Master's Thesis

The Role of Calpains in Enterovirus Infection

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Enteroviruses are a group of non-enveloped positive-sense RNA viruses that cause a variety of diseases ranging from foot-and-mouth disease to meningitis. Previous studies have shown that the inhibition of calpains prevents the enterovirus infection after entry and RNA release into the cytoplasm. Upon protein translation enteroviruses produce a 250 kDa polyprotein that is cleaved into structural and non-structural viral proteins. These cleavages are performed by viral proteases. However, previous studies have shown that the inhibition of the cellular calpains inhibits the enterovirus RNA-replication. Aim of this study is to investigate the role of calpains in Echovirus 1 and Coxsackievirus B3 infections. An array of experiments was performed in order to determine the time window when calpains are required for the infection and to assess the possible mechanism they might have. The inhibition of calpains before 120 min post-infection inhibited both Echovirus 1 and Coxsackievirus B3 infection. The inhibition of calpains prevented the intracellular membrane modifications characteristic to enterovirus infection. In a cleavage experiment calpains were found capable of processing the Coxsackievirus B1 polyprotein. These results suggest that the role of calpains in the enterovirus infection is after entry but before membrane rearrangements and RNA replication, most likely in the polyprotein processing.

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Enterovirukset ovat viruspeitteettömien, positiivisten RNA-virusten ryhmään kuuluva virusperhe, jonka jäsenet aiheuttamat mitä monituisimpia tauteja suu- ja sorkkataudista aina aivokalvontulehdukseen. Aikaisemmat tutkimustulokset ovat osoittaneet, että calpainien toiminnan hiljentäminen solussa estää enterovirusinfektion etenemisen ja uuden virus-RNA:n tuottamisen. Enterovirukset tuottavat proteiininsa yhteenliittyneenä polyproteiinissa, jonka viruksen proteaasit pilkkovat rakenteellisiksi ja toiminnallisiksi proteiineiksi. Tämän Pro Gradu –tutkielman tavoitteena oli selvittää millainen rooli calpaineilla on Echovirus 1 ja Coxsackievirus B3 –infektioissa. Erilaisten kokeiden avulla selvitettiin, missä vaiheessa infektiota calpainit ovat sille tärkeitä ja millä tavalla. Calpainien toiminnan estäminen ennen 120 minuutin aikapistettä esti sekä Echovirus 1, että Coxsackievirus B3 –infektion. Myös enterovirusinfektioille tyypilliset kalvomuutokset solussa estyivät, kun calpainien toiminta estettiin. Pilkkomakokeessa selvisi, että calpainit kykenevät pilkkomaan Coxsackievirus B1:n polyproteiinia. Kokonaisuudessaan nämä tulokset viittaavat siihen, että calpainien toiminta enterovirusten infektiossa tapahtuu viruksen sisäänmenon jälkeen, mutta ennen kalvojen uudelleenjärjestelyä ja RNA:n replikaatiota; calpainit mitä todennäköisimmin osallistuvat polyproteiinin pilkkomiseen.

CONTENTS

1 INTRODUCTION

Enteroviruses are a diverse group of viruses belonging to the family of Picornaviridae that cause a plethora of different clinical syndromes ranging from gastrointestinal tract infections to potentially fatal aseptic meningitis. Enteroviruses are most prominent in children younger than one year and have the highest mortality rate in neonatal babies (Khetsuriani et al. 2006). Probably the most notorious enterovirus is the now eradicated poliovirus. Furthermore, a connection between enterovirus infection and type 1 diabetes mellitus has been suggested. Enteroviruses, coxsacieviruses in particular (Hyöty et al. 1995), have been shown to cause destruction of pancreatic islet cells either by their replication cycle or by overactivating the immune system due to continuous production of viral proteins and alteration of cell function (Soldevila et al. 1991, Jaeckel et al. 2002). Lammi et al. (2005) found that coxsackieviruses 3, 4 and 5 caused the impairment of function in *in vitro* β-cells and eventually cell death. Higher humoral immunity against enteroviruses has been found in patients on the onset of T1DM pointing that these individuals have been previously infected by enteroviruses (Sadeharju et al. 2001). The enterovirus genus contains a total of 15 virus species. Of these enteroviruses A-D and Rhinoviruses A-C are human viruses, while enteroviruses E-L are animal viruses (Lefkowitz 2018, Anonymous 2019). In the current study two viruses from enterovirus B species are used: enteric cytopathic human orphan virus 1 (EV1) and coxsackievirus B3 (CVB3).

1.1 Virus entry

First obstacle on virus's road to replication is the cell membrane. Enteroviruses cross this barrier through specific binding to a cell surface receptor that is utilized to initiate endocytosis. The role of α2β2 (VLA-2) integrin in the EV1 entry has been

previously shown by Bergelson et al. (1992): cells lacking VLA-2 were not infected by EV1. Integrin VLA-2 binds rod shaped collagen under normal conditions, thus in the case of infection EV1 has to compete for integrin binding sites. However, it has been shown that EV1 binds to the receptor more efficiently than its normal ligand: this gives EV1 an advantage in the race for binding sites (Xing 2004). EV1 capsid proteins were observed inside the cells 15 min – 120 min p.i. in vesicular structures without markers of clathrin dependent pathway. Instead electron microscopy showed that EV1 capsid protein co-resided in vesicular structures in the perinuclear area with caveolin-1 suggesting that the internalization of EV1 is caveolin dependent (Marjomäki et al. 2002). Later research has however shown that the colocalization between caveolin-1 containing vesicles and EV1 particles begins only after the internalization of EV1 (Karjalainen et al. 2008). Internalization via caveolae is possible, but recent study has shown that majority of EV1 transport is mediated by lipid raft domains that facilitate the formation of tubulovesicular bodies that engulf EV1 alongside with other liquid phase cargo (Damm et al. 2005, Karjalainen et al. 2008.) These pinocytotic structures do not however contain conventional markers of liquid phase entry, which suggests that the entry route is specific for EV1 entry (Karjalainen et al. 2011, reviewed by Marjomäki et al. 2015).

Once internalized into the cell, the EV1 containing vesicles can be found in the perinuclear area. These vesicles grow in size later on in the infection and form into multivesicular bodies (MVB) (Karjalainen et al. 2008). This maturation is mediated by various endosomal sorting complexes required for transport -complex proteins (Karjalainen et al. 2008). Karjalainen et al. (2008) also found that formation of MVBs is dependent on EV1 infection: just by aggregating integrins the MVBs act differently (Rintanen et al. 2012).

CVB viruses enter the cell by binding to coxsackie-adenovirus receptor (CAR) (Zautner et al. 2003). In polarized cells, where CAR resides in the tight junction (TJ) (Coyne et al. 2007), CVB has been found to initially bind to lipid raft associated decay-accelerating factor (DAF) in the apical surface of the cell (Patel et al. 2009).

Clustered DAFs transit to the TJs and permit the virus attachment to CAR (Patel et al. 2009). Once in contact with CAR the CVB particles undergo capsid reformation into an intermediate particle, where the VP4 is removed from the virion. VP4 however seems to internalize along with the rest of the virus (Coyne et al. 2007).

The specific initiator of enterovirus capsid uncoating is still shrouded by mystery, but in their review Suomalainen and Greber (2013) present some of the uncoating mechanisms of viruses closely related to enteroviruses. The disassembly of the virus particle releases the RNA alongside the internal capsid protein VP4. Panjwani et al. (2014) found that VP4 of rhinovirus facilitates the formation of size specific pores in model membranes. The size of the pores allows the flow of RNA out from the MVBs into the cytoplasm, where RNA is translated and replicated. In addition, study by Soonsawad et al. (2004) showed that larger openings form on the MVB membrane suggesting that RNA might also be released through larger pores.

1.2 Virus genome

Picornavirus genome is a short positive sense single strand RNA genome that consists of one open reading frame (ORF) coding for all viral proteins (Jackobson 1968). In addition to the ORF there are non-translated sections in 3′ and 5′ ends. In the 3′ end resides a poly(A) tail that has been proposed to serve a purpose in the processing of nuclear precursos RNA (Yogo 1972). In the 5′ end resides a genome-linked virusprotein VPg (Lee 1977) that has been shown not to be required for the infectivity of poliovirus (Nomoto 1977). The VPg is proposed to act as an RNA translation primer for poliovirus (Steil 2009) and suspected to also have a role in protecting the viral RNA from detection by antiviral agents that detect capped RNA (Wilkins 2010).

Pelletier et al. (1988) and Jang et al. (1988) both found that in the 5′ end of the poliovirus genome resides a non-coding RNA sequence: the internal ribosome entry site (IRES). They found that it is involved in the initiation of the genome translation. Picornaviruses cause the shutdown of host RNA translation by targeting eukaryotic

translation initiation factor 4 G (eIF4G) and poly(A)-binding protein (PABP) that act in cap-recognition and translation initiation of cellular mRNA (Gingras et al. 1996). This gives the cap-independent picornavirus RNA translation great control over host resources (Reviewed by Martinez-Salas et al. 2015). The ORF can be divided into three portions according to the role of the mature cleaved protein products it codes for: P1 is translated into structural proteins VP1-4 that form the viral capsid; P2 and P3 that are translated into functional proteins that take part in polyprotein processing and RNA replication (Kitamura 1981, reviewed by Goodfellow 2011).

The viral polyprotein translation is initiated by the 5′ IRES entering the ribosome cap-independently. To facilitate the translation initiation IRES elements have been shown to recruit IRES trans-activating factors (ITAF). ITAFs are cellular proteins that are normally used for ribosomal activation: enteroviruses use them for the same purpose. ITAFs induce ribosome recruitment by attaching to the IRES and stabilizing the secondary and tertiary RNA structures therein (Dorner et al. 1984, reviewed by Fitzgerald 2009). After this the translation begins at AUG codon 30–160 nt downstream from the IRES and proceeds as regular cellular translation (Sweeney et al. 2014).

1.3 Polyprotein

In the ribosomes the single ORF of the enterovirus genome is translated into a single polyprotein that is proteolytically cleaved into functional and structural proteins. The enterovirus polyprotein can be divided into three separate parts by the function of the mature proteins that are cleaved from it. These segments are P1, P2 and P3 (Kitamura et al. 1981). In primary cleavage, P1-P2 and P2-P3 are cleaved by 2A and 3C, respectively (Hanecak et al. 1982).

P1 consists of the virion capsid proteins VP1-VP4, named in order of descending molecular weight (Fry and Stuart 2010). P1 is cleaved into VP0, VP3 and VP1 by viral protease 3C (Hanecak et al. 1982). Finally, VP0 is cleaved into VP4 and

VP2 in an intramolecular maturation cleavage that is suspected to be catalysed by a serine residue in VP2 activated by the viral RNA (Rossmann et al. 1985)

P2 is cleaved into the non-structural proteins 2A, 2B and 2C by the 3C protease. Of these 2A is known to conduct the primary cleavage described above. In enteroviruses, 2A is a cis-acting cysteine active proteinase (Toyoda et al. 1986). Mature 2A is formed around a zinc molecule that allows it to fold properly in order to achieve its proteolytic activity (Sommergruber et al. 1994). In addition to its role in the cleavage of P1 and P2 junction 2A is known to inactivate cellular components required for cap-dependent translation processes such as eIF-4G and a subset of nucleoporins (Svitkin et al. 1999). 2B has been shown to have a role in the alteration of cellular membranes to support the viral RNA replication (Cho 1994). It has also been proposed to prevent the cell from entering apoptosis (van Kuppeveld et al. 2005) by disturbing the calcium concentration of Golgi apparatus associated membranes via pore formation (de Jong 2008). Of the P2 proteins 2C is least well known, but it has been suggested to partake in the re-arrangement of cellular membranes required for viral RNA replication (Cho 1994).

