Master's Thesis

Endosomal ions and serum promote enterovirus uncoating

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Echovirus 1 (E1) belonging to the enterovirus B species is an RNA virus that enters non-acidic endosomes and accumulates in neutral multivesicular bodies. There was limited information about physiological triggers leading to uncoating of enteroviruses in the beginning of this study. The aim of this study was to evaluate *in vitro* what combinations of key ions promote uncoating of echovirus 1 at physiological temperature. Another aim of the study was to investigate the effect of serum on uncoating of the virus. Real-time fluorescence spectroscopy was used to measure the binding of SYBR Green II to viral RNA indicating the possible uncoating. End point dilution and cytopathic effect assay were performed to estimate viral infectivity after different treatments. Finally, sucrose and caesium chloride gradient separation of radioactively labeled virus was performed to confirm the state of uncoating. Low sodium concentration together with high potassium concentration induced the uncoating process according to our results. Addition of a small amount of serum enhanced the uncoating leading to the formation of the uncoating intermediate particle and genome egress. In addition, $MgCl_2$ and $CaCl_2$ prevented the uncoating process and, thus had a stabilizing effect in the assay. This study offered new information about molecular cues that trigger the uncoating under physiological conditions. Further studies can be designed based on these findings.

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Enterovirusten B ryhmään kuuluva Echovirus 1 on RNA-virus, jonka tiedetään infektoivan soluja ei-happamien endosomien välityksellä ja kerääntyvän neutraaleihin multivesikulaarisiin kappaleisiin. Tämän tutkimuksen alussa oli olemassa hyvin rajallisesti tietoa fysiologisista tekijöistä, jotka johtavat enterovirusten avautumiseen. Tutkimuksen tarkoituksena oli selvittää *in vitro* millainen yhdistelmä ioneja edistää echovirus 1:n avautumista fysiologisessa lämpötilassa. Päämääränä oli myös tutkia seerumin vaikutusta viruksen avautumiseen. Spektroskooppisissa mittauksissa käytettiin hyväksi SYBR Green II:n kykyä sitoutua viruksen RNA:han ja saada aikaan voimakasta fluoresenssia siinä tilanteessa, kun virus on avautunut vähän tai RNA on kokonaan vapautunut viruksen ulkopuolelle. Viruksen infektiivisyyttä erilaisten käsittelyjen jälkeen arvioitiin muun muassa sytopaattisen määrityksen avulla. Radioaktiivisesti leimatun viruksen avautumisen taso varmistettiin lopulta sakkaroosi- ja cesiumkloridigradienttierottelulla. Matala natriumpitoisuus yhdistettynä korkeaan kaliumpitoisuuteen edisti viruksen avautumista tämän tutkimuksen tulosten mukaan. Seerumilla havaittiin olevan myös viruspartikkelin avautumista ja genomin vapautumista edistävä vaikutus. Lisäksi tutkimuksessa tuli ilmi, että $MgCl_2$ ja $CaCl_2$ vakauttavat viruksen rakennetta. Tämä tutkimus paljasti uutta tietoa fysiologisista tekijöistä, jotka edistävät enterovirusten avautumista. Tutkimuksen tulosten pohjalta on mahdollista suunnitella uusia aihetta syventäviä jatkotutkimuksia.

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ABBREVIATIONS

CsCl	caesium chloride
E1	Echovirus 1
MVBs	multivesicular bodies
VP	viral protein

1 INTRODUCTION

2.1 Enteroviruses

Enteroviruses belong to a family of picornaviruses and they share the common genome structure of picornaviruses, a positive sense single-stranded RNA genome of approximately 7,500 nucleotides. VPg (viral protein genome-linked) is covalently attached to the 5'end and 3'end ends in a poly(A) tract. Untranslated region (UTR) locating at the 5'end is important in the initiation of protein synthesis. Enteroviruses have a non-enveloped icosahedral capsid structure of approximately 30 nm in size. Capsid consists of 12 pentamers, each of which are composed by 5 copies of each structural protein (VP1-4). Capsid proteins VP1, VP2 and VP3 form the external shell of the virus and VP4 is located on the inner surface of the virus particle (Tuthill et al. 2010, Bergelson and Coyne 2013, Marjomäki et al. 2015).

Human enteroviruses consist of enterovirus subgroups and three rhinovirus subgroups (A-C). Most of the enteroviruses infecting humans belong to the four subgroups, *Enterovirus* A-D. These contain poliovirus, coxsackieviruses A and B, echoviruses and several recently identified enteroviruses (EV) (Nikonov et al. 2017, Baggen et al. 2018).

Enteroviral infections are usually transmitted either via the fecal–oral or respiratory route. The primary site of infection is the epithelium of gastrointestinal tract (Wells and Coyne 2019). Enterovirus species are known to cause several mild and severe diseases in humans like poliomyelitis, central nervous system infections (meningitis, encephalitis), skin rashes, myocarditis, and respiratory infections (Pons-Salort et al. 2015). Coxsackievirus B (CVB) viruses (1-5) have been recognized as a common factor contributing to progress of human subacute, acute,

and chronic myocarditis (Gebhard et al. 1998). Enterovirus A71 (EV-A71) has caused several outbreaks in Asia-pacific region affecting especially young children (Puenpa et al. 2019). Roughly 30% of hospitalized hand-foot-and-mouth disease (HMFD) patients with EV-A71 infection develop more severe disease with neurological symptoms like meningitis and cardiopulmonary failure, which can be fatal (Pons-Salort et al. 2015). Poliovirus, the causative agent of poliomyelitis, is one of the most known and studied enteroviruses. Poliovirus cases have not occurred in India since 2011, but there are still challenges with eradication of the disease in Pakistan, Afghanistan, and Nigeria (Lugo and Krogstad 2016). Outbreaks of enterovirus 68 (ED-68) have emerged worldwide in recent years. It is one causative agent of acute flaccid myelitis (AFM) leading to diverse neurological symptoms and limb weakness (Cassidy et al. 2018). Involvement of enteroviral infections has been recognized in the initiation of the disease process leading to the development of type 1 diabetes in genetically susceptible individuals infected at young age (Roivainen and Klingel 2009, Tauriainen et al. 2010, Laitinen et al. 2014). Recent evidence and studies suggest that enteroviral infections are also involved in development of coeliac disease (Kahrs et al. 2019).

2.2 Echovirus 1

Echovirus 1 belongs to Species B enteroviruses. E1 uses $\alpha_2\beta_1$ as its main receptor for attachment (Bergelson et al. 1992, Triantafilou et al. 2001). $\alpha_2\beta_1$ is an extracellular matrix receptor for collagens and lamins and serves as a receptor for many matrix and non-matrix molecules (Madamanchi et al. 2014). The binding affinity of E1 to α_2I domain is 10-fold compared to the binding affinity of collagen to the same domain. Studies have shown that binding of E1 to multiple integrin molecules can cause integrin clustering, possibly leading to cellular signaling and finally internalization of virus-receptor complex in tubulovesicular structures (Xing et al. 2004). β_2 microglobulin is also considered as a possible receptor for E1

since E1 entry is inhibited by antibodies against β_2 microglobulin (Marjomäki et al. 2002).

E1 entry pathway into cells is clathrin-independent and it differs from the usual entry and recycling of $\alpha_2\beta_1$ integrin. (Marjomäki et al. 2015). E1 was originally suggested to internalize in cells using caveola route. Caveolin-1 and $\alpha_2\beta_1$ integrin were shown to colocalize with E1 capsid proteins and migrate into the perinuclear area in the cell during internalization in a study (Marjomäki et al. 2002). However, in a later study it was proposed that initial uptake is independent of caveolin and finally the cargo is delivered into caveosomes (Karjalainen et al. 2008). Several factors regulating the cell entry were revealed in the same study. These included actin, serine/threonine p21-activated kinase (Pak)1, Rac1 , phosphatidylinositol 3-kinase and phospholipase C. Additionally, Na^+/H^+ exchanger appeared to regulate entry of E1, as 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) totally inhibited E1 infection. Many of these regulating factors suggest for macropinocytotic uptake. (Karjalainen et al. 2008, Marjomäki et al. 2015). Presence of cholesterol is also crucial for E1 entry since perturbation of cholesterol inhibits viral entry, uncoating and infection (Siljamäki et al. 2013). It is known that endosomes of the E1 entry pathway are not acidified (Karjalainen et al. 2011, Rintanen et al. 2012).

Tubulovesicular structures (induced by $\alpha_2\beta_1$ clustering) mature finally into non-acidic multivesicular bodies (MVBs) that are different compared to acidic late endosomes and lysosomes in several ways (Karjalainen et al. 2011). Uncoating of E1 starts at 30 minutes after entry and continues up to two hours post-infection (Marjomäki et al. 2002, Soonsawad et al. 2014). Size of MVBs increases due to presence of E1 and breakages in the membranes of MVBs start emerging two hours post-infection. These breakages could facilitate the egress of viral RNA to cytoplasm (Soonsawad et al. 2014).

Internalized integrin does not recycle back to the plasma membrane. Instead it is degraded in MVBs in a process assisted by calpain proteases (Rintanen et al. 2012). It is suggested that calpains may even promote the degradation of the MVBs and participate in the start of E1 replication (Marjomäki et al. 2015). Viral replication starts in cytoplasm three hours post-infection according to previous quantitative PCR study results (Upla et al. 2008).

