.

Fig. 6

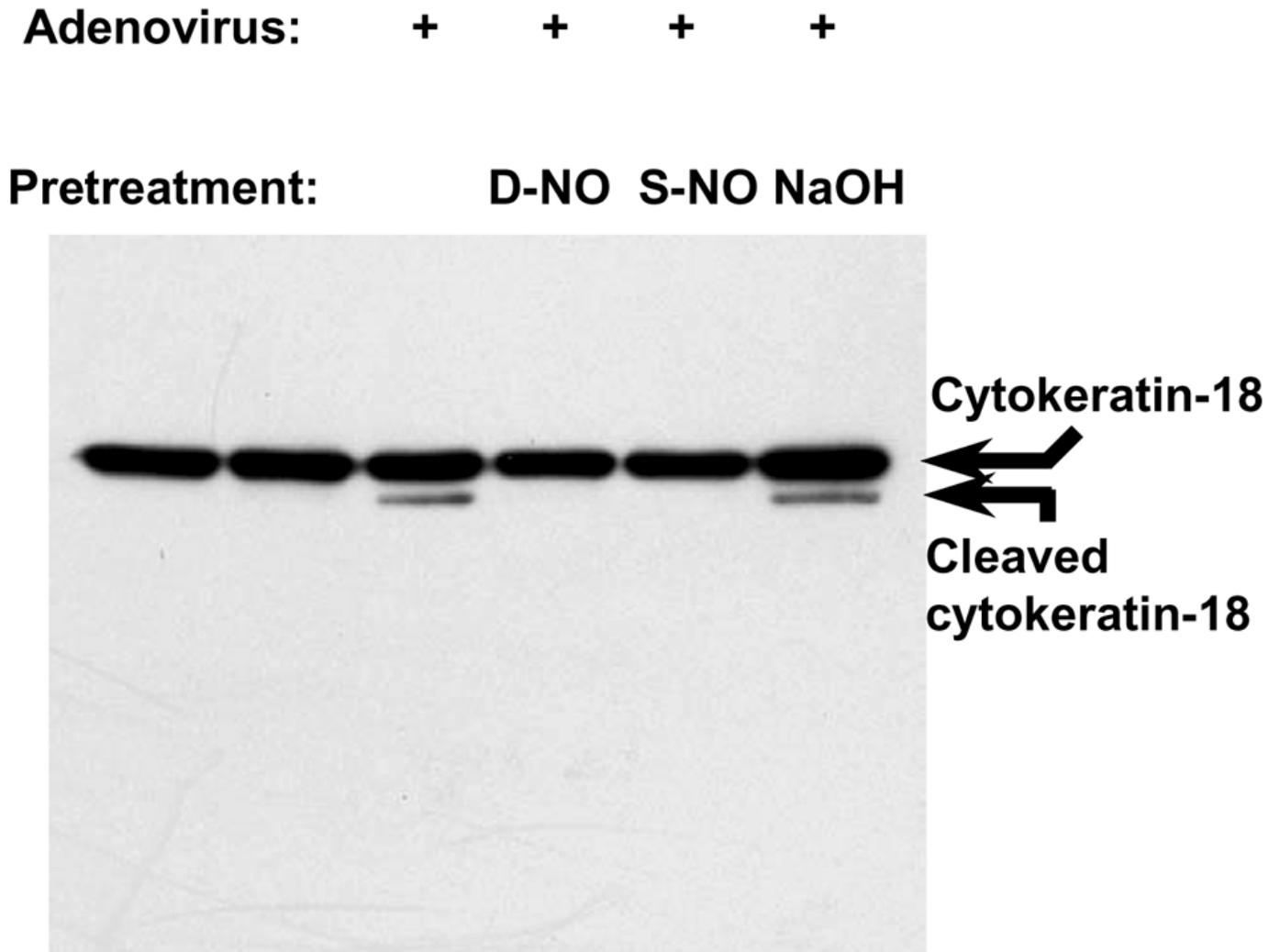


Figure 6. Pretreatment of adenovirus with NO inhibited AVP cleavage of cytokeratin 18 in infected cells. HeLa cells were not infected (lanes 1 and 2) or were infected with adenovirus that had been pretreated with nothing (lane 3), DEA-NONOate (D-NO, lane 4), Sp-NONOate (S-NO, lane 5), or vehicle (NaOH, lane 6). Sixteen hours after infection, the cells were harvested and lysates were fractionated by SDS-PAGE and immunoblotted with antibody to the AVP substrate cytokeratin 18.