Proteins derived from P3 are strongly associated with the genome replication. P3 is cleaved by 3C into 3A, 3B, 3C and 3D. 3A has been proposed to have several different functions: in enterovirus infection 3A and its precursor 3AB have been found to co-localize with transcriptional vesicular structures and thus have been suggested to act by orienting 3B so that the replication machinery can attach to it and initiate transcription of viral RNA (Palmenberg et al. 2010). 3A has been found to inhibit protein transport between ER and Golgi apparatus structures in CVB (Paul 1998). 3B is better known as VPg (Virus protein, genome linked) and it is attached to the 5′ end of the picornavirus RNA. VPg has been found to be crucial factor in the initiation of genome replication (Schein 2006). 3C and its precursor protein 3CD is one of the most conserved protein between picornavirus families (Strauss 2003). Like 2A, 3C is also a cys-reactive proteinase and it performs the secondary cleavages of the polyprotein at Q-C locations with its precursor 3CD: this

includes all cleavages except P1-P2 cleavage and the maturation cleavage of VP0 (Ruecker 1984). 3C has also been found to target such cellular proteins that are involved in cap-dependent translation and those of the actin cytoskeleton (Skern 2002). 3D is the viral polymerase and thus the primary protein associated with the synthesis of plus-strand and minus-strand genome RNA. 3B has been found to assist 3D in attaching to the template RNA (Pathak 2008, Palmenberg et al. 2010).

1.4 RNA replication

Once the RNA is released from the viral capsid and the first polyproteins have been produced and cleaved into functional proteins, RNA replication can commence. After it's initiation, RNA replication and polyprotein production happen simultaneously (Kirkegaard and Semler 2010). Because the cellular environment is not hospitable for viral replication, the viruses have to alter the conditions to be more favourable: one way of doing this is by rearranging the cellular membranes to serve as platforms for viral replication complexes to form (Rozovics and Semler 2010).

Originally the membrane rearrangement was discovered in cells infected with poliovirus (Penman 1964). In poliovirus infection, vesicular formations could be seen in the perinuclear area (Dales et al. 1965). In one of these experiments the viral RNA polymerase could be linked to the smooth membranes in HeLa cells (Caliguiri 1969). Indeed, the initiation of poliovirus RNA transcription has been shown to be dependent on these structures, but the elongation of already initiated RNA chains seems to be independent of them (Bienz et al. 1994), suggesting that the modified membranes are important for the formation of RNA-polymerase complex.

Markers for members of the cellular protein secretory pathway: lysosomes, endoplasmic reticulum and Golgi apparatus associated membranes have been found in association with poliovirus 2C protein (Schlegel et al. 1996). Changes in the morphology of these membranes is suspected to be caused by hydrophobic domain containing viral proteins of P2 and P3. Especially the co-operation of 2BC

precursor protein and 3A has been seen to produce double-layer membranes that resemble the membranes seen in the enterovirus infection in their density and ultrastructure, but are not likely to be cellular stress response (Suhy et al. 2000, Rozovics and Semler 2010)

On their own, 2BC and 2C have been found to cause major deformations in the cytosol including the creation of smoot single-membrane vesicles with electron light contents (Suhy et al. 2000). 2B has been shown to lead to the disassembly of the Golgi apparatus. The COPII associated Golgi entering membranes are most likely recruited by 2B: COPII and 2B have been found co-localizing during infection (Rust et al. 2001). Retrograde trafficking vesicles from Golgi have been found to be vital for poliovirus replication. When COPI associated vesicle transport from Golgi to ER was blocked using Brefeldin A the infection was inhibited (Maynell et al. 1992, Jackson 2014).

In order for the RNA replication to commence on the rearranged membranes the positive RNA has to be modified. VPg is uridylylated into VPguPuPu by the 3D polymerase (Paul et al. 2000). A cis-acting replication element (Cre) is used as template (Paul et al. 2000). Cre is a stable stem-loop structure residing in the 2C region of the viral genome (McKnight & Lemon 1998). After the modification of the VPg the transcription is initiated and 3D proceeds to replicate the positive RNA strand (Paul et al. 2000). The resulting negative RNA strand re-enters the 3D at 3' and the desired positive copy of the original template RNA is acquired.

For the freshly produced genome to exit the cell and propagate to further cells it is encased inside the virus capsid. The capsid is produced from P1 precursor protein, which is cleaved into VP0, 1 and 3 by viral protease 3CD in the presence of chaperone protein Hsp90. After the cleavage Hsp90 dissociates from the structural proteins as they bind together to form a 5S protomer. Five 5S protomers bind together as a 14S pentamer that has been shown to recruit RNA before 12 pentamers are bound together as the provirion. Provirion undergoes still one, apparently spontaneous, cleavage as VP0 is divided into VP2 and VP4 to form the mature virion

(reviewed by Jiang et al. 2014). Enteroviruses egress the cell mainly by cell lysis caused by apoptosis (Carthy et al. 2003, reviewed by Harris and Coyne 2014). Enteroviruses have been found to induce apoptosis by at least three mechanisms. Apoptosis is triggered either by the increase of Ca^{2+} in the mitochondria caused by the virus activity (Brisac et al. 2010), the activation of unfolded protein response due to the accumulation of cellular proteins cleaved by 2A and 3C in the cytosol (Ron and Walter 2007) or the stress activated protein kinases activated by the viral processes (Autret et al. 2007). Some evidence also indicate that enteroviruses might be able to exit the cells by necrosis (Robinson et al. 2014) or by non-lethal mechanisms (Taylor and Jackson 2009), but the apoptotic pathway seems to be the prevalent one.

1.5 Calpains

Calpains are a family of 15 calcium dependent (Khorchid and Ikura 2002) cysteine proteases that in normal cellular conditions take part in highly specific proteolytic processes such as cleavage of membrane-associated proteins such as calcium-ATPase or β-integrins (Goll et al. 2003). Two calpains of this family are considered classical prototype calpains that have been found to be expressed in all cell types. Depending on the concentration of Ca^{2+} required for their activation, these classical calpains are either milli (mCalpain or Calpain 2) or micro (μCalpain or Calpain 1) calpains that are activated at calcium concentrations of 400-800 μM and 3-50 μM respectively (Ono et al. 2012). Classical calpains consist of two subunits: the catalyctical large subunit of 78-80 kDa and the small regulatory subunit of 29 kDa. Knock-out of both mCalpains and μCalpains has been shown to cause embryonic lethality in mouse models, suggesting that they play an important role in the early stages of development (Goll et a. 2003).

In their review of Moldoveanu et al. (2002), Khorchid and Ikura (2002) determined the large calpain subunit to consist of four protein domains: dI-IV and the small subunit of two: dV and dVI. [d]I is a structural protein that forces calpain

to take its circular conformation by anchoring its α-helix tail into a cavity in dVI of the small subunit. [d]II, that can further be divided into dIIa and b contains the catalytic site that has been shown to closely resemble the putative catalytic triad of other cysteine proteinases such as papain (thus the name: Cal(cium dependent)pa(pa)in). [d]II is brought to its catalytically active conformation by binding of Ca2+. After this binding the two free loop regions of dII have been found to form a beta-sheet that pulls the participants of the catalytic triad closer together so that they become active. The catalytic specificity of this triad has been shown to be governed by the 3D conformation of the ligand rather than the precise amino acid composition (Croall et al. 1996).

1.5.1 Calpains and viruses

In their publication Upla et al. (2008) showed that calpains could play a role in the infectious cycle of enteroviruses. They found that the inhibition of calpains by calpeptin, calpain inhibitors 1 and 2 or siRNAs blocked EV1, HPV1 and CVB3 infections. Also previous studies have shown that calpains are involved in the infection of other viruses, such as HIV-1: cleavage of IkBa for the activation of viral transcription (Teranishi et al. 2003) and hepatitis C virus: cleavage of non-structural protein NS5A, is performed by calpains (Kalamvoki and Mavromara 2004). In addition, Harris and Coyne (2014) have suggested increased calpain activity, caused by influx of calcium ions, to facilitate the release of cytochrome C from mitochondria thus causing apoptosis in infected cells. Upla et al. (2008) found that the calpain action occurred only after the viral entry, but the precise role of calpains in enterovirus infection remained unresolved.

1.6 Aim of the study

Aim of this master's thesis was to further elucidate the roles that calpains might play in enterovirus infection (Tab. 1). Main focus of the study was to define the time window when calpains are required for the infection. This issue was addressed by

two experiments. First the actual time series experiment was conducted. In this experiment cells were infected either by EV1 or CVB3. Infected cells were treated with calpain inhibitor at different time intervals. The cells were afterwards labelled for the virus and the infection percentage determined using confocal microscopy and immunoblotting. Knowing the theoretical infection schedule of enteroviruses the likely stage of the replication cycle that the calpains have a role in could be deduced. Hypothesis 1: Inhibition of calpains prevents EV1 and CVB3 infection if done during the early stages of infection.

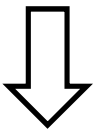
Enteroviruses are known to utilize intracellular membrane structures as a surface for their RNA replication after their reassembly by viral proteins. In order to study to possible role of calpains in the deformation of cytosolic membranes infected cells were treated with calpains at early infection. ER, Golgi and β-COP associated proteins were labelled from calpain inhibitor treated and non-treated cells and observed using confocal microscopy. Hypothesis 2: EV1 infection causes alterations in intracellular membrane structures that can be prevented by calpain inhibition.

The localization of calpains during EV1 infection was observed. A series of infections fixed at different time points were conducted and labelled for calpains and virus protein. This data could be used to determine the change of calpain localization and amount at different stages of infection. Hypothesis 3: During EV1 infection, EV1 VP1 and calpains localize in the same cellular structures.

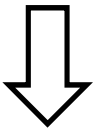
One hypothesis for the role of calpains is, that they might cleave the enterovirus polyprotein. To address this question a cleavage experiment was performed. CVB1 P1+2A mutant protein (with dysfunctional 2A) was introduced to calpains to see whether calpains cleave enterovirus polyprotein at P1-P2 interface. Hypothesis 4: Calpains cleave enterovirus polyprotein.

TABLE 1. Research outline. Research questions are refined into hypotheses that can be tested using the experiments described.

	Research question
1	What is the time frame when calpains are crucial in EV1 and CVB3 infection?
2	How does EV1 infection alter the membrane architecture of the cell?
2.1	How is the alteration affected by calpain inhibition?
3	How does the localization of calpains vary between infected and uninfected cells?
4	How do m- and μCalpains effect the enterovirus polyprotein?



	Hypothesis
1	Inhibition of calpains prevents EV1 and CVB3 infection if done during the early stages of infection
2	EV1 infection causes alterations in intracellular membrane structures that can be prevented by calpain inhibition.
3	During EV1 infection VP1 and calpains localize at same cellular structures.
4	Calpains cleave enterovirus polyprotein.



