Investigation of the Role of PKCα for Influenza A Virus-Induced Signalling and of the Inhibitory Effect of Verapamil on Virus Replication

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Summary

Influenza Virus (IV) activates the Raf/MEK/ERK-(MAPK) cascade late in their replication cycle. This is essential for efficient nuclear RNP-export and therefore for production of infectious IV. To characterize cellular factors involved in MAPK-activation in the context of the viral infection I recently analyzed the role of PKCα. The results so far indicate, that activation of the Ca^{2+} activated PKCα is involved in the IV induced MAPK-signaling and that specific inhibition of this function using a Ca^{2+} channel blocker (Verapamil) at non toxic concentration, negatively affects IV propagation (265). In addition, I have now further analyzed the action of Verapamil for possible additional negative effects on IV replication. Therefore I have investigated viral protein production in IV infected human lung epithelial cell line A549 and found that PB1, NP, and NS1 production is significantly reduced in IV infected and Verapamil treated A549 cells. Cell survival and cellular protein production does not seem to be affected ruling out a general effect of Verapamil on translation. Since PB1 is a functional important subunit of the viral polymerase the activity of the polymerase might be affected. Therefore I analyzed the polymerase activity in Verapamil treated cells for the production of viral mRNA using primer extension analysis of a reporter transcript expressed by either a plasmid based replication system or in virus infected cells.

My current results show that viral mRNA production in the plasmid based replication system is not affected, while it seems to be reduced in virus infected cells. This indicates that Verapamil might alter viral transcription activity in virus infected cells. Taken together, PKCα plays an important role in transmitting the influenza virus induced signal to the MAPK-cascade. As this PKCα-inhibition can be achieved at non-toxic Verapamil concentration, leading to strong reduction of virus titers, inhibition of this cellular activity might be a potential anti-viral therapy.

List of publications:

(1) Henju Marjuki, M. Intakhab Alam, Christina Ehrhardt, Ralf Wagner, Oliver Planz, Hans-D. Klenk, Stephan Ludwig and Stephan Pleschka. "Membrane accumulation of influenza A virus hemagglutinin triggers nuclear export of the viral genome via PKCα mediated activation of ERK signaling". J. Biol. Chem., Vol. 281, Issue 24, 16707-16715, April 2006.

Zusammenfassung

Influenzaviren (IV) aktivieren die Raf/MEK/ERK (MAPK)-Signaltransduktionskaskade in der späten Phase ihres Replikationszyklus. Dies ist essentiell für einen effizienten RNP-Kernexport und damit für die Produktion infektiöser Viren. Zur Charakterisierung zellulärer Faktoren, welche im Kontext der viralen Infektion die MAPK-Kaskade aktivieren, habe ich die Rolle von PKCα analysiert. Die bisherigen Ergebnisse zeigen, daß die Aktivierung der Ca^{2+}-aktivierten PKCα an der virusinduzierten MAPK-Aktivität beteiligt ist, und das die Inhibition dieser Funktion durch einen Ca^{2+}-Kanalblocker (Verapamil) in nicht toxischen Konzentrationen die IV-Vermehrung negativ beeinflußt (265). Zusätzlich habe ich weitere negative Effekte der Wirkung von Verapamil auf die IV-Vermehrung untersucht. Dafür habe ich die Produktion viraler Proteine in der IV-infizierten humanen Lungenalveolar-Epithelzellinie A549 analysiert. Dabei konnte ich zeigen, daß die Expression von PB1, NP und NS1 in infizierten und Verapamil-behandelten A549-Zellen signifikant reduziert ist. Dabei führt die Verapamil-Behandlung nicht generell zu einer Beeinträchtigung des Zellüberlebens oder einer verringerten Zellproteinproduktion. Da PB1 eine funktionell wichtige Untereinheit der viralen RNA-abhängigen RNA-Polymerase (RDRP) ist, könnte die RDRP beeinflußt sein. Deshalb untersuchte ich die Produktion viraler mRNA. Zum einen in transfizierten A549-Zellen in denen mittels eines plasmidgestützen Replikationssystem zur transienten Expression viraler RDRP und eines vRNA-ähnlichen Pol1-Transkriopts virale mRNA gebildet wurde. Zum anderen wurde in transfizierten Zellen nur das vRNA-ähnliche Pol1-Trankript gebildet, welches dann durch die RDRP-Aktivität nach IV-Infektion der transfizierten Zellen repliziert und transkribiert wurde. Die jetzigen Ergebnisse der „Primer-Extension"-Analyse zeigen, daß die Produktion viralen mRNA in dem plasmidgestützten system nicht beeinflußt zu sein scheint, aber in den IV-infizierten Zellen. Das könnte bedeuten, daß Verapamil die virale Transkription in virusinfizierten Zellen beeinträchtigt. Zusammengefaßt kann man sagen, daß PKCα eine wichtige Rolle in der Signaltransmission der IV-induzierten MAPK-Kaskade spielt. Da Verapamil in nicht toxischen Konzentrationen PKCα inhibiert, die IV-Vermehrung hemmt und Verapamil als Pharmazeutika zugelassen ist, könnte Verapamil für einen anti-viralen therapeutischen Ansatz gegen IV nützlich sein.

1. Introduction

1.1 The Causative Agent

1.1.1 Influenza

Influenza is caused by a virus that attacks mainly respiratory tract, the nose, throat, and bronchi and rarely also the lungs. The infection usually lasts for about a week. It is characterized by sudden onset of high fever, myalgia, headache and severe malaise, non-productive cough, sore throat and rhinitis. Most people recover within one to two weeks without requiring any medical treatment. In elderly people suffering from medical conditions, influenza poses a serious risk. In these people, the infection may lead to severe complications of underlying diseases, pneumonia and death.

1.1.2 History of Influenza

The origin of the name influenza is uncertain although the chronicles of a Florentine family used it in reference to the possible influence of the planets at times of respiratory epidemics (1). The Influenza is a distinctive disease and its comings and goings could not be predicted. The term influenza was used in England during the outbreak of 1743 (2). Influenza has no pathognomic features, so a precise picture of its impact was impossible before the first isolation of influenza A virus in 1933 (3), influenza B virus in 1940 (4) and influenza C virus in 1947 (5). Influenza has been termed, 'the last great plaque' (6). Further knowledge of the illness came with the discovery of the hemagglutinating properties of influenza virus in 1941 (7) and development of diagnostic methods based on hemagglutination inhibition. However, in the absence of these tools, a combination of the explosive nature of influenza, its respiratory and systemic features, its tendency for seasonality, and its high attack rates and associated mortality in people of advanced age provides an insight into influenza since ancient times.

1.1.3 Human influenza and transmission

Influenza in man occurs in two epidemiological forms. The first is pandemic influenza which results from the emergence of a new influenza A virus - termed antigenic shift - to which the population possesses little or no immunity, it therefore normally spreads

with high attack rates throughout all parts of the world. The second is interpandemic influenza, occurring as a sporadic infections, a localized outbreak in a given community which usually occurs abruptly, peaks within 2-3 weeks, lasts 5-6 weeks, and is associated with significant 'drift' of the surface antigens. Influenza is spread by virus-laden respiratory secretions from an infected to a susceptible person. The virus is easily passed from person to person through the air by droplets and small particles excreted when infected individuals cough or sneeze. The influenza virus enters the body through the nose or throat. It then takes between one and four days for the person to develop symptoms. Someone suffering from influenza can be infectious from the day before they develop symptoms until seven days after wards (WHO).

The influenza pandemic of 1918-1919 killed more people than the Great War, at somewhere between 20-40 million people. It has been cited as most devastating epidemic in recorded world history. More people died of influenza in a single year than in four-years of the Bleck death Bubonic Plaque from 1347-1351. Known as "Spanish Flu" or "La Grippe" the influenza of 1918-1919 was a global disaster.

The Influenza Pandemics of 1918

1.1.4 Clinical symptoms of influenza virus infection

The replication of the virus leads to the lysis of the epithelial cells and enhanced mucus production causing running nose and cough. Furthermore, inflammation and oedema at the replication site are due to cytokines released. This can lead to fever and related symptoms. Bacterial super-infection of the harmed tissue can further complicate the situation. Normally onset of systemic (fever, myalgia, headaches, severe malaise) and respiratory (coughing, sore throat, rhinitis) symptoms occur after about two days incubation period and can last for about seven to ten days (186).

1.1.5 Different types of influenza viruses

Influenza viruses (IV) belong to the *Orthomyxoviridae* and are subdivided into three genera: type A, B and C (8), due to the characteristic of their nuclear and matrix protein. Currently circulating influenza viruses that cause human disease are divided into two group's A- and B-type. Influenza A viruses (IAV) can infect humans, birds, pigs, horses, seals, whales and other animals (Figure 1.1.), whereas wild aquatic birds are the primary reservoir of these viruses (9). IAV has 2 subtypes, which are important for humans: H1N1 and H3N2, of which the former is currently associated with most deaths (WHO influenza health topic). IV are defined by 2 different protein components, known as antigens, on the surface of the virus. They are spike-like features called hemagglutinin (HA) and neuraminidase (NA) components. Type B influenza viruses are normally found only in humans. Although influenza B viruses can cause human epidemics, they have not caused pandemics. Type C infection usually causes either a very mild respiratory illness or no symptoms at all; it does not cause epidemics or pandemics and does not have the severe public health impact that influenza types A and B do. Unlike IAV, type B and C influenza viruses are not classified according to subtype.

1.2 Influenza A virus

1.2.1 Morphology and genome structure

Influenza A viruses have been the most thoroughly investigated and are best understood genera among A, B and C. The structures of influenza A and B viruses are very similar although the B viruses are somewhat more irregular.

The virion of type A influenza virus is enwrapped by a lipid envelop derived from the plasma membrane of host cells (10) and has a fairly regular appearance in the electron microscope, with sizes that range from 80 to 120 nm in diameter (Figure 1.2.C) (11-12). The genome which is replicated in the nucleus of infected cell, contains eight segmented single-stranded RNAs with negative polarity (ss (-) sense RNA) (13-16) of 890 to 2,341 nucleotides each that code for at least 10 viral structure proteins (Table 1.1. and Figure 1.2.A). The viral genomic RNA (vRNA) of IAV is complexed with the viral RNA-dependent RNA-polymerase (RDRP) in all negative-sense RNA viruses, which comprises three protein subunits (PB1, PB2 and PA) and the nucleoprotein (NP) into biologically active viral ribonucleoprotein complexes (vRNP) (17) assembled in a twisted rod (10-15 nm in width and 30-120 nm in length) (18) (Figure 1.2.B) that is folded back and coiled on itself (19-21). The vRNA serves as a template for both transcription and replication (22). The RNPs are the minimal set of infectious viral structures. The nucleoprotein is a major component of the RNP. The primary function of NP is to encapsidate the virus genome for the purposes of RNA transcription, replication and packaging (23). The outer surface of the virus consists of two glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) embedded in the virus lipid membrane envelope (24, 25). The HA allows the virus to agglutinate red blood cells and is responsible for virus binding to sialic acid (N-acetyl- or N-glycolylneuraminic acid) containing receptors on the cell surface and red blood cells (26, 27) and for fusion of the viral and cellular membrane during virus entry (28-31) to allow the RNPs to enter into the cytoplasm of the host cells. The mature HA forms homotrimers of 220 kDa. Each monomer is initially present as a single polypeptide precursor (HA_0) that is subsequently cleaved into two subunits HA_1 and HA_2 (32). These subunits are linked by a single disulphide bond between residue 14 of HA_1 and residue 137 of HA_2 [23]. The NA is the second subtype-specific glycoprotein and possesses enzymatic activity cleaving sialic acid residues from glycoproteins or glycolipids (33-35), which function as cellular receptors for attachment of influenza virions. The neuraminidase activity of NA mediates the release of newly formed virus particles from the surface of infected cells (36). Without this step, the newly forming virus particles immediately re-bind to their receptor and are not released into the extracellular space. Instead, they remain attached to the cell in large clump (37). The M2 is an integral membrane protein encoded by IAV and possesses an ion channel activity that is required for efficient release of the viral genome into the cell cytoplasm

and for correct maturation of the HA. The viral matrix protein M1 is thought to underlie the lipid bilayer and to associate with the RNP core of the virus. It has been shown that export of vRNP requires the synthesis of M1 protein (13, 39, 40), nevertheless the exact function still remains unknown. The first protein encoded by NS-segment is the non-structural protein NS1. It can only be found in the influenza virus-infected cells and functions as a major pathogenicity factor. It is known that NS1 plays an important role in preventing the PKR-mediated antiviral response (41). Furthermore NS1 protein represses interferon β (IFNβ) synthesis by preventing activation of the NF-kB signalling pathway & transcription of interferon genes (42-47). NS2 or Nuclear Export Protein (NEP) was originally designated as non-structural protein. It possesses a Nuclear Export Signal (NES) sequence, which is essential for export of vRNP from the nucleus (48, 49). M1 and NS1 mRNAs are collinear transcripts, whereas M2 and NS2 protein are generated from a spliced transcript of their genome segments. The PB1 segment contains an alternative +1-reading frame encoding the recently discovered PB1-F2 protein (50), which is the only influenza virus factor identified to act intrinsically by localization and interaction with the mitochondrial-dependent apoptotic pathway. This protein is not essential in cell culture and not strongly conserved (51). Therefore the role of this protein in viral propagation is not well known.
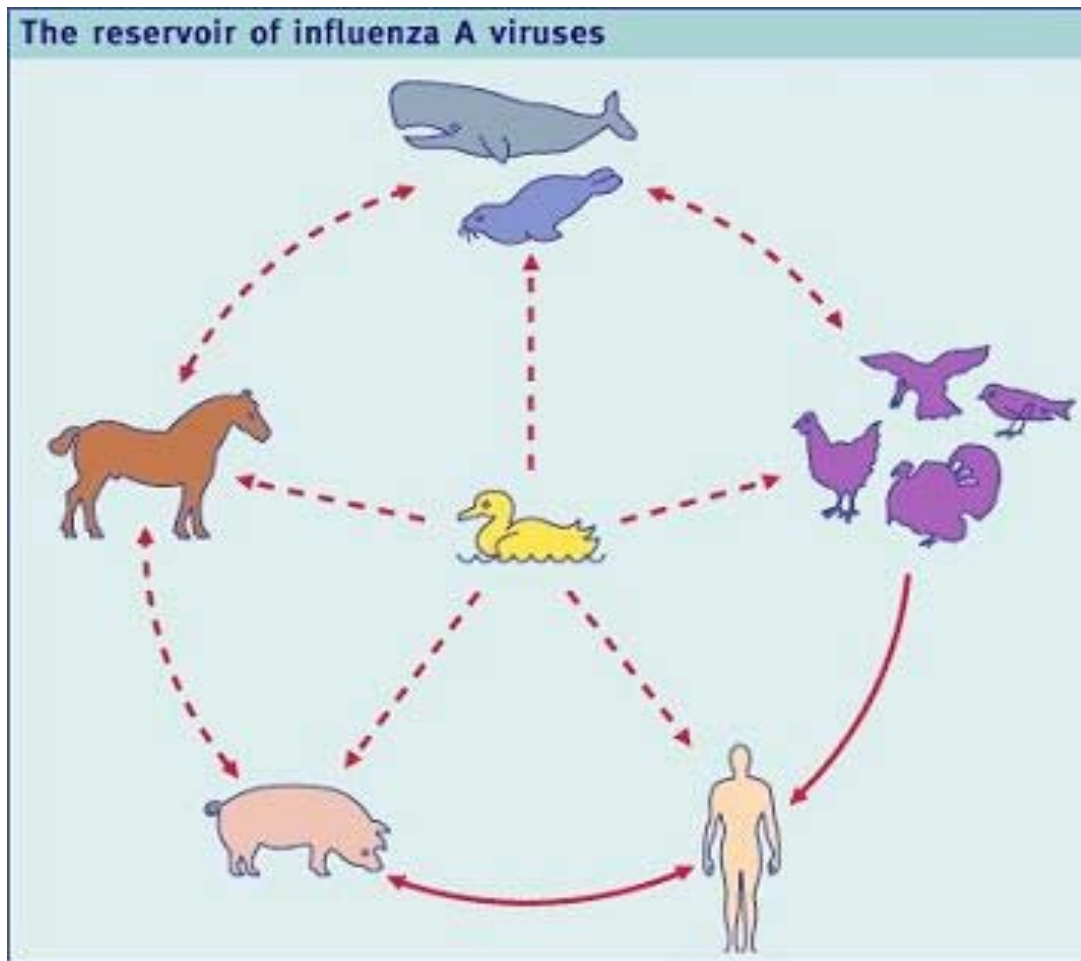
Figure 1.1. The reservoir of influenza A viruses. The working hypothesis is that wild aquatic birds are the primordial reservoir of all influenza viruses for avian and mammalian species. Transmission of influenza has been demonstrated between pigs and humans (solid line). There is extensive evidence for transmission between wild ducks and other species, and the five different host groups are based on phylogenetic analysis of the nucleoproteins of a large number of different influenza viruses. (www.fiav.info).

Table 1.1 Influenza A virus proteins and functions (stem A/PR/8/34, H1N1)

Modified from (256)

Segment	vRNA(nt)	Protein	AA	Functions
1	2341	PB2	759	subunit of RDRP; "Cap-snatching"
2	2341	PB1	757	catalytic subunit of RDRP; elongation
		PB1-F2	87	not known
3	2233	PA	716	subunit of RDRP
4	1778	HA	566	surface-glycoprotein; receptor binding; membrane fusion
5	1565	NP	498	nucleoprotein; encapsidation of v/cRNA; part of transcriptase complex; nuclear/cytoplasmic transport of vRNA
6	1413	NA	454	surface-glycoprotein; neuraminidase; receptor disruption, virus releasing
7	1027	M1	252	matrix protein; ion channel activity;
		M2	97	protecting HA-conformation, RNP-release
8	890	NS1	230	post-transcription regulator; inhibition of (i) pre-mRNA splicing, (ii) polyadenylation (iii) PKR-activation
		NEP/NS2	121	nuclear export factor
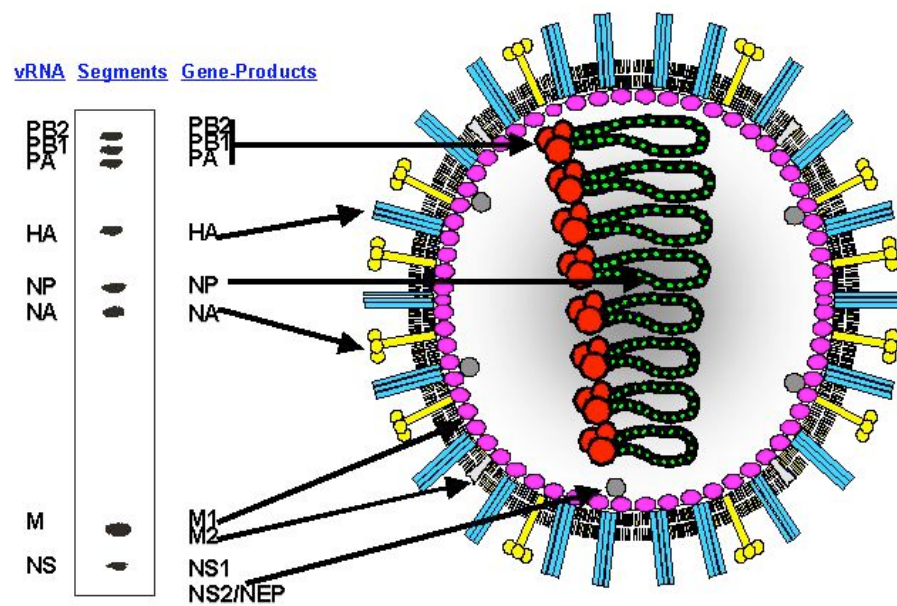
Figure 1.2 A: Schematic diagram of the influenza virion. The eight viral RNA segments were separated in a urea polyacrylamide gel electrophoresis and detected by silver staining (left). The corresponding gene products and their accepted localization in the virion are right indicated. NS1 is not a component of mature virions. (Adapted from S. Pleschka and S. Ludwig, Germany)
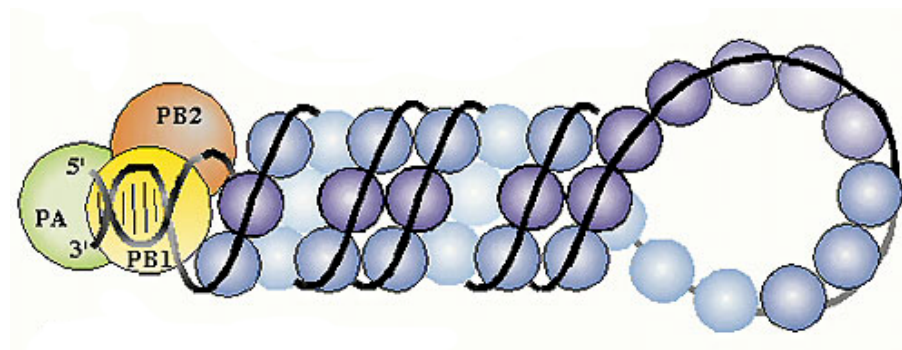
Figure 1.2 B: The structure of influenza virus RNP. The RNP complex is the minimal set of infectious viral structures. It consists of (-) sense single-stranded RNA (black line), nucleoprotein (NP) (blue and purple) and three subunits of RNA-dependent RNA-polymerase (RDRP): PB1 (yellow), PB2 (orange) and PA (green). (Adapted from P. Digard, UK)

Left   **Right**
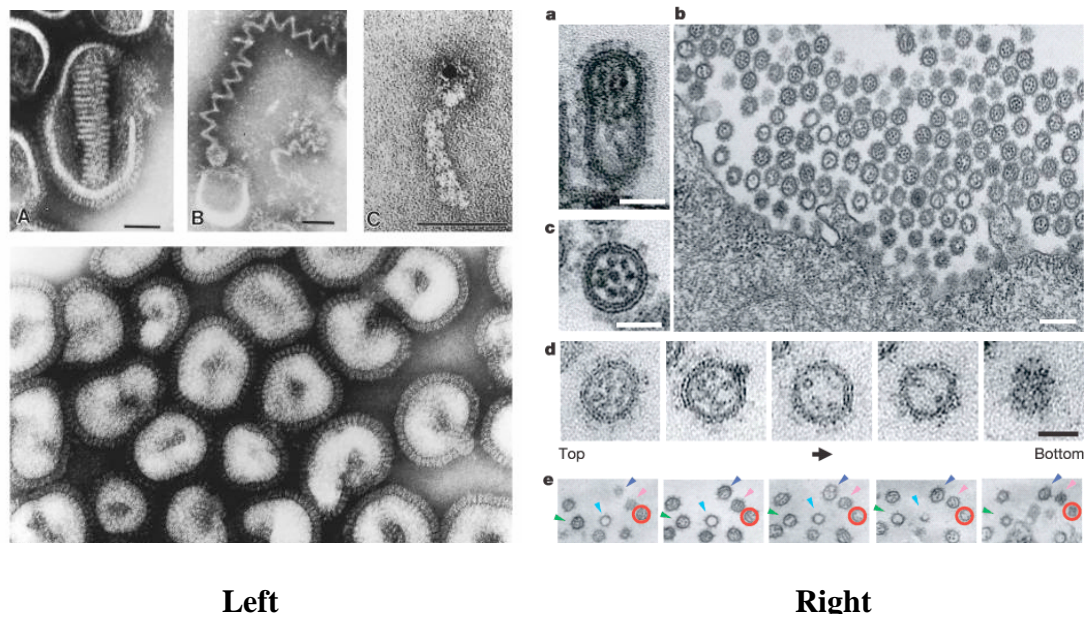
Figure 1.2 C Left: Electron micrographs of influenza virus. Spherical Influenza A virus particle has a diameter of approximately 100 nm. A–C: The structure of the internal components; D: the external view. A substantial fraction (up to 50%) of influenza virions contain large helical internal components (A, B), which may contain individual ribonucleoprotein (RNP) segments (C) linked together. The individual RNPs each contain a binding site for the viral polymerase, as seen by the immunogold labelling of the end of the RNP segment (C). The external view of the virions (D) illustrates the pleomorphic appearance and the surface spikes. Bar in all figures equals 50 nm.