Fig. 7

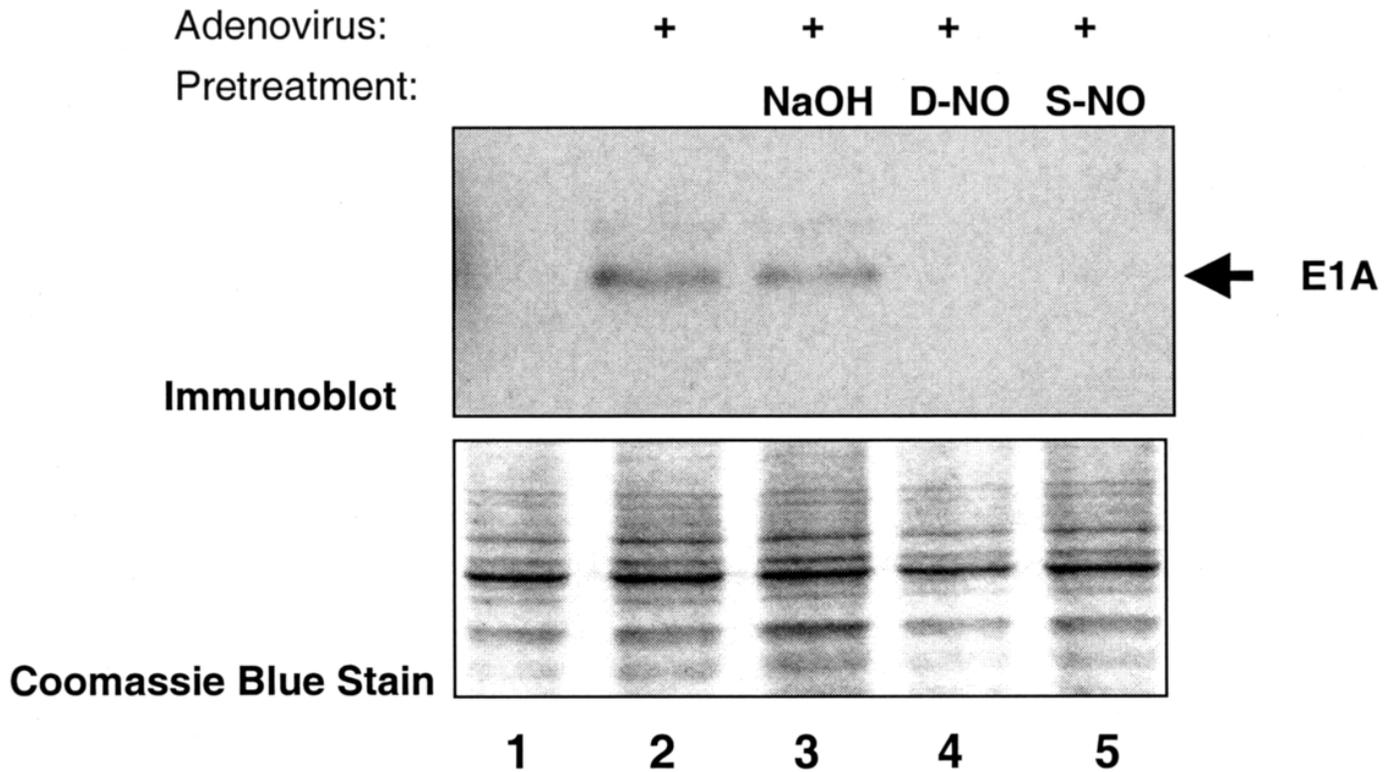


Figure 7. Pretreatment of adenovirus with NO decreased E1A expression in infected cells. HeLa cells were not infected (lane 1) or infected with adenovirus (MOI = 100) that had not been pretreated (lane 2) or had been pretreated with vehicle alone (NaOH, lane 3), DEA-NONOate (D-NO, lane 4), or Sp-NONOate (S-NO, lane 5). Sixteen hours after infection, cells were harvested and lysates were fractionated by SDS-PAGE and immunoblotted with antibody to E1A. Alternatively, 16 h after infection, the same cells were harvested and lysates were fractionated by SDS-PAGE and stained with Coomassie blue.