	Experiment
1	Time series experiment. Calpains are inhibited at different intervals post infection and infection percentage determined using confocal microscopy and immunoblotting.
2	Infected cells and calpain inhibited infected cells labeled for ER, Golgi and β-COP associated proteins and EV1 VP1 Examined with confocal microscopy for changes in membrane architecture.
3	Infected cells and calpain inhibited infected cells labeled for mCalpain and EV1 VP1. Examined with confocal microscopy to determine localization of labeled targets.
4	Treatment of P1+2A mutated protein with m- and μCalpain. Cleavage determined using SDS-PAGE and immunoblotting.

2. MATERIALS AND METHODS

2.1 Choosing the Inhibitor

To choose the best inhibitor for calpain activity, adenocarcinomic human alveolar basal epithelial (A549) cells (ATCC) were cultivated in 10%DMEM (10%FBS, 1%GlutaMAX, 1%Pen-Strep, Gibco, USA). EV1 (Farouk strain, 170000 PFU/µl) dilution was prepared in 1%DMEM (1%FBS and 1%GlutaMAX). The A549 cells were put on ice and introduced to virus dilution. Virus was allowed to bind to the cell surfaces for 1h after which the cells were washed 3 times 5 min each with 0,5%BSA/PBS on ice. The cells were incubated in DMEM with 1%FBS and glutamax at +37C. Calpain inhibitor I (200 µM, Roche, Germany), calpain inhibitor 2 (200 µM, Roche, Germany) and calpeptin (140µM, Calbiochem, USA) were prepared in 1%DMEM and applied to the cells at 1 h post infection (p.i.). The cells were incubated at +37 °C until 6 h p.i.

2.2 Time series experiment

A549 cells (ATCC) were cultivated in 10%DMEM. EV1 (Farouk strain, 170000 PFU/µl) and CVB3 (Nancy strain, 130000 PFU/µl) dilutions were prepared in 1%DMEM. The A549 cells were put on ice and introduced to virus dilutions. Viruses were allowed to bind to the cell surfaces for 1 h after which the cells were washed 3 times 5 min each with 0,5%BSA/PBS on ice. The cells were incubated in 1%DMEM at +37 °C. Calpain inhibitor I (200 µM, Roche, Germany) was prepared in 1%DMEM and applied to the cells in 1 h; 1,5 h; 2 h; 2,5 h; 3 h and 3,5 h time points p.i.. The cells were incubated at +37 °C until 6 h p.i. and fixed for microscopy and immunobloting as described below.

2.3 Cell structure experiment

A549 cells (ATCC) were cultivated in 10%DMEM. EV1 (Farouk strain, 170000 PFU/µl) and CVB3 (Nancy strain, 130000 PFU/µl) dilutions were prepared in 1%DMEM. The A549 cells were put on ice and introduced to virus dilutions. Viruses were allowed to bind to the cell surfaces for 1h after which the cells were washed 3 times 5 min each with 0,5%BSA/PBS on ice. The cells were incubated in 1%DMEM at + 37 °C. Calpain inhibitor I (200 µM, Roche, Germany) was prepared in 1%DMEM and applied to the cells in 1h time point p.i.. The cells were incubated at + 37 °C until 6h p.i. and fixed for microscopy as described below.

2.4 Localization experiment

A549 cells (ATCC) were cultivated in 10% DMEM. EV1 (Farouk strain, 170000 PFU/µl) and CVB3 (Nancy strain, 130000 PFU/µl) dilutions were prepared in 1%DMEM. The A549 cells were put on ice and introduced to virus dilutions. Viruses were allowed to bind to the cell surfaces for 1h after which the cells were washed 3 times 5 min each with 0,5%BSA/PBS on ice. The cells were incubated in 1%DMEM at +37 °C. Calpain inhibitor I (200 µM, Roche, Germany) was prepared in 1%DMEM and applied to the cells at 1 h p.i. and the samples were incubated at +37 °C until 2 h, 3 h, or 4 h p.i.

2.5 P1-2A cleavage with calpain

The preparation of P1-2A construct was done by collaborators in the University of Tampere. Briefly, baculoviral transfer vector (pOET5) was ordered from GeneArt (Regensburg, Germany). Expression cassette for CVB1 capsid proteins VP1-4 and 2A protease with cysteine-to-alanine substitution (resulting in the loss of protease function) was added to the vector to obtain the final construct pOET5-CVB1-P1-2AC>A. FlashBAC baculovirus expression system was used to produce the recombinant baculoviruses by utilizing the flashBAC ULTRA baculovirus genome

(Oxford Expression Technologies, Oxford, UK). CVB1-P1-2AC>A (named P1-2A in the thesis) polyprotein was produced in sf9 cells (Invitrogen). Cells were harvested 3-6 days p.i. by centrifugation and polyproteins were released from the cells by freezing and thawing the cells.

P1+2A protein was exposed to calpain 1 (0,5 U, Calbiochem, USA) and calpain 2 (0,5 U, Calbiochem, USA) and the inhibition effect of calpain inhibitor 1 (200 µM, Roche, Germany) was examined. Cleavage reactions were assembled on ice and incubated at +25 °C for 2 hours. After the incubation the reactions were stopped by adding 5 µl of 4X Laemmli sample buffer (Bio-Rad, USA). The samples were immunoblotted as described below.

2.6 Immunolabeling and confocal microscopy

The cells for confocal microscopy were fixed with 4%PFA for 20 min and stored at +4 °C. The cells were permeabilized with 0,2%Triton X-100 for 5 minutes. The primary antibodies were prepared by diluting them in 3%BSA/PBS (Tab. 2). Primary antibody dilutions were applied on the cells and incubated for 45 min at room temperature. After primary labeling the samples were washed 3 times 5 min each with PBS. Secondary antibodies were prepared by diluting them in 3%BSA/PBS (Tab. 1). The secondary antibodies were applied on the cells and incubated for 30 min at room temperature, covered from the light. The samples were washed 3 times 5 min each with PBS. The second wash included DAPI stain (300 nM, Molecular Probes, USA). The cells were mounted on microscopy coverslips with Mowiol-DABCO (Merck KGaA, Germany), incubated overnight in room temperature and stored at +4 °C. The microscopy was performed using Olympus Fluoview 1000 (2005/2005), Argon-laser, UPLSAPO 60x oil immersion (NA=1,35)

objective. The microscope data was later analyzed using ImageJ (Schindelin et al. 2012) and BioimageXD (Kankaanpää et al. 2012).

TABLE 2. The antibodies used in confocal microscopy. The primary targets, antibodies and secondary antibodies are presented. For the primary antibodies also the concentration of the used antibody is shown, for secondary antibodies the dilution of the commercial antibody used is shown.

EXPERIMENT	SAMPLE	TARGET	PRIMARY AB	C	INFO	SECONDARY AB	DILUTION	INFO
Inhibitor Choosing		VP1	a-EV1	1:200	Affinity purified; Marjomäki et al. 2002	Alexafluor 488, Rabbit	1:200	Life Technologies, USA REF#A11008
		dsRNA	J2	125 ng/mL	Molecular Probes, Eugene, OR, USA	Alexafluor 555, Mouse	1:200	Life Technologies, USA REF#A32727
Time Series	EV1	VP1	a-EV1	1:200	Affinity purified; Marjomäki et al. 2002	Alexafluor 488, Rabbit	1:200	Life Technologies, USA REF#A11008
	CVB3	dsRNA	J2	125 ng/mL	Molecular Probes, Eugene, OR, USA	Alexafluor 555, Mouse	1:200	Life Technologies, USA REF#A32727
Cell Structure	1	PDI, ER	1D3	1:100	Kind gift from prof. Steve Fuller, EMBL, Heidelberg	Alexafluor 555, Mouse	1:200	Life Technologies, USA REF#A32727
	2	VP1	a-EV1	1:200	Affinity purified; Marjomäki et al. 2002	Alexafluor 488, Rabbit	1:200	Life Technologies, USA REF#A11008
		β-COP	E5A3	1:200	Kind gift from prof. Thomas Kreis, University of Geneva	Alexafluor 555, Mouse	1:200	Life Technologies, USA REF#A32727
	3	VP1	a-EV1	1:200	Affinity purified; Marjomäki et al. 2002	Alexafluor 488, Rabbit	1:200	Life Technologies, USA REF#A11008
		CD63	a-CD63	1,23 mg/mL	Zymed, San Francisco, CA, USA	Alexafluor 555, Mouse	1:200	Life Technologies, USA REF#A32727
		VP1	a-EV1	1:200	Affinity purified; Marjomäki et al. 2002	Alexafluor 488, Rabbit	1:200	Life Technologies, USA REF#A11008
Localization	1	μ-Calpain	a-μ-Calpain	100 μg/mL	Sigma Aldrich, Germany, Darmstadt	Alexafluor 555, Mouse	1:200	Life Technologies, USA REF#A32727
		VP1	a-EV1	1:200	Affinity purified; Marjomäki et al. 2002	Alexafluor 488, Rabbit	1:200	Life Technologies, USA REF#A11008
	2	m-Calpain	a-m-Calpain	100 μg/mL	Sigma Aldrich, Germany, Darmstadt	Alexafluor 488, Rabbit	1:200	Life Technologies, USA REF#A11008
		dsRNA	J2	125 ng/mL	Molecular Probes, Eugene, OR, USA	Alexafluor 555, Mouse	1:200	Life Technologies, USA REF#A32727

2.7 Immunoblotting

The cells for immunoblotting were collected into 2X Laemmli sample buffer (Bio-Rad, USA) and stored at -20 °C. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the samples using Miniprotean SDS-PAGE drive. The samples were prepared by boiling them for 9 min and loading them into premade gels (Mini-Protean TGX, Bio-Rad, USA). The drive was filled with running buffer. The samples were first ran with voltage of 100 V until they had reached the bottom of the stacking gel and then with 160 V until the molecular weight marker (Kaleidoscope, Bio-Rad, USA) had reached the bottom of the separation gel. The samples were then blotted onto a polyvinylidene difluoride membrane (Immobilon-P Transfer membrane, Merck KGaA, Germany) using Wester Blotting method for 1 h in 100 V. After blotting the membranes were blocked with 5%BSA/Tween-TBS for 1 h. The primary antibodies were prepared by diluting them in 5%BSA/Tween-TBS (Tab. 3). The membranes were incubated with the primary antibody overnight at 4 °C. The membranes were washed 3 times 5 min each with 0,5%Tween-TBS. The secondary antibodies were prepared by diluting them in 5%BSA/Tween-TBS (Tab. 3). The membranes were incubated with the secondary antibody for 1 h in room temperature. The membranes were washed 5 times 5 min each with 5%BSA/Tween-TBS. Supersignal solution was prepared by mixing reagent 1 (Stable Peroxide Solution, ThermoFisher, USA) and reagent 2 (Luminol, ThermoFisher, USA) 1:1. The membranes were incubated with the supersignal solution for 5 min at room temperature covered from light. The signal from the membranes was imaged onto X-ray film (Super RX, Fujifilm, Japan) and analyzed with ImageJ (Schindelin et al. 2012).

