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Amiloride inhibits the initiation of Coxsackievirus and poliovirus RNA replication by inhibiting VPg uridylylation

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ABSTRACT

The mechanism of amiloride inhibition of Coxsackievirus B3 (CVB3) and poliovirus type 1 (PV1) RNA replication was investigated using membrane-associated RNA replication complexes. Amiloride was shown to inhibit viral RNA replication and VPgpUpU synthesis. However, the drug had no effect on polymerase elongation activity during either (−) strand or (+) strand synthesis. These findings indicated that amiloride inhibited the initiation of RNA synthesis by inhibiting VPg uridylylation. In addition, *in silico* binding studies showed that amiloride docks in the VPg binding site on the back of the viral RNA polymerase, $3D^{pol}$. Since VPg binding at this site on PV1 $3D^{pol}$ was previously shown to be required for VPg uridylylation, our results suggest that amiloride inhibits VPg binding to $3D^{pol}$. In summary, our findings are consistent with a model in which amiloride inhibits VPgpUpU synthesis and viral RNA replication by competing with VPg for binding to $3D^{pol}$.

Introduction

Coxsackievirus B3 (CVB3) and poliovirus type 1 (PV1) are human enteroviruses that belong to the *Picornaviridae* family of (+) strand RNA viruses. The 5′ end of the single-stranded RNA genome is covalently linked to a virus-encoded protein, VPg, and the 3′ end is polyadenylated. The genomic RNA contains a large open reading frame flanked by 5′ and 3′ nontranslated regions (NTRs). Translation of the viral genome results in the synthesis of a polyprotein, which is processed by viral proteases $2A^{pro}$ and $3C^{pro}$/$3CD^{pro}$ into the mature viral proteins. The P1 region of the genome encodes the capsid proteins, and the P2 and P3 regions encode the non-structural proteins that are required for RNA replication including $3D^{pol}$, the viral RNA-dependent RNA polymerase.

The 5′ terminal cloverleaf (5′CL), the 3′NTR including the poly (A) tail and the internal *cre*(2C) hairpin are *cis*-acting elements that are needed for viral RNA replication (Liu et al., 2009; Steil and Barton, 2009a; Ogram and Flanegan, 2011). The 5′CL is a multi-functional element that is required for translation, (−) and (+) strand synthesis and VPg uridylylation (Barton et al., 2001; Gamarnik and Andino, 1998, 2000; Murray et al., 2001; Ogram et al., 2010; Sharma et al., 2005, 2009; Spear et al., 2008; Teterina et al., 2001; Vogt and Andino, 2010). The conserved *cre* hairpin structure in the 2C coding region of the RNA genome serves as the primary template for VPg uridylylation by $3D^{pol}$ to form VPgpUpU (McKnight and Lemon, 1998; Goodfellow et al., 2000; Paul et al., 2000; Gerber et al., 2001). Results of many studies show that VPgpUpU serves as the primer for $3D^{pol}$ to initiate both (−) and (+) strand synthesis (Fogg et al., 2003; Morasco et al., 2003; Murray and Barton, 2003; Takegami et al., 1983a, 1983b; Toyoda et al., 1987; Steil and Barton, 2009b; van Ooij et al., 2006).

The drug, amiloride, was previously shown to inhibit CVB3 replication in infected HeLa cells by inhibiting viral RNA replication without affecting host or viral protein synthesis (Harrison et al., 2008). Amiloride was also shown to increase the mutation frequencies of both CVB3 and PV1 in infected cells. This was shown to be an indirect mutagenic effect that was mediated by an increase in the intracellular concentration of Mg^{2+} and Mn^{2+}, which affected the fidelity of the viral polymerase (Levi et al., 2010). The effect of amiloride on single-nucleotide (AMP) incorporation was investigated in assays containing purified CVB3 $3D^{pol}$ and a 10-nucleotide, self-annealing, RNA primer-template (SSU) (Gazina et al., 2011). A small inhibitory effect on nucleotide incorporation (<9%) was observed in these reactions when amiloride was added with ATP in the presence of Mg^{2+}. Effective inhibition was only observed when $3D^{pol}$ was preincubated with amiloride for several minutes in the absence of ATP or Mg^{2+}.