Right: Budding virions show a specific arrangement of eight rod-like structures of different lengths. (a) Rod-like structures, 12 nm in width, are associated with the viral envelope at the distal end of the budding virion. (b, c) Electron-dense dots, representing transversely sectioned rods, were observed in each virion in a characteristic configuration. (d) Serial section of a virion cut from the distal end. As the distance from the end increased the number of dots decreased, suggesting that the eight rod-like structures differed in length. (e) Lower magnification views of the serial ultra-thin section in (d), demonstrating that the serial section shown in (d) represents the same virion (circled in red). Other virions are indicated by arrowheads. Scale bars, 50 nm (a, c, d); 200 nm (b, e).

1.2.2 Genome replication and propagation

The initial phase of virus infection involves the delivery of transcriptionally active virus RNPs to the nucleus of infected cells to initiate the expression and replication of the virus genome. A single replication cycle of the influenza A virus takes approximately 8-10 hours. It starts when the hemagglutinin- spike of influenza virions binds to the cellular surface receptor containing $\alpha2,3$- or $\alpha2,6$-galactose sialic acid linkages (adsorption) (52-55) and afterwards the particle is engulfed into the cell by endocytosis. Influenza viruses can be internalized into the cells via multiple endocytic pathways,

with both clathrin-mediated as well as clathrin- and caveolin-independent pathway(s) (56). The release of the viral genome (uncoating) takes place in acidic environment of the endocytotic vesicle. At about pH 5.0, a major conformational change in the HA spike is induced (57). This conformational change results in movement of the fusion peptide sequences of HA_2 allowing their insertion into the target membrane (58) . The altered HA draws the membranes of the virus and endosome together and they merge (penetration), creating a hole through which the viral contents are poured into the cytoplasm. The pH-activated ion channels made up of M2 protein are also important for uncoating (Figure 1.3.B). Once a virion particle has been endocytosed, the low pH-activated ion-channel activity of the virion-associated M2 protein (59-61) permits the flow of ions from the endosome to the virion interior, which leads to the reduction of the pH in the virions resulting in disruption the protein-protein interactions and dissociation of RNPs from M1 protein (uncoating) (62-63). Besides its function to release the viral genome into the cell cytoplasm, M2 is also responsible for the correct maturation of the HA. All four proteins of the RNP complex (NP, PB1, PB2 and PA) contain nuclear localization signals (NLS). It has been reported that specific nuclear targeting sequences in the NP result in translocation of the RNPs into the nucleus via nuclear pore complex (NPC) (64-66), where replication and transcription of viral RNA (vRNA) takes place (Figure 1.3.A). Late in the virus replication cycle newly formed RNPs are transported from the cell nucleus to the cytoplasm through the NPC (67) to be prepared for packaging and budding. M1 and NEP/NS2 are known to play an important role in exporting the RNPs and preventing them to be reimported into the nucleus (68-70). After translocation of the RNPs into the nucleus of infected cell, the viral genome is transcribed and replicated by the RDRP (71). The NP encapsidates cRNAs and vRNAs, which is necessary for them to be recognized as templates for the viral polymerase. In the nucleus the (-) sense vRNAs are directly transcribed into viral (+) sense mRNAs and replicated through a complementary (+) sense cRNA intermediate to produce more vRNA molecules (72). Synthesis of these three RNA species requires different modes of initiation and termination (73-75). Synthesis of mRNAs is primed by short-capped RNA fragments (m7GpppNm) that are generated from cellular pre-mRNAs by endonucleolytic cleavage. Consequently, viral mRNA molecules contain a 9 to 17 nucleotide (nt) capped host-derived RNA sequence at their 5' ends. On the other hand, the synthesis of cRNA and vRNA molecules is initiated in a primer-independent manner, resulting in triphosphorylated 5' ends (76). Synthesis of

mRNAs is prematurely terminated 16 to 17 nt from 5' end of the vRNA template at a sequence of 5 to 7 uridines that acts as a polyadenylation signal (77-79). The poly (A) tail is synthesized by the viral RNA polymerase by repeated copying of the U sequence (80). During the synthesis of cRNA molecules, the polyadenylation signal is ignored, resulting in full-length copies of vRNA (81) (Figure 1.3.C). The PB1 subunit forms the core of the complex and is responsible for polymerase activity. The PB2 subunit is involved in generation of capped RNA primers for the initiation of transcription by binding the cap structures of host pre-mRNA (Cap snatching) prior to their endonucleolytic cleavage by PB1. The PA subunit is essential for both transcription and replication, but its exact role in the virus replication cycle still remains unclear (82). Most of the proteins made (e.g. HA, NA) remain in the cytoplasm or become associated with the cell membrane. The PB1, PB2, PA and NP proteins migrate back into the nucleus, where they associate with newly-synthesized vRNA to form new RNP complexes. The level of free NP is thought to control whether mRNA or cRNA is produced, i.e. later in infection when there is a lot of NP, mRNA synthesis stops but cRNA synthesis continues. NP is thus a crucial switch in the replication cycle between expression and assembly (83).

NS1 is a multifunctional protein, which plays a key role in the pathogenesis of influenza A virus (84-86). It inhibits splicing and polyadenlation of the cellular pre-mRNAs and nucleocytoplasmic export of cellular mRNAs and thereby keeps the cellular mRNAs in the nucleus (87-94). The synthesis of cellular proteins is practically switched off approximately three hours after viral infection (host cell shut off mechanism). At the same time viral proteins are efficiently translated. Although the details of this mechanism are not completely understood, it is sure that the NS1 plays a crucial role. The influenza A NS1, which has RNA-binding activity (95-96) binds to double stranded RNA (dsRNA), a viral replication by-product that activates protein kinase R (PKR) (97-99) and thereby represses interferon β (IFN β) synthesis [37], one of the most potent antiviral cytokines (100). Thereby it can prevent activation of the transcription factors NF-kB, IRF-3 and the JNK effectors c-Jun and ATF-2 that are essential for (IFN β)-expression (101-105). The dsRNA-dependent PKR (106-108) is part of interferon-induced host defence against viral infection (109). Normally activated PKR phosphorylates the translation-elongation factor 2 (elF-2α) and thereby stops the translation procedure. Other data show that the IFN antagonistic NS1 protein down-regulates apoptosis, indicating anti-apoptotic potential (110). NA, M2 and the HA

precursor (HA$_0$) are transported via the ERK and the Golgi/trans Golgi-Network to the cell membrane in a signal recognition particle (SRP)-dependent manner (111). In epithelial cells, HA is transported to the apical domain of the plasma membrane (112). In the course of transport to the cell surface (maturation), HA undergoes a series of post-translational processing reactions that include polypeptide folding (113), trimerization (114), N-glycosylation (115), acylation (116) and proteolytic cleavage (117). Infectivity requires proteolytic cleavage of the HA precursor into the disulfide-linked cleavage products HA$_1$ and HA$_2$ (118-124). Due to the newly generated N-terminus of HA$_2$, the fusion peptide is available to trigger the fusion of viral membrane and cellular membrane (125). Normally different proteases are able to cleave the HA$_0$, but it depends on the amino acid (aa) sequence at the HA$_0$ cleavage site. It was proposed that the cleavage site would correlate with the virulence of the virus and that virulent strains (e.g. H5 and H7) would contain a furan intracellular protease recognition motif, whereas the avirulent strains would contain only a single arginine residue used by extracellular proteases (126-127). The mature glycoprotein HA and NA and the nonglycosylated M2 are transferred to the cellular membrane as a trimer (HA) and/or as tetramers (NA, M2). In the nucleus M1 and NEP bind to the RNPs and are exported to the cytoplasm through the NPC. For the further maturation (assembly) the M1 retains the RNPs in the cytoplasm (128). Nuclear export of RNPs has been shown to depend on viral M1 and NEP, and on cellular factors CRM1, Ran-GTP and other potential factors (129-132). Afterwards RNP together with other viral protein in the membrane will be enwrapped with the cellular membrane and released through budding. The enzymatic activity of the NA is required for the virus release from the infected cells by cleaving the sialic acid from virus and cell surface glycoconjugates (receptors) (133-136). The packaging mechanism responsible for sorting eight distinct genome segments into each particle is not a purely random process (137-138). It has been reported that the packaging signal of vRNA molecules already occurs in the nucleus or during nuclear export through the process of vRNP/cRNP discrimination involving the binding interactions of viral polymerase with either of the two differently bulged vRNA and cRNA promoter structures (139. Additionally, an RNA hybridization study showed that only vRNA molecules and no cRNA molecules are found outside the nucleus in the cytoplasm (140). Furthermore it has recently been found that the RNPs of influenza A virus are organized in a distinct pattern, in which the individual RNPs are suspended from the interior of the viral envelope at the distal end of the budding

virion and are oriented perpendicular to the budding tip (141) supporting the data for a model of a selective incorporation of viral RNA segments.
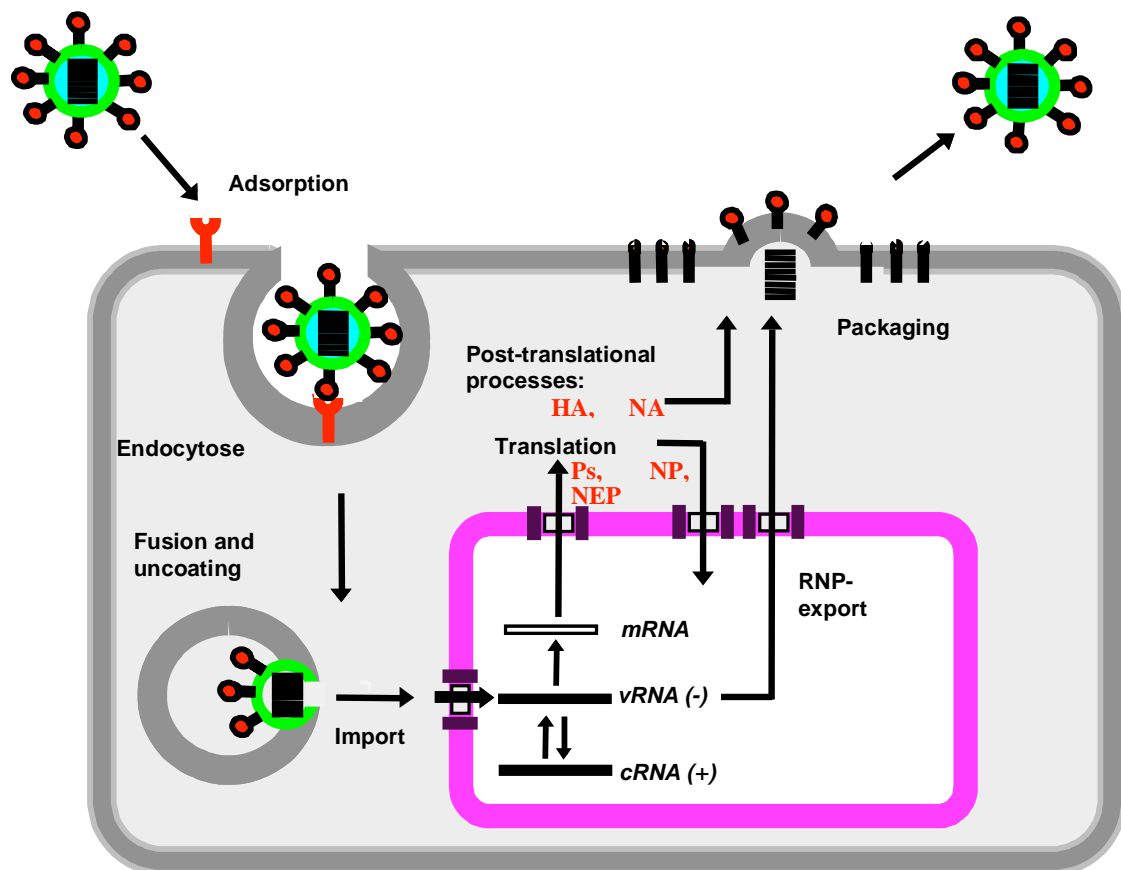
Figure 1.3 A: Replication cycle of influenza virus. It starts when the HA-spike binds to its cellular receptor determinant on cell surface. The virion is then taken into the cell through endocytosis. After the fusion between the viral and the endosomal membrane, the RNPs are released into the cell cytoplasm and afterwards transported into the nucleus, where transcription and replication of the viral RNA takes place. The viral mRNA is subsequently exported to the cytoplasm and translated into viral proteins. The explicative proteins like NP and polymerase proteins are transported back to the nucleus and continue the genome replication. The viral surface-glycoproteins (e.g. HA and NA) are transported to the cell surface and modified by various post-translational processing. Late during virus replication cycle RNPs are exported out of the nucleus and packaged into new enveloped virions that are released from the membrane of infected cell through budding. (Adapted from S. Pleschka, Germany)
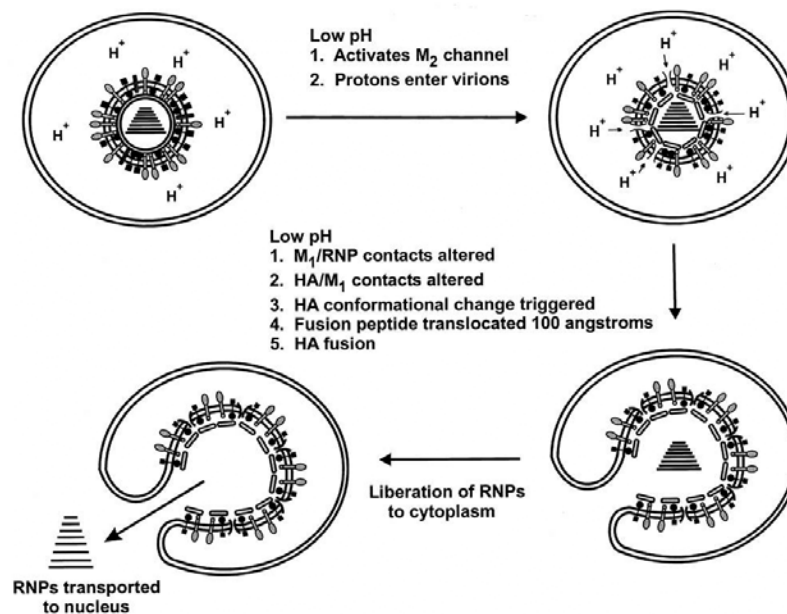
Figure 1.3 B: Schematic diagram of the proposed role of the M2 ion channel activity in virus entry. The M2 ion channel activity is thought to facilitate the flow of protons from the lumen of the endosome into the virion interior, bringing about dissociation of protein-protein interactions between the HA cytoplasmic tail and M1, M1, and lipid or RNPs and M1 from the RNPs.
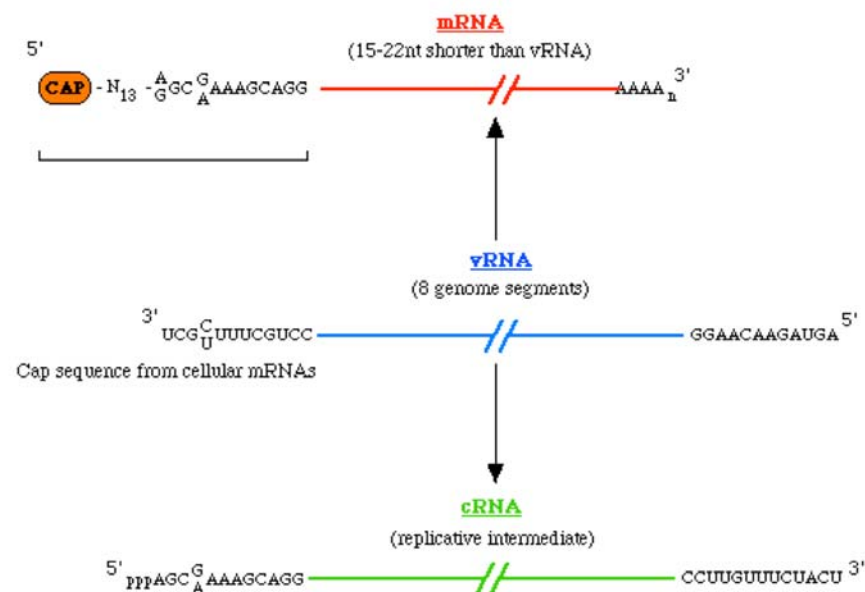
Figure 1.3 C: Replication and translation process of vRNA. PB2 attaches to the m7G cap of host mRNAs. This structure is then cleaved from the cellular mRNA by PB1, remaining attached to PB2. The cap serves as a primer for RNA synthesis. The (-) sense vRNAs are directly transcribed into (+) sense mRNAs and replicated through producing (+) sense cRNAs to synthesize more vRNA molecules.

1.2.3 Antigenic variation of influenza virus infection (antigenic shift and drift)

The three types of influenza viruses (A, B and C) vary greatly in their epidemiological significance. The epidemiology of influenza A viruses has been well studied in relation to the occurrence of epidemics and pandemic episodes of disease in humans. The continuing occurrence of influenza epidemics is caused by rapid evolution of the viral genome. This can be monitored by antigenic analyses and sequence analysis of genes, particularly the hemagglutinin gene.

Influenza A virus infection has two phenomena; the first being the almost identical annual epidemics which occur in most countries and the second are the extensive pandemics which occur approximately every 10-30 years (NIH, Report on Flu, 2005). It is apparent that pandemics are due to the appearance of new influenza A subtypes against which the population has no immunity. This phenomenon is known as "antigenic shift" (187-188). As immunity to the new subtype by producing virus-neutralizing antibodies against the hemagglutinin (HA) protein builds up, further epidemics are more limited. The HA antigen is always involved in antigenic shift as it is responsible for eliciting virus-neutralizing antibodies (189-191). There are three possibilities as to how antigenic shift can occur (CDC, Focus on Bird Flu, 2005).

(I) Reassortment: The most widely held view is that the new virus subtypes are reassortant viruses resulting from double infection, where eight RNA segments of each swap with each other, producing a new virus. Pigs are thought to provide the "mixing vessel" for AIV and human influenza virus where genetic reassortment may occur (192-195).