2.3 Uncoating of enteroviruses

After binding to cell receptors and entry by endocytosis, enteroviruses undergo uncoating in order to release the viral RNA to the cytoplasm and reach cellular translation and replication machinery. Three distinct particle types have been shown to occur during the entry process. These three forms have differences in their sedimentation coefficients and antigenic properties. The 160S particle presents the native, and infective form of the virion. The 135S or A-particle is the destabilized, uncoating intermediate form of the virion. The 80s particle is the empty, genome-free end-product of the uncoating process (Tuthill et al. 2010, Baggen et al. 2018, Wells and Coyne 2019). These three distinct particle types are represented in Figure 1.

Uncoating is either triggered by low pH in the endosomes or for some enteroviruses by receptor binding (Kaplan et al. 1990, Prchla et al. 1994, Coyne and Bergelson 2006, Liu et al. 2018, Zhao et al. 2019, Zhao et al. 2020). These cellular uncoating cues are leading to conformational changes and formation of the A-particle. Uncoating of enteroviruses is demonstrated to require a removal of a fatty acid called “pocket factor” from the hydrophobic pocket within VP1 as a prerequisite step. This step leads to destabilization of the particle and formation of the A-particle (Smyth et al. 2003, Marjomäki et al. 2015). The formed A-particle is expanded compared to the native form, the N-terminus of VP1 is externalized and the subunit VP4 is lost during the uncoating process (Fricks and Hogle 1990,

Greve et al. 1991). Recently, a novel uncoating intermediate particle of E1 was characterized. This particle is naturally produced during infection and it is highly infective. Additionally, it is capable of receptor binding and still contains VP4 (Myllynen et al. 2016). N-terminus of VP4 has been demonstrated to have an ability to permeabilize membranes leading to pore formation for genome egress in a study with human rhinovirus (Panjwani et al. 2016). It has been demonstrated that RNA is finally released near a twofold symmetry axis (Bostina et al. 2011). A lipid-modifying enzyme PLA2G16 has been identified as a factor that facilitates the viral genome transfer into the cytoplasm. PLA2G16 could also be involved in membrane pore formation and maintenance for genome release (Staring et al. 2017).

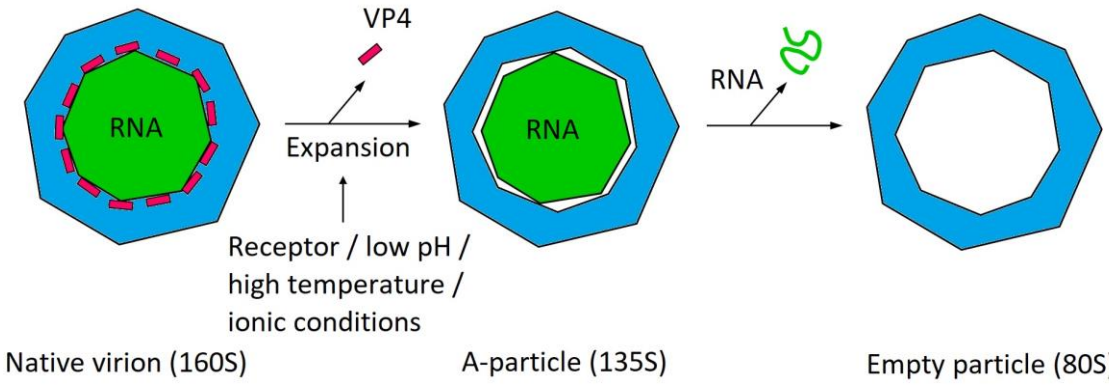

Figure 1. Particle types of enterovirus during the uncoating process. Three distinct particle types are observed respective to their sedimentation coefficients and antigenic properties. Formation of A-particle is triggered by uncoating cues including low pH in the endosomes, receptor binding, ionic conditions or high non-physiological temperatures. The formed A-particle is expanded compared to the native form, the N-terminus of VP1 is externalized and the subunit VP4 is usually lost during the process. Finally, viral RNA is released into the cytosol leading to the formation of the empty 80S particle. Modified from Baggen et al. (2018).

Structural studies have provided a lot of information about uncoating of enteroviruses. Acid treatment was shown to induce uncoating in a study

concerning human enterovirus D68 (EV-D68). Results achieved by high-resolution cryo-electron microscopy showed dramatic conformational changes of full native virions to form A particles caused by acid treatment; the particle was expanded, VP1 N termini from the capsid interior was externalized and pores were formed around the icosahedral twofold axes (Liu et al. 2018).

Some possible uncoating receptors for enteroviruses have already been identified. It has been demonstrated in a study utilizing colocalization study and sucrose gradient separation that Group B coxsackieviruses (CVBs) require interaction with the coxsackievirus and adenovirus receptor (CAR) for A-particle formation (Coyne and Bergelson 2006). Human neonatal Fc receptor (FcRn) has been identified as a universal uncoating receptor for a large group of EV-B viruses (including echoviruses and CV-A9) by performing CRISPR-Cas9 library screening in a recent study (Zhao et al. 2019). FcRn-decorated liposome model experiments confirmed FcRn-mediated uncoating. Finally, cryo-electron microscopy structures revealed that binding of the identified FcRn uncoating receptor triggered the release of pocket factor from virion in acidic environment (Zhao et al. 2019). Another recent study has suggested that KREMEN1 (KRM1) receptor binding to coxsackievirus A10 (CV-A10) releases pocket factor and triggers the conversion of viral particles to the altered state (A-particle). The results were obtained by comparing cryo-EM model structures (Zhao et al. 2020).

A-particles of different enteroviruses have been produced *in vitro* by heating a virus in non-physiological high temperatures. In a study focusing on enterovirus 71 (EV-71) uncoating, a purified EV71 mature virus was heated to 56°C for 12 minutes. Cryo-EM study revealed formation of A-particle and genome-free empty capsids (Shingler et al. 2013). Poliovirus 135S particles have also been produced by heating the native virus at 50°C for cryo-EM reconstruction study (Butan et al. 2014). However, a recent study has demonstrated that heating E1 particle to non-physiological temperatures produces a more fragile particle that is distinct from

the natural uncoating intermediate particle (Myllynen et al. 2016). Thus, it is important to study the uncoating of E1 at physiological temperature based on this information.

Effect of serum albumin on uncoating process of enteroviruses has been investigated in a few studies. One earlier study suggested that fatty acid depleted bovine serum albumin induces the formation of echovirus 12 A-particle (Ward et al. 2000). It was recently demonstrated that extracellular serum albumin and endosomal ions induce the formation of a metastable uncoating intermediate of E1 by using real-time spectroscopy and sucrose gradient separation with radioactively labelled virus (Ruokolainen et al. 2019). Another recent study demonstrated that albumin enhances the rate of coxsackievirus B3 strain 28 conversion to non-infectious A-particles (Carson and Cole 2020).

2.4 Aim of the study

It is known that E1 accumulates in neutral multivesicular bodies and uncoating of the virus starts 30 minutes post-infection. There was limited information about molecular cues leading to uncoating of enteroviruses at physiological conditions in the beginning of this study in 2016. In particular, there was little information about effects of ionic conditions on stability and structure of a viral particle (Wetz and Kucinski 1991, Cords et al. 1975). The aim of this study was to investigate if there was a certain ionic combination that induces the formation of uncoating intermediate particles and genome release *in vitro* at 37°C. Another aim of this study was to examine the effect of serum on uncoating of E1.

Main research questions of this study:

1. What kind of ionic conditions impact on virion stability at physiological temperature?

2. Is there a certain ionic composition of the physiologically relevant ions (Na^+, K^+, Mg^{2+} and Ca^{2+}) that promotes the uncoating of the virus at physiological temperature?

3. Does serum (FCS) induce the uncoating process?

It was hypothesized that certain conditions exist that either stabilize the virus or promote uncoating. It was also hypothesized that serum might induce uncoating of the virus. The possible component of the serum that promotes uncoating could be determined in further studies.

Real-time spectroscopy was used in this study in order to reveal if uncoating of the virus occurred. The state of uncoating was determined by sucrose and CsCl gradient separation of radioactively labelled virus. Infectivity of the virus was tested by end-point dilution and cytopathic effect assay (CPE).

2 MATERIALS AND METHODS

2.1 Virus preparations

E1 and radioactive 35S-labelled E1 used in this study were previously produced and purified. The purification was performed either by 10-40% sucrose gradient or CsCl gradient separation. The infectivity of the virus was approximately 1,09E+12 pfu/ml.

2.2 Cytopathic effect assay (CPE)

The CPE assay was carried out in confluent monolayers of A549 cells. The cells were cultivated in DMEM supplemented with 10% FBS, 1% GlutaMAX and 1% penicillin-streptomycin on a 96-well microtiter plate for 24 hours at 37°C, after which the cells were infected with treated virus (incubated 1 or 3 hours at 37°C in different solutions) diluted in 1% DMEM. The amount of added virus was 5 nanograms. Each sample had four replicates and non-infected cell control was included. The cells were infected for 24 hours at 37°C. After infection, the cells were washed twice with PBS, and the infected cells were stained with CPE stain containing crystal violet for 10 minutes at room temperature. Excess stain was washed away with water. The cells were then treated with lysis buffer to homogenize the sample. Finally, the absorbance of the stain was measured at 570 nm to determine the number of non-infected cells (Victor X4 2030 multilabel reader, PerkinElmer).

