Amiloride Is a Competitive Inhibitor of Coxsackievirus B3 RNA Polymerase[▽]

Elena V. Gazina,^{1*} Eric D. Smidansky,² Jessica K. Holien,³ David N. Harrison,^{1‡} Brett A. Cromer,⁴ Jamie J. Arnold,² Michael W. Parker,^{3,5} Craig E. Cameron,² and Steven Petrou^{1,6}

Florey Neuroscience Institutes, The University of Melbourne, Victoria 3010, Australia¹; Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802²; Biota Structural Biology Laboratory, St. Vincent's Institute of Medical Research, Fitzroy, Victoria 3065, Australia³; Health Innovations Research Institute, RMIT University, Bundoora, Victoria 3083, Australia⁴; Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Victoria 3010, Australia⁵; and Centre for Neuroscience, The University of Melbourne, Victoria 3010, Australia⁶

Received 4 May 2011/Accepted 15 July 2011

Amiloride and its derivative 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) were previously shown to inhibit coxsackievirus B3 (CVB3) RNA replication in cell culture, with two amino acid substitutions in the viral RNA-dependent RNA polymerase 3D^{pol} conferring partial resistance of CVB3 to these compounds (D. N. Harrison, E. V. Gazina, D. F. Purcell, D. A. Anderson, and S. Petrou, *J. Virol.* 82:1465–1473, 2008). Here we demonstrate that amiloride and EIPA inhibit the enzymatic activity of CVB3 3D^{pol} *in vitro*, affecting both VPg uridylylation and RNA elongation. Examination of the mechanism of inhibition of 3D^{pol} by amiloride showed that the compound acts as a competitive inhibitor, competing with incoming nucleoside triphosphates (NTPs) and Mg²⁺. Docking analysis suggested a binding site for amiloride and EIPA in 3D^{pol}, located in close proximity to one of the Mg²⁺ ions and overlapping the nucleotide binding site, thus explaining the observed competition. This is the first report of a molecular mechanism of action of nonnucleoside inhibitors against a picornaviral RNA-dependent RNA polymerase.

The family *Picornaviridae* is a family of positive-sense RNA viruses which contains numerous human pathogens, causing poliomyelitis, myocarditis, meningitis, hepatitis, the common cold, and other diseases. The viral genomic RNA is ~7,500 nucleotides (nt) long and contains a 22-amino-acid peptide, VPg, covalently linked to the 5' end and a poly(A) tail at the 3' end. Genome replication occurs via synthesis of a complementary, negative-sense RNA strand, catalyzed by the viral RNA-dependent RNA polymerase, 3D^{pol}, in association with a number of viral and host proteins. It is a complex process taking place in membrane-associated replication complexes in the cytoplasm of infected cells (reviewed in references 5, 7, and 23). The synthesis of both complementary and genomic RNA strands is initiated by attachment of two UMP nucleotides to a tyrosine residue of VPg, resulting in the production of VPg-pUpU. VPg uridylylation requires a template. In the case of genomic strand synthesis, an internal stem-loop in the genomic RNA strand (*cis*-acting replication element [CRE]) is used as a template, with subsequent translocation of VPg-pUpU to the 3' end of the complementary strand and its elongation into a full-length genomic strand (9, 15, 16, 26). The complementary strand synthesis does not absolutely depend on CRE—it can

also be templated by the poly(A) tail of the genomic strand (9, 15, 16, 26).

VPg uridylylation and RNA elongation have been reproduced successfully *in vitro* by use of purified components. VPg uridylylation assays require 3D^{pol}, VPg, CRE or poly(A), UTP, and Mg²⁺ or Mn²⁺ (19, 20), with CRE-templated reaction stimulated by viral proteins 3CD or 3C (18, 19), whereas an elongation assay mix contains an RNA primer instead of VPg (21).

Coxsackievirus B3 (CVB3) is a picornavirus responsible for 14 to 32% of human myocarditis cases (1). Amiloride and its derivative 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) inhibit CVB3 propagation in cell culture by inhibiting viral genome replication (11). Two amino acid substitutions in 3D^{pol} (S299T and A372V) confer partial resistance of the virus to the compounds, suggesting that amiloride analogues may act as inhibitors of CVB3 3D^{pol} (11). Here we show that amiloride and EIPA inhibit VPg uridylylation and RNA elongation by CVB3 3D^{pol} *in vitro*, acting as competitive inhibitors with respect to nucleoside triphosphates (NTPs) and Mg²⁺. We further demonstrate that the S299T substitution in 3D^{pol} reduces the extent of inhibition of RNA elongation by amiloride and EIPA, thus recapitulating its effect on CVB3 inhibition in cell culture. This is the first report of a molecular mechanism of action of nonnucleoside inhibitors against a picornaviral RNA-dependent RNA polymerase.

MATERIALS AND METHODS

Reagents. Expression and purification of wild-type (WT) and S299T CVB3 3D^{pol} and CVB3 3C have been described previously (14, 17, 26). The 3D^{pol} concentration was measured as described previously (26). CVB3 CRE was pro-

* Corresponding author. Mailing address: Florey Neuroscience Institutes, The University of Melbourne, Victoria 3010, Australia. Phone: 61-3-8344 9678. Fax: 61-3-9348 1707. E-mail: egazina@unimelb.edu.au.

‡ Present address: Burnet Institute, Melbourne, Victoria 3004, Australia.

[▽] Published ahead of print on 27 July 2011.

duced as described in reference 17. CVB3 VPg was synthesized by the Peptide Laboratory of the Howard Florey Institute. Gel-purified 10-nt RNA (symmetrical substrate [SSU]) was purchased from Dharmacon. [γ -³²P]ATP and [α -³²P]UTP were purchased from Perkin Elmer, and all other reagents were purchased from Sigma. Amiloride was dissolved in dimethyl sulfoxide (DMSO) at a 500 mM concentration, and EIPA was dissolved in ethanol at a 50 mM concentration. Final concentrations of DMSO and ethanol in reaction mixtures containing the compounds were 0.1% and 2%, respectively. Equal concentrations of the solvents were present in the no-compound controls.