(II) Recirculation of existing subtypes: In 1977 H1N1 reappeared which was very similar to the strain which circulated before 1957. It was widely believed that it may have escaped from a laboratory (196). As a sizable proportion of the population had already been exposed to the H1N1 virus before 1957, it did not cause a pandemic.

(III) Gradual adaptation of animal viruses to human transmission: These are small changes in the virus that happen continually over time caused by missense mutation. One reason for this is the lack of proofreading mechanism in the RNA replication, resulting in error rate of one in 10,000 nucleotides (197). Spontaneous mutations in the HA (or NA) gene give their owners a selective advantage as the host population becomes increasingly immune to the earlier strains. These antigenic changes are known as "antigenic drift" (Figure 1.5.). Epidemics due to new virus strains arising from antigenic drift are not as severe as for those showing antigenic shift, since partial

immunity is present in persons with cross-reacting antibody induced by previous infection.

1.3 Avian influenza viruses

The first avian influenza (AI) virus was isolated in 1902 (142), although it was not identified as a member of influenza A virus family until 1955. Influenza viruses have been isolated from domestic poultry suffering from highly pathogenic avian influenza (fowl plague) and from apparently healthy wild birds, including waterfowl.

Fowl plague viruses (FPV) were used for this work representing a model of type A influenza viruses. AI is a highly contagious viral infection, which can affect all species of birds and can manifest itself in different ways depending mainly on the pathogenicity of the virus involved and on the species affected. IV that infects birds are called "avian influenza viruses" (AIV) and only IAV can infect birds. In waterfowl, influenza is always asymptomatic and birds regularly shed large amounts of the virus. They seldom get sick, while other small mammals that contact the influenza virus are infected and may die. It appears that birds have established a coexistence with the influenza virus. Through co-evolution, birds and the influenza virus are optimally adapted to one another.

1.3.1   History of avian influenza

The AI has captured considerable international attention over the past years with serious epidemics of this disease affecting almost all areas in Southeast Asia since 1997. Now considered an avian pandemic, serious outbreaks of AI had also affected the Netherlands, Belgium, and Germany in 2003 (149-150). The AI was first recorded in Italy more than 100 years ago in 1878 as described by Perroncito et al. This highly pathogenic avian influenza is characterized by sudden onset of severe disease, rapid contagion, and a mortality rate that can approach 100% within 48 hours (151-153). As the cause of massive poultry epidemics, this disease was then known as "Fowl Plague". The causative agent of this disease was isolated from a chicken in 1902, marking the first documented isolation of influenza virus (154). In 1955, it was determined that the virus causing Fowl Plague is influenza A virus (157). The AIV particle is characterized by the surface glycoproteins hemagglutinin (HA) of the H7 or H5 type.

1.3.2 Direct transmission

Direct transmission from birds to human is also possible. In 1997 HPAI H5N1 was isolated for the first time from a human patient in Hong Kong (176-178). The virus infected 18 patients after close contact with poultry, resulting in six deaths (176, 178,179). Within three days, Hong Kong's entire chicken population was slaughtered to prevent further outbreak (180). Recently HPAI H7N7 broke out in Netherlands in 2003 (176, 181) and spread to Belgium and Germany and resulted in the culling of 30 million chickens. The virus infected 83 people causing conjunctivitis and flu-like symptoms, and caused the death of one veterinarian (182-183). After 1997 and 2003, a major outbreak of HPAI subtype H5N1 surfaced again in Vietnam's and Thailand's poultry industry in the early 2004 accompanied with human cases (183). Within a few weeks, the disease had spread to other ten countries and regions in Asia. From the 173 cases of avian flu in humans, 93 were fatal (WHO, 2007). Vietnam has seen the most cases thus far 93 in total with 42 fatal infections. Cumulative no. of H5N1 cases are 50 as a total and 33 death reported by WHO (2007)

Though scientists determined that the spread of the AIV from birds to humans are rare occurrences, they were also quick to express grave caution that this problem could become significantly worse if the virus mutated into a more lethal form, or a form that could pass easily from humans to humans. The WHO is particularly concerned about the avian virus' potential to swap genes with a common influenza virus, creating a lethal form of the virus that could spread around the globe within months. From 1997 to 2007 human cases have been reported in more than 15 countries, most of which are in Asia (WHO, April 2007). Taken together, the death toll associated with the 1918 influenza virus correlated to the current population could be between 180 million and 360 million human deaths globally (184-185).
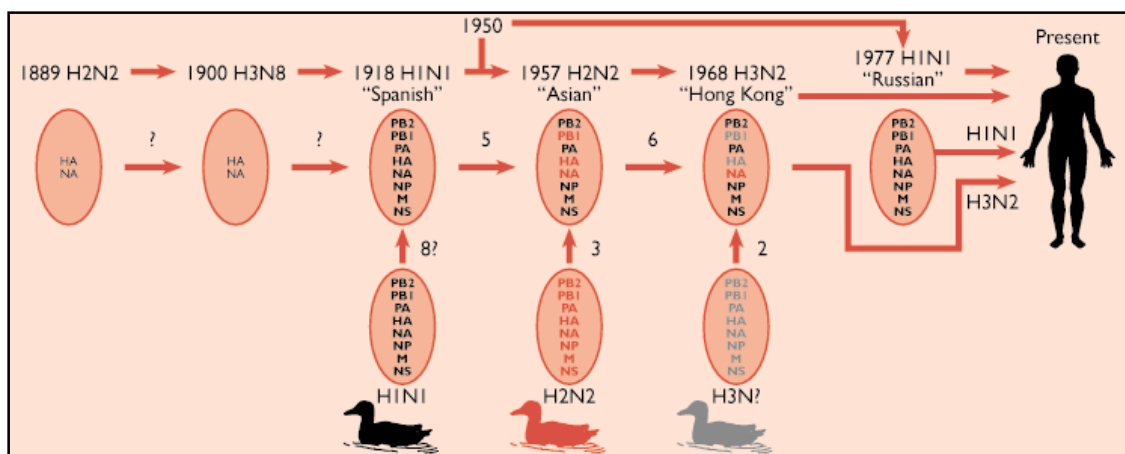
Figure 1.4.: Postulated evolution of the influenza A viruses currently circulating in humans. Serological archaeology assumes that H2N2 and H3N8 influenza viruses circulated in humans in 1889 and 1900, respectively. Phylogenetic evidence suggests that an influenza virus possessing eight gene segments from avian influenza reservoirs was transmitted to humans and pigs before 1918 and replaced the 1900 strain. This virus was probably carried from North America to Europe by American troops and caused the catastrophic influenza pandemic of 1918. In 1957 the Asian pandemic virus acquired three genes (PB1, HA, and NA) from the avian influenza gene pool in wild ducks by genetic reassortment and kept five other genes from the circulating human strain. After the Asian strain appeared, the H1N1 strains disappeared from humans. In 1968 the Hong Kong pandemic virus acquired two genes (PB1 and HA) from the duck reservoir by reassortment and kept six genes from the virus circulating in humans. After the appearance of the Hong Kong strain, the H2N2 Asian strains were no longer detectable in humans. In 1977 the Russian H1N1 influenza virus that had circulated in humans in 1950 reappeared and spread in children and young adults. This virus probably escaped from a laboratory and has continued to co-circulate with the H3N2 influenza viruses in human population.

1.4 The progress of reverse genetic systems for influenza viruses

The reverse genetics techniques for influenza viruses have enabled the construction of IV that contains altered genomes. The ability to introduce attenuation characteristics into the viral genome holds the promise for developing a live attenuated influenza virus for vaccination

Reverse genetics techniques that permit the site-specific genetic manipulation of negative-strand viruses were first established the group of P. Pales in 1989 (143). In the initial experiment, *in vitro* reconstituted NA-specific RNPs were transfected into cells which were then infected with helper influenza A virus (144). Specifically, the RNPs were generated in vitro by mixing synthetic RNA transcribed from cDNA and purified NP protein and viral polymerase proteins.

An alternative method developed by S. Pleschka of introducing site-specific mutations into the influenza virus genome is based on the intracellular reconstitution of RNP complexes derived from plasmid-based expression vectors. In this system, influenza virus RNA transcripts are derived from plasmids, which are transfected into cells. Genomic or antigenomic viral transcripts are generated via cellular polymerase promoter at the 5$^{'}$ end of the viral gene (145), and a ribozyme sequence that generates the desired 3$^{'}$ end by autocatalytic cleavage. Co-transfection with plasmids expressing the viral polymerase proteins PB1, PB2, PA and NP protein results in the encapsidation, transcription and replication of the expressed viral RNAs. The co-transfected cells are then infected with helper influenza virus, which takes up the

intracellularly reconstituted RNPs. In contrast to the RNP transfection system this plasmid-based transfection system eliminates the need for purification of the viral NP and polymerase proteins which is required for in vitro reconstitution of RNP complexes (146).

It has been shown that co expression in mammalian cells of all eight influenza virus genes and viral NP and polymerase proteins from plasmids results in the generation of recombinant viruses. Due to the segmented nature of the genome of influenza viruses, more than one plasmid is required to express the eight viral RNA segments.

1.5 Influenza vaccines and antivirals

Yearly vaccination with the flu vaccine is the primary strategy for preventing complications of influenza virus infections. It is remarkable that the first attempts to inject an influenza virus into humans were made only 2 years after the first isolation of human influenza virus from an infected ferret in 1933 (3). These studies were not originally designed to develop a vaccine, but were to investigate whether live influenza virus grown in chick embryos would infect humans and induce antibody after subcutaneous injection (4). It was observed that neutralizing antibodies developed in serum, peaked after 2 weeks and persisted for unto 6 months.

Antiviral medications with activity against influenza viruses can be effective for the prevention and treatment of influenza. They are relatively harmless to the host, and therefore can be used to treat infections. There are adamantanes (amantadine and rimantadine) that are licensed in the U.S for the treatment and prevention of influenza.

The mechanism of action of these two drugs is inhibition of acid activation of the M2 ion channel that normally results in acidification of the interior of the virion with dissociation of the M1 matrix protein from the ribonucleoprotein complex (uncoating) so that transport of the ribonucleoprotein complex to the nucleus that is a requisite step for genome transcription, translation and assembly, can not occur. Resistance of influenza A viruses to admantanes in vitro was described in the initial reports of the antiviral effects of these drugs (267,268). In 1981, two strains of H3N2 virus were relatively resistant to amantadine and rimantadine.

However, because a high proportion of circulating influenza viruses in the U.S. in recent years have been resistant to the adamantanes, CDC recommends that neither amantadine nor rimantadine be used for the treatment or chemoprophylaxis of influenza

in the United States during the 2007-08 influenza season. Two licensed influenza antiviral agents are recommended for use in the United States during the 2007-08 influenza seasons (1) Oseltamivir and (2) Zanamivir. These are chemically related antiviral medications known as neuraminidase inhibitors that have activity against both influenza A and B viruses.

These drugs have mechanism of action towards the inhibition of virus release by destroying neuraminidase, which is required for the release of the new progeny infectious virus. Oseltamivir is approved for treatment of persons aged 1 year and older. Zanamivir is approved for treatment of persons aged 7 years and older. The two medications differ in pharmacokinetics, adverse events, and routes of administration, dosages, and costs.

1.6 Signal transduction and influenza viruses

1.6.1 Mechanisms of intracellular signal transduction

There are approximately 10^{14} cells in human body. In order to keep each organ functional, every single cell must be able to react both to the external stimulus including physical and mechanical signals and to the signals from other cells. This mechanism requires the recognition, processing and passing on signals via intracellular signalling pathways that regulate the cell metabolism, differentiation and proliferation (201-203).

The cellular response to growth factors, inflammatory cytokines, and other mitogens is often mediated by receptors that either are G-protein-coupled receptor (GPCR) or intrinsic protein receptor tyrosine kinases (RTK) (204). The binding of the ligand to receptor tyrosine kinases induces dimerization and autophosphorylation (activation) of the kinases. The activated tyrosine kinase binds to and phosphorylates an adaptor protein, such as Grb2, that, in turn, activates a guanine-nucleotide exchange factor, such as mammalian son-of-sevenless (mSOS), that, in turn, activates a small GTP-binding protein, such as Ras or Rac (202). The GTP-binding proteins then transmit the signal to one of several cascades of protein serine/threonine kinases that utilize the sequential phosphorylation of kinases to transmit and amplify the signal (205). These kinase cascades are collectively known as mitogen-activated protein kinase (MAPK) signaling cascades (206-108). ERK has been the best characterized MAPK and the

Raf/MEK/ERK pathway represents one of the best characterized MAPK signaling pathways (208, 209) over a dozen MAPKs organized in at least four different families have been identified in mammals (208). They are activated via phosphorylation catalysed by distinct upstream kinases on both tyrosine and threonine residues within their catalytic domains. These processes are vital to the cells; therefore a high degree of homology exists between divergent species. In mammalian cells, three distinguishable MAPK modules have been well described now known as the ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) and the p38/MAPK pathways (208). The MAP kinase lies within protein kinase cascades. Each cascade consists of no fewer than three enzymes that are activated in series: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAP kinase (MAPK) (Figure 1.6.). Currently, at least 14 MAPKKKs, 7 MAPKKs and 12 MAPKs have been identified in mammalian cells (208) (Table 1.2.). These pathways regulate a variety of responses including cell growth, differentiation, adaptation to the environment, as well as apoptosis (210-212). The MAPKs also control numerous regulatory processes during development and homeostasis (213-215). The mammalian ERK module, also known as the classical mitogen cascade, consists of serine/threonine kinase Raf, the dual-specificity kinase MEK and the classical MAPK ERK. For both MEK and ERK, there exist two isoforms (MEK1/2 and ERK1/2) while three isoforms, A-, B- and C-Raf are known for the Raf kinase.

Table 1.2: Components of MAPK pathways in mammalian cells

MAPKKK	MAPKK	MAPK
Raf-1, A-Raf, B-Raf, Mos, TAK1, MUK, SPRK, MST, MEKK1, MEKK2, MEKK3, MEKK4, Tpl-2, ASK	MEK1 (MKK1), MEK2 (MKK2), MEK5, MEK3, MEK4, MEK6, MKK7	ERK1, ERK2, p38α, p38β, p38γ, p38δ, JNK1, JNK2, JNK3, ERK3, ERK3, ERK4, ERK5
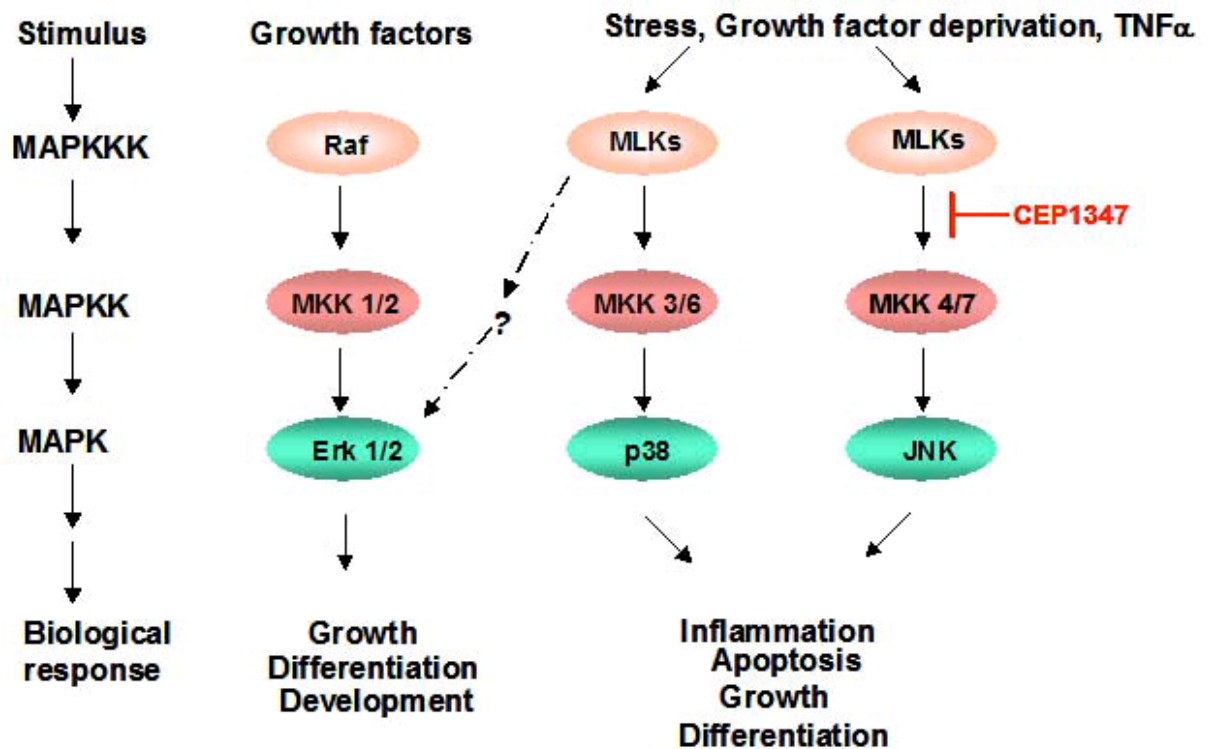
Figure 1.5.: MAP kinase cascade in mammalian cells. Mitogen-activated protein kinases (MAPK) are a family of Ser/Thr protein kinases widely conserved among eukaryotes and are involved in many cellular programs such as cell proliferation, cell differentiation, cell movement and cell death. MAPK signaling cascades are organized hierarchically into three-tiered modules. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs). The MAPKKKs are in turn activated by interaction with the family of small GTPases and/or other protein kinases, connecting the MAPK module to cell surface receptors or external stimuli.

1.6.2 The Raf/MEK/ERK pathway (MAPK signalling cascade)

The Raf/MEK/ERK signalling cascade becomes strongly activated by mitogenic stimuli such as growth factors (210-212). This signalling pathway transduces signals leading to growth or differentiation in cells. Binding of a growth factors e.g. epidermal growth factor EGF) to the corresponding receptor tyrosine kinase RTK) leads to its oligomerisation and subsequent autophosphorylation (202, 216). Phosphorylated tyrosines on the intracellular part of receptor, serve as docking sites for the SH2-domain (Src homology) of adapter protein the GrB2, which itself binds via its SH3-domain to the proline rich motif of the GDP-GTP exchange factor Sops (son of sevenless). SOS interacts with Ras and activates the exchange of GDP to GTP (217-