Fig. 8

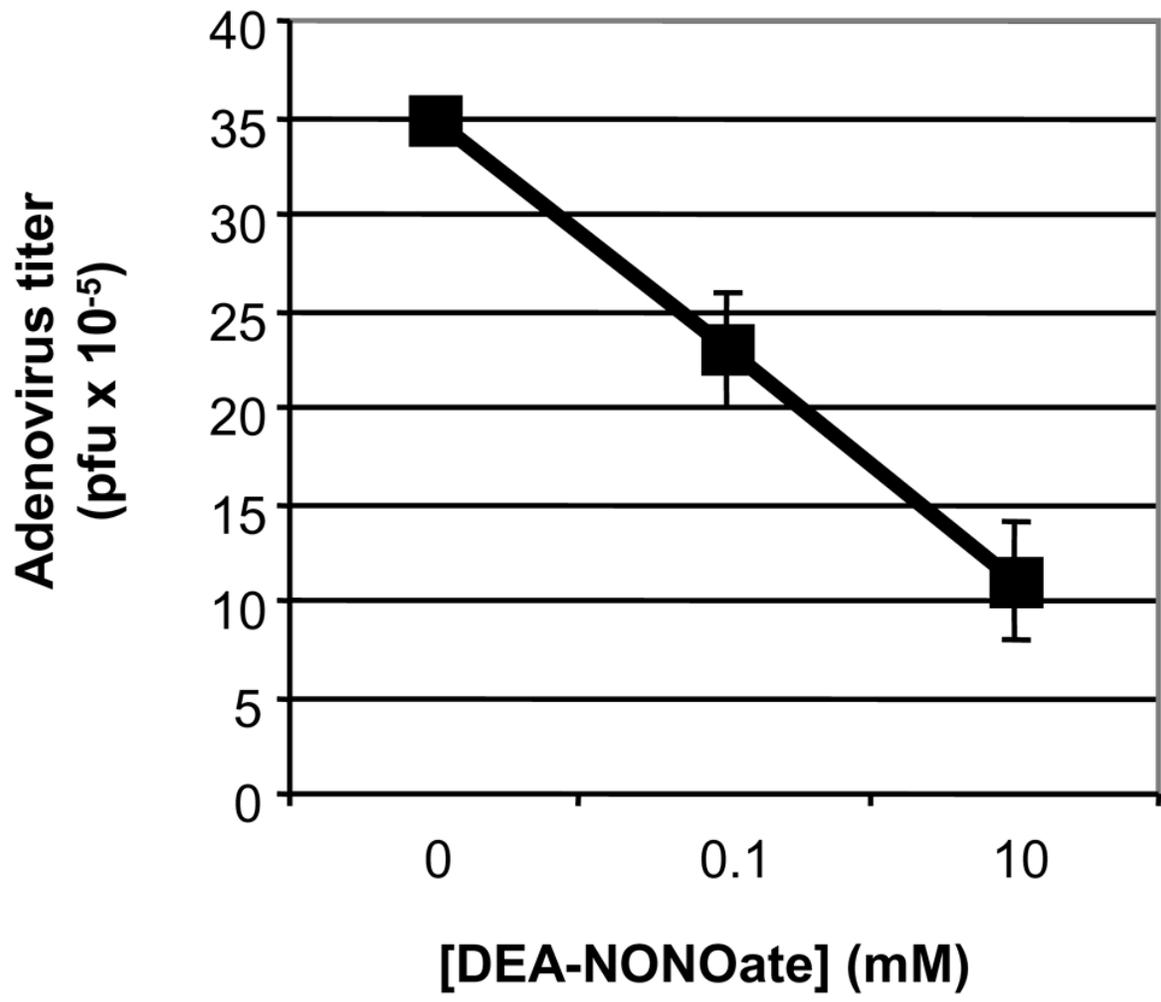


Figure 8. Pretreatment of adenovirus with NO decreased adenovirus infectivity. Purified adenovirus was pretreated with vehicle alone (NaOH) or with DEA-NONOate for 10 min, and virus infectivity was measured by a plaque-forming assay.