2.3 End-point dilution assay

Monolayers of GMK cells were cultured in 96-well microtiter plates in MEM (containing 10% FBS, 1% GlutaMAX and 1% penicillin/streptomycin). The cells were incubated overnight. Cell monolayers were infected with treated virus

(incubated 1 or 3 hours at 37°C in different solutions) by preparing a dilution series in 1% MEM starting with 10^{-4} virus solution. The cells were then infected for 3 days at 37°C. Confluence of the cells was followed during the infection. After 3 days the cells were stained with 50 µl of crystal violet stain (8.33 mM crystal violet, 45 mM CaCl2, 10% ethanol, 18.5% formalin, and 35 mM Tris base) by incubating for 10 minutes at room temperature. The excess stain was washed with water, after which the 96-plates were dried in an oven for 2 hours. The infectivity was then determined by calculating the number of dyed and non-dyed wells. The dyed wells represent non-infected cells. The 50% tissue culture infective dose (TCID50) was calculated by comparing the number of infected and uninfected wells for eight replicates in one 96- well microtiter plate at each dilution. PFU/ml was calculated by multiplying the TCID50/ml value by 0.7 according to the Poisson distribution estimation.

2.4 Caesium chloride gradient centrifugation analysis

100 ng of 35S E1 together with 900 ng of nonradioactive E1 was treated for 1 or 3 hours at 37°C in 100 µl of a chosen buffer. The samples were then cooled on ice before loading them onto a 10-ml gradient of 24% CsCl in TNE buffer. The gradients were then centrifuged using SW41 rotor (30,000 rpm, 24 h, 10°C, Optima LE-80k Ultracentrifuge, Beckman), after which 500-µl fractions were collected. Eppendorf containers (empty and then filled with fractions) were weighed for density determination. Fractions were then added onto scintillation cocktail (Ultima Gold MW; Perkin Elmer). The radioactivity of the samples was analysed by Liquid Scintillation Analyzer (Tri-Carb 2910 TR, PerkinElmer). Finally, the results were plotted in Microsoft Excel.

2.5 Sucrose gradient centrifugation analysis

100 ng of 35S E1 together with 900 ng of nonradioactive E1 was treated for 1 or 3 hours at 37°C in 100 µl of a chosen buffer. The samples were then cooled on ice before loading them onto a 10-ml 5% to 20% sucrose gradient. The gradients were balanced with R-buffer and then centrifuged using SW41TI rotor (35,000 rpm, 2 h, 4°C, Beckman Ultracentrifuge), after which 500-µl fractions were collected directly onto scintillation cocktail (Ultima Gold MW; Perkin Elmer). The radioactivity of the samples was analysed by Liquid Scintillation Analyzer (Tri-Carb 2910 TR, PerkinElmer). Finally, the results were plotted in Microsoft Excel.

2.6 Real-time fluorescence measurements

1 µg of E1 virus was added onto 100 µl of chosen buffer/solution in 96-well plate for treatment. For each sample there was also a corresponding blank (containing all the other factors except the virus) in order to eliminate any fluorescence originating from other factors than the virus. 10X SYBR-Green II (Sigma-Aldrich) was added in order to determine whether viral RNA was accessible and uncoating of the virus was induced during fluorescence measurements. The samples were cooled on the ice until the measurement was started. Each sample well was measured for fluorescence with 485 nm and 535 nm excitation and emission filters once in every minute for 180 minutes at 37°C using Victor X4 2030 Multilabel reader (PerkinElmer). The results were plotted in Microsoft Excel.

3 RESULTS

3.1 E1 remains in a stable form in physiological buffers

The uncoating of E1 due to physiological and ionic components was investigated in this study using real-time fluorescence measurement and gradient analysis. At first it was examined if E1 remains in stable condition in different storage buffers at physiological temperature. The exact compositions of buffers used in the study are represented in Table 1. After incubating E1 in PBS-$MgCl2_2$ for 3 hours at 37°C, only a minor increase in fluorescence was observed during real-time fluorescence measurement (Figure 2). This tentatively indicated that uncoating of E1 did not occur in the storage buffer. Similar results were observed after incubating E1 in DPBS (Figure 2). We composed endosomal buffer (Endo PBS) based on endosomal measurements in the literature (Tosteson and Chow 1996, Gerasimenko et al. 1998, Christensen et al. 2002, Hara-Chikuma et al. 2005, Steinberg et al. 2010, Scott and Gruenberg 2011, Albrecht et al. 2015). Endo PBS contained 20 mM NaCl, 5 mM K, 0.5 mM $MgCl_2$ and 0.2 mM $CaCl_2$. Endo PBS did not induce uncoating according to fluorescence measurements (Figure 2). Incubation of E1 in H_2O produced mainly linear curve with slightly higher increase in fluorescence compared to physiological buffers (Figure 2).

Table 1. Compositions of buffers used in the study.

	Concentration (mM)								
Buffer or solution	**NaCl**	**Na_2HPO_4**	**Total Na**	**KCL**	**K_2HPO_4**	**KH_2PO_4**	**Total K**	**$MgCl_2$**	**CaCl2**
PBS-$MgCl_2$	137	8	145	3		2	5	2	
DPBS	138	8	146	2,7		1,5	4,2	0,5	0,9
Endo PBS	20		20		2	1	5	0,5	0,2
20 mM Nacl + 30 mM K	20		20		12	6	30		
20 mM Nacl + 10 mM K	20		20		4	2	10		

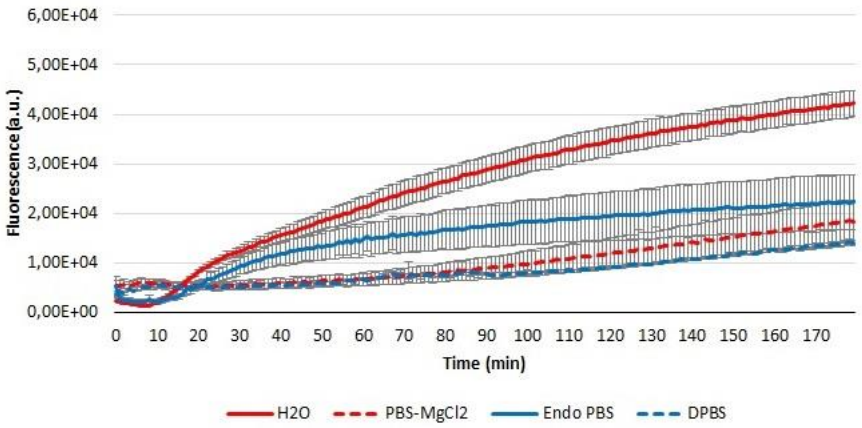

Figure 2. Real-time measurement of SYBR green II fluorescence at +37°C for 3 hours in the presence of E1 treated in water, PBS-$MgCl_2$ (2 mM), Endo PBS and DPBS. The fluorescence level of the dye is increased when the dye is bound to available RNA. Thus, the level of fluorescence indicates the state of uncoating process. The results are averages from a minimum of four replicates and the error bars ± are presenting standard errors of the means. a.u., arbitrary units.

When ^{35}S-labeled E1 was incubated in PBS-$MgCl_2$ and DPBS for three hours and then subjected to CsCl gradient centrifugation analysis, the results showed the highest radioactivity values peaking at a density of approximately 1.30 g cm^{-3} (Figure 3A and 3B). This density is known to represent the density of the intact and native form of E1. These results supported the results of real-time fluorescence measurements (Figure 2). E1 incubated in Endo PBS for 3 hours was analyzed by both sucrose and CsCl gradient centrifugation. Sucrose gradient showed a broad peak with a visible neck (fractions 12-19) suggesting that both native and intermediate uncoating particles were present (Figure 3C). A lower peak (fractions 6-9) represents empty RNA-free particles according to several earlier articles on sucrose gradient separation of enterovirus particles (Breindl 1971, Marjomäki et al. 2002). CsCl gradient analysis confirmed that most of the particles were in native form at a density of 1.30 g cm^{-3} (Figure 3D). Additionally, a

smaller number of particles were observed at a density of 1.38 g cm^{-3} representing the intermediate uncoating form of virus. These results indicate that E1 was mainly in stable form in physiological buffers and in Endo PBS at physiological temperature.

E1 incubated in H_2O for 3 hours at 37°C was analyzed by sucrose gradient analysis (Figure 3E). The results showed a high, relatively narrow peak at fractions 13-17. This most likely represented the intact form of the virus. Also, a broad and low peak was observed at fractions 6-10 representing the empty capsids.