220). GTP loaded Ras leads to the recruitment to the cell membrane of the cytosolic serine/threonine kinase Raf, a membrane shuttle kinase identified as an oncogenic product of the murine sarcomavirus (221-225). This translocation results in the activation of the membrane associated Raf. Ras-Raf interaction alone, however, is not sufficient to activate Raf completely (226-228). Activation mediated by multiple kinases like Src, PKC (protein kinase C) and PAK (p21 (Rac/Cdc42)-activated protein kinase) leads to phosphorylation and positive regulation of Raf (230-234), whereas other kinases like PKA (protein kinase A) down regulate Raf activity (235). The first characterized substrate of Raf isoenzymes was the dual specificity kinase MEK (236). Once activated, MEK transduces the signals through phosphorylation on T-E-Y-motifs in the MAP-kinases ERK1 (p44) and ERK2 (p42) (237, 238) (Figure 1.7.). ERK phosphorylation increases its catalytic activity, mediates oligomerisation and alleviates the shuttling of the kinase to the nucleus. ERKs are nuclear shuttle kinases and have several described substrates in contrast to its upstream activators Raf and MEK. ERK target proteins are e.g. serine/threonine kinases like 3pK, ribosomal S6 kinase (RSK1p90) also known as MAPKAP-kinase 1, RNA-polymerase II, phospholipase A2 and several transcription factors like, Elk-1 and c-Jun (226, 239-242). ERKs are essential elements of mitogenic signalling. Prolonged activation and nuclear retention of ERKs is required for transcription of the cyclin D1 gene (243), suggesting a mechanism of ERK-mediated enhancement of cell cycle entry. ERKs are also associated with cytoskeleton (244), playing a putative role in cytoskeleton reorganisation. Expression of constitutively active components of the pathway causes cell transformation (245).
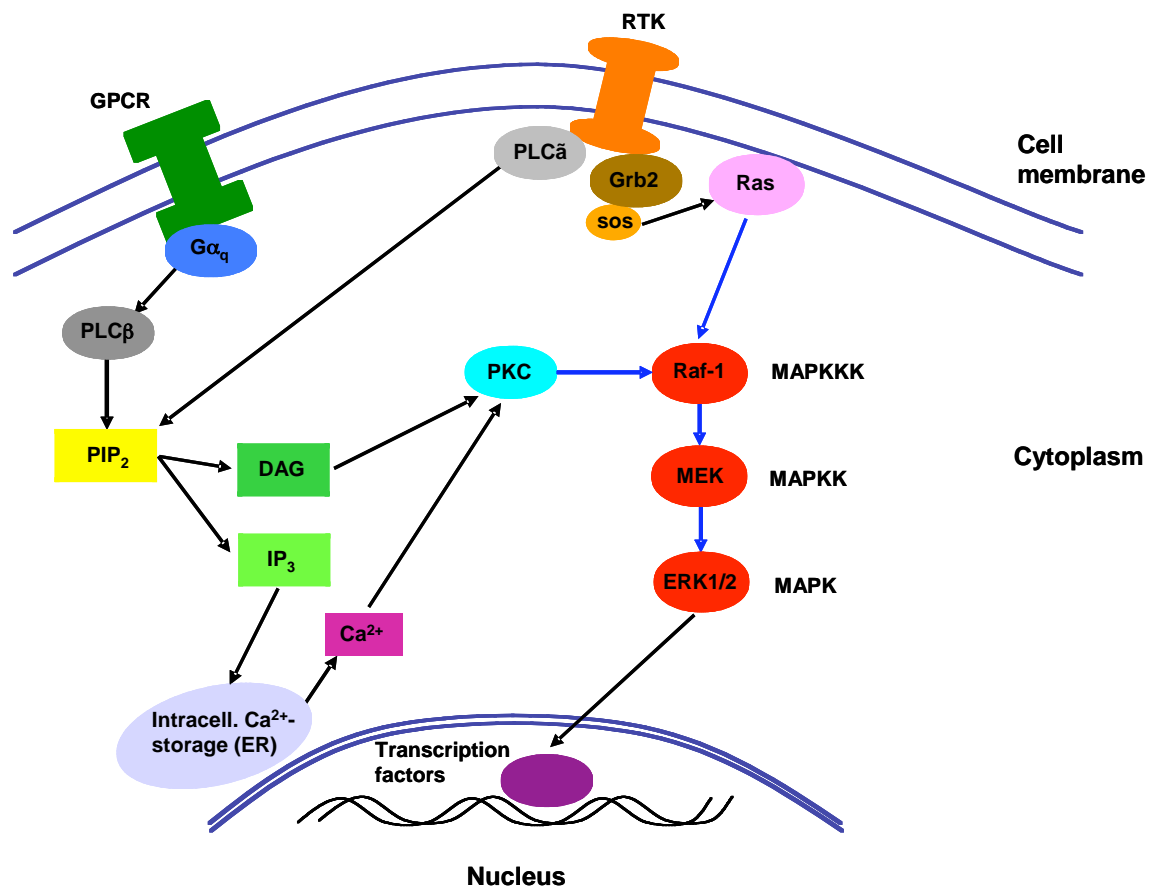
Figure 1.6.: Activation of Raf/MEK/ERK (MAPK) signaling cascade (simplified).
The G-protein coupled receptors (GPCR) and receptor tyrosine kinase (RTK) are the
acceptor of extra-cellular stimuli localized in the plasma membrane. After cell receptor
stimulation the signal from GPGR is transmitted via G-Protein (Gαq) to phospholipase
C_β (PLC$_\beta$), which afterwards cleaves phosphatidylinositol-4, 5-Bisphosphat (PIP$_2$) in
inositol-1, 4, 5-trisphosphat (IP$_3$) and Diacylglycerol (DAG). IP$_3$ diffuses into cytosol
and binds on its receptors that are localized on endoplasmic reticulum. This leads to
release of calcium-ion (Ca^{2+}) from intra-cellular storage. The Ca^{2+}-channel (CRAC) in
the plasma membrane is consequently activated and contributes to the increase of
cytosolic Ca^{2+}-ion. The Protein-Kinase C (PKC) is subsequently activated by DAG and
increased Ca^{2+}-concentration. Moreover the extra-cellular stimuli are conveyed via
Grb2/sos complex to Ras. Both, activated Ras as well as PKC can lead to an activation
of Raf/MEK/ERK (MAPK) signalling cascade (blue lines). Afterwards ERK1/2 is
translocated to the nucleus and interacts with transcription factors to change the gene
expression (249-251). (Figure adapted from S. Kötter, Germany).

1.6.3 Virus-induced Raf/MEK/ERK (MAPK) signalling cascade is essential host function for influenza A virus propagation

Replication and transcription of the influenza virus genome takes place exclusively within the nucleus of the infected cells (252). Late in the infectious cycle the RNPs are exported from the nucleus to the cell membrane to be enwrapped into budding progeny virions (252-254). During their replication influenza viruses interacts with many different cellular functions to promote their propagation (255, 256). Among the different signalling processes activated by influenza virus infection, the Raf/MEK/ERK (MAPK) signalling cascade plays a crucial role for efficient virus propagation. This pathway is activated late during influenza virus replication cycle and is required for an efficient nuclear RNP export (Figure 1.7) (252-254). Analysis of this MAPK signalling cascade has been facilitated by the use of highly specific MEK-inhibitors (257, 258). It was shown that a block of this signalling cascade impairs nuclear RNP export and virus replication without the emergence of resistant variants (254, 255). In addition, influenza virus replicate more efficiently upon selective induction of Raf/MEK/ERK signalling cascade in vivo by expression of a constitutively active version of Raf (259).
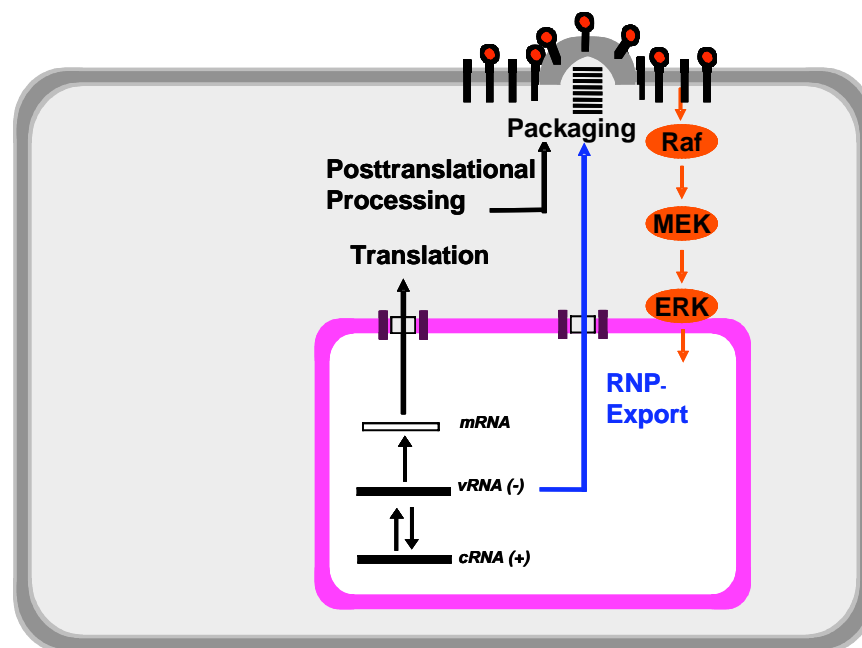
Figure1.7: Raf/MEK/ERK (MAPK) cascade is activated by influenza virus. Virus-induced MAPK signalling pathway is induced late during viral replication cycle. Nuclear RNP export is dependent on this cellular mechanism [196-198]. (Adapted from S. Pleschka, Germany).

1.6.4 Role of virus induced calcium dependent PKCα signal transmission

Data in the literature suggests that after IAV infection influx of Ca^{2+} is accelerated and Ca^{2+} accumulates in the infected cells (260). The virus induces a respiratory burst in which H2O2 (but not O2-) are formed. Preceding the respiratory burst, a rise in intracellular calcium (Ca^{2+}) is noted, but both responses are nearly independent of extra cellular Ca^{2+}. The Ca^{2+} increase is paralleled by inositol-1, 4, 5-triphosphate (IP_3) generation, implying that it is the result of phospholipase C (PLC) activation. The virus also elicits membrane depolarization, which is independently mediated from the Ca^{2+} increase and respiratory burst and may reflect protein kinase C (PKC) activation (261). There are 12 isoforms of PKCs and these are classified in three categories (1) Classical or Conventional (α, β, γ) (2) Typical and (3) Atypical. Classical PKCs are calcium dependent kinases and PKCα is one of them. Ca^{2+} and DAG activate classical PKCs. PKCα and or β are activated when human polymorphonuclear leukocytes (PMNL) are incubated with influenza virus (262).

PKCβII isoform is activated in early Influenza viral replication but not PKCα (263). PKC-inhibitors reduce virus entry if added early in infection (Root et al., 2000). Calcium increase is blocked by L-type calcium channel blockers (CCB) Verapamil which inhibits influenza virus replication if added late in replication cycle (264). CCB blocks calcium channels in the endoplasmic reticulum therefore calcium influx from internal calcium store to cytoplasm is inhibited and therefore PKCα mediated signaling might be negatively effected. We have recently shown that viral hemagglutinin membrane accumulation and its tight association with lipid-raft domains triggers activation of the MAPK cascade, which seems to involve protein kinase Cα activation (265).

1.6.5 Protein kinase C as a therapeutic target

PKC is a viable target for development of novel therapeutics against cardiac diseases, cancer, viral infections or other diseases as it is intricately involved in the processes that mediate disease pathogenesis. PKC is a family of serine/threonine kinases that regulate a variety of cell functions including proliferation, gene expression, cell cycle,

differentiation, cytoskeletal organization cell migration, cell behaviors and apoptosis. The PKC family was the first recognized receptor of diacylglycerol. Aberrant PKC activation can lead to diseases of cellular dysregulation such as cancer and diabetes. Activation of PKC by phorbol esters promotes tumor formation, and from that it is concluded that inhibitors of classical PKC might prevent carcinogenesis or inhibit tumor formation (198). Strategies that serve to alter classical PKC function may prove to be useful in the treatment of numerous disease states. Further examination of downstream effectors of PKC showed that extra cellular signal-regulated kinase (ERK) is critical for angiogenesis, and is accordingly phosphorylated in response to PMA. Surprisingly however, phosphorylation of ERK is dependent of PKC activity (200).

1.6.6 Calcium channel blocker (Verapamil)

Verapamil is a calcium channel blocker. Increased concentrations of cytosolic Ca^{2+} causes increased contractions of the myocardium and vascular smooth muscle. The entry of extracellular Ca^{2+} is more important in initiating the contraction of myocardial cells, while the release of Ca^{2+} from intracellular storage sites also participates in vascular smooth muscle, particularly in some vascular beds. In addition, the entry of extracellular Ca^{2+} can trigger the release of additional Ca^{2+} from intracellular stores. Verapamil not only reduces the magnitude of Ca^{2+} current through the slow channel, but also decreases the rate of recovery of the channel. In addition, channel blockade caused by Verapamil is enhanced as the frequency of stimulation increases. Verapamil depresses the rate of the sinus node pacemaker and slow atrio-ventricular conduction; the later effect is the basis for its use in the treatment of supraventricular tachyarrhythmias. Therefore Verapamil is used in cardiovascular diseases (heart attack, high blood pressure and angina pectoris) therapy in humans.

1.7 Aim of the project

The aim of my project was to investigate the role of PKCα for efficient influenza virus replication and its part in activation of influenza virus-induced Raf/MEK/ERK (MAPK) signalling cascade which is essential host function for RNP-export and therefore for the production of infectious progeny virus. This signalling cascade is activated late in the influenza virus replication cycle and nuclear RNP export is

dependent on this cellular function. In influenza virus infected cells calcium concentration (Ca^{2+}) is increased and therefore the calcium dependent cellular factor PKCα could be one of the upstream kinases responsible for the activation of Raf/MEK/ERK signalling. Therefore I wanted to investigate the inhibitory effect of Verapamil on virus-induced calcium-dependent PKCα transmitted Raf/MEK/ERK signalling, RNP-export and virus replication. Additionally I wanted to analyze the effect of Verapamil on viral protein production and viral transcription.

2. Materials and Methods

2.1 Materials

2.1.1   Chemicals and reagents

Acrylamide	Bio-Rad, USA
Acrylamide/bisacrylamide 30, 30%, 37.5:1	Roth, Germany
Acrylamide/bisacrylamide 40, 40%, 19:1	Roth, Germany
Acetic acid	Roth, Germany
Agarose ultra pure	Roth, Germany
Ammonium persulfate (APS)	Serva, Germany
Ampicillin	ICN, USA
Agar high-gel strength	Serva, Germany
Boric acid	Roth, Germany
Bromophenol blue	Merck, Germany
Chloroform	Roth, Germany
Coomassie brilliant blue R 250	Merck, Germany
Dimethylsulfoxid (DMSO)	Sigma, USA
1,4-Diazabicyclo [2,2,2]octane (DABCO)	Merck, Germany
DEAE Dextran (Mw, 500,000)	Pharmacia Biotech
Di-Sodium hydrogen phosphate anhydrous (Na_2HPO_4)	Merck, Germany
Ethanol (absolute)	Roth, Germany
Ethidium bromide	Roche, Germany
Ethylenediamine tetraacetic acid (EDTA)	Fluka, USA
Extract of yeast powder	Merck, Germany
Formamide	Fluka, USA
Glycerol	Sigma, USA
Glycogen	Roche, Germany
Isoamyl alcohol	Roth, Germany
Isopropanol	Roth, Germany
Magnesium chloride ($MgCl_2$)	Merck, Germany
Mowiol 40 - 88	Aldrich, Germany
Methanol	Roth, Germany
N-2-hydroxyethylpiperazine (HEPES)	Sigma, USA
Oxoid Agar	Oxoid, England

Peptone from meat trypsin-digested   Merck, Germany

Potassium chloride (KCl)   Roth, Germany

Potassium dihydrogen phosphate (KH_2PO_4)   Roth, Germany

Roti-phenol   Roth, Germany

Roti-phenol/ chloroform   Roth, Germany

Sodium acetic acid (NaAc)   Roth, Germany

Sodium chloride (NaCl)   Roth, Germany

Sodium dodecyl sulfate (SDS)   Merck, Germany

Sodium hydroxide (NaOH)   Merck, Germany

Sodium borohydride $NaBH_4$)   Roth, Germany

Sodium hydrogen carbonate ($NaHCO_3$)   Fluka, USA

Sodium citric acid   Roth, Germany

Silver nitrate   Merck, Germany

Tris base   Roth, Germany

N, N, N', N'-Tetramethyl-ethylene diamine (TEMED)   Serva, Germany

Urea   BRL, USA

ß-mercaptoethanol   Roth, Germany

Xylene cyanole   Sigma, USA

2.1.2 Instruments

Bio Imaging Analyzer (BAS 2000)   Fuji Film

Bacterial incubator and shaker (CH 4103)   Infors AG

Cell culture microscope   Hund Wetzlar

Cell culture incubator Heraeus;   Nuaire

Confocal laser scanning microscopy (DM IRBE)   Leitz

Culture Hood (HB2448)   Heraeus

Developing machine Optimax,   Protec

DNA-Spectrophotometer GeneQuant II   Pharmacia Biotech

Electrophoresis apparatus system   Institute for Medical
                                                            Virology

Electrophoresis power supply (EPS500/400   Pharmacia

ELITE analyzer   Beckman Coulter

ELISA reader (Type LP 400)   Diagnostic Pasteur

FACS Caliber	Becton Dickinson
Fine scale (Mettler PM460)	Mettler Waagen
Gene Pulser	Biorad
GeneQuant II (RNA/DNA calculator)	Pharmacia Biotech
Heat block	Jumotron
Magnetic stirrer	IKA Labortechnik
Mega centrifuge (J-TB-024D)	Beckman
Megafuge 1.0 R	Heraeus
Microplate Luminometer	Molecular Devices
Microwave oven	Quelle
Mini centrifuge Biofuge 13,	Heraeus
PCR machine (GeneAmp, PCR system 2400)	Perkin Elmer
pH meter (Type 632)	Metrohm
Photo system for gel (P93D)	Mitsubishi
Shaker (Type 301)	MSGV GmbH
Scale (P1200)	Mettler
Scanner Canoscan LiDE30/N1240U	Canon
SDS-PAGE system	Institute for Medical Virology
Sonicator (Type HD70)	Bondelin Sonoplus
Spectrophotometer (DU-70)	Beckman
Speed Vac (SPD111V)	Savant
Thermomixer	Eppendorf
UV light source	Vilber Lourmat
Vortex (Vibrofix VF1)	IKA Labortechnik
Western-Blot chamber	Institute for Medical Virology
Water bath (SW-20C)	Julabo

2.1.3 Enzymes and enzyme inhibitor

Restriction endonucleases	NEB, England
RNase inhibitor (RNasin, 40U/μl)	Promega, USA

2.1.4 Nucleotides and reaction buffer

DNA ladder mix	PEQLAB, Germany

10x restriction enzyme buffer 1, 2, 3 and 4	NEB, England
10x RQ1 DNase buffer	Promega, USA
Tris-HCl buffer for flash cat assay (pH 7.4)	Stratagene, Germany
Bio-Rad protein assay buffer	Bio-Rad, USA
Cell lysis buffer	Promega, USA

2.1.5 Plasmids

Dn-PKCα

Dn-Ras

pPOL1-GFP-RT

pPOL1-CAT-RT

pHMG-PB1

pHMG-PB2

pHMG-PA

pHMG-NP

2.1.6 Kits

QIAGEN plasmid maxi kit	QIAGEN, Germany
QIAGEN plasmid midi kit	QIAGEN, Germany
QIAGEN RNA/DNA mini kit	QIAGEN, Germany
QIAprep spin miniprep kit	QIAGEN, Germany
Flash CAT Non-Radioactive CAT Assay Kit	Stratagene, USA

2.1.7 Solutions for plasmid DNA isolation

Solution I

Glucose	50 mM
Tris.Cl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

Autoclaved for 15 min at 10 lb/sq.

Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)

1% SDS

Solution III

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml

2.1.8 Materials for cell culture

Dulbecco's Modified Eagle's medium (DMEM)	Invitrogen, USA
Fetal calf serum (FCS)	PAN, Germany
Opti-MEM	Invitrogen, USA
100x penicillin-streptomycin solution (P/S)	PAA, Austria
1x trypsin-EDTA (0.5 g/L trypsin, 0.2 g/L EDTA)	PAA, Austria
Tissue culture dish	Greiner, Germany
Tissue culture flask	NUNC, USA
Bovine Albumin (BA)	MP Biomedical

2.1.9 Methylcellulose (MC) media, 100 ml (1.75%)

MEM (10x)	10 ml
P/S (100x)	1 ml
BA (30%)	1 ml
$NaHCO_3$ (7.5%)	4 ml
MC (3%)	58.3 ml
DEAE Dextran (1%)	1 ml
dH_2O	24.7 ml

2.1.10 Preparation of all kind of buffers

2.1.11 Preparation of TLB buffer

Tris HCl, pH 7.4	20 mM
NaCl	137 mM
Glycerol	10% (v/v)
Triton X-100	1% (v/v)
EDTA	2 mM
Sodium-β-glycerophosphate	50 mM
Sodium pyrophosphate	20 mM

(Protease inhibitors are added in lysis buffer during making lysates)

2.1.12 Lysis Buffer

TLB buffer (see TLB buffer)	10 ml
Pefablock	200 mM
Aprotinin	5 mg/ml
Leupeptin	5 mg/ml
Sodium orthovanadate	100 mM
Benzamidine	1 M

2.1.13 5x SDS-PAGE buffer

SDS	5 g
Tris-base	30 g
Glycine	144 g

Dissolved in 5 L dH$_2$O.

2.1.14 Transfer buffer (Semi-dry)

Tris-base	5.8 g
Glycine	2.9 g
SDS	0.17 g
Methanol	200 ml

Dissolved in 1 L dH$_2$O.

2.1.15 10x TBS (Tris Buffer Saline)

24.2 g	Tris
80 g	NaCl

Adjusted pH to 6.7 with HCl filled to 1 L with dH$_2$O. 1x TBS buffer was used for washing membrane and diluting antibody.

2.1.16 1x TBST buffer

100 ml	10x TBS
900 ml	dH$_2$O
0.5 ml	Tween 20

2.1.17 blocking buffer

1g	nonfat dry milk powder
1g	BSA
100 ml	1x TBST

2.1.18 SDS-PAGE buffer and gel

0.5 M Tris pH 6.8 (100 ml)

Tris-base	6.05 g
dH$_2$O	50 ml

Adjusted pH to 6.8 with HCl filled to 100 ml with dH$_2$O

1.5 M Tris pH 8.8 (100 ml)

Tris-base	18.15 g
dH$_2$O	50 ml

Adjusted pH to 8.8 with HCl filled to 100 ml with dH$_2$O.