TABLE 3. The antibodies used in immunoblotting. The primary targets, antibodies and secondary antibodies are presented. For the primary antibodies also the concentration of the used antibody is presented, for secondary antibodies the dilution of the commercial antibody used is presented.

EXPERIMENT	TARGET	PRIMARY AB	C	INFO	SECONDARY AB	Dilution	INFO
Time series	EV1	a-EV1	1:200	Affinity purified; Marjomäki et al. 2002	Anti-rabbit IgG HRP-linked Antibody	1:3000	Cell signalling, The Netherlands
	CVB3	DAKO	47 µg/mL	DAKO, Glostrup, Denmark	Anti-mouse IgG HRP-linked Antibody	1:3000	Cell signalling, The Netherlands
	Actin	a-actin	3,75 µM	Sigma Aldrich, Germany	Anti-mouse IgG HRP-linked Antibody	1:3000	Cell signalling, The Netherlands
Cleavage experiment	EV1	a-EV1	1:200	Affinity purified; Marjomäki et al. 2002	Anti-rabbit IgG HRP-linked Antibody	1:3000	Cell signalling, The Netherlands
	CVB3	DAKO	47 µg/mL	DAKO, Glostrup, Denmark	Anti-mouse IgG HRP-linked Antibody	1:3000	Cell signalling, The Netherlands

3 RESULTS

3.1 Calpain inhibitor 1 inhibited EV1 infection most efficiently

In order to conduct calpain inhibition in the experiments described, the optimal inhibitor for calpain was determined. Different calpain inhibitors were compared in an infection experiment and the results were obtained by counting the percentage of infected cells using confocal microscopy. The addition of Calpain inhibitor 1 prevented EV1 infection more efficiently than Calpeptin or Calpain inhibitor 2 (Fig. 1), therefore it was chosen to be used in further experiments.

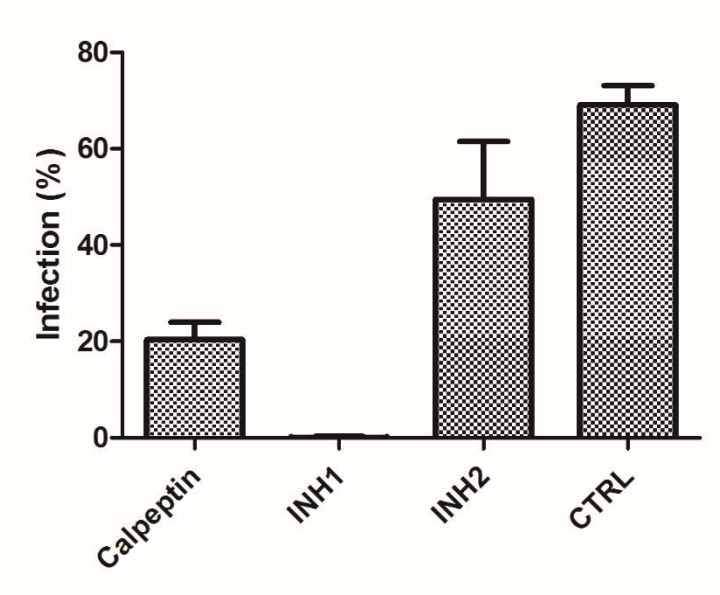


FIGURE 1. The effect of different calpain inhibitors on EV1 infection. Calpain inhibitor 1 proved to be the most efficient inhibitor in preventing EV1 infection. The infection percentages of samples treated with different calpain inhibitors or control with no inhibitors (CTRL) are shown. The inhibitors were added at 60 min p.i. and the total infection time was 360 min. Infection percentages were determined by counting immunofluorescently labeled infected cells from confocal micrographs. Pearson standard deviation is shown. INH1=Calpain inhibitor 1, INH2=Calpain inhibitor 2. The results are means from three different experiments (±SD).

3.2 Calpain inhibitor prevents infection if added at an early stage

To find the time frame where calpains are required for the progression of enterovirus infection, a time series experiment was conducted. The activity of calpains was inhibited by introducing calpain inhibitor 1 at different time points in an infection that was followed until 360 min. Afterwards the samples were observed by confocal microscopy using antibodies against dsRNA for CVB3 and VP1 for EV1 as an indicator of infection. In order to determine the whole population infection level the amount of VP1 was also determined using Western blot.

In confocal microscopy it was observed that if the calpain inhibitor was added at 60 min p.i. in CVB3 infection, only few cells showed signs of virus replication and cells appear healthy and normal. At 90 min and 120 min time points some replication could be seen and many nuclei were beginning to adopt a crescent form indicating an infection. After 150 min the cells were fully infected, nuclei had shrunk and the dsRNA was observed in almost all of the cells (Fig. 2, A).

The microscopy data was quantified by counting the number of infected cells and comparing it to total cell count based on the number of DAPI stained nuclei. The quantified data showed that the addition of calpain inhibitor 1 decreases the number of infected cells. The effect was most potent when the inhibitor was added at 60 min p.i. The effect decreased steadily until 150 min p.i. after which inhibitor 1 had no effect on the infection (Fig. 2, B). A statistically significant difference was found between the infection percentage of the control infection and the infection percentages of 60 min (Mann-Whitney; n=79; U=3; p<0,00), 90min (Mann-Whitney; n=82; U=76,0; p<0,00), 120 min (Mann-Whitney; n=80; U=282,0; p<0,00) and 180 min (Mann-Whitney; n=70; U=428; p<0,041) inhibitor addition time points.

CVB3 infected A549 cell lysates were also analysed by western blotting. The data was analysed by comparing the band intensities of VP1 and actin that was used as loading control. This data showed that the addition of calpain inhibitor 1 decreased the amount of VP1 if added at 60 or 90 min p.i. After that the addition of calpain inhibitor 1 had no inhibitory effect on the infection. There was no

statistically significant difference observable between the control infection sample and the samples treated with calpain inhibitor 1. This is probably due to the fact that the intensity of the virus control band was low compared to samples of late calpain inhibitor 1 addition. However, when comparing the band of the highest intensity (180 min) to others, statistically significant differences were found. The time points of 60 min (Mann-Whitney; n=6; U=0,00; p=0,050) and 90 min (Mann-Whitney; n=6; U=0,00; p=0,050) differ from the 180 min time point. (Fig. 2, C)

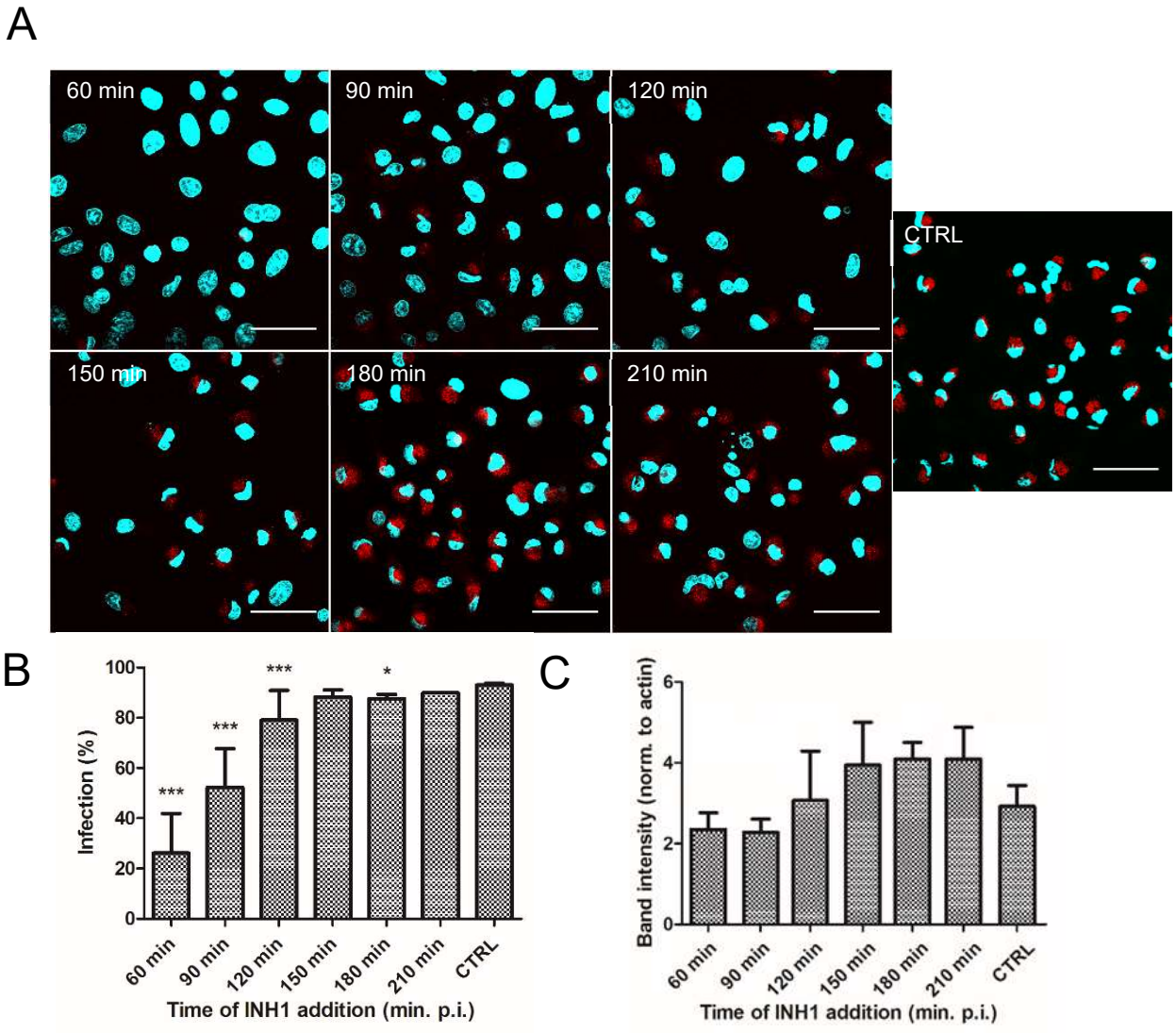


FIGURE 2. The effect of calpain inhibitor 1 on CVB3 infection. (A) Confocal microscopy. The number of cells containing viral dsRNA (red) increases as the calpain inhibitor 1 is added later in the infection. After 120 min time point the calpain inhibitor 1 has no effect on the infection. The scale bar is 50 µm, times shown are the calpain inhibitor 1 addition times. Nuclei are presented in cyan. **(B) Quantified microscopy data.** Calpain inhibitor 1 decreased the number of infected cells when added before 2 h p.1. If added after 2 h p.i. the inhibition of calpains has no effect on the CVB3 infection. Infection percentages were determined by confocal microscopy. The results are means from three different experiments (±SD). Mann-Whitney-U: * P<0,05; ** P<0,01; *** P<0,001 to control. **(C) Western blot.** The amount of CVB3 VP1 decreases if the calpain inhibitor 1 is added at early stages of infection. If calpain inhibitor 1 is added at 60 min or 90 min p.i. the amount of viral protein in the samples is lower than if the inhibitor is added at 120 min or later. However, there was no statistically significant difference found between the samples and control. The results are means from three different experiments (±SD).