VPg uridylylation assay using poly(A) template. The VPg uridylylation assay was conducted essentially as described previously (20). Reaction mixtures contained 1.2 μ M CVB3 3D^{pol} (WT or S299T mutant), poly(A) (equivalent of 70 μ M AMP), 50 μ M VPg, 10 μ M ³²P-labeled UTP (0.1 μ Ci/ μ l), 50 mM HEPES-NaOH, pH 7.5, 6% glycerol, 11 mM 2-mercaptoethanol, 0.5 mM Mn(CH₃COO)₂, and the indicated concentrations of amiloride or EIPA. Reaction mixtures were assembled on ice without UTP and then incubated at 30°C for 5 min. Reactions were then started by addition of ³²P-labeled UTP and continued for 1 h at 30°C. The reactions were quenched by addition of EDTA to a final concentration of 50 mM, and reaction products were separated by 15% Tris-Tricine SDS-PAGE and quantified using a BAS-5000 phosphorimager (Fujifilm) with Multi Gauge software.

RNA elongation assay. SSU RNA was end labeled using [γ -³²P]ATP and T4 polynucleotide kinase (New England BioLabs), followed by purification on a Zeba 7K spin column (Pierce) equilibrated with water, as specified by the manufacturers' instructions. The resulting 20 μ M ³²P-labeled SSU was mixed with unlabeled SSU to a 200 μ M concentration and annealed by heating to 90°C for 1 min, followed by cooling to 10°C at 5°C/min.

Unless specified otherwise, reaction mixtures contained 1.8 μ M CVB3 3D^{pol} (WT or S299T mutant), 20 μ M annealed ³²P-labeled SSU (10 μ M duplex), 10 μ M ATP, 5 mM MgCl₂, 50 mM HEPES-NaOH, pH 7.5, 2% glycerol, 11 mM 2-mercaptoethanol, and the indicated concentrations of amiloride or EIPA. Reaction mixtures were assembled on ice and then incubated at 30°C. The orders of assembly of reaction mixtures and incubation times are indicated. Reactions were quenched by addition of an equal volume of 100 mM EDTA, 68% formamide, 0.02% bromophenol blue, and 0.02% xylene cyanol in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and then heated to 65°C for 5 min prior to loading on a 20% polyacrylamide (1.5% bisacrylamide) gel containing TBE and 7% urea. The gels were visualized and quantified using a BAS-5000 phosphorimager (Fujifilm) with Multi Gauge software.

VPg uridylylation assay using CVB3 CRE template. Reaction mixtures contained 1 μ M 3D^{pol} (WT or S299T mutant), 1 μ M CVB3 3C, 1 μ M CVB3 CRE, 50 μ M CVB3 VPg, 250 μ M ³²P-labeled UTP (0.1 μ Ci/ μ l), 50 mM HEPES-NaOH, pH 7.5, 10% glycerol, 12 mM 2-mercaptoethanol, 30 mM NaCl, 0.7 mM Mg(CH₃COO)₂, and the indicated concentrations of amiloride. Reaction mixtures were assembled on ice without 3D^{pol} and incubated at 30°C for 5 min. Reactions were initiated by addition of 3D^{pol}, continued for 5 min at 30°C, and quenched by addition of EDTA to a final concentration of 50 mM. Reaction products were separated by 15% Tris-Tricine SDS-PAGE and quantified using a Typhoon phosphorimager (GE Healthcare).

Molecular modeling. CVB3 and poliovirus 3D^{pol} structures were downloaded from the Protein Data Bank (PDB; <http://www.pdb.org/>) and analyzed for docking suitability (i.e., model completeness, resolution, and visual analysis of conformational changes) by using Pymol (<http://pymol.org/>). Four alternate structures were used in the docking studies based on their docking suitability and the presence of cocrystallized ligands (i.e., Mg²⁺, ATP, or primer). These four had PDB codes 3CDW (10), 2ILY (24), 3OL7, and 3OL6 (8). All structures were aligned by their α -carbons, and for each structure, water and nonphysiological ligands (i.e., detergents, salts, etc.) were removed. While aligned, the coordinates of the two Mg²⁺ ions (crystallized in structure 3OL7 [8]) and part of the VPg primer (crystallized in structure 3CDW [10]) were extracted from their native crystal structure and merged into each alternate protein structure to create four different 3D^{pol} models for each X-ray structure: apo, with Mg²⁺, with VPg, and with Mg²⁺ and VPg. Each model was then minimized until gradient convergence was reached under the Merck molecular force fields (MMFFs).

The unprotonated form and the three protonated tautomers of amiloride and EIPA were drawn and minimized under the Tripos force field in Sybylx1.2 (Tripos). ATP was extracted from structure 2ILY and minimized under the Tripos force field in Sybylx1.2.

Docking protomers were constructed for each of the 16 alternate models in Surflex-Dock (Tripos). Each protomer was constructed by selecting a 15-Å sphere around Ser299, adding a bloat of 3 Å and a threshold of 0.36 (blind docking using the entire 3D^{pol} molecule produced similar results). All ligands were docked into each protomer, with 5 additional starting conformations per

molecule, ring flexibility considered, the density of search increased to 9.0 (default, 3.0), and hydrogen and heavy atom protein movement of the receptor allowed. All other parameters used were the defaults. The top 10 solutions were analyzed visually in Sybylx1.2 and Pymol, with all figures generated in Pymol.