Loading buffer (10 ml)

8 M urea solution	5 ml
120 mM Tris (pH 7.6)	2 ml
20% (w/v) SDS	1 ml
Methanol	0.5 ml
Glycerin	1.5 ml
Bromphenol blue	0.05 g
Autoclaved	

Resolving gels (10%):

30% Acrylamide	3.3 ml
1.5 M Tris pH 8.8	2.5 ml
10% SDS	100 µl
dH$_2$O	4 ml
10% APS	50 µl
TEMED	5.5 µl

Stacking gels

30% Acrylamide	0.75 ml
0.5 M Tris pH 6.8	1.25 ml
10% SDS	50 µl
dH_2O	2.9 ml
10% APS	50 µl
TEMED	3 µl

Rainbow protein size marker	Biorad
PVDF-Membrane Immobilon-P transfer membrane	Millipore
STOP SOLUTION	USB
Blotting papers	
Kinase Buffer (ICA)	
DAB-substrate (Sigma FAST™)	
FAST™ UREA H_2O_2)	
True blue	

2.1.19 Primer extension sequencing gel (6%)

Rotiphorese® sequencing gel concentrate	Roth
Rotiphorese® sequencing gel buffer concentrate	Roth
Rotiphorese® sequencing gel diluents	Roth

2.1.20 Materials for cell viability (MTT) test

Thiazolyl blue tetrazolium bromide powder	Sigma USA
Disposable cell scraper	SARSTEDT

2.2 E. coli strains and cell lines and virus strains

XL1-Blue	Stratagene, Germany
The following three cell lines were kindly provided by	Prof. Dr S. Pleschka:
293T (Human embryonic kidney cells)	
MDCK (Madin-Darby canine kidney cells)	
A549 (Human lung epithelial cells)	
A/FPV/Bratislava/79 (H7N7)	Strain collection Giessen
A/PR/8/34, (H1N1), (PR8)	

2.3 Agarose gel electrophoresis

Ethidium bromide

20000x ethidium bromide stock solution (10 mg/ml ethidium bromide)

6x loading buffer

0.025% (w/v) bromophenol blue

30% (v/v) glycerol

50x TAE

242 g	Tris base
57.1 ml	glacial acetic acid
100 ml	0.5 M EDTA (pH 8.0)

2.4 Monoclonal and polyclonal antibodies

Anti-P-ERK (E-4) mouse monoclonal IgG	(1:500)	Santa Crutz
Anti-ERK2 (C-14) mouse monoclonal IgG	(1:1000)	Santa Crutz
Anti-P-PKCα rabbit	(1:500)	Santa Crutz
Anti-PKCα mouse	(1:500)	Santa Crutz
Anti-P-Tyrosine mouse monoclonal IgG	(1:200)	Upstate
HRP conjugated goat anti- mouse IgG	(1:1000)	Santa Crutz
HRP conjugated bovine anti-rabbit	(1:1000)	Santa Crutz
HRP conjugated donkey anti-goat	(1:1000)	Santa Crutz
Anti-PB1 goat polyclonal IgG	(1:200)	Santa Crutz
Anti-NS1 goat polyclonal IgG	(1:500)	Santa Crutz
Anti-NS2 goat polyclonal IgG		Santa Crutz
Anti-NP mouse monoclonal IgG	(1:200)	Serotec
Anti-M1 mouse monoclonal IgG	(1:500)	Serotec
Anti-flu A NP mouse (clone N7C4)	(1:1000)	L. Stitz
Anti-flu A NP mouse (clone 1331)	(1:1000)	Biozol
Anti-flu A NP (FPV) goat	(1:300)	R. G. Webster
Anti-H7HA mouse monoclonal IgG		Dr. R. Wagner
FITC conjugated goat anti- mouse IgG	(1:100)	Dianova
FITC conjugated rabbit anti- goat IgG	(1:100)	Dianova
FITC conjugated goat anti -rabbit IgG	(1:100)	Dianova
Cy5 conjugated sheep anti- mouse IgG	(1:100)	Dianova

Cy5 conjugated rabbit anti -goat IgG	(1:100)	Dianova
Cy5 conjugated donkey anti -rabbit IgG	(1:100)	Dianova
Texas Red conjugated rabbit anti-mouse	(1:100)	Dianova

2.5 Buffers for Immunoflorescence assay

100x Ca^{2+}/Mg^{2+} solution

1 g	$MgCl_2$
1.32 g	$CaCl_2$
100 ml	dH_2O

Autoclaved, then filtered with the 0.2 μm filter column (SARSTEDT, Germany).

1x PBS^{++} buffer

500 ml	1x PBS (autoclaved)
5 ml	100x Ca^{2+}/Mg^{2+} solution

10 x PBS

8 0 g	NaCl
2 g	KCl
14.4 g	Na_2HPO_4
2.4 g	KH_2PO_4

Dissolved in 800 ml of H_2O, and then adjust the pH to 7.4 with HCl. Finally add H_2O to 1 L and autoclaved.

Cell fixing buffer (100 ml)

95 ml	1xPBS++
4 ml	Formaldehyde (PFA)
1 ml	Triton X-100 (t-Octylphenoxypolyethoxyethanol)

Or only 4% paraformaldehyde (4ml paraformaldehyde dissolved in 96ml 1xPBS++) used as a fixing solution when intracellular molecules are prevented for diffusion.

2.6 Mowiol DABCO

2.4 g	Mowiol
6 g	Glycerol
6 ml	dH_2O

Mixed by magnetic stirring over night. Next day 12 ml 0.2 M Tris-HCl (pH 8.5) was added and incubated at $50^{o}C$ for 30 min. The viscous mixture was centrifuged at 6000

rpm at room temperature for 15 min, and the supernatant was mixed with DABCO (final concentration 2.5%). The solution can be stored at $-20^{\circ}C$ for several months.

2.7 Stimulators and Inhibitors

12-O-tetradecanoyl-phorbol-13-acetate (TPA)	Sigma
Calcium channel blocker (Verapamil)	Biomol, Germany

2.8 Other materials

Deoxynucleosidetriphosphate set (dNTP, 100mM)	Promega, USA
Eppendorf tube	Eppendorf, Germany
X-ray film	Kodak X-OMAT
Syringe (5ml, with 25G needle)	Braun, Germany
Falcon centrifuge tube	Falcon, Germany
Lipofectamine	Invitrogen, USA
Lipofectamine 2000	Invitrogen, USA
Plus Reagent	Invitrogen, USA
Microtiter plate (96 wells)	Greiner, Germany
TLC plate	Merck, Germany
SOC medium	Invitrogen, USA

2.9 Methods

2.9.1   Maintenance of cell culture

Mardin-Darby canine kidney cells (MDCK), Human lung epithelial (A549) and Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco modified Eagles medium (DMEM) containing phenol red as a pH-indicator, supplemented with 10% heat inactivated fetal calf serum (FCS) and 1x penicillin /streptomycin. The cells were incubated at 37°C with 5% CO_2 and 95% humidity. They were cultured routinely to a 100% confluence and than passaged according to the need.

2.9.2   Storage and thawing cell cultures

To freeze confluent cells ($75cm^2$ flask), the cells were washed with 1x PBS and 5 ml of 1x trypsin-EDTA was added and incubated at 37°C with 5% CO_2 for 3-5 min. Cells were checked for cell detachment (incubate longer if the cells have not

completely detached). After the cells have detached 5 ml fresh medium was added and the detached cells were collected and centrifuged at 1,000 rpm (Megafuge 1.0R, 350 g), 4°C for 5 min. Supernatant was discarded and pellet was gently resuspended in 1 ml freeze medium (complete DMEM media with 10% DMSO) and transferred into labelled (cell name, passage No., date and name) cryovials. These vials were set into a styropore box and left to freeze gradually in the -80°C freezer. The DMSO prevents ice crystal formation and allows the cells to remain intact. After 24 h, frozen vials were placed into liquid nitrogen where they can be stored for a longer period of time.

To thaw the cells, they were taken out of the liquid nitrogen and placed immediately into 37°C water bath for 5 min. Afterwards cells were resuspended carefully and transferred into cell culture flask filled with prewarmed complete DMEM (20 ml for A549 and MDCK and 25 ml for 293T cells because 293T cells grow very fast and require more energy). On the next day cells will reach a 100% confluency and should be passaged for further propagation.

2.9.3 Infection of cells

A/FPV/Bratislava/79(H7N7) virus strain was used for infection of A549, MDCK and 293T cells. The virus inoculum was made by diluting the virus stock with certain volume of 1x PBS/BA/P/S/Ca^{2+}/Mg^{2+} depending on the desired multiplicity of infection (MOI) used for experiment. A549, MDCK and 293T cells were seeded with 2 ml volume cell suspension into 3.5 cm dishes and grown to confluency by incubating at 37°C with 5% CO_2 overnight or at least 16-18 h. The cells were washed with 1x PBS++, and then 100 µl of viral inoculum (virus stock diluted in 1x PBS/BA/P/S/Ca^{++}/Mg^{++}) was laid on the cells by creating bubbles or foam in the centre of the dish to ensure a consistent virus distribution. The cells were incubated at room temperature for 1h. There after inoculum was aspirated and 2 ml of DMEM/BA/P/S was added. Cells were then further incubated at 37°C with 5% CO_2 as needed/indicated.

The calculation of m.o.i. is done as follows:

$$\frac{1000 \ \mu l}{\text{Virus titer (PFU/ml)}} = \frac{X \ \mu l \ virus}{\text{MOI} \ \ x \ \ \text{cell amount in the culture}}$$

2.9.4 Preparation of cell lysates for Western blot analysis

At indicated time post stimulation or infection cells, were washed with cold 1x PBS++. Thereafter-another 1 ml of cold 1x PBS++ was added to the cells. Then with the help of cell scraper the cells were scraped off and transferred into eppendorf cups. Scraped cells were collected in a falcon and centrifuged at 13,000 rpm (Biofuge 13, 25,000 g) for 1 min. Supernatant was discarded and cell pellet was resuspended in 75 μl lysis buffer. The lysis was done by incubating cells for 20-30 min on ice with vortexing at every 5 min interval. The lysed cells were centrifuged at 13,000 rpm (Biofuge 13, 25,000 g), at 4°C for 15 min. The supernatant, which contains desired proteins, was finally transferred into new eppendorf and was either used or stored at -70°C.

2.9.5 Western blotting (Semi-dry)

2.9.5.1 Measurement of protein concentration (Bio-Rad protein assay)

The Bio-Rad Protein Assay is based on the observation that when Coomasie Brilliant Blue G250 binds to the protein the absorbency maximum shifts from 450 nm to 595 nm [250]. 5 μl of cell lysate was added into diluted Bio-Rad Dye Reagent (1:4 dilution of Dye Reagent concentrate in ddH$_2$O) and mixed well. After a period of 10 min, the protein content was determined by measuring the absorption at wavelength 595 versus reagent blank (containing the lysis buffer only). This was done to apply an equal protein amount of all samples onto the SDS-PAGE gel.

2.9.5.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis apparatus was assembled according to manufacturer's (Bio-Rad) instruction, and the resolving gel was poured in between the two glass plates. Leave a space about 1 cm plus the length of the teeth of the comb. Isopropanol, 100% ethanol or ddH$_2$O was added to the surface of the gel to apply the same distribution along the

gel surface. After the resolving gel was polymerized, isopropanol was removed and the stacking gel was poured on top of the resolving gel. Insert the comb and let the gel polymerise. 75 µl of sample (after determining the protein concentration) with 35 µl laemlli buffer containing 10% β-mercaptoethanol to reduce disulphide bonds were incubated for 5 min at 95^oC and cooled on ice for 1 min, then shortly centrifuged and the calculated amount was loaded into wells of gel. Rainbow protein marker (2 µl marker + 8 µl laemlli buffer) or odyssey machine specific protein marker was loaded as control. Before running, 1x SDS-PAGE running buffer was added to the chamber, a cover was placed over the gel chamber. Gels should be run at about 20 V/cm gel length. The negatively charged SDS-proteins complexes migrate in the direction of the anode at the bottom of the gel. Small proteins move rapidly through the gel, whereas large ones stay at the top. Proteins that differ in mass by about 2% can be distinguished with this method. The electrophoretic mobility of many proteins in SDS-polyacrylamide gels is proportional to the logarithm of their mass.

2.9.5.3 Transfer membrane in "Semi-dry" electroblotter

After the cell extracts were subjected to SDS-PAGE, the proteins were transferred by electroblotting onto a PVDF-Membrane, which was incubated before in 100% Methanol for 2-3 min, and washed three times (5 min each) in ddH_2O, then equilibrated in the transfer buffer for 2-3 min. On the layer of 2 blotting papers the PVDF-Membrane was laid. The polyacrylamide gel and again 2 blotting papers were then placed on the PVDF-Membrane, making a "sandwich" (without any bubbles), and laid in "Semi-dry" Electroblotter (Scheicher & Schuell, Germany). The current was set to 0.8 mA/cm^2 for 1 blot for 90 min for transfer.

2.9.5.4 Immunodetection of proteins on PVDF-Membrane

After transferring the proteins, the PVDF-Membrane was washed for 5 min in 1 x TBST buffer, then the membrane was blocked in blocking buffer (1% milk powder + 1% BSA in 1x TBST) for 2 h at room temperature or overnight at 4°C on flip flop shaker (for best result). There after the membrane was washed for 5 min in 1x TBST buffer, then incubated with the primary antibody for 1 h at room temperature or 4^oC on flip flop shaker or overnight at 4^oC on flip flop shaker depending on the conditions

of antibodies binding with proteins prescribed by company. After washing three times (5 min each) in 1 x TBST buffer, the membrane was incubated with the secondary antibody solution for 1 h at room temperature or 4^0C on flip flop shaker.

2.9.5.5 Enhanced Chemiluminescence (ECL) reaction

As mentioned above the membrane was washed three times (5 min each) in 1x TBST buffer and once in 1x TBS, then incubated in ECL (enhanced chemiluminescence, 1:1 ratio of reagent 1 and 2 i.e. 4 + 4 = 8 ml/blot or according to manufacture (Amersham) protocol) for 1 min in a plastic tray. After 1 min incubation in ECL solution, membrane was transferred between a glass plate and a clear plastic membrane and was laid into a photo cassette, and then a light sensitive film (Bio Max Film MR1, Kodak) was placed on top of the membrane and exposed for 1.5-5 min or depending on the desired intensity of bands. The film was then developed (Protec, Processor-Technology, Germany).

2.9.5.6 Striping of bound antibodies from the PVDF Membrane

In order to detect the ERK2 protein (as a loading control), the attached antibody was striped from membrane by incubating with a 1:10 diluted strong stripping solution (Re-Blot Plus Western Blot Recycling Kit, Chemicon) for 45-60 min at room temperature or Roth striping solution 20-25 ml/blot for 1-2 h at 37^oC. After washing with 1x TBST buffer for 5 min, the membrane was incubated in blocking buffer for 2 h at room temperature or 4^oC on flip flop shaker or overnight at 4^oC on flip flop shaker. Then it was washed for 5 min in 1x TBST buffer and incubated with the anti-ERK2 mouse monoclonal antibody solution for 1 h at room temperature or 4^oC followed by secondary antibodies incubation for 1 h. Subsequently the membrane was washed three times in 1x TBST buffer and washed once in 1x TBS, then incubated for 1 min in ECL solution and proceeded as mentioned above. The bands were then detected by ECL.

2.9.5.7 Quantification of protein bands

Protein bands exposed on the film were scanned (Cano scan LiDE 30/N1240U) at 800 dpi and the picture was saved in grey scales as TIFF file. The intensity of protein

bands was densitometrically determined by means of PC-BAS software (Fuji film). To determine the relative amount of the protein of interest protein bands were quantified and normalized in relation to the loaded ERK2 protein. The lowest value of the loading control (PKCα or ERK2) was set to one and divided by the value of the other bands of the loading control to get a factor for each corresponding band of the protein of interest and this factor was multiplied with the value of the band of the protein of interest. In case of viral proteins detection, the lowest ERK2 value was also set to one and divided by the other ERK2 values to get a factor that was multiplied with the value of the viral proteins. This calculation was done to normalize the amount of P-PKCα, P-ERK and viral proteins by the amount of ERK2 protein loaded.

2.9.6 Detection of influenza A viral proteins

A549 cells were grown to confluence in T75 flask. The following day, cells were washed with 1x PBS and trypsinized in 5 ml 1x Trypsin by incubating at 37°C in 5% CO_2 incubator. After complete detachment of the cells from the wall of the flask, 5 ml complete DMEM (DMEM /10%FCS/P/S) was added into flask and cells were mixed properly by pipetting in and out with glass pipette to make a clear cell suspension. In these 10 ml cell suspension 38 ml complete DMEM media was added to make the total cell suspension about 48 ml. From this cell suspension 3.5 cm dishes were seeded by distributing 2 ml cell suspension in each dish and incubated at 37°C in 5% CO_2 incubator for overnight. Next following day after confluency (90-100%), cells were washed with 1x PBS++ and infected at (MOI=1) with 100μl virus dilution in 1xPBS++/BA/P/S/Ca^{2+}/Mg^{2+} by creating a bubble in the centre of the dish to hold the liquid and the dish was incubated at room temperature for 1h. 1h post incubation virus dilution in the dishes were aspirated and 2 ml DMEM/BA/P/S media was added in each dish and incubated at 37°C in 5% CO_2 incubator for the indicated time points (4, 6, 8 and 10 h). After 2 h p.i cells were treated with 100 μM Verapamil by adding 20 μl Verapamil (from the stock 10 mM) solution in 1 ml media taken out from the respective dishes and was mixed properly and again transferring back to the same dish and incubated at 37°C in 5% CO_2 incubator. At every indicated time points dishes were taken out from the incubator and washed with ice-cold 1x PBS++ or 1x PBS. Further in 1 ml ice-cold 1x PBS++ or 1x PBS, cells were scraped (by disposable cell scraper, SARSTEDT) and transferred into new eppendorf. Cells were centrifuged at

13000 rpm for 30-60 seconds and pellets were dissolved in 75 μl Tris lysis buffer (TLB) containing protease inhibitors. Cell lysates were incubated at ice for 20 min and at every 5 min interval cell lysates were vortexed. After 20 minutes incubation at ice cell lysates were centrifuged at 13000 rpm for 15 min at 4ºC. The supernatant which is a lysate is transferred into new eppendorf and stored at -20ºC or -70ºC or until required for the use.

2.9.6.1 Western blot analysis of viral proteins

Calculated protein amount was loaded onto 10% Polyacrylamide gel. After clear separation of proteins and the loading dye about to touch the bottom, the gel was taken out from the western blot chamber and transferred on nitrocellulose membrane for 90 min (48 mA current/gel). Blot was washed twice with 1x TBST and then blocked in blocking buffer (1% milk powder +1% BSA) either for 2 h or overnight (4ºC on flip flop shaker in plastic bag). Later blots were incubated overnight with primary antibodies (anti-NP mouse, 1:200, anti-M1 mouse 1:500, anti-PB1 rabbit 1:200 and, anti-NS1 rabbit 1:500 in blocking buffer) at 4ºC on flip flop shaker in plastic bag. Blots were washed thrice in 1x TBST washing buffer and then incubated with secondary antibodies (HRP conjugated anti-mouse and anti-rabbit) for 1 h at 4ºC on flip flop shaker in plastic bag. The bands were then detected by ECL.

In order to detect the ERK2 protein as the loading control, the attached antibody was striped from the membrane and blocked in blocking solution. The membrane was incubated with the primary mouse anti-ERK2 monoclonal antibody solution for 1 h at room temperature followed by 1 h secondary antibodies incubation. Subsequently the membrane was washed three times in 1x TBST buffer and washed once in 1x TBS, then incubated for 1 min in ECL solution and proceeded as described above. Finally the bands were quantified and the values were normalized to the loading control.

2.9.7 Immuncomplex kinase assay (ICA)

The kinase activity can be measured in vitro by radioactivity of (γ^{32}P) ATP labelled substrate of the kinase. To determine PKCα and ERK activity, A549-cells were infected with FPV (MOI=1) and either left untreated or were treated with Verapamil (100μM/ml, 2 h p.i.), cell lysates were made at 8 h p.i and 10 h p.i. and used for ICA,

using myelin basic protein (MBP) as substrate indicating kinase-activity. Loading was controlled with anti PKCα and ERK-specific mAbs in WB-analysis.

 After infection and Verapamil treatment at indicated time points, cells were washed with cold 1x PBS++. Thereafter another 1 ml of cold 1x PBS++ was added to the cells, then they were scraped off and transferred into eppendorf cups. These were then centrifuged at 13,000 rpm (Biofuge 13, 25,000 g) for 1 min and the cell pellet was resuspended in 75 µl lysis buffer. The lysis was done by incubating cells for 20-30 min on ice and intermittent vortexing every 5 min interval. The lysed cells were centrifuged at 13,000 rpm (Biofuge 13, 25,000 g), 4°C for 15 min. The supernatant was finally transferred into new eppendorf cups and stored at -70°C till required. The immunoprecipitation was performed by incubating the 12CA5 anti HA monoclonal antibody and protein A agarose for 2 h at 4°C. Immunoprecipitated kinase was washed twice first with TLB buffer and then with Kinase buffer. 5µCi (γ32P) ATP, 2-5 µg of substrate (2mg/ml dephosphorylated myelin basic protein (MBP) in 20mM MOPS, pH 7.2, 25mM β-glycerophosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol; Upstate cell signalling solution) was added, 0.1 mM ATP, and 20 µl Kinase buffer were added. The mixture was incubated at 30°C for 30 min essentially as described [251]. The reaction is then stopped by adding SDS-loading buffer and incubated at 95°C for 5 min. Immune complexes were used for in vitro kinase assays with purified MBP as substrate for ERK as previously described [186]. The proteins are separated by SDS-PAGE and then blotted onto nitro-cellulose membranes. After Western blot proteins were detected by a BAS 2000 Bio Imaging Analyzer (Fuji film) and autoradiography. Quantification of specific bands was done with the PC-BAS software package. Western blot analysis with the anti ERK2 or 12CA5 anti HA monoclonal antibodies was performed to monitor equal loading of kinases. This assay was performed in collaboration with Centre for Molecular Biology (ZMBE), Münster.