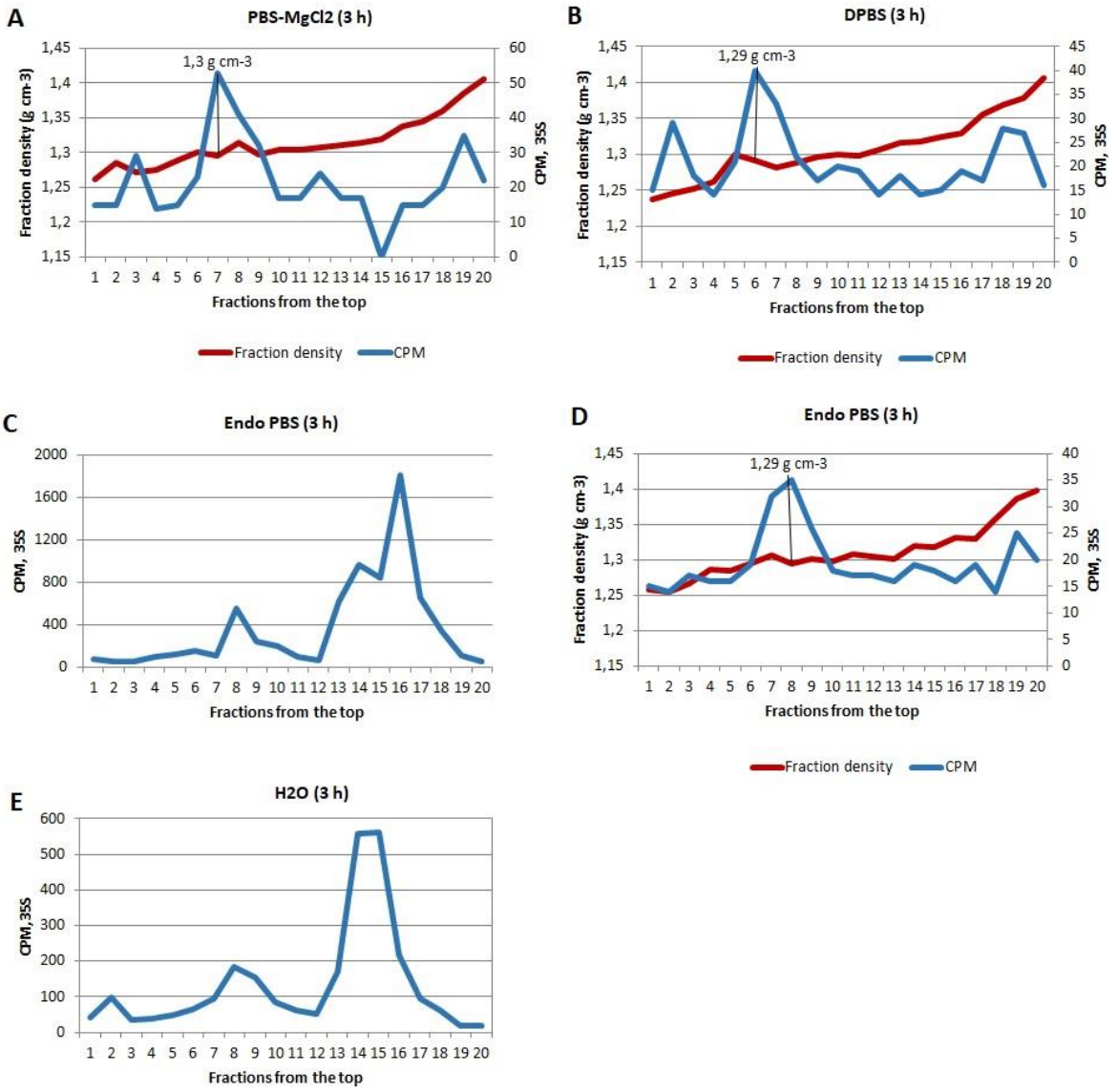

Figure 3. Stability of echovirus 1 in physiological buffers and water after 3-hour incubation at 37°C determined by sucrose and CsCl gradient separation. Gradients A, B and D are CsCl gradients and gradients C and E on the right are sucrose gradients. Density of 1.3 g cm^{-3} represents the intact form of E1 in CsCl gradient separation. In sucrose gradient a narrow band around 12-17 fractions represents intact form of E1 and broadening of the band represents formation of the intermediate uncoating particle. A band at fractions 5-10 represents empty capsids. (A) CsCl gradient separation of E1 treated in PBS-$MgCl_2$ for 3 hours at 37°C. (B) CsCl gradient separation of E1 treated in DPBS for 3 hours at 37°C. (C) Sucrose gradient (5% to 20%) separation of E1 treated in Endo PBS for 3 hours at 37°C. (D) CsCl gradient separation of E1 treated in Endo PBS for 3 hours at 37°C.

(E) Sucrose gradient (5% to 20%) separation of E1 treated in water for 3 hours at 37°C.

3.2 Presence of serum induces the uncoating of E1 at physiological temperature

In order to test the effect of serum in the uncoating process of E1, we added different dilutions of foetal calf serum (FSC) to MEM (0, 0.1, 1 and 10 %) and incubated E1 for 3 hours at 37°C in these dilutions. Real-time fluorescence measurement showed a significant increase in fluorescence with E1 incubated in 0.1% and 1% MEM after 30 minutes (Figure 4). The level of fluorescence stabilized in 80–90 minutes post infection. With 10% MEM the fluorescence increase was lower than with smaller serum concentration and lowest with 0% of serum in MEM (Figure 4). These results strongly suggested that a small amount of serum might induce the uncoating process of E1 *in vitro.*

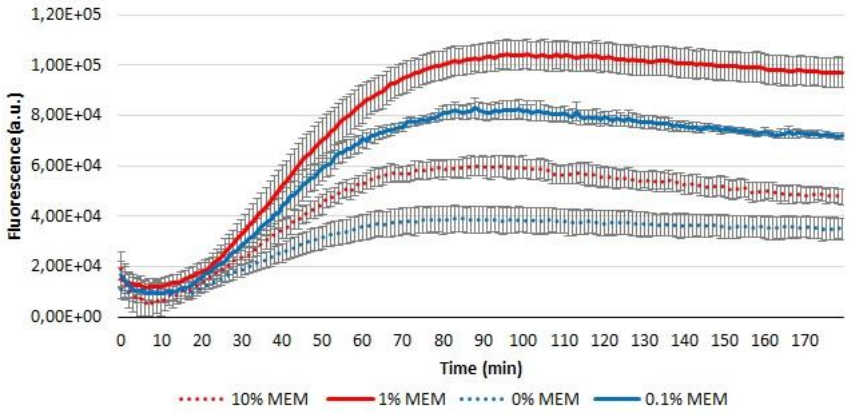

Figure 4. Real-time measurement of SYBR green II fluorescence at +37°C for 3 hours in the presence of E1 treated in MEM containing 0, 0.1, 1 or 10 % of FCS. The fluorescence level of the dye is increased when the dye is bound to available RNA. Thus, the level of fluorescence indicates the state of the uncoating process. The results are averages from a minimum of four replicates and the error bars ± are presenting standard errors of the means. a.u., arbitrary units.

Next, the gradient analysis was performed in order to confirm the state of uncoating. E1 incubated in 1% MEM for three hours was analysed with both sucrose and CsCl gradient centrifugation. Sucrose gradient showed a high and broad peak (fractions 12-18) and a smaller peak at fractions 6-9 (Figure 5A). All forms of E1 were present (empty, native, and intermediate). CsCl gradient analysis showed a high band peaking at a density of 1.28 g cm-3 suggesting that these were empty E1 particles (Figure 5B). A small band peaking at a density of 1.40 g cm- 3 suggested that these were intermediate uncoating particles. Results confirmed that 1% MEM induced the uncoating process. E1 incubated in 0.1% MEM for one hour at 37°C was analysed by sucrose gradient centrifugation (Figure 5C). The results showed a broad band with neck (fractions 12-18) and a very low band at fractions (6-9) suggesting that already after one-hour incubation some uncoating had occurred. E1 incubated in 10% MEM for three hours at 37 °C was analysed by CsCl gradient centrifugation (Figure 5D). The results showed a high band peaking at a density of 1.24 g cm-3 suggesting that these were empty E1 particles. Additionally, another nearly as high band appeared peaking at a density of 1.40 g cm-3 presenting intermediate uncoating particles. Determined by CsCl gradient centrifugation analysis it was observed that also a higher amount of serum (10% FCS in MEM) induced uncoating of E1, but not with as high intensity as with lower amount (1% FSC in MEM).