RESULTS

Amiloride and EIPA inhibit poly(A)-templated VPg uridylylation. To examine the hypothesis that amiloride and EIPA act as CVB3 3D^{pol} inhibitors (11), we assessed whether these compounds inhibit VPg uridylylation and/or RNA elongation by CVB3 3D^{pol} *in vitro*.

First, we conducted a VPg uridylylation and RNA elongation assay by using a poly(A) template (20). The assay was performed on WT and S299T 3D^{pol} in the presence of Mn²⁺ (no product was observed in the presence of Mg²⁺), using various concentrations of amiloride or EIPA or no compound.

Amiloride and EIPA reduced both VPg-pU(pU) and VPg-poly(U) production by WT and S299T 3D^{pol}, in a concentration-dependent manner (Fig. 1), demonstrating that they inhibit VPg uridylylation. EIPA had a stronger inhibitory effect than amiloride (Fig. 1B), consistent with its stronger effect on CVB3 replication in cell culture (11). S299T 3D^{pol} was less susceptible to inhibition than WT 3D^{pol} (Fig. 1), consistent with the previous finding that the S299T mutation confers partial resistance of CVB3 to the compounds (11).

The effect of the compounds on VPg-poly(U) production appeared stronger than that on VPg-pU(pU) production (Fig. 1B), which could mean that they inhibit RNA elongation. However, amiloride and EIPA had no obvious concentration-dependent effect on the length of the resulting poly(U) chain (Fig. 1A), which is contrary to what is expected in the case of inhibition of RNA elongation. These results were therefore inconclusive regarding the inhibition of RNA elongation.

Amiloride and EIPA inhibit RNA elongation. To test whether amiloride and EIPA inhibit RNA elongation, we used a symmetrical, self-annealing, 10-nt RNA primer and template with uridine as the first templating base (SSU) (2):

5'-GCAUGGGGCC

CCCGGGUACG-5'

ATP was used as the only nucleotide substrate to analyze single-nucleotide incorporation into SSU. In the presence of Mg²⁺, 3D^{pol} binds to the SSU duplex and incorporates a single AMP at the 3' terminus of one RNA strand, with rapid, pre-steady-state burst kinetics (2, 3). This is followed by slow dissociation of 3D^{pol} from the product (rate-limiting step of the steady-state phase) and subsequent rebinding to a new SSU duplex followed by AMP incorporation (2).

We measured the steady-state kinetics of 11-nt RNA production (formed by the addition of one AMP molecule to the 10-nt RNA primer) by WT and S299T 3D^{pol} in the absence or presence of various concentrations of amiloride or EIPA. 3D^{pol} was preincubated with SSU duplex (used in a 5.5-fold excess of 3D^{pol}) and the test compounds in the presence of Mg²⁺ for 6 min at 30°C, after which reactions were initiated by addition of ATP, continued for 10, 18, 26, or 34 min, and then quenched (Fig. 2A). The amount of AMP incorporated into SSU during the pre-steady-state burst was determined by plotting 11-nt RNA production in each reaction as a function of

FIG. 1. Inhibition of VPg-pU(pU) and VPg-poly(U) production by amiloride and EIPA. Reaction mixtures contained 1.2 μM CVB3 3D^{pol} (WT or S299T), poly(A) (equivalent of 70 μM AMP), 50 μM VPg, 10 μM ³²P-labeled UTP, 0.5 mM Mn(CH₃COO)₂, and the indicated concentrations of amiloride or EIPA. Reactions were started by addition of UTP, and mixtures were incubated at 30°C for 1 h. Reaction products were then separated by gel electrophoresis, and VPg-pU(pU) and VPg-poly(U) production was quantified using a phosphorimager. (A) Representative gel images. Positions of UTP, VPg-pU(pU), and VPg-poly(U) are marked. (B) Dose-response curves for amiloride (left) and EIPA (right). ■, VPg-pU(pU) production by WT 3D^{pol}; ▲, VPg-poly(U) production by WT 3D^{pol}; □, VPg-pU(pU) production by S299T 3D^{pol}; △, VPg-poly(U) production by S299T 3D^{pol}. IC₅₀, 50% inhibitory concentrations calculated using the equation $y = 100 / [1 + 10^{(\log \text{IC}_{50} - \log X) \times \text{hill slope}}]$ (GraphPad Prism). The data points are means $\pm$ standard errors of the means (SEM) for 3 independent experiments.

the reaction time and fitting the data points to a line. The y intercept of each line defined the amount of AMP incorporated during the pre-steady-state burst (Fig. 2B). Both compounds inhibited the amplitude of the pre-steady-state burst in a concentration-dependent manner (Fig. 2C), thus demonstrating that they inhibited RNA elongation.

EIPA had a stronger effect on the pre-steady-state burst of AMP incorporation than did amiloride, and WT 3D^{pol} was

more susceptible to the inhibition than S299T 3D^{pol} (Fig. 2C), consistent with the data on CVB3 replication in cell culture (11). The difference in susceptibility to inhibition between WT and S299T 3D^{pol} (3.6-fold difference in 50% inhibitory concentration [IC₅₀] for amiloride and 4.4-fold difference for EIPA) (Fig. 2) was significantly more pronounced than that observed for VPg-pU(pU) production (1.3- and 2-fold differences in IC₅₀ for amiloride and EIPA, respectively) (Fig. 1).