2.9.8 Cell viability test (MTT Assay)

Verapamil is a calcium channel blocker and indirect inhibitor of PKCα. I used 100 µM/ml Verapamil in my studies. In order to determine whether this concentration has any detrimental affect on cell viability, the MTT Formazan-assay was performed. This assay measures the activity of mitochondrial dehydrogenase in the living cells. 3-

(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is taken by the cells and reacts with NADH, which is reduced to NAD. This reaction produces blue formazan crystals in living cells.

A549 cells were seeded in 96 well cell culture plates (200 µl/well from 10ml cell suspension diluted in 45 ml complete 1x DMEM and grown overnight at 37°C with 5% CO_2 so that they were confluent on the next day. Confluent cells were either left untreated or were treated with Verapamil (100µM mixed in DMEM) and incubated for an additional 4, 6, 8 and 10 h at 37°C with 5% CO_2 incubator. Then the medium was replaced with 200 µl of complete DMEM media and incubated for 1 hour to allow cell proliferation. 7 µl of 5 mg/ml MTT stock solution was diluted in 193 µl complete DMEM media (175 µg/ml final concentrations) and added into each well after aspirating the old media. Cells were incubated further for 90 min and subsequently fixed with 4% paraformaldehyde (PFA, in 1x PBS) at room temperature for 30 min. The fixing solution was aspirated and the plates were air dried under the hood for 10-15 min. The tetrazolium crystal was dissolved by adding 200 µl of isopropanol to each well and shaking them by placing the plates on 96-well plate shaker for 10 min. The plates were analyzed photometrically at 550 nm excitation in an enzyme-linked immunosorbent assay (ELISA) reader.

2.9.9 Analysis of infectious virus titres

2.9.9.1 Immunohistochemistry (Focus forming units, FFU)

Immunohistochemistry is the localization of antigens or proteins in tissue sections by the use of labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, or colloidal gold.

Virus dilution was made in the 96 well U-bottom microtiter plates. First 180 µl of 1xPBS++/BA/P/S/Ca^{2+}/Mg^{2+} was pipetted into each well of the first row and then 20 µl of the virus stock or virus supernatant collected from A549 infected cells was added into the wells of the first row. The virus dilution (200 µl) was mixed by pipetting in and out and 20 µl of it was transferred into subsequent wells of the second

row. The same steps were repeated up to the last row to get a 10^{-1} to 10^{-8} dilution series.

The MDCK cells were seeded in 96 well plate (10 ml cell suspension + 45ml DMEM complete media = 55 ml, distribute 150 µl/well) and grown over night at 37°C with 5% CO_2 so that they reach 90-100% confluency on the day of infection. The next day, cells were washed once with 200 µl 1x PBS++, then infected with 50 µl of various virus dilutions and incubated at room temperature for 1 h. Virus inoculum was aspirated and 150 µl or 100 µl methylcellulose media was added into each well. The plate was placed at 37°C with 5% CO_2 for 30h in case of FPV and 48 h in case of other viruses like PR8. After the incubation time, methylcellulose media was removed by aspiration. Cells were washed twice with 1x PBS++ and fixed as well as permeabilized with 100 µl of 4% PFA/1% TritonX-100 in 1x PBS++ for 25 min at room temperature or overnight at 4°C. Later cells were washed three times with 1x PBS++/0.05% Tween20 and incubated with 50 µl of primary antibody (anti NP-mAb, 1:1000 diluted in 1x PBS++/3% BSA) for 45 min or 1 h at room temperature. After aspirating the primary antibody dilution, cells were washed three times with PBS++/0.05% Tween20, followed by incubation with secondary antibody (Horse Radish Peroxidase (HRP)-conjugated anti mouse, 1:1000 diluted in PBS++/3% BSA) for 45 min at room temperature. Cells were washed twice with 1x PBS++/0.05% Tween20 and 100 µl DAB-substrate (first, one silver pill Sigma FAST^{TM} DAB) was dissolved in 15 ml 1x PBS followed by dissolving one golden pill (Sigma FAST^{TM} UREA H_2O_2) or True blue was added into each well and incubated at room temperature for 5-10 min. When brown or blue stained foci's respectively were observed under the microscope the cells were washed with normal water to remove the rest of salts and air dried at room temperature until the next procedure of analysis. After scanning and counting of foci's, the viral titre was determined as follows:

FFU/ml = number of foci's x volume factor x dilution factor
Volume factor: FFU (Foci forming unit) is related to 1 ml. If a dish was infected with 50 µl viral dilution solution, the factor is 20.

2.9.10 Immunoflorescence assay (IFA)/Laser scanning confocal microscopy

The confluent cells in a culture flasks were washed with 1x PBS and trypsinized by adding 5 ml 1x trypsin-EDTA and incubated at 37°C with 5% CO_2 incubator for 5-10 minutes or until cells were completely detached. 5ml complete DMEM media was added in the trypsinized cells and resuspended by pipetting in and out. These 10 ml cell suspensions were resuspended in 45 ml complete DMEM media and seeded 2 ml in each 3.5 cm dish containing 2x sterile glass coverslips (12 mm) and incubated at 37°C with 5% CO_2. On the next day, the cells were confluent. Cells were washed with 1xPBS++ and infected with IV (MOI=1 or 5) or treated with TPA (100ng/ml) and infected cells were treated with Verapamil at non toxic concentration (100μM). Depending on the purpose of the experiment, the growth medium was removed from the culture dish, and the cells were washed once with 1x PBS++, then the cells were fixed with 1 ml 4% PFA in 1x PBS++ without or with 1% Triton X-100 (depending on the aim of the experiment) for 30 min at room temperature or over night at 4°C. After fixation, cells were washed twice with 1x PBS^{++} and subsequently incubated with 25 μl of the primary antibody (1:200 dilution in 1x PBS++/3% BSA) for each coverslip for 1 h at room temperature or 37°C with 5% CO_2 incubator. Afterwards cells were washed twice as mentioned above, then incubated with 25 μl of the either, FITC, CY5, or Texas red conjugated secondary antibodies (1:100 dilution in PBS++/3% BSA) for 1 h at room temperature under dark conditions. Then the cells were washed twice as mentioned above, and once with ddH$_2$O. The glass coverslip was fixed on the glass carrier with Mowiol, and hardened overnight by incubating at 4°C. The analysis was done on next day with a confocal laser scanning microscope.

2.9.11 Preparation of plasmid DNA

Plasmid DNA mini preparation was normally done according to the following protocol: a single bacterial colony or 5 μl glycerol stocks was transferred into 5 ml of LB medium containing 100 μg/μl ampicillin in a loosely capped 15 ml glass tube. The tube was incubated overnight at 37°C with vigorous shaking. 1.5 ml of culture was poured into a microfuge tube and centrifuged at 6000 rpm (Biofuge 13, 5,000 g) for 4 min at 4°C in a microfuge. The medium was removed by aspiration, leaving the bacterial pellet as dry as possible. The pellet was resuspended in 100 μl of solution I

by vigorous vortexing, until the pellet was dispersed. Then 200 µl of prepared solution II was added, the contents were mixed by inverting the tube rapidly six times and left at room temperature for max 5 min. 150 µl ice-cold solution III was subsequently added, the tube was vortexed gently for 10 seconds and stored on ice for 10 min. The tube was centrifuged at 13,000 rpm (Biofuge 13, 25,000 g) for 10 min at 4°C in a microfuge and the supernatant was transferred to a new tube. The DNA was precipitated by adding 250 µl of isopropanol at room temperature and the contents were mixed by vigorously vortexing. The tube was centrifuged at 13,000 rpm (Biofuge 13, 25,000 g) for 15 min at 4°C in a microfuge. After centrifugation the supernatant was removed by gentle aspiration, and then the pellet was rinsed with 500 µl of chilled 70% ethanol and centrifuged for 5 min at 13,000 rpm (Biofuge 13, 25000 g). The supernatant was removed and the pellet of DNA was air-dried for 10 min at room temperature or exposing to heated lamp. Then the DNA was redissolved in 100 µl TE (pH 8.0) containing DNAase-free pancreatic RNAase (20 µg/ml) or ddH20.

Plasmid DNA maxi preparation was preformed using QIA prep plasmid midi or maxi kit. The 100 µl (glycerol stock) cultures of bacteria containing plasmids were grown in 100 ml of LB media overnight in 37°C incubator with shaking at 200 rpm. The bacteria were collected by centrifuging the overnight culture at 6,000 rpm (Beckman JA14, 7,500 g) and 4°C for 15 min. There after the bacterial pellet was resuspended in 10 ml of buffer P1 and 10 ml buffer P2 was added. The whole mixture was mixed gently and incubated at room temperature for 5 min. 10 ml of chilled buffer P3 was added, mixed immediately and incubated on ice for 20 min. The mixture was centrifuged at 4,000 rpm (Megafuge 1.0R, 5,400 g) for 30 min at 4°C. The supernatant was applied to an equilibrated QIAGEN-tip 500 and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice with 30 ml buffer QC. DNA was eluted with 15 ml buffer QF. The DNA is precipitated by adding 0.7 volumes (10.5 ml) of isopropanol and centrifugation at 12,000 rpm (Beckman JA20, 21,000 g) for 30 min at 4°C. The resulting pellet was washed twice with 70% ethanol and air-dried at room temperature. The pellet is then carefully resuspended in certain volume of TE buffer depending on the size of DNA pellet. The concentration of plasmid DNA was measured photometrically and adjusted to 1 µg/µl. The plasmid DNA can be stored at 4°C for a short time only or at -20°C over a longer period.

2.9.11.1 Measurement of plasmid DNA concentration

The DNA concentration is determined by using a DNA photometer (GeneQuant II, Pharmacia Biotech) at wavelength of 260 nm. The absorption of 1 at 260 nm corresponds to a concentration of 50 µg/ml double stranded DNA. First of all an appropriate DNA dilution (normally 1:100 in H20 or TE buffer) was made, transferred into a quartz crystal cuvette and placed into the photometer, which has been calibrated before to the solvent (either H_2O or TE buffer). The desired OD should lie between 0.2 and 0.4 to ensure correct measurement.

2.9.11.2 Restriction endonuclease digestion

Digestion of DNA with restriction endonuclease was performed by pipetting DNA sample, 10x restriction buffer, restriction enzyme, and distilled and deionised water (ddH_2O) in a 1.5 ml eppendorf tube. The reaction mixture was incubated at the recommended temperature (usually at 37^oC). Normally 1-5 U of restriction enzyme was used to digest 1 µg DNA. Restriction endonucleases were inactivated by heating 65^oC for 15 min.

2.9.11.3 Agarose gel electrophoresis

1% agarose gels were routinely used for analyzing DNA. The agarose was melted in 1x TAE buffer using a microwave oven, and cooled to about 55^oC or pleasant warm to touch. Before the gel was poured into a casting platform, ethidium bromide (8 µl (stock 10 mg/ml) per 100 ml of dissolved 1% agarose) was added. DNA samples were mixed with appropriate amount of 6x DNA loading buffer before loading into the wells. Normally, the gel was run at 100-120 V and 300-400 mA. DNA was visualized by placing gel on a UV transilluminator and picture was captured directly by a photo system.

2.9.12 DNA-transfection of eukaryotic cell cultures

2.9.12.1 Transfection of adherent 293T cells

293T cells were split 1:2 into a new T-75 flask one day prior to transfection to promote cell growth. The next day, 2 ml cell suspension was seeded in 3.5 cm dishes in such a way (1:3 dilutions) that cells reach up to approximately 90% confluency

over night for the transfection. To transfect the cells 7µg DNA (2:1:1:1:2 ratio of pPOL1-CAT-RT, pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP plasmid DNA of the stock 1 µg/µl) was diluted in 90 µl 1x DMEM (without serum and antibiotics) in a tube A (5 ml polystyrene Round-Bottom Tube, Falcon), then 10 µl Plus Reagent was added into the mixture and gently mixed by pipetting up and down and incubated at room temperature for 15 min. Just before completion of 15 min incubation of tube A, 7 µl Lipofectamine Reagent was diluted in 93 µl 1x DMEM (without serum and antibiotics) in another tube B (5 ml polystyrene Round-Bottom Tube, Falcon) and mixed by pipetting up and down. The diluted Lipofectamine Reagent (7 + 93 = 100 µl) in tube B was added to the tube A containing pre-complexed DNA and Plus Reagent in 1x DMEM without serum and antibiotics (7 + 10 + 90 = 107 µl). Then the mixture A and B was mixed gently and incubated for 30 min at room temperature. While complexes were forming, the media in each dishes was replaced with 800 µl 1x DMEM (without serum and antibiotics). The DNA-Plus-Lipofectamine Reagent in 1x DMEM without serum and antibiotics complexes (total 107+100= 207 µl volume) were added drop wise into the dish containing fresh medium. The dropwise added complexes were mixed with the medium by gentle shaking the dishes and incubated at $37^{\circ}C$ at 5% CO_2 for minimum 6 h. Thereafter, the media containing DNA-Plus-Lipofectamine Reagent in 1x DMEM (without serum and antibiotics) complexes was removed and replaced with 2 ml of complete DMEM media. The cells were further incubated at $37^{\circ}C$ at 5% CO_2 for 24-48 h.

2.9.12.2 Transfection of suspended MDCK cells

The day before transfection, MDCK cells were split 1:2 into a new flask to promote cell growth. On the day of transfection, confluent cells in 75 cm^2 culture flask were washed with 1x PBS, trypsinized by 5 ml Accutase I and incubated at $37^{\circ}C$ for 15 min. During incubation time, DNA and Lipofectamine 2000 (L2000) mix were prepared as follows: 4 µg DNA was diluted in 50 µl of OPTI-MEM reduced serum medium and mixed gently in a 5 ml polystyrene round-bottom tube. L2000 was mixed gently before using, than 12 µl of L2000 was diluted in 238 µl of OPTI-MEM in another 5 ml polystyrene round-bottom tube, mixed gently and incubated not longer than 5 min at room temperature. The diluted L2000 was added to the tube containing the diluted DNA, then mixed gently and incubated for 20 min or longer at room

temperature to allow forming of the DNA-L2000 complexes. After cell trypsinization, 5 ml medium without antibiotics (DMEM with 10% FCS) was added into the flask, the mixture was transferred into a 15 ml Falcon centrifuge tube and subsequently centrifuged at 1,000 rpm (Megafuge 1.0R, 350 g) for 5 min. The supernatant was removed by aspiration, and then the cell pellet was resuspended by adding 7 ml medium (DMEM with 10% FCS). 700 µl of cell suspension was mixed with 300 µl of DNA-L2000 complexes and reseeded onto 3.5 cm dishes. The cells were incubated at 37^oC with 5% CO_2 for 6 h, and then 1 ml of media (DMEM with 10% FCS) was added. 24 h after transfection the media containing DNA-L2000 complexes was replaced with 2 ml of complete DMEM media.

2.9.13 Primer Extension

The primer extension reaction was used to quantify the amount of viral cRNA, vRNA, and mRNA. One uses a radiolabeled primer that is complementary to a region towards the 5' end of the transcript, to generate a single stranded DNA molecule towards the 5' end of the RNA. The size of the labeled single-stranded DNA is then determined on a 6% sequencing gel. I used total RNA of transfected 293T cells.

2.9.13.1 Transfection and RNA Isolation

Two different approaches were used to test the effect of Verapamil on viral polymerase for the production level of cRNA, mRNA and vRNA either in 5 plasmid based replication system (mini genome replication system) or pPOL1-CAT-RT + virus replication system.

293T cells were grown overnight or until confluency in 3.5 cm dishes. Confluent cells were washed with 1x PBS and replaced with 800 µl fresh serum and antibiotic (pen/strep) free media. In the case of "5 plasmid based" replication system, pPOL1-CAT-RT, pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP plasmid were diluted in the ratio (2:1:1:1:2 ratio of pPOL1-CAT-RT, pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP plasmid DNA of the stock 1µg/µl) and transfected using lipofectamine and plus reagent. In the case of "5 plasmid based" replication system, 24 h.p.t cells were directly treated with Verapamil as indicated and incubated at 37^0C at 5% CO2 incubator for 8 h.

In the case of "pPOL1-CAT-RT+ virus" replication system, 293T cells were transfected with only 2 μl ((2μg) from the stock of 1μg/μl plasmid DNA and after 24 h p.t. (hours post transfection) cells were infected with FPV (MOI=1). After 2 h p.i. cells were treated with Verapamil (100μM/ml) and incubated at 37˚C at 5% CO2 incubator for 8h.

After 32 h.p.t and treatment in both the case media from cells was replaced by 1 ml trizol and incubated at room temperature in the laminar hood for 15 min. After 15 min cells with trizol are pipetted in and out several times until the trizole cells mix becomes liquid. These trizole treated samples are transferred in a new RNAase free eppendof and incubated at -70^0C for overnight or it is used directly for RNA isolation.

2.9.13.2 RNA isolation

To each eppendorf tube 200 μl of chloroform was added and mixed properly and incubate for 5 min at room temperature with occasional shaking. Later the tubes were centrifuged for 20 min at 4^0C at 13000 rpm. The upper phase (app. 0.6ml) was transferred later into fresh RNAase free eppendorf. 0.5 ml isopropanol was added to this upper phase supernatant and mixed properly and incubated at room temperature for 10 min. Centrifuge at 13000 rpm for 20 min at 4^0C. The supernatant is removed carefully and 1 ml of freshly made 70% ethanol was added to the pellet and centrifuged at 13000 rpm for 5-10 min at 4^0C. Without dislodging the pellet, alcohol content was discarded and pellet was air dried for 5-10 min and dissolved in 50 μl RNAase free (DEPC) $H_2$0 and used directly for the experiment or frozen at -70^0C.

2.9.13.3 DNA marker labeling

Two types of DNA marker (Bioline and DNA hyper ladder IV) are labeled with γ^{32}P. Mixture of 13.5 μl autoclaved $dH_2$0, 5 μl each of DNA ladder marker, 2.5 μl 10x PNK buffer (Roche), 3μl γ^{32}P (30 co of the stock of 10μC/ μl) and 1 μl T4 polynucleotide kinase (PNK, 10U/ μl) were properly mixed by pipetting in and out and incubated at 37^0C (PCR machine) for 1h. After 1h incubation reaction was stopped by adding 5 μl STOP SOLUTION (USB). 5-10 μl were loaded onto the 6% seq. gel.

2.9.13.4 Primer labeling

CAT-89: 5'ATGTTCTTTACGATGCGATTGGG3'

CAT-2:   5'CGCAAGGCGACAAG GTGCTGA3'

5S-rRNA: 5'TCCCAGGCGGTCTCCCATCC3'

Two types of primers (CAT-89 and CAT-2) are used for the detection of cRNA, mRNA and vRNA. **CAT-89 primer** anneals with positive sense cRNA and mRNA but the product length of the two (cRNA -89 nucleotides an mRNA -106 nucleotides) differs by a factor of 17 nucleotide. mRNA is longer than cRNA due to CAP structure at 5'end and polyadenylation signal at 3'end. **CAT-2 primer** anneals with negative sense vRNA and gives the product size of 158 nucleotides. **5S-rRNA primer** is used to anneal internal loading control of 5S-rRNA and gives 100 nucleotide length products.

Each primer stock is 100 pmol/µl. Before labeling these primers (each primer) is diluted in DEPC water to a working concentration of 10 pmol/ µl from the stock.

2.9.13.5 Primer labeling reaction

Primer	3 µl
DEPC H20	2 µl
10xPNK buffer (Roche)	1 µl
γ^{32}P- ATP (30µCi)	3 µl
T4 polynucleotide kinase (PNK, 10U/ µl)	1 µl

Mixed well and incubated at 37^0C for 1h in PCR thermoblock. After 1h incubation, samples are taken out of the block and 40 µl of DEPC water is added into each eppendorf. This 50 µl mixture is transferred into new 1.5 ml RNAase-free eppendorf and further 450 µl PN-buffer (Qiagen Nucleotide removal kit) is added, mixed well and placed over the Qiagen Nucleotide removal column with the help of a pipette. The column is spun for 1 min at 6000 rpm and the collection is discarded. The column is washed 2 times with 500 µl PE-buffer. Further spun down for 1 min at 6000 rpm and the collection is discarded followed by one more time spinning down for one minute at 13000 rpm to remove residual alcohol content in the column. Fresh 1.5 ml RNAase free eppendorfs are labeled clearly and the column is placed onto it. 40 µl

EB-buffer or DEPC H$_2$0 is added on the column and incubated for 2 min at room temperature. Finally the tube is centrifuged for 1 min at 13000 rpm and the eluate (40 µl) is collected in the eppendorf. This can be stored at -20^{0}C in isotope room.

2.9.13.6 Primer Extension (Reverse Transcription)

Primer-Mix : Before RT (cDNA preparation), mix the following primers as follows according to the no. of samples and always make surplus than the need.