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Taken together, these results indicate that the kinetics of amiloride inhibition of $3D^{pol}$ catalytic activity is very slow compared to the rapid rate of ATP incorporation in the presence of Mg^{2+}. Finally, amiloride was shown to inhibit VPgpUpU synthesis in reconstituted reactions containing purified CVB3 $3D^{pol}$, VPg and *cre* hairpin RNA (Gazina et al., 2011). Interestingly, the inhibition of VPgpUpU synthesis by amiloride was not dependent on preincubating $3D^{pol}$ with the drug prior to adding UTP to start the reaction.

In the current study, we examined the effect of amiloride on CVB3 and PV1 RNA replication in preinitiation replication complexes (PIRCs) isolated from HeLa cell-free reactions (Barton and Flanegan, 1997; Lyons et al., 2001; Morasco et al., 2003; Sharma et al., 2009). Our findings showed that amiloride had no measurable effect on the elongation activity of the viral polymerase, but specifically inhibited the initiation of RNA replication and VPg uridylylation. Furthermore, in silico binding studies showed that amiloride docks at the VPg binding site previously identified at the back of $3D^{pol}$. Since previous genetic studies indicate that VPg binding to this site is required for efficient VPgpUpU synthesis (Lyle et al., 2002), our results suggest that amiloride inhibits VPg binding to $3D^{pol}$. Based on these findings, we propose a model in which amiloride competes with VPg for binding to the site on the back of $3D^{pol}$, which in turn inhibits VPgpUpU synthesis and viral RNA replication.

Results

Effect of amiloride on viral RNA replication.

To investigate the underlying mechanism of amiloride inhibition of CVB3 RNA replication, we used membrane-associated preinitiation replication complexes (PIRCs) isolated from HeLa S10 reactions. In previous studies, we showed that PIRCs support efficient VPgpUpU synthesis and (−) and (+) strand synthesis (Sharma et al., 2009). CVB3 P23 RNA is a subgenomic RNA that contains sequences for the 5′NTR, the P23 coding region that encodes the replication proteins, the 3′NTR and poly(A) tail. P23 RNA contains two non-viral G residues at the 5′ end which supports only (−) strand RNA synthesis. This RNA was added to HeLa S10 reactions, and PIRCs were isolated from the reactions at 3 h. The PIRCs were resuspended in replication assay buffer containing all four NTPs, [α-^{32}P] CTP and Mg^{2+}. Labeled (−) strand synthesis was measured in the presence or absence of amiloride as described in *Materials and methods*. The amount of labeled (−) strand RNA synthesized in each reaction was determined by electrophoresis in a denaturing agarose gel. In the presence of 0.4, 0.8 and 1.6 mM amiloride, (−) strand synthesis was 49%, 25% and 2%, respectively of the level observed in the absence of the drug (Fig. 1A). The concentration of amiloride required to observe 50% inhibition of (−) strand synthesis (IC_{50}) was calculated and shown to be 0.41 mM in these reactions (Fig. 1B). These results demonstrated that amiloride inhibited (−) strand synthesis in a concentration dependent manner in PIRCs.