FIG. 2. Inhibition of AMP incorporation into SSU by amiloride and EIPA. A total of 1.8 μM CVB3 3D^{pol} (WT or S299T) was preincubated with 20 μM ³²P-labeled SSU, 5 mM MgCl₂, and the indicated concentrations of amiloride or EIPA for 6 min at 30°C. Reactions were started by addition of 10 μM ATP and continued for 10, 18, 26, or 34 min. The 11-nt product was then separated from 10-nt SSU by gel electrophoresis, and its amount was quantified using a phosphorimager. (A) Representative gel images for amiloride treatment. Positions of 10-nt and 11-nt RNAs are marked. (B) Steady-state kinetics of 11-mer production in reactions shown in panel A. Data points are fitted to lines. The y intercept of each line defines the amplitude of AMP incorporation into SSU during the pre-steady-state burst. Amiloride concentrations: ●, 0 μM ; ■, 62.5 μM ; ▲, 125 μM ; ◆, 250 μM ; △, 500 μM . (C) Dose-response curves for inhibition of pre-steady-state AMP incorporation into SSU by amiloride and EIPA. ■, WT 3D^{pol} plus amiloride; ▲, WT 3D^{pol} plus EIPA; □, S299T 3D^{pol} plus amiloride; △, S299T 3D^{pol} plus EIPA. IC₅₀, 50% inhibitory concentrations calculated using the equation $y = 100 / \{1 + 10^{[(\log \text{IC}_{50} - \log X) \times \text{hill slope}]}\}$ (GraphPad Prism). The data points are averages $\pm$ SEM for 4 independent experiments.

In contrast to inhibition of the pre-steady-state burst amplitude, the effects of the compounds on the steady-state rate constant were small (data not shown) and difficult to interpret due to the occurrence of multiple events (i.e., dissociation, association,

and nucleotide incorporation). Our following studies were therefore focused on the pre-steady-state burst amplitude.

Inhibitory effect on RNA elongation depends on the order of addition. As a first step toward elucidation of the mechanism

FIG. 3. Effect of order of Mg^{2+} addition on inhibition of pre-steady-state AMP incorporation into SSU by amiloride and EIPA. A total of 1.8 μM CVB3 $3D^{pol}$ (WT or S299T) was preincubated with 20 μM ^{32}P -labeled SSU and the indicated concentrations of amiloride or EIPA for 6 min at 30°C. Reactions were started by addition of 10 μM ATP and 5 mM $MgCl_2$. The pre-steady-state burst amplitude of AMP incorporation into SSU was measured and quantified as described in the legend to Fig. 2. IC_{50} , 50% inhibitory concentrations calculated using the equation $y = 100/[1 + \Delta^{10}[(\log IC_{50} - \log X) \times \text{hill slope}]]$ (GraphPad Prism). ■, WT $3D^{pol}$ plus amiloride; ▲, WT $3D^{pol}$ plus EIPA; □, S299T $3D^{pol}$ plus amiloride; △, S299T $3D^{pol}$ plus EIPA. The data points are averages $\pm$ SEM for 3 independent experiments.

FIG. 4. Dependence of inhibitory effect of amiloride on incubation time before ATP addition. Amiloride was added to preassembled SSU-3D^{pol} complexes and incubated at 30°C for the indicated periods prior to the start of reactions by addition of 5 mM ATP. The reactions were allowed to proceed for 20 s, after which the 11-nt product was separated from SSU by gel electrophoresis and its amount was quantified using a phosphorimager. Product formation, normalized to no-amiloride controls, was plotted as a function of incubation time before ATP addition. Lines are fits to the single exponential model $P = A \times \exp(-k_{\text{obs}} \times t) + C$, where A is the amplitude, k_{obs} is the observed rate constant, t is the independent variable time, and C is the endpoint. Inhibition half-times were calculated as $\ln 2/k_{\text{obs}}$. ▲, WT 3D^{pol} plus 250 μM amiloride; ■, WT 3D^{pol} plus 500 μM amiloride; □, S299T 3D^{pol} plus 500 μM amiloride; ◇, S299T 3D^{pol} plus 1 mM amiloride. The data points are averages $\pm$ SEM for 2 independent experiments.

3D^{pol} gradually increased with the increase in incubation time (Fig. 4). The calculated inhibition half-time values were ~35 s for WT 3D^{pol} with 500 μM amiloride and S299T 3D^{pol} with 1,000 μM amiloride and ~70 s for WT 3D^{pol} with 250 μM amiloride and S299T 3D^{pol} with 500 μM amiloride (Fig. 4). These data demonstrated that amiloride inhibits 3D^{pol} slowly, possibly due to steric constraints of the binding site or to a conformational change occurring in the process. They also showed that S299T 3D^{pol} is inhibited 2-fold slower than WT 3D^{pol} by the same concentration of amiloride, consistent with the partial resistance to inhibition of RNA elongation conferred by this mutation.

Amiloride competes with ATP and Mg²⁺. To test the hypothesis that amiloride may compete with NTPs for the binding site on 3D^{pol}, we conducted a competition assay with the WT enzyme, using various concentrations of ATP and amiloride. We incubated 3D^{pol}, SSU, amiloride, and ATP for 5 min at 30°C in the absence of Mg²⁺ to allow binding, after which reactions were started by the addition of 5 mM MgCl₂. The amplitude of pre-steady-state AMP incorporation into SSU was quantified and plotted as a function of ATP concentration (Fig. 5A and B). The data showed that the inhibitory effect of amiloride decreased with increases in ATP concentration (Fig. 5B), suggesting that amiloride competes with ATP for the binding site. This result also demonstrated that Mg²⁺ is not required for binding of SSU, ATP, and amiloride to 3D^{pol} (otherwise competition between ATP and amiloride would not be observed under these conditions).