No.of samples	x1	x12	x14	x16
Labeled **Cat 2**	0,25 µl	3 µl	3,5 µl	4 µl
Labeled **Cat 89**	0,25 µl	3 µl	3,5 µl	4 µl
DPEC H$_2$O	0,5 µl	6 µl	7 µl	8 µl

No.of samples	x1	x12	x14	x16
Labeled **5srRNA**	0,25 µl	3 µl	3,5 µl	4 µl
DPEC H$_2$O	0,75 µl	9 µl	10,5 µl	12µl

Note: 5s rRNA was diluted 1:100 before use

200 µl PCR eppendorfs were labeled. For 1 sample reaction, 10 µl trizol-purified RNA was mixed with 1 µl primer mix and placed in PCR machine. Water control was also used for this experiment and instead of 10 µl RNA, 10 µl water was mixed with 1 µl primer mix. The PCR reaction was run at 95°C for 3 min, 50°C for 92 min and 70°C for 3 min, 4°C ~ . In the mean time prepare Superscript II or III-mix as follows:

Superscript II-Mix

	x1	x12	x14	x16
5x buffer	2 µl	24 µl	28 µl	32 µl
0,1M DTT	1 µl	12 µl	14 µl	16 µl
10mM dNTPs	0,5 µl	6 µl	7 µl	8 µl
DPEC H$_2$O	1,25 µl	15 µl	17,5 µl	20 µl
Superscript II (rev. transcriptase)	0,25 µl	3 µl	3,5 µl	4 µl
RNAase inhibitor	0.5 µl	< 3 µl	< 3 µl	< 3 µl

After 3 min running at 95°C, if the temp. in PCR machine has reached to 50°C, then Add 5µl Superscript mix into RNA-Primer-mix ad then mix it well. Incubate further at 50°C for 90 min and let it run till it's gone through. Newly produced cDNA was either used for loading on 6% sequencing gel or was stored at -20°C.

2.9.13.7 8% sequencing gel

Following components of 8% sequencing gel was mixed properly and poured in between the sequencing gel plates followed by placing 16 well combs. After adding TEMED in the gel mixture, it is essential to pour the gel as soon as possible otherwise it can solidify before pouring into the gel plates.

23.3 ml sequencing gel Verdünner (Roth)

12.7 ml sequencing gel concentrate (Roth)

4 ml sequencing gel-buffer (Roth)

500 µl 6% APS (Ammonium persulfate)

40 µl TEMED

The polymerized gel was prewarmed up to 50°C before loading the samples for the best result. The voltage was supplied up to maximum but current was maintained up to 40W. When the gel temperature was reached up to 50°C the 5 µl STOP-Solution was added to each sample (cDNA) and incubated at 95°C for 3 min (in PCR machine). 20 µl of each samples were loaded into the wells. Labeled marker (10 µl was also loaded for the size control. Gel was run at 1500 volt and 25W till the dark blue dye reaches the end of the gel and the approximate time required was 2 h.

When gel run was over then the glass plate was separated very carefully and the gel was placed on wattman-paper (right size) and was wrapped with plastic foil (plastic foil side at the top. Care is always exercised during the whole experiment because of radioactive hazardous material $\gamma^{32}P$. The wrapped gel was dried in vacuum drier for 90-120 min. When the gel was dried then it was placed onto the screen cassettes and make sure that gel side facing towards the white side of screen platter and incubated over night or as desired at room temperature. Next day the gel was scanned on the typhoon and the band was quantified using GenQuant software.

2.9.14 Chloramphenicol Acetyl Transferase (CAT Assay)

Aim of the CAT assay is to analyze the effect of Verapamil on CAT protein expression of the plasmid containing CAT gene (pPOL1-CAT-RT) in the presence of 4 other helper plasmids pHMG-PB1, pHMG-PB2, pHMG-PA, and pHMG-NP derived from PR8 virus system that supports CAT gene expression. CAT assay was performed to check the acetylating activity of CAT protein expressed in 293T cells. Confluent 293T cells were distributed in 3.5 cm dishes and incubated overnight at 37^0C in CO_2 incubator. Next day confluent (80-90%) cells were transfected with 7 μg plasmid DNA (2:1:1:1:2 ratio of pPOL1-CAT-RT, pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP plasmid respectively of the stock 1μg/μl) using lipofectamine and plus reagent. Between 6-8 h.p.t media was changed with complete media or only 1xDMEM with P/S (without FCS) and incubated overnight or until 48 h at 37^0C in CO_2 incubator.

In the same way of transfection method for pPOL1-CAT-RT along with pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP, 293 T cells were also transfected with plasmid containing GFP gene described as pPOL1-GFP-RT along with pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP used as a positive control to analyze the transfection efficiency. After 48 h.p.t pPOL1-GFP-RT transfected cells were analyzed microscopically. If the transfection efficiency for GFP control was good then pPOL1-CAT-RT plasmid transfected cells were either left untreated or treated with Verapamil (100 μM) after 48 h.p.t and incubated at 37^0C with 5% CO_2 incubator for further 8 h.

2.9.14.1 Lysis

48 h.p.t cells were washed with 1x PBS^{++} and cells were scraped in 500μl 1x PBS^{++} and transferred in new eppendorf (on ice). Cells were centrifuged at 3000 rpm at 4°C for 1 min. Pellet was resuspended in 100 μl of 0.25M Tris (pH-7.5). Resuspended pellet was incubated for 2 min in liquid nitrogen followed by 5 min incubation at 37°C in water bath. This process was repeated for two times and centrifuged at 3000 rpm for 1 min at 4°. 100μl supernatant, which is enzyme extract, was transferred in new eppendorf on ice and was used for CAT assay or was stored at -70°C.

2.9.14.2 Determination of protein amount

5 µl sample from the cell lysate was taken to measure the protein concentration. Briefly, 200 µl of Bio-Rad protein assay buffer and 800 µl of ddH$_2$O were mixed with 5 µl of sample in a 1.5 ml eppendorf tube. The concentration of protein was calculated by measuring the absorbance at 595 nm in a DU-70 spectrophotometer (Beckman). According to the indicated absorbance, the protein concentration of different samples could be calculated and compared and equal amounts used for the enzyme assay.

2.9.14.3 Enzyme dilution: 1:10, 1:100, and 1:1000

Enzyme extract (prediluted equal amounts, see above) was diluted in 1:10, 1:100, and 1:1000 ratios. 10 µl of enzyme extract was diluted in 90 µl tris buffer in eppendorf that gives 1:10 dilution. From this mixture, 10 µl was further diluted in 90 µl tris buffer in eppendorf that gives 1:100 dilutions. And finally 10 µl of this mixture was further diluted in 90 µl tirs buffer in eppendorf that ends in 1: 1000 dilution of enzyme extract. This can be either used for the experiment or can be stored at -20˚C.

2.9.14.4 Chloramphenicol reaction

50 µl of each prediluted enzyme (1:10, 1:100 and 1:1000) extract was incubated with 20 µl (4mM) AcCoA and 5 µl BODIPY. It was mixed properly and this 75 µl reaction mixture was incubated at 37˚C for 2.30 h in a water bath. In the mean time running chamber was set up. Running buffer was made by mixing 26 ml methanol and 174 ml chloroform (13:87) and poured into the chamber and covered with lid for overnight to generate vapours. If the mixture does not dissolve after addition of BODIPY then 1.5 ml methanol was added.

After 2.30 h incubation of reaction mixture, the substrate was extracted by adding 500 µl ethyl acetate to the mixture by vigorously vortexing for 5 min followed by 5 min centrifugation at 13000 rpm at room temp. Two phases will be created upper ethyl acetate layer (450 µl) and lower water layer. Ethyl acetate layer was transferred into new eppendorf (with holes made in the lid) and were concentrated in the form of dried pellet for 5-20 min in speed vac.

2.9.14.5 Loading samples on TLC plate

Speed vac dried pellet was dissolved in 20 µl ethyl acetate and was loaded in the form of little dots on TLC plate with the help of air gas supply. Air supply helps minimizing spread of ethyl acetate on TLC plate during sample loading. The sample loaded TLC plate was placed in pre-equilibrated running buffer in an inclined position. As soon as TLC plate touches the buffer, migration of samples starts by capillary action and is completed in about 30 min. The presence of acetylated chloramphenicol was visualized as a light green color spot on the chromatogram in UV-light. The TLC plate was removed from the buffer at the end of 30 min and allowed to dry. Later the spots were scanned by typhoon machine and quantified by Genuine software.

2.9.15 Verapamil doses preparation for animal experiments

To investigate the inhibitory effect of Verapamil on virus replication in either of infected mouse, ferret or guinea pig, drug dosage can be prepared according to pharmaceutical and dispensing law. In animal experiment we prefer to use mouse as a model for infection and subsequently drug treatment because certain strain of mouse is adapted for influenza virus infection. Verapamil-HCl powder has Molecular weight 491.6g/l=1M (491.6mg/l=1mM, 49.1mg/l=100µM). In order to achieve the same non toxic concentration (100µM) which was used for in vitro studies, the Verapamil doses are calculated depending on the body weight or volume of the animal and in this case mouse. The body weight of mouse varies from 25 to 35 gm but 30 gm is most likely. Verapamil is water soluble. Either non-pyrogenic water for injection or non-pyrogenic saline water (water containing 0.8% NaCl) are recommended for diluting Verapamil for animal experiment. Normal water is not recommended for diluting Verapamil or any drug because it causes fatality and serious illness to the animal. Calculated drug dosage could be administered in the mouse by various modes but intraperitoneal injection is most preferred. Administration of drug through injection should be continued depending on the availability of the drug in the body, metabolic rate of the drug in the body or maintaining the continuous supply of constant concentration of drug in the body. Above 5 mg/mouse is not recommended. Intra peritoneal (1P) injection is recommended in most of the cases but other routes of drug administration can be also used.

Formulae of pharmaceutical doses calculation: Drug doses for humans are calculated by this formula:

$$\frac{70\text{kg (human weight)}}{200\text{gm rat weight}} = \frac{50}{x}, \text{ Where x is the human dose.}$$

Verapamil Acute Toxic Doses (LD50)

Mouse:

i.v 7.6mg/kg   (Windholz1983)

s.c 68mg/kg   (Windholz1983)

i.p 68mg/kg   (Windholz1983)

p.o 114mg/kg (Windholz1983)

Rat:

i.v   16mg/kg   (Windholz1983)

s.c 107 mg/kg (Windholz1983)

i.p   68mg/kg (Windholz1983)

p.o 114mg/kg (Windholz1983)

3. Results

3.1 Analysis of the role of tyrosine kinase activity in influenza virus infected cells

Previous studies (254), demonstrate that Raf/MEK/ERK (MAPK)-cascade is an essential host function for efficient influenza virus replication. Using MEK inhibitor (U0126) at non toxic concentration resulted into the blocked of RNP-export from nucleus to the cytoplasm leading to reduced virus titers. To demonstrate that the influenza virus induced Ras-activation could be an upstream kinase for the transmission of signals to the Raf/MEK/ERK (MAPK) cascade. Ras is a GTP-binding protein that can be activated by a number of external stimuli including viruses. The activation of Ras can lead to activation of the MAP kinase signalling pathway. I wanted to analyze the phosphorylation of tyrosine residues in virus infected MDCK cells. Therefore I infected MDCK cells with IV (MOI=1). At indicated time points cells were fixed permeabilized and stained with primary mouse anti-NP (green) and mouse anti-phosphor tyrosine specific antibodies (red) followed by FITC conjugated anti-mouse and Texas red conjugated anti-mouse secondary antibodies. The confocal laser scanning microscope result shows that receptor tyrosine kinase activity (which could activate MAPK via Ras) is not significantly elevated in virus infected cells. This indicates that therefore PKCs might also play a role.
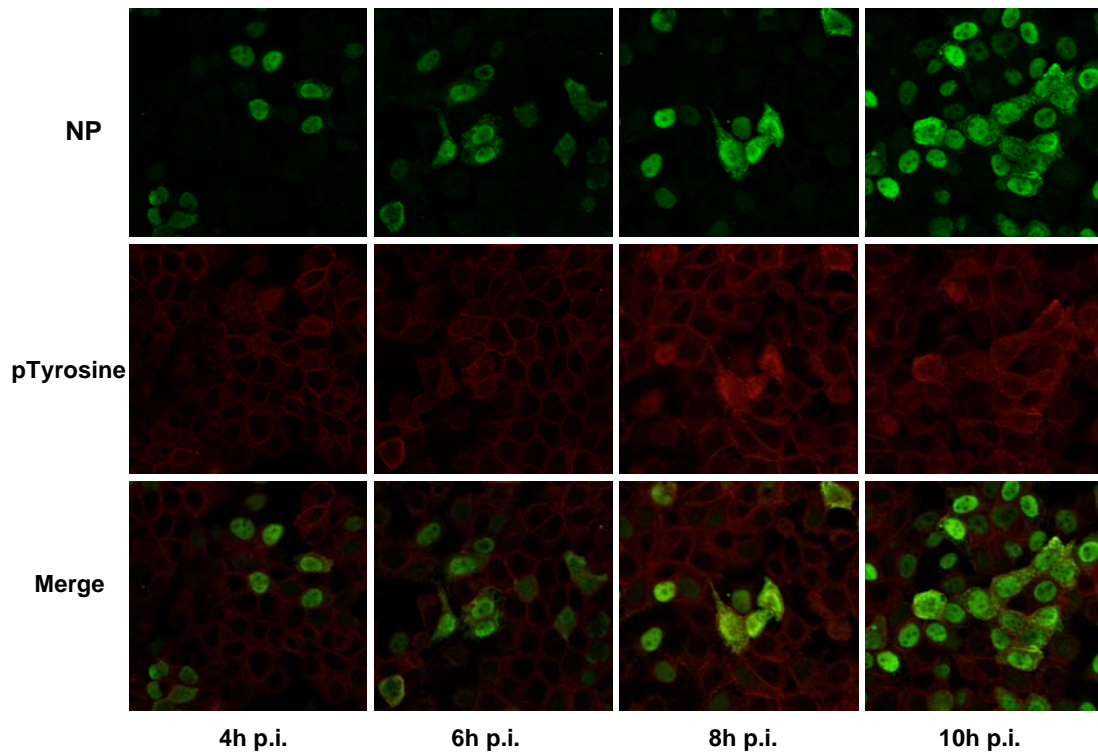
Fig 3.1: Confocal laser scanning microscopic analysis of tyrosine activity in Influenza virus (IV) infected cells: MDCK cells were infected with IV (MOI=1). At indicated time points cells were fixed permeabilized and stained with primary mouse anti-NP (green) and mouse anti-phosphor tyrosine specific antibodies (red) followed by FITC conjugated anti-mouse and texas red conjugated anti-mouse secondary antibodies.

3.2 Analysis of the importance of PKCα-activity versus Ras-activity in influenza virus infected cells.

It was also not determined yet that, what could be the upstream kinases for activating Raf/MEK/ERK signalling. There are two possible upstream kinases PKCα or Ras which could be involved in the virus induced MAPK activation. Previous studies (262), suggests that PKCα and or β are activated when human polymorphonuclear leukocytes were incubated with influenza virus. PKCβII isoform is activated in early influenza viral replication but not PKCα (263). On the basis of above mentioned literature, I wanted to compare the importance of PKCα or Ras on virus replication by analyzing virus titers. Therefore MDCK cells were transfected with plasmids expressing either dominant negative (DN-PKCα) or (DN-Ras) which reduces signalling via PKCα or Ras. 24 h p.t cells were infected with IV (MOI=1) and at indicated time points (6 and 8 h.p.i.) supernatant were collected and virus titer was determined. My comparative analysis shows that PKCα is more important for virus replication, while Ras play's a minor role in virus replication. These data argue against an important role of Ras for MAPK- activation in IV infected cells (265).
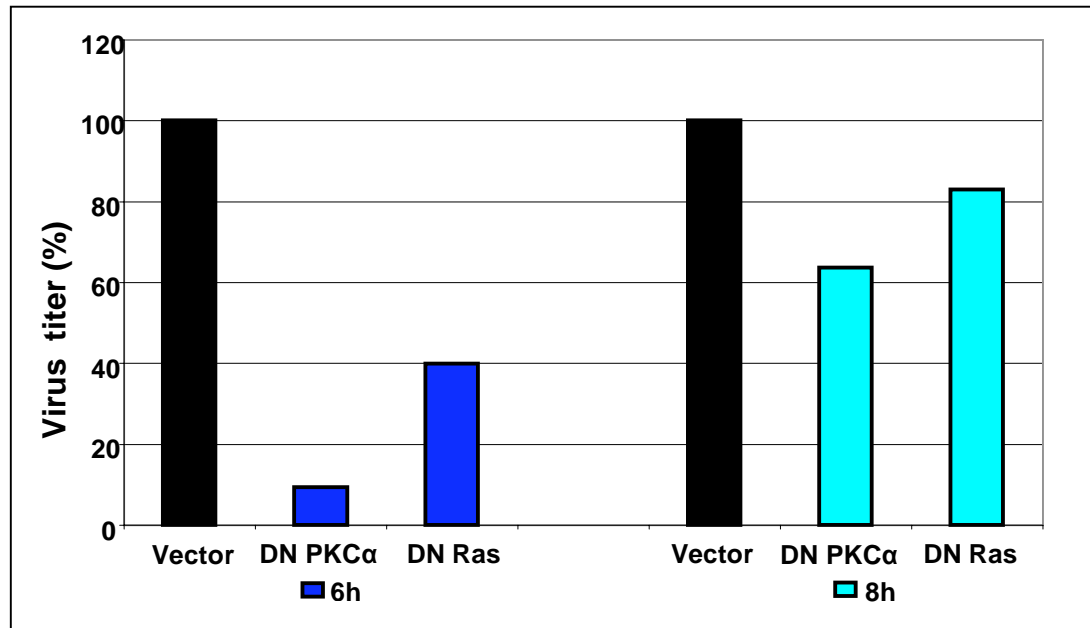
Fig 3.2: Comparative study of virus titration from the supernatants of FPV infected cells expressing Dn-PKCα or Dn-Ras (n=3): MDCK cells were transfected with plasmids expressing either DN-PKCα or DN-Ras. 24 h p.t cells were infected with IV (MOI=1) and at indicated time points (6 and 8 h.p.i.) supernatant were collected and virus titer was determined.

3.3 Analysis of the cell viability (+/-) Verapamil treatment

In order to assure that Verapamil (which is a calcium channel blocker and indirect inhibitor of PKCα) has no detrimental effect on cell survival at concentration (100μM) used in the experiments I investigated the cell viability by the MTT-Formazan test. This assay measures the activity of mitochondrial dehydrogenase in the living cells. 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is taken up by the cells and reacts with NADH, which is reduced to NAD. This reaction produces blue formazan crystals in living cells. The MTT assay shows that Verapamil has no significant toxic effect on cell survival.
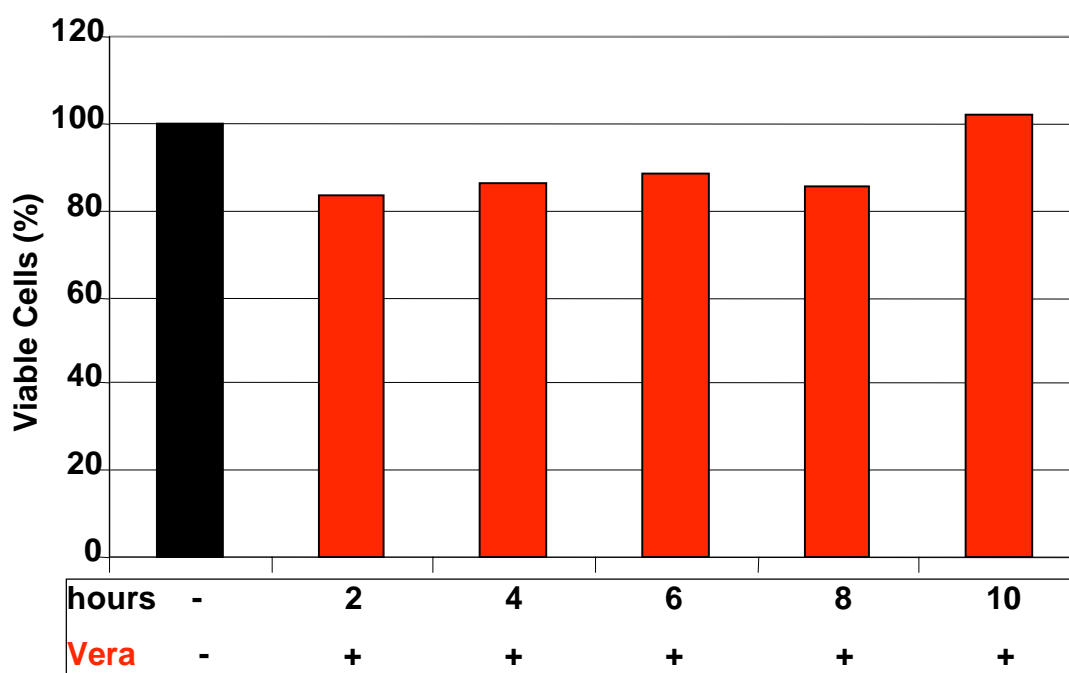
Fig 3.3: Analysis of the effect of Verapamil on cell survival or cell viability test (MTT) (4): A549 cells were treated with non-toxic concentration of Verapamil (100μM/ml) for different time points. MTT assay was performed.