In EV1 infection, a minute amount of VP1 was seen if Calpain inhibitor 1 was added at 60 min and 90 min time points. The intensity of the infection increased significantly if the inhibitor was added later in the infection. From 180 min time point on, the inhibition seemed to have only little effect on the infection. The nature of the inhibition seemed to change: in very early addition of the inhibitor the infection was weak in individual cells. VP1 was not diffuse in the cytoplasm, but instead aggregated in a small volume, suggesting that the infection had started locally. At later time points, VP1 appeared to be more evenly distributed in the cytoplasm: only in the 210 min time point VP1 could again be seen congregated in ball like shapes near the nuclei. (Fig. 3, A).

The quantification of microscopy data showed that the addition of calpain inhibitor 1 decreased the number of infected cells if added before 90 min p.i. (Fig. 3, B). After 90 min p.i. there was clear increase in infection percentage and a steady increase from 120 min to 210 min. There was a statistically significant difference between the infection control and 60 min (Mann-Whitney; n=59; U=32,5; p<0,00), 90 min (Mann-Whitney; n=62; U=114,00; p<0,00), 120 min (Mann-Whitney, n=60; U=298,500; p=0,025) and 150 min (Mann-Whitney, n=63; U=300,00; p=0,007).

When EV1 infected cell lysates were analyzed by western blotting the results showed that the addition of calpain inhibitor 1 decreases the amount of VP1 if added at 120 min p.i. or earlier. A statistically significant difference was found between the infection control and 60 min (Mann-Whitney; U=0,00; n=8; p=0,021), 90 min (Mann-Whitney; U=0,00; n=8; p=0,021) and 120 min (Mann-Whitney; U=0,00; n=8; p=0,021) samples. (Fig. 3, C).

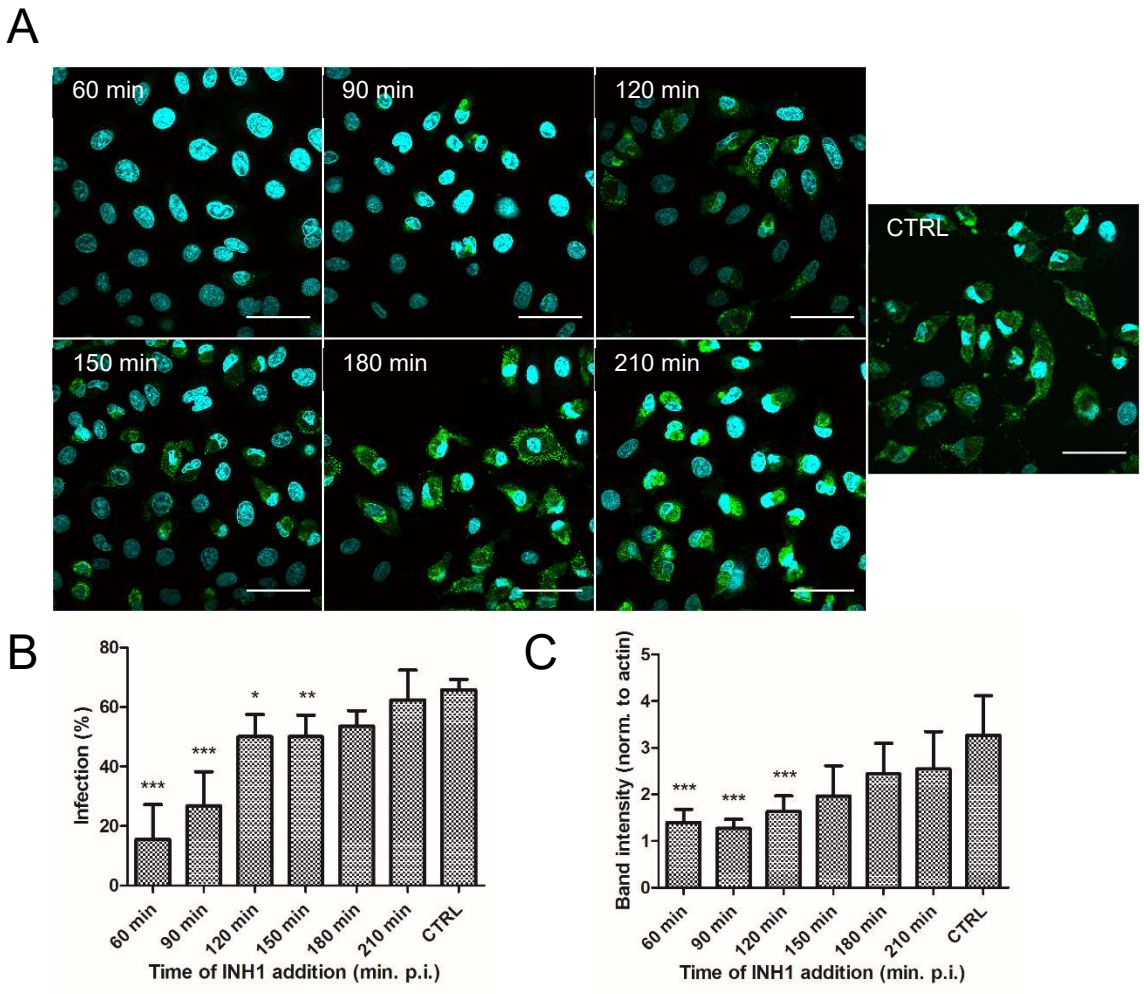


FIGURE 3. The effect of calpain inhibitor 1 on EV1 infection. (A) Confocal microscopy. The amount of VP1 (green) containing cells increases as the calpain inhibitor 1 is added at later time points. After 90 min time point the amount of VP1 seems to gradually increase until 210 min. The scale bar is 50 µm, the times shown are the calpain inhibitor 1 addition times. Nuclei are presented in cyan. **(B) Quantified microscopy data.** Calpain inhibitor 1 (INH1) decreased the number of infected cells most effectively when added before 120 min p.i. If added after 120 min p.i., the inhibition of the infection is not as effective. After 180 min p.i. the addition of calpain inhibitor 1 has no effect on the number of infected cells. Infection percentages were determined by confocal microscopy. The results are means from three different experiments (±SD). Mann-Whitney-U: * P<0,05; ** P<0,01; *** P<0,001 to control. **(C) Western blot.** The amount of VP1 decreases if the calpain inhibitor 1 is added at early stages of infection. If calpain inhibitor 1 is added at 120min or earlier p.i. the amount of viral protein in the samples is significantly lower than if the inhibitor is added at later time. The results are means from four different experiments (±SD). Mann-Whitney-U: * P<0,05; ** P<0,01; *** P<0,001 to control.

3.3 Calpain inhibitor 1 inhibits structural changes of membranous structures in EV1 infection

Enteroviruses are known to utilize membranous structures of the cell as platforms for their genome replication. In order to assess how calpain inhibition during infection affects some of these structures, an infection assay of 6 h was conducted with and without the inhibitor. From the fixed samples marker proteins for endoplasmic reticulum (Protein disulphide isomerase, PDI), ER-Golgi route (β-COP) and intraluminal vesicles of late endosomes (CD63) were immunolabeled for confocal microscopy.

EV1 infection caused the endoplasmic reticulum to shrink and form concentrated structures. This was detected by observing changes in the localization of immunolabeled ER associated protein PDI. This effect was prevented by the addition of calpain inhibitor 1 at 60 min p.i. (Fig. 4). The amount of β-COP associated membranes increased in EV1 infection, but there was no change in the spatial distribution. The addition of inhibitor to non-infected cells increased the level of β-COP signal. Inhibitor treatment decreased the β-COP signal in infected cells to a level closer to the before mentioned control. (Fig. 5). In EV1 infection, CD63 associated membranes localized near the nucleus in a ball like formation in cells that were strongly infected; in less infected cells the ball like structures were not as apparent. When treated with calpain inhibitor 1, these structures did not form and CD63 resided evenly distributed in the cytosol. (Fig. 6).

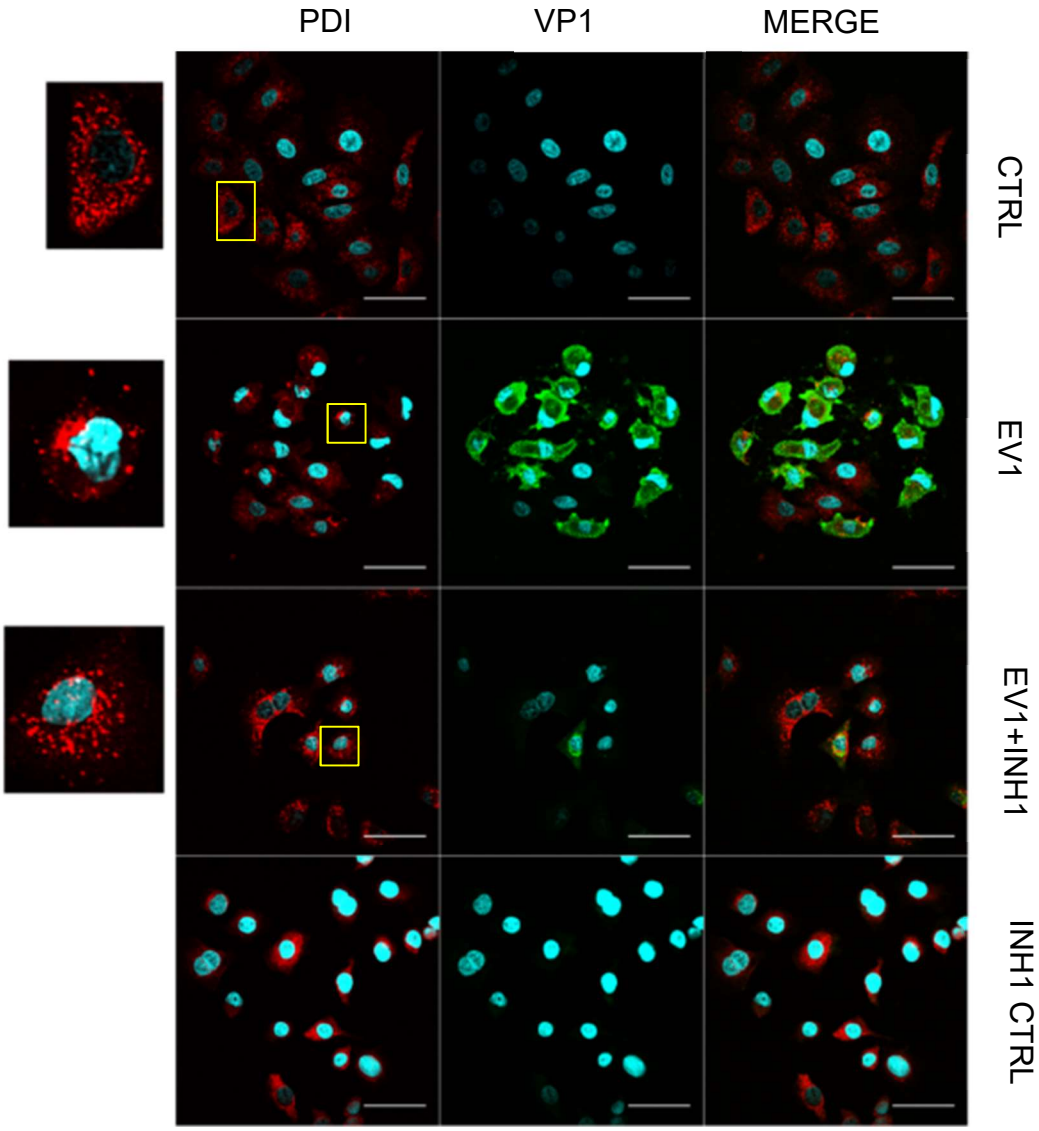


FIGURE 4. The effect of EV1 infection and calpain inhibitor 1 on PDI. In EV1 (VP1=green) infection ER associated PDI (red) is concentrated into dense structures indicating their usage in virus replication. In samples with calpain inhibitor 1 the ER remains evenly distributed throughout the cell. Length of the scale bar is 50 µm.