A similar competition assay was conducted using various concentrations of Mg²⁺ and amiloride to test the hypothesis that amiloride may compete with Mg²⁺ for the binding site. WT 3D^{pol}, SSU, amiloride, and Mg²⁺ were incubated for 5 min at 30°C, after which reactions were started by the addition

of 10 μM ATP (Fig. 5C and D). The results were more complicated to interpret than those from the ATP competition assay, perhaps because the stoichiometry of the complexes involves two Mg²⁺ ions at two functionally distinct sites (see Fig. 8). With amiloride concentrations of 50 and 125 μM, the inhibition decreased with an increase in Mg²⁺ concentration from 0.5 to 3.5 mM (Fig. 5D). The response was saturated above 3.5 mM Mg²⁺, as evidenced by the leveling off of the line (Fig. 5D). A 500 μM amiloride concentration was too high to observe a significant effect of Mg²⁺ concentration on the level of inhibition by amiloride (Fig. 5D). In total, these data are consistent with competition between amiloride and Mg²⁺ for binding to 3D^{pol}. Note that the effect of Mg²⁺ concentration on the enzymatic activity of 3D^{pol} in the absence of amiloride (i.e., a concentration-dependent increase in AMP incorporation followed by a decrease [Fig. 5C]) is a pattern for optimum Mg²⁺ concentration observed in nucleic acid polymerases, including CVB3 3D^{pol} (10).

Amiloride inhibits CRE-templated VPg uridylylation. The above results suggested that amiloride inhibits RNA elongation by acting as a competitive inhibitor with respect to NTPs and Mg²⁺. The VPg uridylylation assay using a poly(A) template indicated that amiloride also inhibits VPg uridylylation, but that assay could be done only by using Mn²⁺ instead of Mg²⁺. We therefore examined inhibition of VPg uridylylation by amiloride under more biologically relevant conditions by using CRE template in the presence of Mg²⁺ (17). The assay was conducted on WT and S299T 3D^{pol}. Reaction mixtures including CVB3 3C, CVB3 CRE, CVB3 VPg, 250 μM UTP, 0.7 mM Mg²⁺, and various concentrations of amiloride were incubated at 30°C for 5 min. Reactions were initiated by the addition of 3D^{pol}, incubated for 5 min, and then quenched. Concentrations of 250 μM UTP and 0.7 mM Mg²⁺ correspond

FIG. 5. Competition assays. (A and B) Amiloride-ATP competition assay. Concentrations of 1.8 μM WT 3D^{pol}, 20 μM SSU, 0.1 to 5 mM ATP, and 0 to 500 μM amiloride were incubated for 5 min at 30°C in the absence of Mg²⁺. Reactions were then started by addition of 5 mM MgCl₂. (C and D) Amiloride-Mg²⁺ competition assay. Concentrations of 1.8 μM WT 3D^{pol}, 20 μM SSU, 0.5 to 5 mM MgCl₂, and 0 to 500 μM amiloride were incubated for 5 min at 30°C. Reactions were then started by addition of 10 μM ATP. (A to D) Pre-steady-state burst amplitudes of AMP incorporation into SSU were measured and quantified as described in the legend to Fig. 2. (A and C) Concentrations of AMP incorporated during the pre-steady-state burst, plotted as a function of ATP and MgCl₂ concentrations, respectively. (B) For each ATP concentration, AMP incorporation in the presence of amiloride was calculated as percentages of the untreated values, using the average values in panel A. The data for each concentration of amiloride were fit to a line. (D) For each MgCl₂ concentration, AMP incorporation in the presence of amiloride was calculated as percentages of the untreated values, using the average values in panel C. Amiloride concentrations: ●, 0 μM ; ◆, 50 μM ; ■, 125 μM ; ▲, 500 μM .

to the physiological concentrations of UTP and free Mg²⁺ in mammalian cells (22, 25). The results demonstrated that amiloride effectively inhibited VPg uridylylation under these conditions (Fig. 6), with the IC₅₀ being lower than that for VPg uridylylation using the poly(A) template (Fig. 1). Surprisingly, there was no difference in inhibitory effect between WT and S299T 3D^{pol} (Fig. 6). Changing the experimental conditions, such as the incubation time, UTP and Mg²⁺ concentrations (i.e., using 10 μM UTP and 5 mM Mg²⁺), and order of addition (i.e., starting the reaction with UTP and Mg²⁺), produced similar results to those shown in Fig. 6 (data not shown).

Molecular basis of inhibition. CVB3 and poliovirus 3D^{pol} structures (3CDW, 2ILY, 3OL6, and 3OL7) were downloaded

from the Protein Data Bank and analyzed for docking suitability. Similar docking results were obtained irrespective of which 3D^{pol} structure was used or whether unprotonated amiloride and EIPA or their protonated tautomers were docked. Thus, for simplicity, only the unprotonated forms of EIPA and amiloride docked into structures based on the 3CDW entry are described in detail.

Amiloride and EIPA were able to dock into each of the four 3D^{pol} structures: apo, with Mg²⁺, with VPg, and with Mg²⁺ and VPg (Table 2). Notably, when Mg²⁺ ions were present, a consistent binding pose was not observed for either compound, suggesting that Mg²⁺ inhibits compound binding. Also, when Mg²⁺ was present, the Surflex scoring function, the C score (a unitless con-

FIG. 6. Inhibition of CRE-templated VPg-pU(pU) production by amiloride. Reaction mixtures contained 1 μM CVB3 3D^{pol} (WT or S299T), 1 μM CVB3 3C, 1 μM CRE, 50 μM VPg, 250 μM ³²P-labeled UTP, 0.7 mM Mg(CH₃COO)₂, and the indicated concentrations of amiloride. Reactions were started by addition of 3D^{pol} and incubated at 30°C for 5 min. Reaction products were then separated by gel electrophoresis, and VPg-pU(pU) production was quantified using a phosphorimager. (A) Representative gel images. Positions of UTP and VPg-pU(pU) are marked. (B) Dose-response curves for WT (●) and S299T (○) 3D^{pol}. IC₅₀, 50% inhibitory concentrations calculated using the equation $y = 100/[1 + 10^{(\log \text{IC}_{50} - \log X) \times \text{hill slope}}]$ (GraphPad Prism). The data points are averages $\pm$ SEM for 3 independent experiments.

sensus score which integrates a number of popular scoring functions to indicate the affinity of ligands bound to a receptor), was significantly reduced for the top docked solution (Table 2).