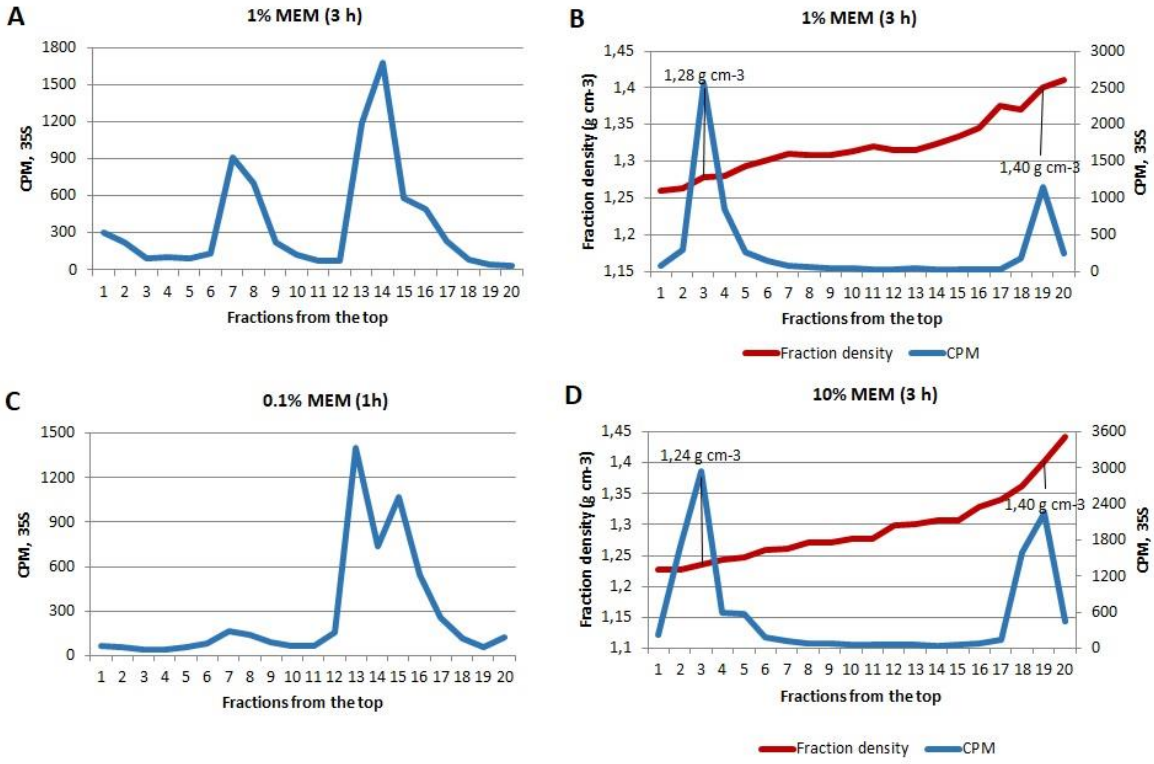

Figure 5. Stability of echovirus 1 in MEM supplemented with FCS after 1- or 3-hour incubation at 37°C determined by sucrose and CsCl gradient separation. Sucrose gradients are presented on the left and CsCl gradients are presented on the right. Density of 1.3 g cm^{-3} represents the intact form of E1 in CsCl gradient separation. Empty capsids form a band at the density of 1.24 – 1.28 g cm^{-3} and intermediate uncoating particles at the density of 1.4 g cm^{-3}. In sucrose gradient a narrow peak around 12-17 fractions represents intact form of E1 and broadening of the band represents formation of the intermediate uncoating particle. A band at fractions 5-10 represents empty capsids. (A) Sucrose gradient (5% to 20%) separation of E1 treated in MEM supplemented with 1% of FCS for 3 hours at 37°C. (B) CsCl gradient separation of E1 treated in MEM supplemented with 1% of FCS for 3 hours at 37°C. (C) Sucrose gradient (5% to 20%) separation of E1 treated in MEM supplemented with 0.1% of FCS for 1 hour at 37°C. (D) CsCl gradient separation of E1 treated in MEM supplemented with 10% of FCS for 3 hours at 37°C.

3.3 Optimal endosomal ionic conditions promote uncoating of E1

Next, we wanted to resolve whether the estimated optimal endosomal ionic conditions and finally addition of a small amount of serum would induce the

uncoating of E1 *in vitro.* When E1 was incubated in physiological concentration of NaCl (138 mM) for 3 hours, a moderate increase in fluorescence was noted during real-time fluorescence measurement (Figure 6). Yet there was a slightly faster increase in fluorescence in the treatment with 20 mM NaCl (Figure 6). Interestingly, the final level of fluorescence was higher in treatment with a high concentration on NaCl. When 10 mM potassium was added to 20 mM NaCl solution there was again an increase in fluorescence and even significantly higher increase upon treatment with 30 mM potassium added to 20 mM NaCl (Figure 6). So, now it seemed that a high amount of potassium combined with a low sodium concentration might induce uncoating of E1 at physiological temperature. When 0.1% of FSC was added to 20 mM NaCl + 30 mM potassium solution we observed yet the highest increase in fluorescence suggesting that this environment was favorable for uncoating and addition of serum enhanced the uncoating (Figure 6).

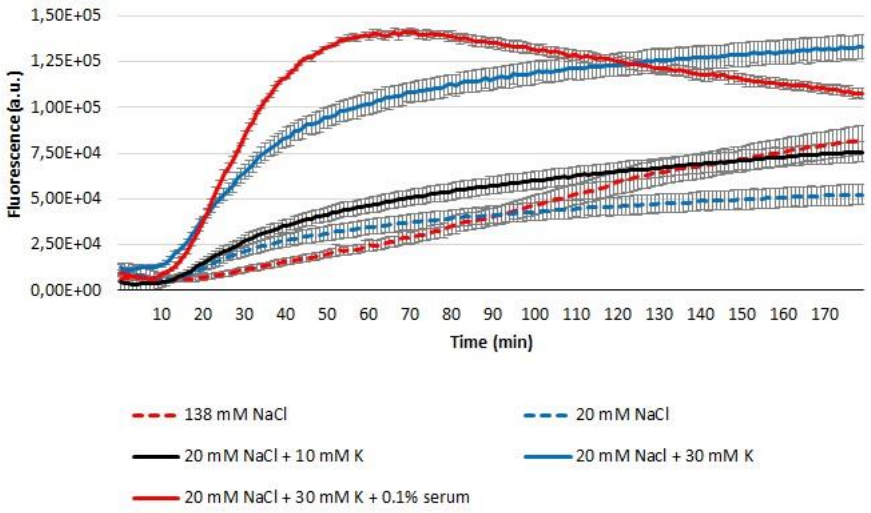

Figure 6. Real-time measurement of SYBR green II fluorescence at +37°C for 3 hours in the presence of E1 treated in 138 mM NaCl, 20 mM NaCl, 20 mM NaCl + 10- or 30-mM potassium and 20 mM NaCl + 30 mM potassium supplemented with + 0.1 % of FCS. The fluorescence level of the dye is increased when the dye is bound to available RNA. Thus, the level of fluorescence indicates the state of the uncoating process. The results are averages from a minimum of four replicates

and the error bars ± are presenting standard errors of the means. a.u., arbitrary units.

E1 incubated in physiological concentration of NaCl (138 mM) was analyzed by sucrose gradient centrifugation (Figure 7A). Results showed a relatively narrow and high band at fractions 15-18 representing intact E1 particles and a lot smaller band at fractions 5-10 representing a small number of empty particles. For 20 mM NaCl + 30 mM potassium solution the sucrose gradient analysis revealed a high and slightly to the left shifted band at fractions 14-18 indicating that most E1 particles were intact (Figure 7B). A lower and broad band at fractions 2-12 represented a smaller number of empty particles. CsCl gradient analysis showed a slightly different result (Figure 7C). The highest band peaked at a density of 1.42 g cm^{-3} representing intermediate uncoating particles and a bit lower band peaking at a density of 1.3 g cm^{-3} representing intact particles. Also, a low band peaked at a density of 1.23 g cm^{-3} representing empty particles.

E1 incubated in 20 mm NaCl + 10 mM K + 0.1% FSC for 1 hour showed a relatively narrow and high band with a small neck on the left side at fractions 12-16 in sucrose gradient analysis (Figure 7D). Interestingly when the amount of potassium was increased to 30 mM there was only a small band at fractions 12-16 and a wide and high band at fractions 2-9 indicating that most E1 particles were empty (Figure 7E). This indicated that a high amount of potassium (together with low sodium and small amount of serum) is favorable for the uncoating of E1 at physiological temperature. When E1 incubated in 20 mM NaCl + 30 mM K + 0.1% serum for three hours was analyzed by sucrose gradient centrifugation there was not much difference to one-hour incubation (Figure 7F). Most particles were empty as expected. CsCl gradient analysis showed a high and broad peak at a density of 1.27 g cm^{-3} indicating that most particles were empty and a small peak at a density of 1.4 g cm^{-3} representing uncoating intermediate particles (Figure 7G).