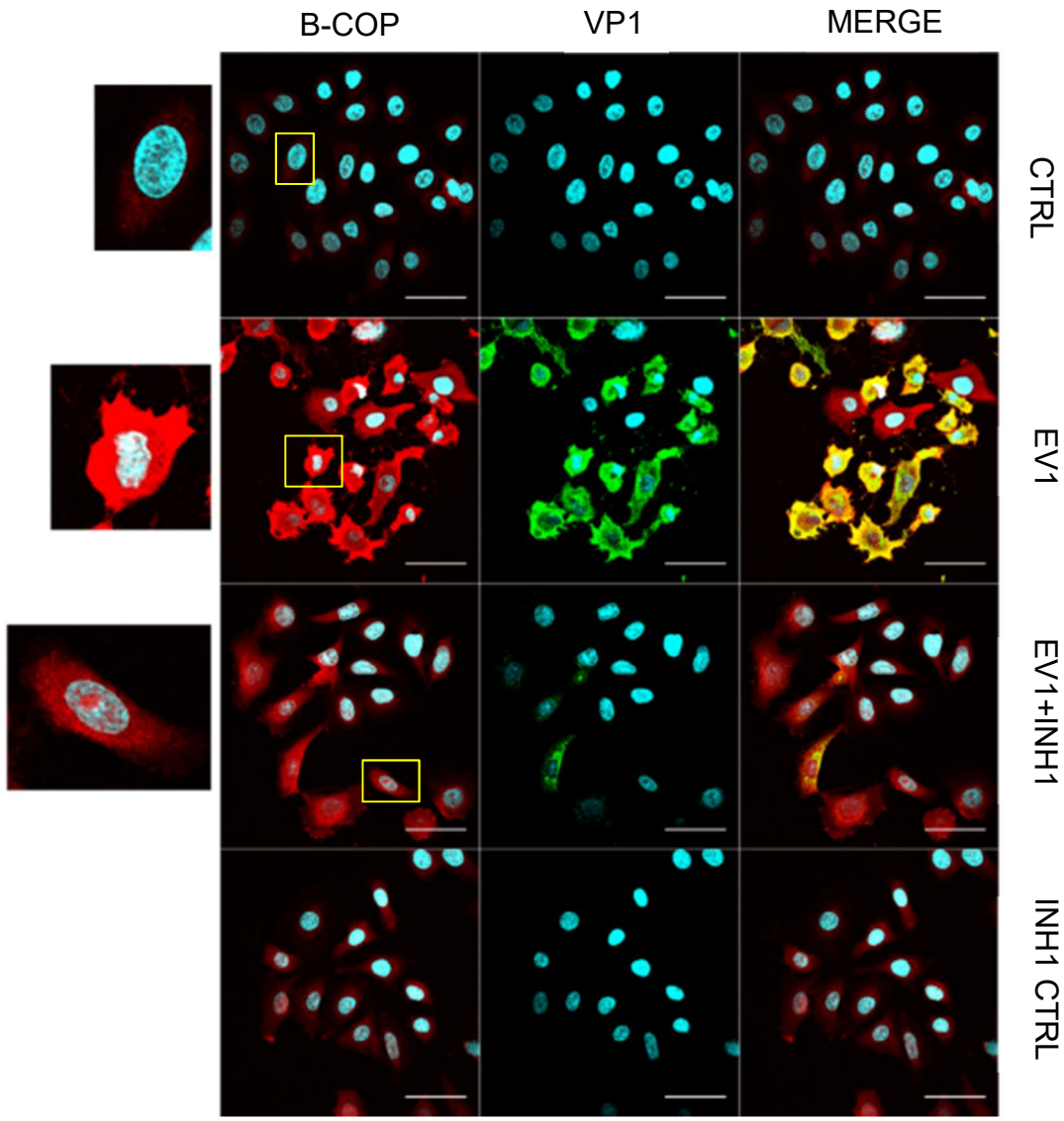
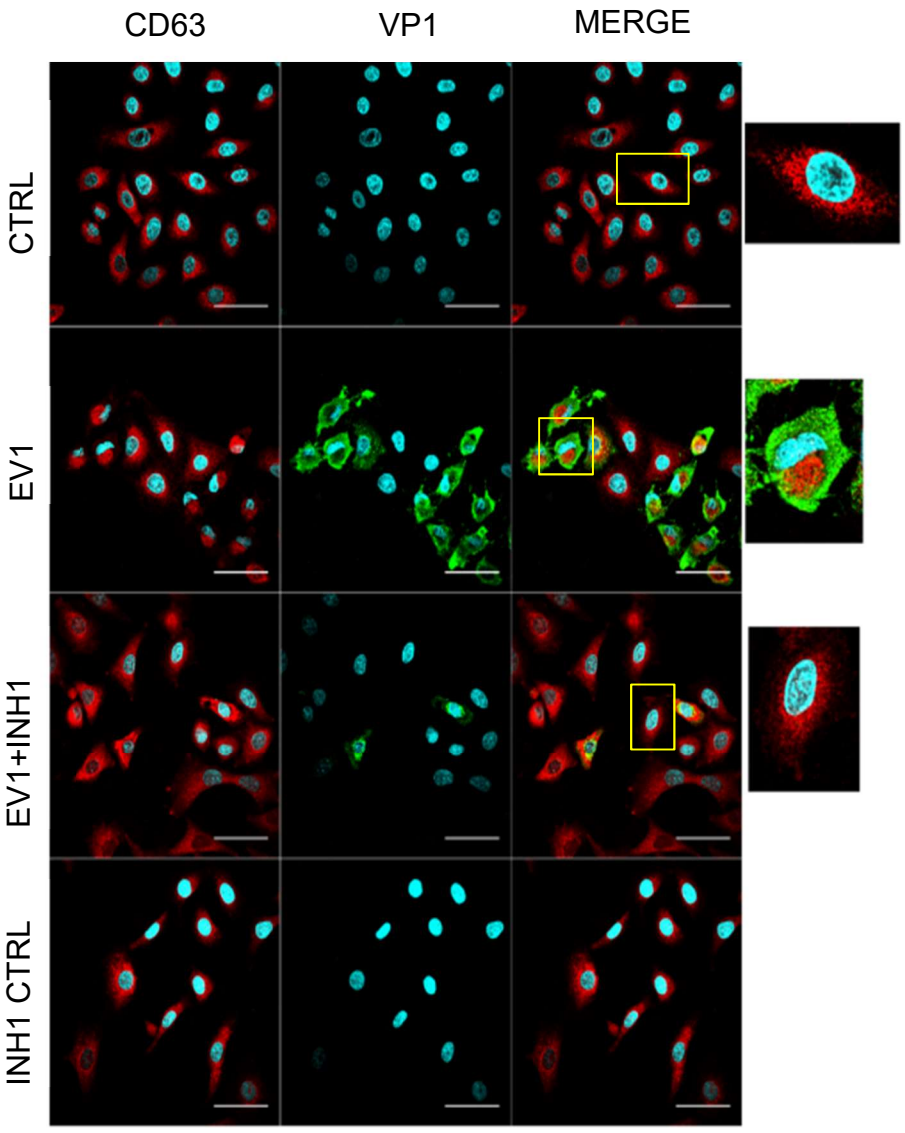


FIGURE 5. The effect of EV1 infection and calpain inhibitor 1 on β-COP. The amount of ER-Golgi route associated β-COP (red) increases in EV1 (VP1=green) infection. This increase is inhibited to some degree by the addition of calpain inhibitor 1: to a level resembling the non-infected cells treated with inhibitor. Length of the scale bar is 50 µm.



F**IGURE 6. The effect of EV1 infection and calpain inhibitor 1 on CD63.** The normally diffusely distributed late endosome associated CD63 (red) form ball like structures near the nucleus (cyan) in EV1 infection (VP1=green). When treated with calpain inhibitor 1 lysosomes retain their normal distribution. Length of the scale bar is 50 µm.

3.4 The localization of calpain does not change in EV1 infection, but becomes more abundant after 240 min p.i.

Previous study (Upla et al. 2008) has suggested that calpain 1 would co-localize with VP1. In order to ascertain this and explore the overall localization of calpain in infection, a localization experiment was conducted. EV1 infections of 120, 180 and 240 min were fixed, stained for calpain 1 and VP1 and examined in confocal microscopy.

EV1 infection appears to increase the amount of calpain 1. At 120 min p.i. there was no infection to be seen in the cells and calpain 1 could be seen everywhere in the cytoplasm. At 180 min p.i. the EV1 infection was prevalent and some minute co-localization between EV1 VP1 and calpain 1 could be seen. At 240 min p.i. the amount of calpain 1 had increased compared to the earlier time points. Calpain 1 was also more abundant in infected cells than non-infected cells at 240 min time point (Fig. 7).