Since amiloride inhibited CVB3 (−) strand synthesis, a corresponding decrease in (+) strand synthesis should also be observed in the presence of the drug. To determine if amiloride differentially inhibited CVB3 (+) strand synthesis, we measured the effect of amiloride on the ratio of (+)/(−) strand synthesis (Sharma et al., 2009). A decrease in the (+)/(−) strand ratio should be observed if amiloride differentially inhibits (+) strand synthesis. The (+)/(−) strand ratio was calculated by measuring labeled RNA synthesis in reactions containing CVB3 P23 RNA or CVB3 RzP23 RNA as previously described (Sharma et al., 2009). (−) strand RNA is synthesized in reactions containing P23 RNA. In contrast, both (−) strand and (+) strand RNAs are synthesized in reactions containing RzP23 RNA. This RNA contains a 5′ hammerhead ribozyme (Rz) which upon cleavage generates an authentic 5′ terminus which supports both (−) and (+) strand synthesis. The effect of amiloride on labeled RNA synthesis was determined in separate reactions containing either P23 or RzP23 RNA. The results showed that amiloride inhibited (−) strand synthesis (Fig. 1C left) and overall synthesis (both (+) and (−) strands) (Fig. 1C right) by similar amounts. The amount of labeled RNA synthesized in each reaction was then used to calculate the ratio of (+)/(−) strand synthesis as described in *Materials and methods*. In the absence of the drug, the (+)/(−) strand ratio was 14 consistent with the previous studies (Fig. 1C) (Sharma et al., 2009). In reactions containing 0.4 mM and 0.8 mM amiloride, the (+)/(−) strand ratio was 14 and 10, respectively (Fig. 1C). These results indicated that amiloride had no significant effect on the ratio of (+)/(−) strand synthesis and therefore, amiloride did not differentially inhibit CVB3 (+) strand synthesis.

We next determined if amiloride also inhibited PV1 RNA replication in PIRCs. The synthesis of (−) strand RNA was measured in the presence or absence of amiloride as described above. In the presence of 0.4, 0.8 and 1.6 mM amiloride, (−) strand synthesis was 58%, 28% and 2%, respectively, of the level observed in the absence of the drug (IC_{50}=0.48 mM) (Fig. 2A and B). These results showed that amiloride inhibited PV1 (−) strand synthesis at levels similar to that observed with CVB3 P23 RNA (Fig. 1A).

To determine the effect of amiloride on (+) strand synthesis in reactions containing PV1 RNA, we calculated the (+)/(−) strand ratio by measuring labeled RNA synthesis in reactions containing PV1 P23 RNA or PV1 RzP23 RNA. Amiloride inhibition of (−) strand synthesis (Fig. 2C left) and overall synthesis (Fig. 2C right) was similar in these reactions. The ratio of (+)/(−) strand synthesis was 20 in the absence of the drug (Fig. 2C). In reactions containing 0.4 mM or 0.8 mM amiloride, the (+)/(−) strand ratio was 18 and 23, respectively (Fig. 2C). As previously observed with CVB3, amiloride had no significant effect on the ratio of (+)/(−) strand synthesis during PV1 RNA replication and did not differentially inhibit (+) strand synthesis.

Effect of amiloride on (−) strand elongation

We next investigated if amiloride inhibited the elongation of CVB3 (−) strand RNA in replication complexes. To do this, we measured the effect of amiloride on the time required to synthesize full-length (−) strand RNA in PIRCs. In these reactions, removal of guanidine–HCl allows for the synchronous initiation of (−) strand synthesis. If amiloride inhibits the elongation rate of $3D^{pol}$, then the time required to synthesize full-length (−) strand RNA will increase in the presence of the drug. In contrast, if amiloride inhibits the initiation of RNA synthesis, the total amount of RNA synthesized will decrease but the time required to synthesize full-length RNA will be the same. PIRCs were isolated from reactions containing CVB3 P23 RNA and resuspended in replication buffer in the presence or absence of amiloride. Aliquots were removed at the indicated times and labeled product RNA was analyzed by denaturing agarose gel electrophoresis. In the untreated reactions, the growing nascent RNA chains were observed at 5, 6 and 7 min and full-length (−) strand RNA was observed at 8 min (Fig. 3A). In the presence of 0.8 mM amiloride, full-length (−) strand RNA was detected at 8–9 min although the overall intensity of the product RNA was significantly reduced (Fig. 3A). These results indicated that amiloride had no significant effect on the time required to synthesize full-length product RNA even though the overall level of (−) strand synthesis was inhibited by four-fold (Fig. 1A). Taken together, these results indicated that