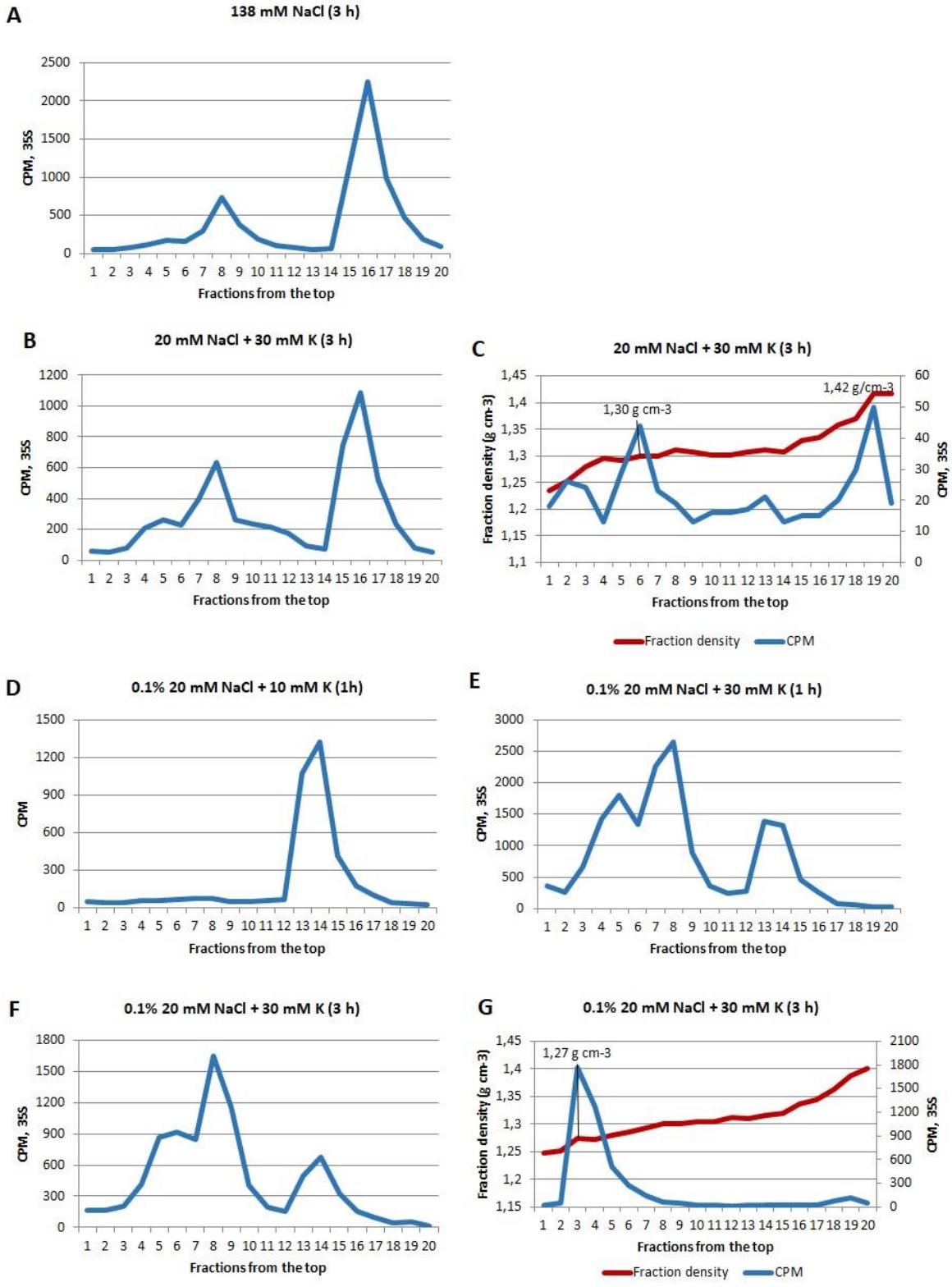
A
138 mM NaCl (3 h)
CPM, 35S
Fractions from the top
B
20 mM NaCl + 30 mM K (3 h)
CPM, 35S
Fractions from the top
C
20 mM NaCl + 30 mM K (3 h)
1,30 g cm-3
1,42 g/cm-3
Fraction density (g cm-3)
CPM, 35S
Fractions from the top
Fraction density
CPM
D
0.1% 20 mM NaCl + 10 mM K (1h)
CPM
Fractions from the top
E
0.1% 20 mM NaCl + 30 mM K (1 h)
CPM, 35S
Fractions from the top
F
0.1% 20 mM NaCl + 30 mM K (3 h)
CPM, 35S
Fractions from the top
G
0.1% 20 mM NaCl + 30 mM K (3 h)
1,27 g cm-3
Fraction density (g cm-3)
CPM, 35S
Fractions from the top
Fraction density
CPM

Figure 7. Stability of echovirus 1 in the presence of different concentrations of NaCl and potassium and a low concentration of FCS after 1 or 3-hour incubation at 37°C determined by sucrose and CsCl gradient separation. All the gradients on the left and gradient E on the right are sucrose gradients. All the other gradients on the right are CsCl gradients. Density of 1.3 g cm^{-3} represents the intact form of E1 in CsCl gradient separation. Empty capsids form a band at the density of 1.24 – 1.28 g cm^{-3} and intermediate uncoating particles at the density of 1.4 g cm^{-3}. In sucrose gradient a narrow band around 12-17 fractions represents intact form of E1 and broadening of the band represents formation of the intermediate uncoating particle. A band at fractions 5-10 represents empty capsids. (A) Sucrose gradient (5% to 20%) separation of E1 treated in 138 mM NaCl for 3 hours at 37°C. (B)) Sucrose gradient (5% to 20%) separation of E1 treated in 20 mM NaCl + 30 mM potassium for 3 hours at 37°C. (C) CsCl gradient separation of E1 treated in 20 mM NaCl + 30 mM potassium for 3 hours at 37°C (D) Sucrose gradient (5% to 20%) separation of E1 treated in 20 mM NaCl + 10 mM potassium supplemented with 0.1% of FCS for one hour at 37°C. (E) Sucrose gradient (5% to 20%) separation of E1 treated in 20 mM NaCl + 30 mM potassium supplemented with 0.1% of FCS for one hour at 37°C. (F) Sucrose gradient (5% to 20%) separation of E1 treated in 20 mM NaCl + 30 mM potassium supplemented with 0.1% of FCS for 3 hours at 37°C. (G) CsCl gradient separation of E1 treated in 20 mM NaCl + 30 mM potassium supplemented with 0.1% of FCS for 3 hours at 37°C.

3.4 Estimated endosomal concentrations of calcium and magnesium have a stabilizing effect on E1.

One open question was still whether the estimated endosomal concentrations of calcium or magnesium were optimal for the uncoating of E1 in the presence of 20 mM NaCl, 30 mM K and 1% serum. While the actual lowest levels of calcium and magnesium are not known, we used 0.2 mM calcium and 0.5 mM magnesium as average values, respectively. The real time fluorescence measurement suggested that 0.2 mM calcium and 0.5 mM magnesium were able to stabilize the virions against uncoating in the presence or absence of 0.1% serum (Figure 8A and 8B).

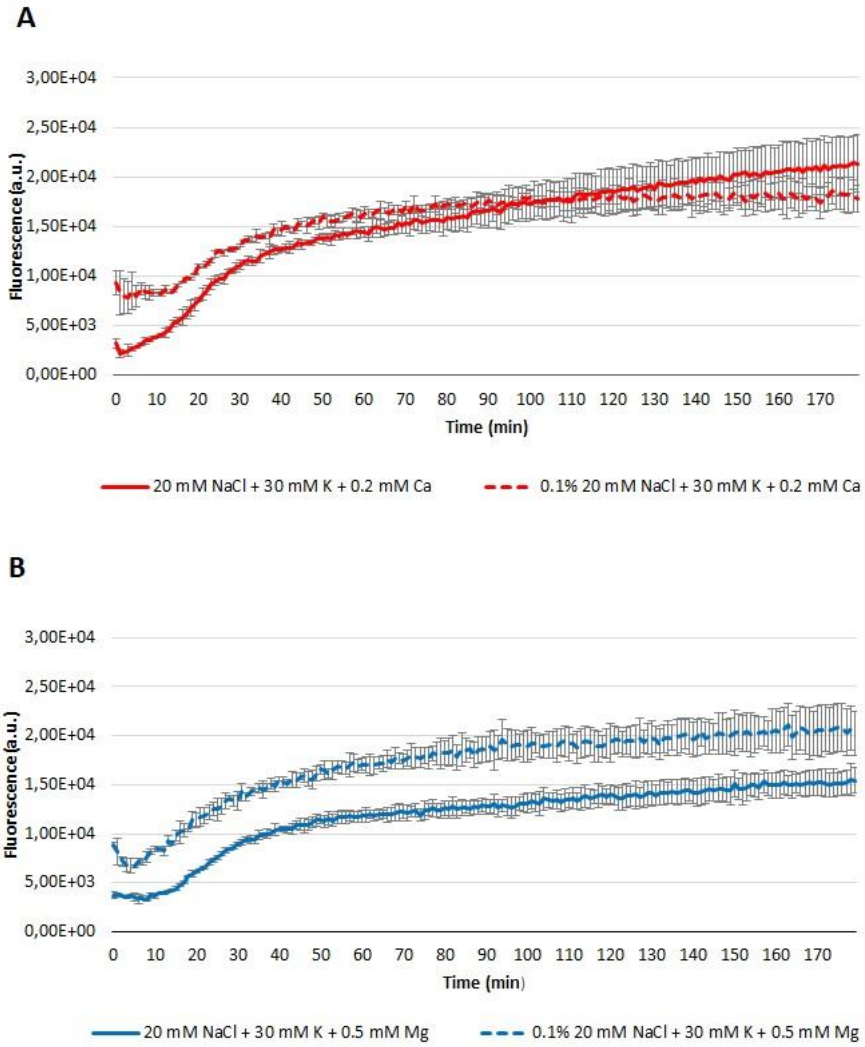

Figure 8. (A) Real-time measurement of SYBR green II fluorescence at +37°C for 3 hours in the presence of E1 treated in 20 mM NaCl + 30 mM potassium + 0.2 mM calcium either with or without 0.1% of FCS. (B) Real-time measurement of SYBR green II fluorescence at +37°C for 3 hours in the presence of E1 treated in 20 mM NaCl + 30 mM potassium + 0.5 mM magnesium either with or without 0.1% of FCS. The fluorescence level of the dye is increased when the dye is bound to available RNA. Thus, the level of fluorescence indicates the state of the uncoating process. The results are averages from a minimum of four replicates and the error bars ± are presenting standard errors of the means. a.u., arbitrary units.