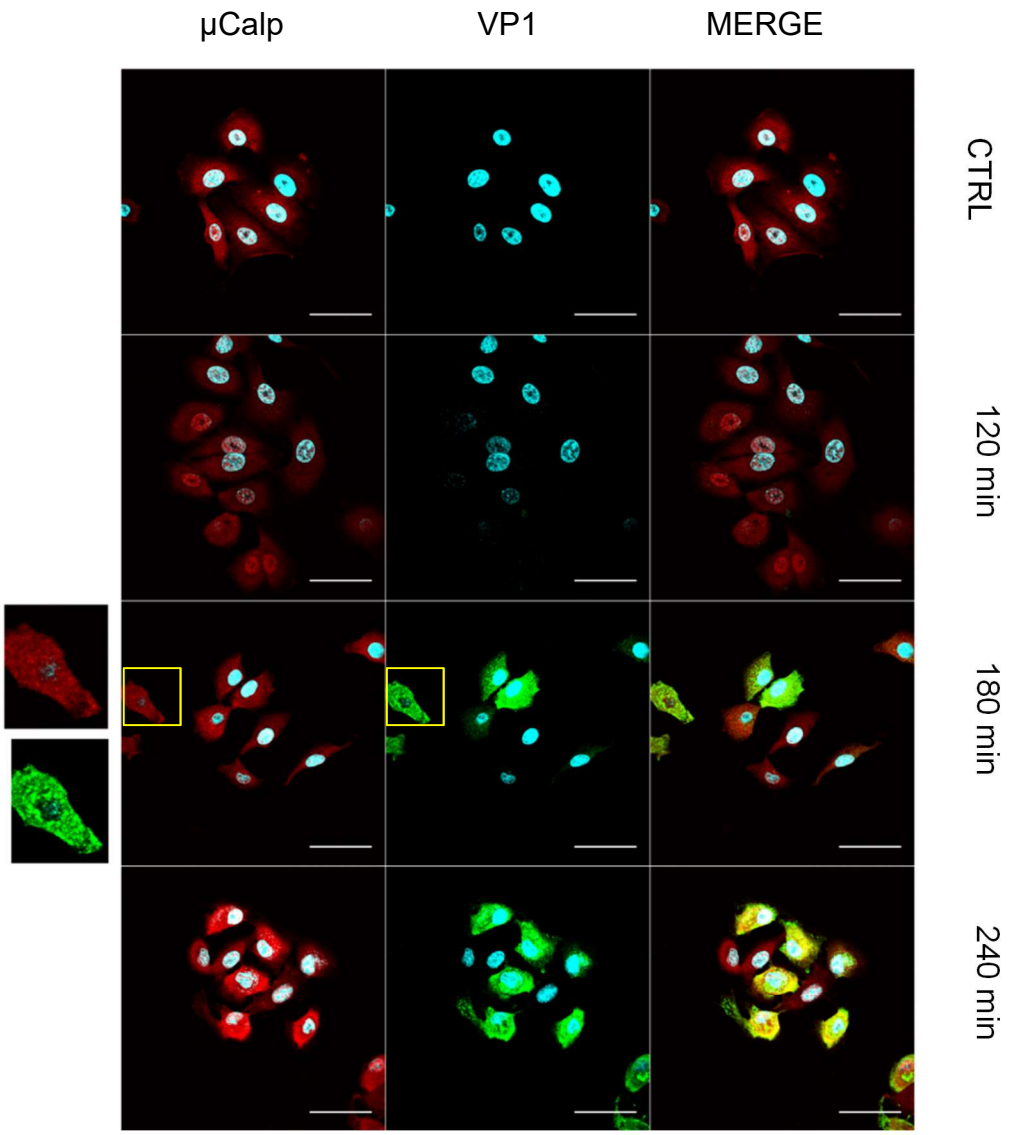


FIGURE 7. Localization of calpain 1 in EV1 infection. The localization of calpain 1 (µCalp, red) is shown at 120, 180 and 240 min time points p.i.. There is some co-localization observable by eye between VP1 (green) and calpain 1 at 180 min time point. At 240 min time point the amount of calpain 1 has increased and is greater in infected than non-infected cells. Nuclei can be seen in cyan in the pictures. Length of the scale bar is 50 µm.

3.5 Calpain 1 and 2 cleave P1 at VP0-VP3 junction

Since earlier and our results here showed that the time action of calpains is 2-3 h p.i. and takes place after RNA release into the cytoplasm, but before membrane rearrangements and RNA replication, we wanted to study the effect of calpain proteases on viral polyprotein processing. This was assessed in a cleavage experiment, where calpains 1 and 2 were introduced to a P1-2A protein construct with or without calpain inhibitor 1. In the P1-2A construct, 2A was rendered dysfunctional by mutation to allow the observation of calpain induced cleavage only.

The cleavage reactions were analyzed using western blotting, where the results were detected with an antibody against VP1. The results showed that calpain 1 and 2 cleave the P1-2A mutant protein (Fig. 8, A). The cleavage was inhibited by the addition of Calpain inhibitor 1. The cleavage action performed by Calpain 2 was reversed more efficiently by the inhibitor than that of Calpain 1. Calpain 1 cleaved the P1-2A construct to some degree also in the presence of the inhibitor. Inhibition of calpains did not return the P1-2A band intensity to same level with the control sample. (Fig. 8, C). Both calpains produced a cleavage product of approximately 75 kDa in size (Fig. 8, D). This cleavage product did not appear in samples that had calpain inhibitor 1 included. When examining the possible cleavage outcomes, the cleavage at VP0-VP3 produces a 75 kDa product (Fig. 8, B). This suggests that calpains 1 and 2 cleave the P1-2A construct at VP0-VP3 bond. In one of the replicates calpain inhibitor 1 control sample slightly heavier band also appeared. This larger band was very close to 75 kDa and caused a distortion in the quantification of inhibitor control lane (data not shown).

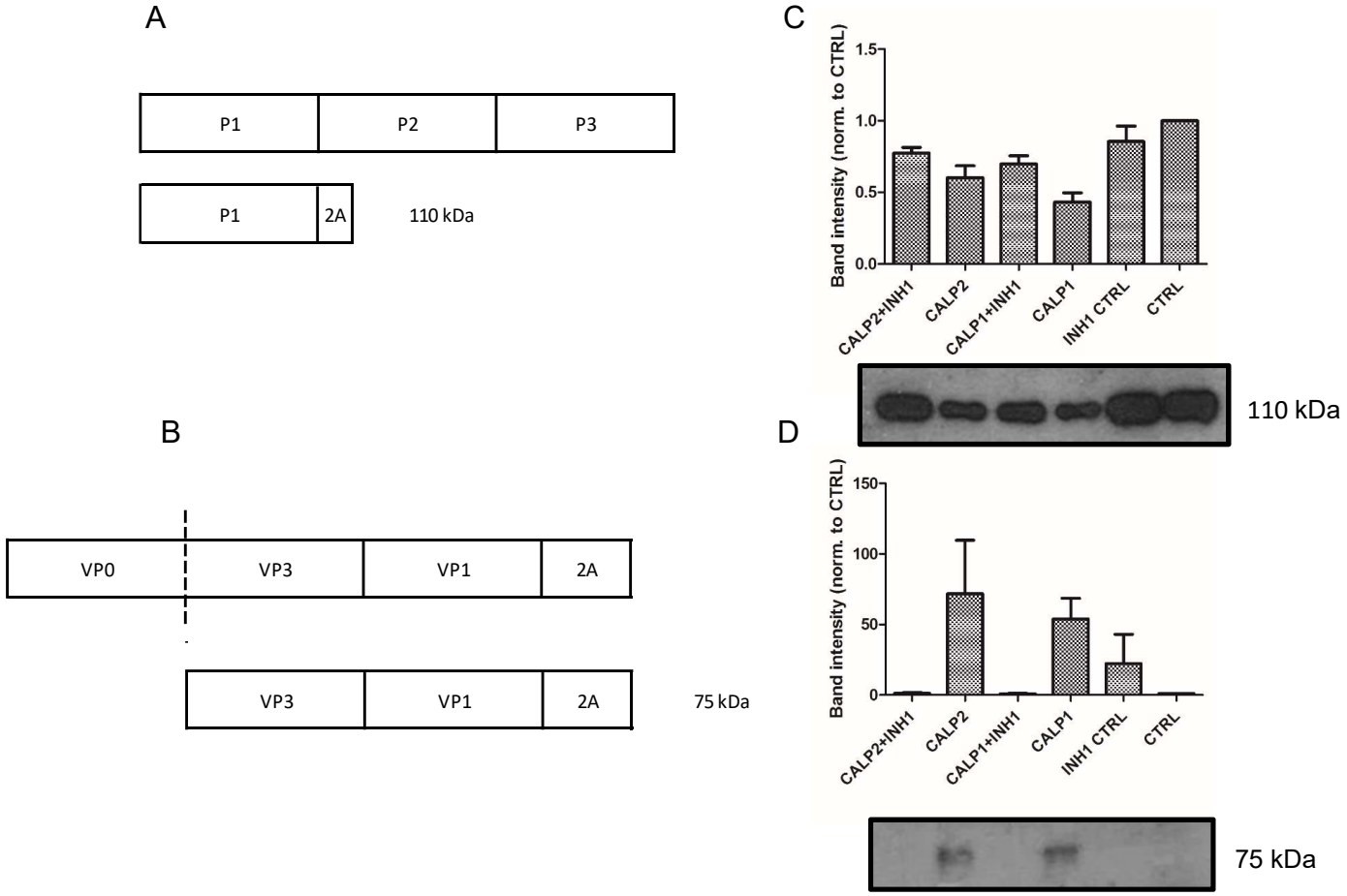


FIGURE 8. The effect of calpains on VP1-2A conjugate shown in WB. (A) The P1-2A conjugate is constructed of the structural part of polyprotein, P1 and the ineffective protease 2A belonging to P2. The size of this conjugate is 110 kDa. **(B)** When VP0 is cleaved of the construct, weight of the remaining product detected with antibody for VP1 is 75 kDa. **(C)** The addition of Calpain 1 and 2 causes the cleavage of P1-2A conjugate. This effect is reversed by the addition of Calpain inhibitor 1. **(D)** In samples treated with calpain 1 or 2, a cleavage product of 75 kDa that is equal to the product described above (B) was produced. The results were normalized by dividing each band intensity by control band intensity (with no added proteases). The results were quantified from two separate experiments. Calp2 = Calpain 2, INH1 = Calpain inhibitor 1, Calp1 = Calpain 1. The results are means from two different experiments (±SD).

4 DISCUSSION

Previous work has shown that the inhibition of the cellular calpain system completely blocks EV1 and CVB3 infection (Upla et al. 2008, Coyne…). Upla et al. (2008) also found that EV1 infection increases the calpain activity and causes the co-localization of EV1 and calpain. Upla's research gave strong evidence that calpains are crucial for the enterovirus infection but left the question of what precise function calpains serve open. The current research aimed to further elucidate the part that calpains are playing in enterovirus infection. This was done by conducting four experiments. Time series experiment, which revealed that calpains are essential for EV1 and CVB3 infection during the first two hours of infection. Calpain localization experiment, which showed that the overall localization of calpains does not change in enterovirus infection. Cell structure experiment, which showed that membrane modifications typical for enterovirus infection were prevented by calpain inhibition. And last, polyprotein cleavage experiment, which revealed that enterovirus polyprotein can be cleaved by both m- and μCalpain. Together the current findings confirm that calpains do have a role in enterovirus infection and suggest that it takes place during 120 first minutes of infection, possibly in the polyprotein processing step.

4.1 The time window

In order to narrow down the processes in which calpains could be involved during enterovirus infection, a time series where calpain activity was inhibited at different time points p.i. was performed. Earlier findings by Upla et al. (2008) suggested that calpains are required for processes that take place late in the infection. The current time series experiment showed that the time-period when calpain is crucial for CVB3 and EV1 infection was between 0—120 min p.i. After this, the omittance of calpain did not cause an important decrease in infection levels. The change in infection percentage was different between the viruses. CVB3 infection seemed to

increase very rapidly, if the calpain inhibitor was not added right at the beginning of the infection. In contrast, in EV1 infection the change in infection percentage was more gradual: a clear increase was observed after 90 minutes, but the climb to control level happened only after 150 min. During the 6 h infection EV1 control infection reached only 60—70% infection percentage, whereas CVB3 reached almost 100% infectivity. This observation suggests that CVB3 infection that clearly proceeded faster, passed the stages where calpains are required for the infection more rapidly than the slower EV1, thus having a steeper change in infection percentage.