Where a consistent solution was obtained (apo and VPg configurations of 3D^{pol}), both amiloride and EIPA docked with hydrogen bonds to amino acid residues Ala57, Ser173, Ser60, and Lys61 (Fig. 7). The amino group of amiloride, which is capped by a branched carbon chain (i.e., *N*-ethyl-*N*-isopropyl group) in EIPA, hydrogen bonded to Ser289. Hydrophobic interactions were found between EIPA or amiloride and Ala57, Ile58, Lys172, Ser173, and Arg174. Additional hydrophobic interactions were found between the *N*-ethyl-*N*-iso-

propyl group of EIPA and Lys61, Asp238, and Ser289 (Fig. 7). The carbonylguanidino moiety of each compound was found in two possible orientations due to its flexibility. The C scores for both orientations were very similar, and thus both are plausible solutions. However, one orientation (Fig. 7A) had a higher C score for EIPA (7.7121 versus 7.217), whereas the alternative orientation (Fig. 7B) had a higher C score for amiloride (7.3372 versus 7.087).

The position of docked amiloride or EIPA was distinct from where VPg and the RNA template interact with 3D^{pol} (8), suggesting that the presence of RNA template and/or VPg should not have a significant effect on binding (Table 3 and Fig. 8). This was consistent with our finding that amiloride can bind to preassembled SSU-3D^{pol} complexes. Amiloride and EIPA were also distant from Ser299 (and Ala372) (Table 3). Our data showing that the S299T mutation affects inhibition of RNA elongation but not CRE-templated VPg uridylylation by amiloride are consistent with Ser299 not being part of the compound's binding site.

The pyrazine ring of amiloride and EIPA was very close to the binding site of one of the Mg²⁺ ions causing repulsive interactions (Table 3 and Fig. 8), consistent with the difficulty of the docking program in obtaining a consistent, high-scoring

TABLE 2. C scores for docking of ligands into alternate 3D^{pol} configurations^a

3D ^{pol} configuration	C score for top solution		
	EIPA	Amiloride	ATP
Apo	7.7121	7.3372	9.8231
Mg ²⁺	5.5377	4.3044	12.3604
Mg ²⁺ and VPg	5.1489	5.0479	9.6415
VPg	7.7121	7.2949	9.7121

^a A higher C score is a reflection of a higher affinity of the ligand for 3D^{pol}.

FIG. 7. Stereo views of docked solutions of EIPA (A) and amiloride (B) with the apo form of 3D^{pol}, displaying the residues involved in important interactions. (A) Hydrogen bonds are displayed as yellow dashed lines between EIPA (purple sticks) and Ala57, Ser60, Lys61, and Ser173. (B) Hydrogen bonds are displayed as yellow dashed lines between amiloride (magenta sticks) and Ala57, Ser60, Lys61, Ser173, and Ser289.

docking pose in the presence of Mg²⁺ (Table 2). This was enhanced for EIPA because its *N*-ethyl-*N*-isopropyl group encroached on the position of the Mg²⁺ ion (Table 3 and Fig. 8).

ATP docked into the same position in all 3D^{pol} configurations (i.e., apo, with Mg²⁺, with VPg, and with VPg and Mg²⁺), in an orientation observed in the crystal structures (Fig. 8). There was overlap between the binding site of ATP and the binding sites of amiloride and EIPA (Fig. 8), suggesting that the compounds compete with NTPs for binding to 3D^{pol}. The docking score of ATP was significantly higher than that of amiloride or EIPA (Table 2), suggesting that ATP binds to 3D^{pol} with a higher affinity than that of amiloride or EIPA.

TABLE 3. Distances from ligands to substructures^a

Residue or other structure	Distance (Å) to amiloride	Distance (Å) to EIPA
Ala57	2.4	2.6
Ser60	2.5	3.3
Lys61	3.2	2.9
Ser173	2.8	2.2
Ser289	3.3	3.1
Ile58	3.2	3.0
Lys172	2.9	3.1
Arg174	2.4	2.7
Asp238	3.8	3.5
Ser299	13.0	13.3
Ala372	20.0	21.0
VPg	28.0	29.4
RNA template	7.3	6.4
Mg ²⁺ ion	5.3	2.5

^a All measurements are an indication of heavy atom distances from the ligand to the closest heavy atom in the substructure.

DISCUSSION

Amiloride and EIPA have been shown to inhibit CVB3 RNA replication in cell culture, with amino acid substitutions in 3D^{pol} (S299T or A372V) conferring partial resistance of CVB3 to the compounds, suggesting that the antiviral effect of the compounds may be due to 3D^{pol} inhibition (11).

Here we demonstrated that amiloride and EIPA inhibit VPg uridylylation and RNA elongation by CVB3 3D^{pol} *in vitro*. Examination of the mechanism of inhibition of 3D^{pol} by amiloride showed that the compound acts as a competitive inhibitor, competing with incoming NTPs and Mg²⁺ (Fig. 5). Docking analysis suggested the most likely binding site for amiloride and EIPA in 3D^{pol} (Fig. 8). Binding of the compounds at this site would interfere with binding of NTPs and one of the Mg²⁺ ions, thus explaining the observed competition (Fig. 8).

Previous studies have shown that amiloride inhibits a number of protein kinases via competition with ATP (4, 6, 12). Thus, this compound has affinity for the nucleotide binding sites of different enzymes.