Sucrose gradient analysis was performed for these samples (in the presence of 1% FSC). After one-hour incubation at 37°C, there was not much difference between the treatments in the presence of 0.2 mM calcium or 0.5 mM magnesium (Figure 9A and 9B). In both cases only one relatively narrow band was observed in sucrose gradient results at fractions 13-17 probably presenting intact E1 particles. CsCl gradient analysis did not either show significant difference between the samples, but the results were different compared to sucrose gradient analysis results (Figure 9C and 9D). With 0.2 mM calcium the results showed a high band peaking at a density of 1.26 g cm^{-3} and with 0.5 mM magnesium a high band peaking at a density of 1.27 g $cm^{-3.}$ These both were most likely representing empty capsids. With 0.2 mM calcium there was also a lower band peaking at a density of 1.38 g cm^{-3} and with 0,5 mM magnesium there was a relatively high peak at density of 1.4 g cm^{-3}. These were representing the uncoating intermediate particles. Together, the real time fluorescence measurement and sucrose gradient results suggested that the used concentrations of magnesium and calcium were not inducing uncoating.

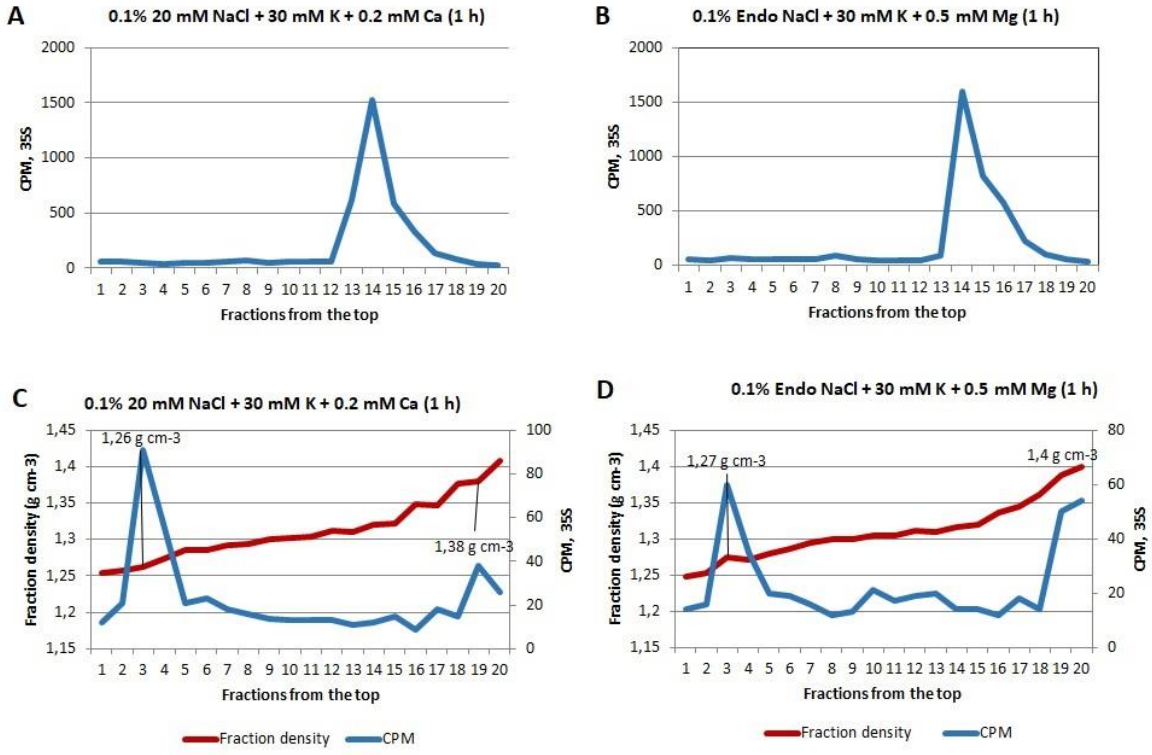

Figure 9. The effect of addition of either 0,2 mM calcium or 0,5 mM magnesium on the stability of echovirus 1 after 1-hour incubation at 37°C determined by sucrose and CsCl gradient separation. Sucrose gradients are presented above and CsCl gradients are presented below. Density of 1.3 g cm^{-3} represents the intact form of E1 in CsCl gradient separation. Empty capsids form a band at the density of 1.24 – 1.28 g cm^{-3} and intermediate uncoating particles at the density of 1.4 g cm^{-3}. In sucrose gradient a narrow band around 12-17 fractions represents intact form of E1 and broadening of the band represents formation of the intermediate uncoating particle. A band at fractions 5-10 represents empty capsids. (A) Sucrose gradient (5% to 20%) separation of E1 treated in 20 mM NaCl, 30 mM potassium and 0.2 mM calcium in the presence of 0.1% of FSC for one hour at 37°C. (B)) Sucrose gradient (5% to 20%) separation of E1 treated in 20 mM NaCl, 30 mM potassium and 0.5 mM magnesium in the presence of 0.1% of FSC for one hour at 37°C. (C) CsCl gradient separation of E1 treated in 20 mM NaCl, 30 mM potassium and 0.2 mM calcium in the presence of 0.1% of FSC for one hour at 37°C. (D) CsCl gradient separation of E1 treated in 20 mM NaCl, 30 mM potassium and 0.5 mM magnesium in the presence of 0.1% of FSC for one hour at 37°C.

3.5 E1 uncoating intermediate particles are still highly infective after tested treatments

Infectivity of the E1 after different treatments (with incubation at 37°C for 3 hours) was determined with end point dilution and/or cytopathic effect assay (CPE). When determined with end point dilution there was not any significant decrease in infectivity with treatments in tested buffers (Table 2). The infectivity of E1 was highest in treatment with PBS-MgCl2 for 3 hours at 37°C (Table 2). The treatment with water caused a decrease of a few logarithms in infectivity (Table 2). Interestingly the treatment with MEM with or without serum caused a decrease of 3-4 logarithms in infectivity (Table 2). Yet the virus was still quite infective.

Table 2. The infectivity of E1 after 3-hour incubation at 37°C in different buffers and solutions determined by end-point dilution assay. The results are averages from a minimum of 8 technical replicates.

Sample	Infectivity (pfu/ml)
0% MEM	7,43E+08
1% MEM	7,43E+08
0.1% MEM	1,60E+08
10% MEM	5,87E+08
H20	7,43E+09
PBS-MgCl2	1,09E+12
Endo PBS	1,60E+11
DPBS	1,19E+11
138 mM NaCl	1,19E+11
20 mM NaCl	2,35E+11
20 mM NaCl + 10 mM K	1,94E+11

Cytopathic effect assay (CPE) confirmed that E1 was mainly highly infective after every different treatment. None of PBS-MgCl2, Endo PBS and DPBS treatments caused any notable decrease in infectivity compared to virus control. (Figure 10A)

According to CPE results the treatment with water caused a slight decrease in infectivity (Figure 10A). CPE results suggested that the infectivity was somewhat decreased in E1 samples treated in MEM with or without serum compared to virus control, but the infectivity was still high (Figure 10B). Neither treatment with high (138 mM) nor low (20 mM) concentration of NaCl decreased the infectivity (Figure 10C). Treatment with low concentration (20 mM) of NaCl and high concentration of potassium (30 mM) with or without serum (0.1%) did not affect the infectivity either (Figure 10C). When either 0.2 mM calcium or 0.5 mM magnesium was added there was no significant effect on the infectivity (Figure 10D).

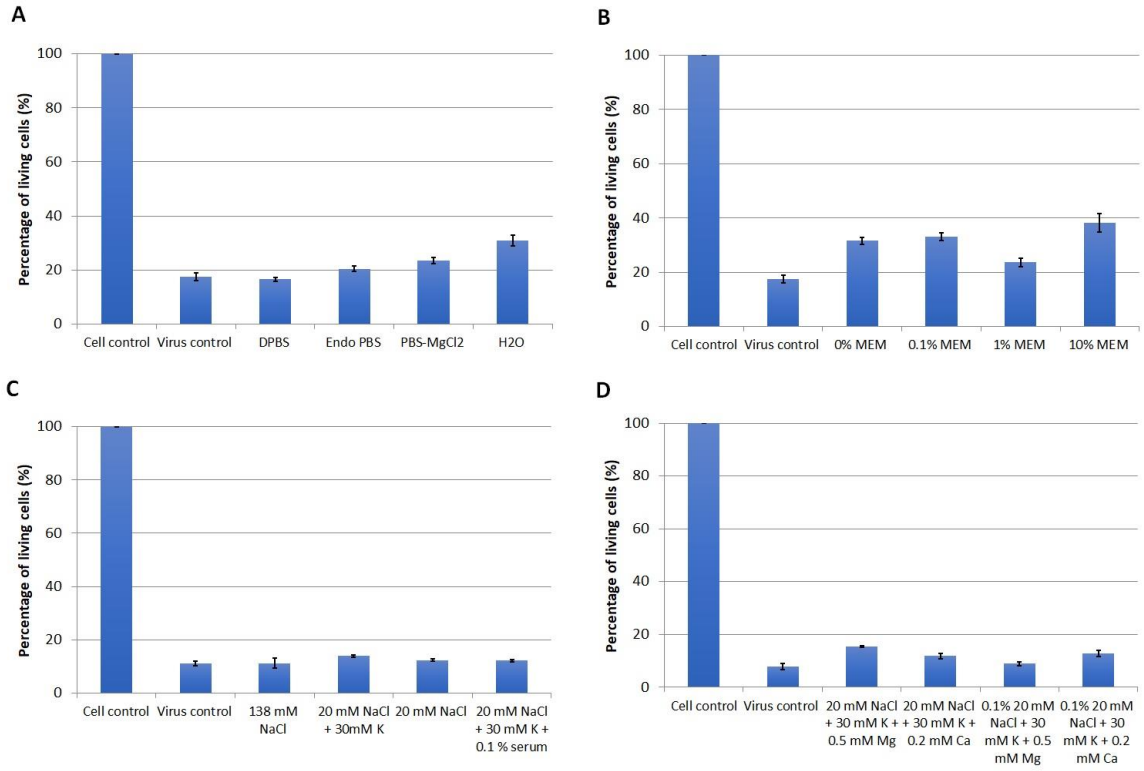

Figure 10. The number of living cells after infection with 5 ng of E1 treated in different solutions and buffers for 3 hours at 37°C. Results were achieved by cytopathic effect assay (CPE). Cell control represents non-infected cells and virus control represents infection of cells with intact non-treated virus. (A) A representation of the infectivity of E1 after treatment with DPBS, Endo PBS, PBS-$MgCl_2$ and water for 3 hours at 37°C. (B) A representation of the infectivity of E1