Whatever calpains are doing for the benefit of the virus, it happens during 120 minutes at the beginning of the infection. Many important processes take place during the first two hours of enterovirus infection. Upla et al. (2008) found that calpain inhibition did not prevent EV1 from entering SAOS-cells. They also found that calpain inhibition could prevent the infection when EV1 genome was microinjected into the cells and concluded that calpains have a role after the viral genome release. Their finding that calpain inhibition performed as late as 240 min p.i. inhibits enterovirus infection could not, however, be replicated in the current study. This is most likely due to different virus generation used and the variation in infection properties they possess. In addition, the cell line in the current study was A549, whereas most of the studies carried out by Upla et al. included SAOS cells. The virus batch that was used in the current research was evidently faster in its replication cycle, which might be due to different cell line used. Another possibility is, that the viral concentration added was higher or the cell population on the plates was lower than those in Uppla et al. (2008).

Interestingly, the time window when calpains are required for the infection could be related to the release time of the viral genome. Huttunen et al. (2014) performed an experiment, where they created a neutral-red-CVA9 (NR-CVA9). The genome of this NR-CVA9 could be irreversibly cross-linked by light exposure and

thus rendered un-infectious. By treating the infected cells at different time points in a 6 h infection with light exposure, a time window, where the genome had been released and protein production had started could be determined.

Huttunen et al. (2014) found that the opening of the virus capsid and the genome release takes place during the first 120 minutes of infection. A similar experiment was also conducted by Soonsawad et al. (2004) using EV1. They found that the RNA release takes place during the first hour of the infection. Knowing that calpains have no part in the opening of the virus capsid (Upla et al. 2008) and seeing how the current results determine the necessity period of calpains to the first 120 minutes of infection, it seems plausible that calpains act right after the genome release, possibly in the production of the first polyprotein. These results support hypothesis 2: Inhibition of calpains prevents EV1 and CVB3 infection if done during the early stages of infection.

4.2 Calpain localization

Earlier studies have shown (Upla et al. 2008) that calpain localizes discretely in cellular structures containing VP1. In the current study, m- and μCalpains were labeled from different length infections alongside with VP1 to determine if the enterovirus infection causes changes in calpain localization. In EV1 infection the localization of total calpain did not change nor did it exclusively localize in same cellular loci as VP1. This result is in disagreement with the results that Upla et al. (2008) showed: in their study both m- and μCalpains were discretely co-localized in the same intracellular locations as VP1. This observation was done at 2 h p.i. In the current study, no infected cells could be even found in the samples that were drawn at 2 h p.i. timepoint. However, in the 3 h and 4 h samples the distribution of calpains within the cell did not change, merely the signal intensity increased. The difference between the current study and Upla's findings can be due to mere chance: fixed microscope samples are only a snapshot in time, providing the viewer with a highly

restricted temporal resolution. If the process where calpains localize exclusively with VP1 is brief, it can be difficult to capture in one microscope slip.

Comparing the signal intensities between different microscope slips by eye is more or less arbitrary, so very little can be said about the calpain amounts between time points. However, in 240 min infection the amount of calpain was greater in cells that had been infected than in those that were not infected. This result suggests that in enterovirus infection the recruitment of calpain is increased. It is widely known that enterovirus infection silences the host transcription and translation processes by complex cascades (Dougherty et al. 2010). It would thus seem unlikely, that during enterovirus infection the production of calpains could be upregulated, as the production of other host proteins is clearly inhibited.

When studying calpains' possible role in the α2β1 degrading pathway Rintanen et al. (2011) found calpains to be activated in infected cells. A similar 1,4-fold increase in calpain activity following calcium release from ER was earlier observed by Rosser et al. (1993) using a t-BOC assay. Calpains have been suggested to relocate to the cell periphery upon cell division, possibly in response to a Ca2+ flux (Saido et al. 1993, Goll et al. 2015). A similar Ca2+ flux to the cytoplasm is also known to occur in enterovirus infection (Van Kuppeveld et al. 2005). As the first observations of viral RNA in the cell have been seen in the cell periphery in MVBs, from which it is released to the cytoplasm (Karjalainen et al. 2008), one could suspect that the calpains could be transferred to this location or activated there to assist in the early stages of infection. Calpains are by default diffusely distributed in the cytoplasm and the previous findings suggest that calpain localization should be seen changing in enterovirus infection and their activity increased in areas of early infection. In the current study only the total distribution of calpains was observed, and it could not be seen changing during the infection. It would therefore be more informative to additionally examine the activity distribution of calpains to see, if calpains are more active when localized in same compartments with virus proteins.

These results do not give a definitive answer to hypothesis 4: During EV1 infection VP1 and calpains localize in the same cellular structures, since calpains clearly localize in all cellular structures, but not exclusively those containing VP1.

4.3 Membrane modifications

Enteroviruses are known to utilize cellular membranes as platforms for their replication processes. These platforms are constructed by viral proteins 3A, 2BC and 2B by modifying ER (Suhy et al. 2000) and Golgi apparatus (Rust et al. 2002) associated membranes. Protein associated with these membranes, and MVBs linked to virus entry were labelled and change of their shape assessed.

β-COP signal was greater in infected than non-infected cells, within samples treated with EV1 and between EV1 treated samples and control samples. This suggests that in enterovirus infection β-COP associated membranes grow in number in the cytoplasm. β-COP associates with endosomes (Aniento et al. 1996). Suggesting the possibility that late enterovirus infection could have an arresting effect on endocytosis, causing the accumulation of β-COP associated endosomes in the cytosol. β-COP has additionally been shown to associate with ER-Golgi traffic associated membranes (Peter et al. 1993). However, Golgi structures have been shown to disassemble during enterovirus infection (Sandoval and Carrasco 1997) partly due to the calcium releasing pores formed on their surface by viral protein 2B. This process has also been shown to degrade ER structures (van Kuppeveld et al. 2005). The release of calcium has been shown to result in the activation of calpains causing the release of cytochrome C and resulting in apoptosis. Broken down Golgi and ER structures have been shown to accumulate in the cytoplasm, transformed by viral protein 2BC to act as replication sites of the viral genome (Bienz et al. 1994). The activity increase caused by calcium escaped from the ER could be one of the borders lining the necessity period of calpain in the enterovirus infection: one could

suspect that in order for calpains to have marked effect on the infection they must be in active state.

Distribution changes that were seen in the current research were most likely reorganized Golgi and ER structures that are used as viral replication platforms. Whether the disassembly and reorganization of these membranes is directly caused by calpain action cannot be commented based on the current research. However, the inhibition of calpains clearly prevented the hijacking of these membranes, suggesting that the viral proteins 3A, 3BC and 3C among others could not perform their tasks. This in turn might suggest, that the release of these viral proteins from the polyprotein would somehow be dependent on calpain activity.

In the EV1 treated sample in cells with advanced infection, CD63 associated membranes seem to form a ball like structure in the crescent cavity near the nucleus. This formation was not seen in cells that were treated with calpain inhibitor. CD63 has been shown to associate with late endosomes, MVBs and especially ILVs therein (Edgar et al. 2014). These findings would therefore suggest that MVBs gather in the vicinity of the nucleus during enterovirus infection. Enterovirus infection has been shown to depend on the biogenesis of such multivesicular bodies (Karjalainen et al. 2011). These ball like structures were not seen by Karjalainen et al. (2011) at 2 h p.i., instead in their studies CD63 seemed to be diffusely distributed throughout the cytoplasm. It would thus seem that the ball like shape is adapted only in late infection when the virus production is in full power. It is also noteworthy that CD63 does not seem to colocalize with VP1, but instead, there is much more virus outside the region where the CD63 associated membranes congregate. CD63 is known to be important factor in exosome formation and vesicles associated with it can be considered as late endosomes or MVBs (Hessvik and Llorente 2018). Enteroviruses have been shown to utilize exosomes as means of shedding their next generation out of the cell before killing the cell (Inal and Jorfi 2013).

This result supports hypothesis 3: EV1 infection causes alterations in intracellular membrane structures that can be prevented by calpain inhibition. In the light of earlier data by Upla et al. (2008) and our data shown here, calpain action takes place after RNA release into the cytoplasm and before the rearrangement of cellular membranes. In other words, the inhibition of calpains inhibited an infection step that happens before and is crucial to the membrane reassembly.

4.4 The polyprotein cleavage

Calpains are calcium dependent cysteine proteases (Khorchid and Ikura 2002), and thus one could suspect that their role in the infection might be to cleave something, for example the viral polyprotein, as is their natural function. In a simple cleavage experiment P1-2A mutant with dysfunctional 2A proteinase was introduced to calpains to determine if calpains are capable of processing the polyprotein.

M- and μCalpain were found to cleave the P1-2A mutant protein in a fashion that was inhibited by the addition of calpain inhibitor 1. The resulting cleavage product was 75 kDa in size, equal to P1-2A mutant without VP0. This would suggest that the cleavage took place at VP0-VP3 bond. This process is considered to be performed by the viral protease 3C (Hanecak et al. 1982). Since VP0 is a capsid protein and serves no known functional purpose in the enterovirus replication, it would seem unlikely that the inhibition of VP0-VP3 bond cleavage would hinder the progression of the infection in the magnitude and time period observed. However, although not pointing out a precise process that calpains capacitate by polyprotein cleavage, this result shows that calpains are indeed capable of partaking in the polyprotein processing. Whether calpains can also cleave other parts of the polyprotein and release proteins needed for replication, remains to be studied. Knowing that calpains differentiate ligands by their three dimensional structure (Goll et al. 2015) the idea of calpains cleaving the polyprotein at multiple locations, especially those that are canonically considered to be cleaved by 3C, does

not sound farfetched but remains to be tested. These results support hypothesis 1: Calpains cleave enterovirus polyprotein.

As discussed in section 4.3 the actions performed by 3A, 3BC and 3C were prevented by calpain inhibition, suggesting that they were not released from the polyprotein. Knowing that calpains are capable of cleaving enterovirus polyprotein and seeing how the omittance of calpains prevents the activities performed by these proteins, it would seem plausible that calpains might contribute to the release of these proteins from the polyprotein.

4.5 Conclusion

In this study an array of experiments was performed to clarify the roles that calpains might have in enterovirus infection. Upla et al. (2008) clearly showed that without calpain activity, enterovirus infection cannot proceed to RNA replication. Thus, the aim of this study was to determine the time window when calpains are crucial for the progression of the infection. By knowing the epoch of calpain activity, it is possible to narrow down the possible processes they might be associated with.

Overall picture was obtained from the time-series experiment. It showed that after 2 h p.i. the activity of calpains was not required anymore and the infections could proceed normally. This picture was enhanced by examining the effect that omittance of calpains had on the intracellular membrane alterations. Calpains clearly inhibited the membrane processing, meaning that a process prior to this, perhaps the release of membrane modifying viral proteins, is dependent on calpains. Lastly a proof of concept for calpains cleaving the polyprotein was obtained from the cleavage experiment, where they were seen to target VP0-VP3 junction in P1-A2 conjugate protein. Taken together, these results suggest that calpain activity avails enterovirus infection in the early stages of infection, after RNA release into the cytoplasm, possibly in the polyprotein processing.

Further experiments are required to determine the exact sites on the enterovirus polyprotein that can be cleaved by calpains. In addition to the additional cleavage experiments, in order to give definitive evidence of the membrane alterations and calpain localization during infection, quantitative imaging and analysis should be performed. The results from the membrane structure and calpain localization experiments in this study should be interpreted with caution for they were conducted only once and there was no quantification of the results, all conclusions are done merely by eye. Most of the changes seen are fairly obvious, but especially differences in brightness of the signal between treatments needs further quantification.

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