Compared to amiloride, EIPA was a more potent 3D^{pol} inhibitor (Fig. 1 to 3), consistent with its stronger effect on WT CVB3 replication in cell culture (11). The docking analysis suggested that this was due to the additional hydrophobic interactions between the branched carbon chain of EIPA and three amino acid residues of 3D^{pol} (Fig. 7). The difference between amiloride and EIPA in the inhibition of CVB3 replication in cell culture (30-fold difference in IC₅₀ [11]) was more pronounced than that in the inhibition of CVB3 3D^{pol} in the *in vitro* assays, likely due to different intracellular accumulation of the two compounds.

FIG. 8. 3D^{pol} structure (gray cartoon) showing the positions of the RNA template (red sticks), VPg (orange sticks), Mg²⁺ ions (purple spheres), ATP (green sticks), and residues Ala372 and Ser299 (blue sticks) in relation to the docking positions of amiloride (magenta sticks) and EIPA (mauve sticks). Amiloride and EIPA are distant from the RNA template and VPg binding sites, and thus both are not expected to have an effect on binding. However, the close proximity of amiloride and EIPA to one of the Mg²⁺ ions and an overlap with the nucleotide binding site suggest that the presence of Mg²⁺ and/or ATP would affect compound binding.

CRE-templated VPg uridylylation was inhibited by amiloride in the presence of physiological concentrations of UTP and Mg²⁺ (Fig. 6), with an IC₅₀ similar to that observed for CVB3 inhibition in cell culture (i.e., 60 μ M [11]). This suggests that inhibition of VPg uridylylation is part of the mechanism of inhibition of CVB3 RNA replication in cell culture.

Single AMP incorporation into SSU was inhibited by amiloride more effectively than VPg uridylylation when the compound was allowed to bind to 3D^{pol}-SSU complexes prior to the addition of ATP and Mg²⁺ (Fig. 3). However, the inhibitory effect of amiloride was strongly reduced when the compound was added to 3D^{pol}-SSU complexes together with ATP in the presence of Mg²⁺ due to slow inhibition kinetics compared to rapid nucleotide incorporation (Fig. 4). Because a small inhibitory effect on single-nucleotide addition can be magnified greatly over the >7,400 nucleotide additions in the course of RNA elongation, we concluded that it is likely that amiloride and EIPA inhibit CVB3 RNA elongation in cell culture.

Additional evidence supporting this conclusion comes from inhibition of S299T 3D^{pol} compared to the WT enzyme. The S299T mutation confers partial resistance of CVB3 to amiloride and EIPA in cell culture (11). Furthermore, this mutation was shown to reduce the inhibition of CVB3 RNA synthesis in cell culture by amiloride (14). Single AMP incorporation into SSU by S299T 3D^{pol} was less inhibited by amiloride and EIPA than by the WT enzyme (Fig. 2 and 3), recapitulating the cell culture data. In contrast, the extents of inhibition of CRE-templated VPg uridylylation by amiloride were the same for the two enzymes (Fig. 6). When poly(A) was

used as a template for VPg uridylylation, a small difference in IC₅₀ between S299T 3D^{pol} and the WT was observed for amiloride (1.3-fold), with a larger one observed for EIPA (2-fold) (Fig. 1), but this assay is less biologically relevant than the assay using the CRE template. Our overall conclusion, therefore, is that both VPg uridylylation and RNA elongation are inhibited by amiloride and EIPA in cell culture, with RNA elongation being the main process rescued by the S299T mutation in 3D^{pol}.

Our finding that VPg uridylylation is less influenced by the S299T mutation than is RNA elongation in terms of resistance to amiloride is not surprising in light of the data on the related poliovirus 3D^{pol} demonstrating that VPg uridylylation activity is distinct from RNA elongation activity, with differing responses to a mutation. Substitution of N297 in poliovirus 3D^{pol} (homologous to N298 in CVB3 3D^{pol}, next to S299) had different effects on VPg uridylylation and RNA elongation, revealing the distinct specificities of initiation and RNA elongation complexes (13).

An additional factor contributing to virus resistance conferred by the S299T mutation is the fidelity of CVB3 3D^{pol}. Amiloride increases the error rate of WT 3D^{pol}, whereas S299T 3D^{pol} has a lower fidelity than the WT but remains unaffected by the compound (14). Interestingly, virus resistance conferred by A372V substitution is due solely to the higher fidelity of A372V 3D^{pol} than that of the WT enzyme, because this mutation does not affect the inhibition of RNA synthesis by the compound (14).

In summary, our data strongly suggest that amiloride and EIPA inhibit CVB3 replication in cell culture by inhibiting

VPg uridylylation and RNA elongation, with RNA elongation being the main process rescued by the S299T mutation in 3D^{pol}. The compounds act as competitive inhibitors of CVB3 3D^{pol}, competing with NTPs and Mg²⁺ for binding to the active site of the enzyme. This is the first report of a molecular mechanism of action of nonnucleoside inhibitors against a picornaviral RNA-dependent RNA polymerase.

ACKNOWLEDGMENTS

This work was supported by ARC grant DP0987855. E.D.S., J.J.A., and C.E.C. were supported by a grant from NIAID, NIH (AI045818). S.P. is an NHMRC fellow. M.W.P. is an ARC Federation fellow and an NHMRC honorary fellow.

We thank I. Moustafa and B. Jarrott for critical readings of the manuscript.