after treatment with MEM supplemented with 0, 0.1, 1 and 10% of FCS for 3 hours at 37°C. (C) A representation of the infectivity of E1 after treatment with 138 mM NaCl, 20 mM NaCl and 20 mM NaCl + 30 mM potassium with and without 0.1% of FSC. (D) A representation of the infectivity of E1 after treatment with 20 mM NaCl + 30 mM potassium and either 0,2 mM calcium or 0.5 mM magnesium in the presence and absence of 0.1% FCS. All the results are averages from a minimum of five technical replicates and the error bars ± are presenting standard errors of the means.

4 DISCUSSION

4.1 Serum and endosomal ions induce the uncoating of E1

Uncoating of E1 is known to begin at 30 minutes post-infection and continue for at least two hours post-infection with the virus ending up into pH-neutral multivesicular bodies. However, there was little information about the physiological factors that trigger the uncoating process of enteroviruses at the beginning of this study in 2016. In this study we wanted to investigate if there was a certain ionic condition that induces uncoating of E1. Also, the effect of serum in the uncoating process was in our interest.

Throughout this study, we confirmed that E1 remains mainly in intact form in physiological buffers at physiological temperature (Figure 2 and 3). Similar results were obtained later in a published study by the group (Ruokolainen et al. 2019). In this study the Endo PBS, containing the originally estimated endosomal ionic composition, did not induce uncoating according to real-time fluorescence measurement and CsCl gradient separation (Figure 2 and 3D). Instead, sucrose gradient suggested for some uncoating intermediate formation (Figure 3C). In the later study the endosomal buffer solution promoted a slow formation of the uncoating intermediate over three hours at 37°C (Ruokolainen et al. 2019). However, the potassium concentration of the buffer was 6-fold higher in the study by Ruokolainen et. al (2019) compared to 5 mM potassium concentration of Endo PBS in this study.

We demonstrated that serum is a significant factor that triggers the uncoating of E1. The enhancement of uncoating was recognised as most powerful with a low serum concentration in MEM (Figure 4 and 5). It was later confirmed that 1% bovine serum effectively induces formation of the uncoating intermediate and empty capsids at 37°C (Ruokolainen et al. 2019). Ruokolainen et al. (2019)

demonstrated that serum albumin, in the balance with fatty acids, triggers the formation of uncoating intermediate particle. Serum albumin is the most abundant protein in plasma that is essentially required for maintenance of oncotic pressure and distribution of fluid between body compartments. Serum albumin is also the main carrier for fatty acids (Fanali et al. 2012). In an earlier study it was demonstrated that serum albumins, HSA and BSA, block echovirus 7 infection by inhibiting the uncoating step of the viral life cycle (Ward et al. 1999). Later it was suggested that fatty acid depleted albumin induced the formation of echovirus 12 A-particle (Ward et al. 2000). It was discovered in a recent study that albumin enhances the rate of coxsackievirus B3 strain 28 conversion to non-infectious A-particles at least 2-fold (Carson and Cole 2020).

Even though the real-time fluorescence measurement showed a moderate increase in fluorescence for E1 incubated in 138 mM NaCl, the sucrose gradient separation results did not suggest for uncoating in our study (Figure 6 and 7A). The linearity of the curve in real-time fluorescence results may indicate that it does not represent true uncoating of the virus. High physiological concentration of NaCl may cause interference with real time fluorescence measurement.

We made a significant observation that a low concentration of NaCl (20 mM) and high concentration of potassium (30 mM) in the presence of low amount of serum (0.1%) induces the uncoating of E1 producing mainly empty capsids (Figure 6 and 7F and G). 20 mM NaCl + 30 mM potassium without serum produced both empty and uncoating intermediate particles (Figure 7B and C). Mainly empty capsids were formed due to similar treatment in the later study (Ruokolainen et al. 2019). The study by Ruokolainen et al. (2019) still strongly supported the observations of this study, underlining the role of high potassium and low sodium concentration in inducing the uncoating process. Measurements performed by others on endosomes suggest that sodium ion concentration decreases along the endocytic pathway, while potassium ion concentration increases (Scott and Gruenberg 2011).

It was suggested in an earlier study with poliovirus that low ionic strength buffers induce the formation of empty 80S capsids (Wetz and Kucinski 1991). Another study with poliovirus demonstrated that potassium concentration affects the appearance of ion channels formed by intact viruses. It was suggested that these ion channels could participate in the transportation of viral RNA into the cytosol (Tosteson and Chow 1996). Influenza A virus has been shown to require high potassium ion concentration for efficient uncoating process (Stauffer et al. 2014).

E1 remained in a stable form due to addition of 0.2 mM calcium and 0.5 mM magnesium (into 0.1% 20 mM NaCl + 20 mM potassium) based on real-time fluorescence measurement and sucrose gradient separation in our study (Figure 8 and 9A and B). According to CsCl gradient results, uncoating was induced but it must be considered that CsCl gradient separation is a very harsh treatment for virus and the virus had already been purified once by CsCl gradient before treatment (Figure 8 C and D). In the study by Ruokolainen et al. (2019) the addition of divalent cations Mg^{2+} and Ca^{2+} in same concentrations had a stabilizing effect as well. When the concentration of calcium and magnesium were diluted to 100-fold or 10-fold, the uncoating process was more efficient. Ruokolainen et al. (2019) demonstrated that uncoating still occurs slowly in the presence of endosomal values of magnesium and calcium while the virus remained mainly in a stable form in this study in similar conditions. It has been suggested that Ca^{2+} concentration is lowest in early endosomes and increases along the endocytic pathway (Albrehct et al. 2015). Stabilizing effect of divalent cations on viral structure has been observed in several studies. Both magnesium and calcium ions effectively reduced uncoating in a study with parvovirus minute virus of mice (Cotmore et al. 2010). Similar effect was observed with human parvovirus B19 (Caliaro et al. 2019). It is possible that depletion of divalent cations (Mg^{2+} and Ca^{2+}) triggers the egress of viral RNA in echovirus 1.

We observed that E1 was still highly infective after all treatments (Figure 10 and Table 2). The highest decrease in infectivity (up to three logs) was detected in E1 treated in MEM (with or without serum). These results were supported by findings in other studies where the infectivity of uncoating intermediate particle was high (Myllynen et al. 2016, Ruokolainen et al. 2019).

4.2 Remarks

In some cases, the CsCl gradient separation produced results suggesting for stronger uncoating process than sucrose gradient separation results. As already mentioned, CsCl gradient separation is a harsh treatment for virus and virus had been already purified by the procedure before treatment and another CsCl gradient separation. Sucrose gradient separation might be better and more reliable method to study the uncoating process. E1 is known to accumulate in pH-neutral multivesicular bodies during uncoating. pH values of the buffers used in treatments were not constantly measured and controlled so the conditions did not completely mimic natural environment of the virus during uncoating. It is also possible that pH value has some effect on the function of SYBR Green II dye during real-time fluorescence measurement. The fluorescence originating from the dye bound to RNA inside and outside of the virus was not differentiated in this study. It was impossible to recognize the amount of fluorescence originating from released RNA and RNA still locked inside the virus in porous intermediate uncoating particle based on our real time fluorescence measurement results. Addition of RNase would have solved this problem since released free RNA is sensitive to RNase treatment. The improvement was performed in a later study (Ruokolainen et al. 2019).

4.3 Conclusions

In this study we demonstrated the inducing effect of high potassium and low sodium concentration in the formation of highly infective E1 uncoating intermediate particle and genome egress at physiological temperature *in vitro*. We also stated that $CaCl_2$ and $MgCl_2$ have a stabilizing effect on E1 particle. Depletion of divalent Mg^{2+} and Ca^{2+} cations may induce the uncoating process. Our results suggest that addition of a small amount of serum enhances the uncoating of E1. It was later confirmed that serum albumin is the component of serum that induces the uncoating of E1. Observations of this study offer new information about triggers that lead to uncoating of enteroviruses under physiological conditions. Further research is still required to find out if these results can be repeated with other enteroviruses. Designing of new experiments to measure the exact ionic composition of endosomes that are targeted by virus for uncoating is also needed. In addition, there are still many unidentified molecular triggers that lead to the uncoating of E1 and other enteroviruses that are regularly posing a threat in form of many infections and diseases.

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