REFERENCES

- Andreoletti, L., N. Leveque, C. Boulagnon, C. Brasselet, and P. Fornes. 2009. Viral causes of human myocarditis. *Arch. Cardiovasc. Dis.* **102**:559–568.
- Arnold, J. J., and C. E. Cameron. 2000. Poliovirus RNA-dependent RNA polymerase (3D(pol)). Assembly of stable, elongation-competent complexes by using a symmetrical primer-template substrate (sym/sub). *J. Biol. Chem.* **275**:5329–5336.
- Arnold, J. J., and C. E. Cameron. 2004. Poliovirus RNA-dependent RNA polymerase (3Dpol): pre-steady-state kinetic analysis of ribonucleotide incorporation in the presence of Mg²⁺. *Biochemistry* **43**:5126–5137.
- Besterman, J. M., W. S. May, Jr., H. LeVine III, E. J. Cragoe, Jr., and P. Cuatrecasas. 1985. Amiloride inhibits phorbol ester-stimulated Na⁺/H⁺ exchange and protein kinase C. An amiloride analog selectively inhibits Na⁺/H⁺ exchange. *J. Biol. Chem.* **260**:1155–1159.
- Cameron, C. E., H. Suk Oh, and I. M. Moustafa. 2010. Expanding knowledge of P3 proteins in the poliovirus lifecycle. *Future Microbiol.* **5**:867–881.
- Davis, R. J., and M. P. Czech. 1985. Amiloride directly inhibits growth factor receptor tyrosine kinase activity. *J. Biol. Chem.* **260**:2543–2551.
- Ferrer-Orta, C., R. Agudo, E. Domingo, and N. Verdaguier. 2009. Structural insights into replication initiation and elongation processes by the FMDV RNA-dependent RNA polymerase. *Curr. Opin. Struct. Biol.* **19**:752–758.
- Gong, P., and O. B. Peersen. 2010. Structural basis for active site closure by the poliovirus RNA-dependent RNA polymerase. *Proc. Natl. Acad. Sci. U. S. A.* **107**:22505–22510.
- Goodfellow, I. G., C. Polacek, R. Andino, and D. J. Evans. 2003. The poliovirus 2C cis-acting replication element-mediated uridylylation of VPg is not required for synthesis of negative-sense genomes. *J. Gen. Virol.* **84**:2359–2363.
- Gruez, A., et al. 2008. The crystal structure of coxsackievirus B3 RNA-dependent RNA polymerase in complex with its protein primer VPg confirms the existence of a second VPg binding site on Picornaviridae polymerases. *J. Virol.* **82**:9577–9590.
- Harrison, D. N., E. V. Gazina, D. F. Purcell, D. A. Anderson, and S. Petrou. 2008. Amiloride derivatives inhibit coxsackievirus B3 RNA replication. *J. Virol.* **82**:1465–1473.
- Holland, R., J. R. Woodgett, and D. G. Hardie. 1983. Evidence that amiloride antagonises insulin-stimulated protein phosphorylation by inhibiting protein kinase activity. *FEBS Lett.* **154**:269–273.
- Korneeva, V. S., and C. E. Cameron. 2007. Structure-function relationships of the viral RNA-dependent RNA polymerase: fidelity, replication speed, and initiation mechanism determined by a residue in the ribose-binding pocket. *J. Biol. Chem.* **282**:16135–16145.
- Levi, L. I., et al. 2010. Fidelity variants of RNA dependent RNA polymerases uncover an indirect, mutagenic activity of amiloride compounds. *PLoS Pathog.* **6**:e1001163.
- Morasco, B. J., N. Sharma, J. Parilla, and J. B. Flanagan. 2003. Poliovirus cre(2C)-dependent synthesis of VPgUpU is required for positive- but not negative-strand RNA synthesis. *J. Virol.* **77**:5136–5144.
- Murray, K. E., and D. J. Barton. 2003. Poliovirus CRE-dependent VPg uridylylation is required for positive-strand RNA synthesis but not for negative-strand RNA synthesis. *J. Virol.* **77**:4739–4750.
- Pathak, H. B., J. J. Arnold, P. N. Wiegand, M. R. Hargittai, and C. E. Cameron. 2007. Picornavirus genome replication: assembly and organization of the VPg uridylylation ribonucleoprotein (initiation) complex. *J. Biol. Chem.* **282**:16202–16213.
- Pathak, H. B., et al. 2002. Structure-function relationships of the RNA-dependent RNA polymerase from poliovirus (3Dpol). A surface of the primary oligomerization domain functions in capsid precursor processing and VPg uridylylation. *J. Biol. Chem.* **277**:31551–31562.
- Paul, A. V., E. Rieder, D. W. Kim, J. H. van Boom, and E. Wimmer. 2000. Identification of an RNA hairpin in poliovirus RNA that serves as the primary template in the in vitro uridylylation of VPg. *J. Virol.* **74**:10359–10370.
- Paul, A. V., J. H. van Boom, D. Filippov, and E. Wimmer. 1998. Protein-primed RNA synthesis by purified poliovirus RNA polymerase. *Nature* **393**:280–284.
- Plotch, S. J., O. Palant, and Y. Gluzman. 1989. Purification and properties of poliovirus RNA polymerase expressed in *Escherichia coli*. *J. Virol.* **63**:216–225.
- Romani, A. 2007. Regulation of magnesium homeostasis and transport in mammalian cells. *Arch. Biochem. Biophys.* **458**:90–102.
- Steil, B. P., and D. J. Barton. 2009. Cis-active RNA elements (CREs) and picornavirus RNA replication. *Virus Res.* **139**:240–252.
- Thompson, A. A., R. A. Albertini, and O. B. Peersen. 2007. Stabilization of poliovirus polymerase by NTP binding and fingers-thumb interactions. *J. Mol. Biol.* **366**:1459–1474.
- Traut, T. W. 1994. Physiological concentrations of purines and pyrimidines. *Mol. Cell. Biochem.* **140**:1–22.
- van Ooij, M. J., et al. 2006. Structural and functional characterization of the coxsackievirus B3 CRE(2C): role of CRE(2C) in negative- and positive-strand RNA synthesis. *J. Gen. Virol.* **87**:103–113.