3.4 Analysis of IV titers from infected A549 cells (+/-) Verapamil treatment

Literature shows that PKC-inhibitors reduce influenza virus entry if added early in infection. Also other mentioned literature suggests that Ca^{++} influx is increase in IV infected cells and PKCα is a calcium dependent kinase therefore I wanted to investigate the effect of Verapamil on virus replication by analyzing virus titers. Therefore A549 cells were infected with IV (MOI=5) and 2 h.p.i. cells were either left untreated or treated with Verapamil at non toxic concentration (100μM). At indicated time points virus supernatants were collected and virus titration was performed. The result shows that Verapamil leads to strong reduction in virus titers, which suggests that Verapamil is a potent inhibitor of virus replication. Therefore Verapamil can be used as antiviral therapeutic intervention against influenza. Verapamil is an already marketed and licensed drug for the treatment of the cardiovascular diseases like, heart

attack, high blood pressure, angina etc. Hence this drug could be interesting in the direction of antiviral approach.
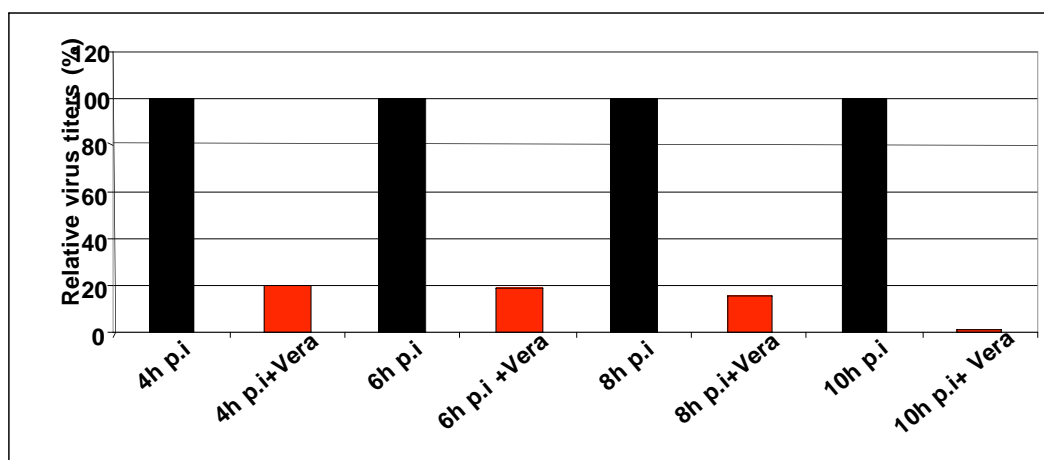

Fig 3.4: Plaque titration analysis of the inhibitory effect of Verapamil on virus replication (n=4): A549 cells were infected with IV (MOI=5) and 2 h.p.i. cells were either left untreated or treated with Verapamil at non toxic concentration (100μM). At indicated time points virus supernatants were collected and virus titration was performed.

3.5 & 3.6 Analysis of the effect of Verapamil on cellular protein production and on host cell shut-off mechanism respectively

Since Verapamil is a potent inhibitor of IV replication and might be used as antiviral drug. In order to make sure that Verapamil does not interfere with cellular protein production. I wanted to investigate the total protein amount in cells either left untreated or treated with Verapamil. Equal amount of A549 cells were seeded. After confluency cells were either left untreated or treated with Verapamil at non toxic concentration (100μM). At indicated time points cell lysates were made. Spectrophotometric analysis of each sample at 595 nm wavelength shows that there is no significant variation in the total amount of protein, which suggests that Verapamil does not inhibit cellular protein production (translation).

In virus infected cells ERK2 protein production seems to be reduced at late time points (8-10 h) due to host cell shut-off mechanism. Verapamil treatment seems to reduce this effect.
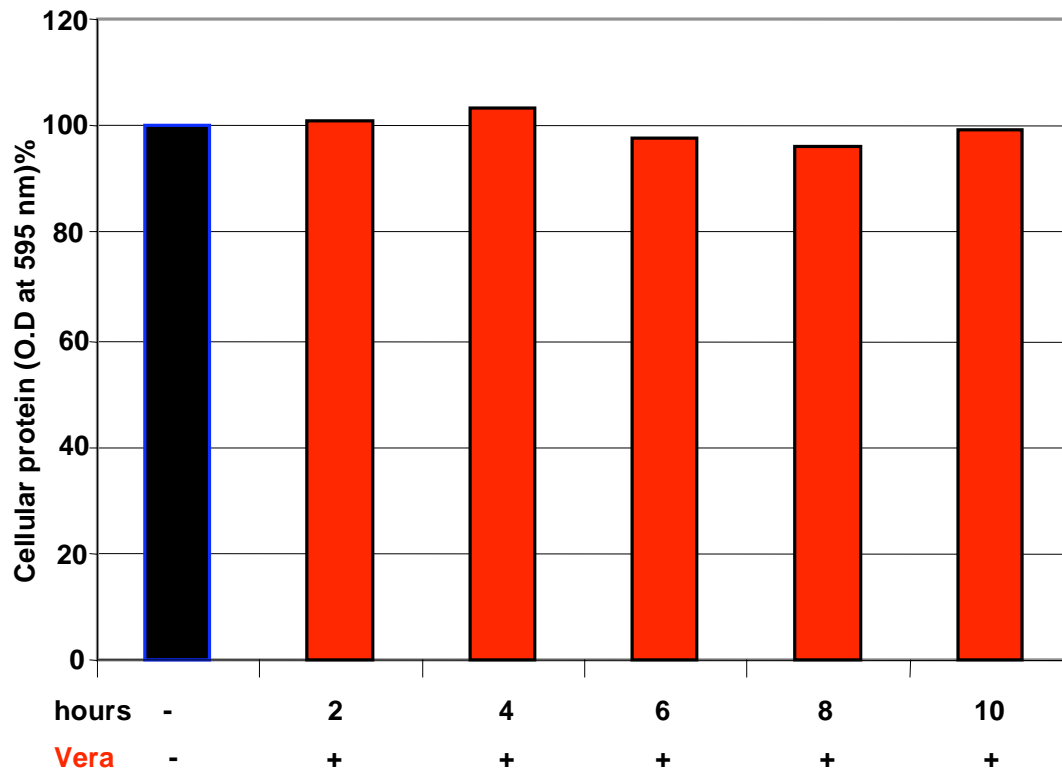
Fig 3.5: Spectrophotometric analysis of the effect of Verapamil on cellular protein production (n=4): Equal amount of A549 cells were seeded in 3.5 cm dishes. After confluency cells were treated with Verapamil at non toxic concentration (100μM). At indicated time points cell lysates were made. Spectrophotometrically O.D of each samples were measured.
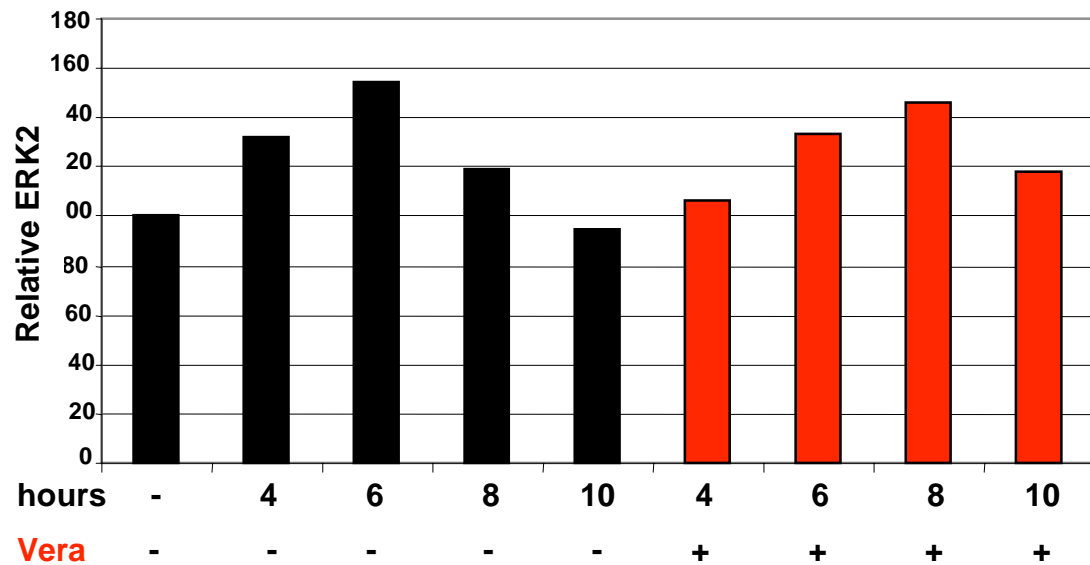
Fig 3.6: Western blot analysis of the effect of Verapamil on ERK2 protein production in IV infected A549 cells (n=3): A549 cells were infected with IV and either left untreated or treated with Verapamil at non toxic concentration (100μM). At indiacted time point's, cell lysates were made and analzed for ERK2 protein production.

3.7 Analysis of the PKCα-and ERK-activation in virus infected A549 (+/-) Verapamil

As mentioned in the literature that Raf/MEK/ERK (MAPK)-cascade is essential host function required for virus for its efficient replication and this cascade is activated at late time replication cycle. (254) indicates that IV infection leads to strong ERK-activation and MEK inhibitor (U0126) strongly reduces ERK-activation. In order to establish the connection between PKCα and ERK-activation (phosphorylation), I wanted to investigate the effect of Verapamil on virus induced Raf/MEK/ERK induction. Therefore A549-cells were infected with IV (MOI=1) and either left untreated or were treated with Verapamil (100μM/ml, 2 h p. i.), cell lysates were made at 8 h p.i and 10h p. i. and used for western blot-analysis using phosphor-specific mAbs. Loading was controlled using anti PKCα and ERK-specific mAbs.

The effect of Verapamil on virus-induced ERK-activation indicates that signal to the Raf/MEK/ERK (MAPK)-cascade is dependent on PKCα-activation.
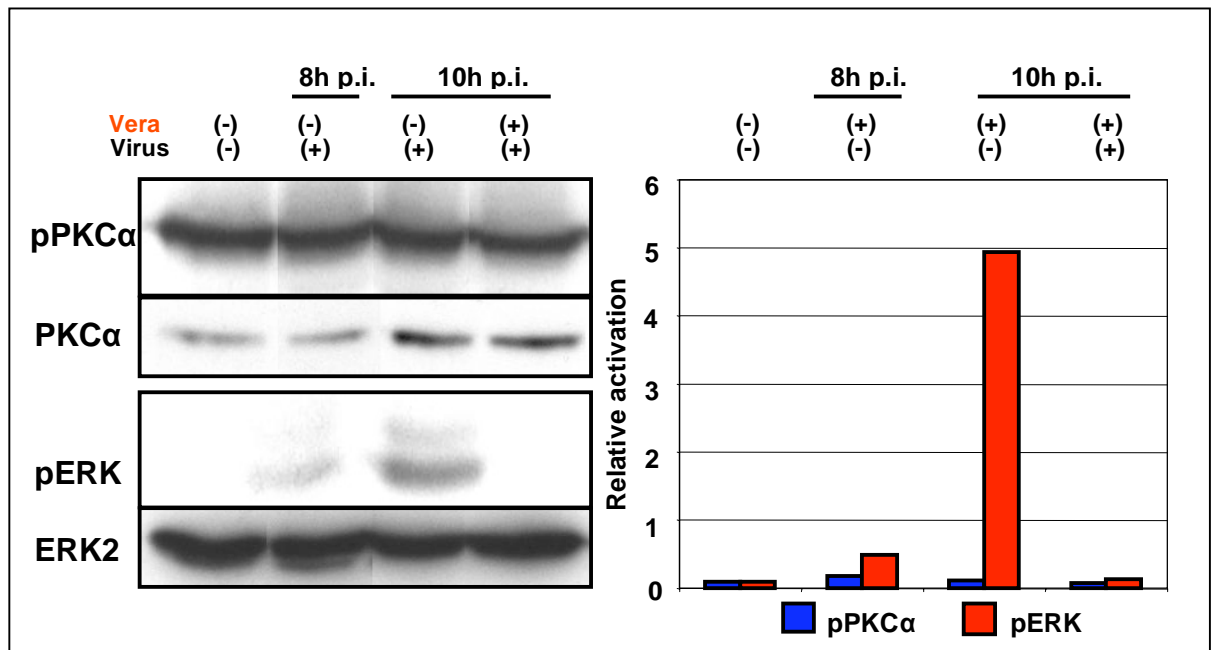

Fig 3.7: Western blot analysis of the PKCα- & ERK-activation (n=3): A549-cells were infected with IV (MOI=1) and either left untreated or were treated with Verapamil (100μM/ml, 2 h p. i.), cell lysates were made at 8 h p.i and 10h p. i. and used for WB-analysis using phosphor-specific mAbs. Loading was controlled using anti PKCα and ERK-specific mAbs.

3.8 Analysis of the PKCα-and ERK-activity in virus infected A549 (+/-) Verapamil

The previous result shown in the above experiment suggests that ERK is activated via PKCα signalling. The negative effect of Verapamil was strong on ERK-activation (phosphorylation) while the effect was weak on PKCα-activation (phosphorylation). Therefore the idea came that Verapamil might not block the activation of PKCα but PKCα-activity might be blocked. Hence I wanted to investigate the activity of PKCα and ERK using MBP as a substrate by immune complex kinase assay (ICA). A549-cells were infected with IV (MOI=1) and either left untreated or were treated with Verapamil (100μM/ml, 2h p.i.), cell lysates were made at 8 h p.i and 10 h p.i. and used for ICA, to analyze PKCα- and ERK kinase-activity respectively. Loading was controlled with anti PKCα and ERK-specific mAbs in WB-analysis. The result shows that Verapamil strongly reduces ERK activity and to a minor extent PKCα-activity. This indicates that virus induced Raf/MEK/ERK-signal transmission is dependent on PKCα-activity. (This work was performed in collaboration of Prof. Dr. Stephan Ludwig, University of Munster, Germany).
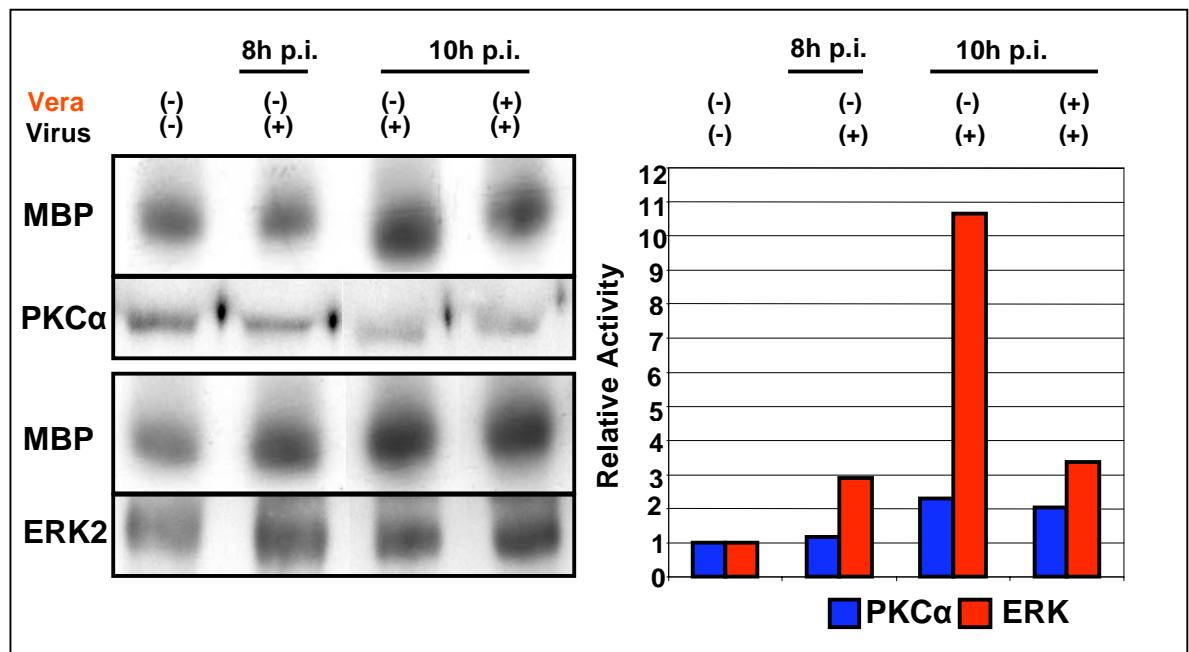
Fig 3.8: Immune complex assay (ICA) analysis of PKCα- & ERK-activity (n=2):
A549-cells were infected with IV (MOI=1) and either left untreated or were treated with
Verapamil (100µM/ml, 2h p.i.), cell lysates were made at 8 h p.i and 10 h p.i. and used for
ICA, using myelin basic protein (MBP) as substrate indicating PKCα- and ERK kinase-
activity respectively. Loading was controlled with anti PKCα and ERK-specific mAbs in
WB-analysis.

3.9 Analysis of the intracellular localization of pPKCα in TPA activated A549 cells (Control experiment)

In normal state PKCα is cytoplasmic. Literatures also suggest that TPA induced PKC -α is translocated to the plasma membrane. This experiment was used as positive control to demonstrate virus induced PKCα localization in virus infected cells. The confocal result indicates that TPA induced phosphorylated PKCα (pPKCα) is localized into plasma membrane from the cytoplasm.
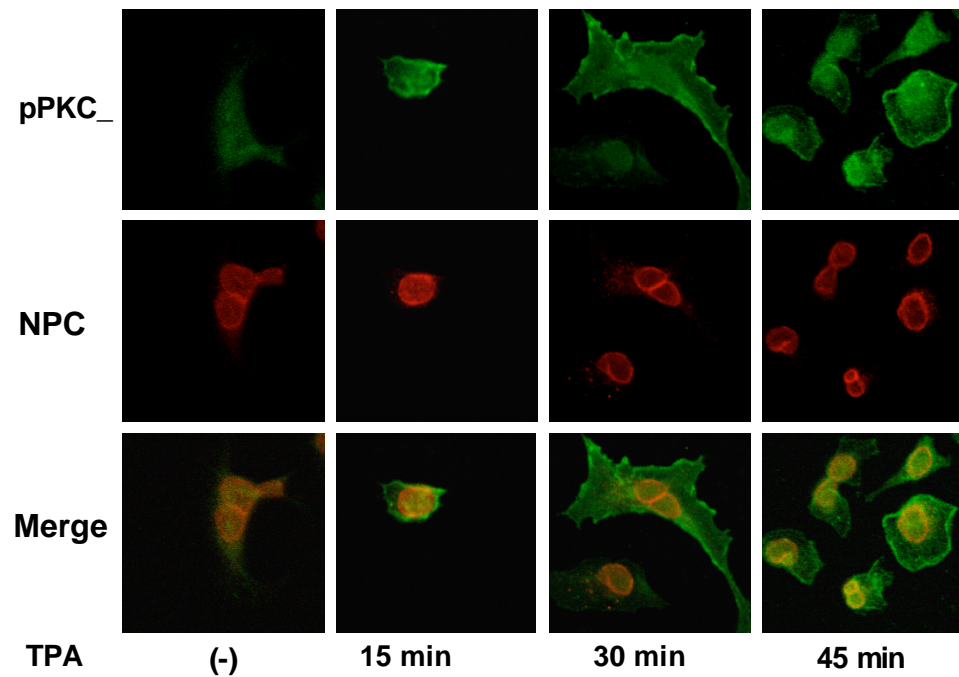
Fig 3.9 : Confocal laser scanning microscopic analysis of intracellular localization of phosphorylated PKCα: A549 cells were stimulated by TPA (200ng/ml). At indicated time points post treatment cells were fixed and stained with primary goat anti-pPKCα and mouse anti-NPC. Cells were then washed and incubated with secondary FITC conjugated anti-goat and texas red conjugated anti-mouse antibodies. The result indicates that TPA induced phosphorylated PKCα (pPKCα) is localized into plasma membrane from the cytoplasm. Nuclear pore complex (NPC) to visualize the nucleus is detected by using mouse anti-NPC antibodies (red).

3.10, 11 & 12 Analysis of the RNP-export in virus infected A549 cells (+/-) Verapamil treatment

I have shown in my previous result, that Verapamil strongly reduces virus titers. Literature indicates that RNPs of influenza virus are essentially exported out from nucleus to the cytoplasm for the production of new infectious progeny virus. The inhibitory effect of Verapamil on virus replication raised interesting question regarding PKCα-activation and RNP-export. Therefore I wanted to investigate the PKCα-activation and RNP-export in untreated and treated cells. To this point A549 cells were infected with IV (MOI=1). 2 h.p.i cells were either left untreated or treated with Verapamil. At indicated time point's p.i, cells were fixed permeabilized and stained with primary mouse anti-NP and goat anti-pPKCα antibodies followed by secondary FITC conjugated anti-mouse and CY5 conjugated anti-goat antibodies. Confocal laser scanning microscope result shows that late in the virus replication cycle (8-10 h), PKCα is activated at a time point when RNPs are exported out from nucleus to the cytoplasm. That suggests that RNP-export is connected to PKCα activation and that PKCα-activation and RNP-export are strongly blocked by Verapamil leading to inefficient virus replication.

NP

pPKCα

Merge

Virus   **(-)**   **4h**   **6h**   **8h**   **10h**

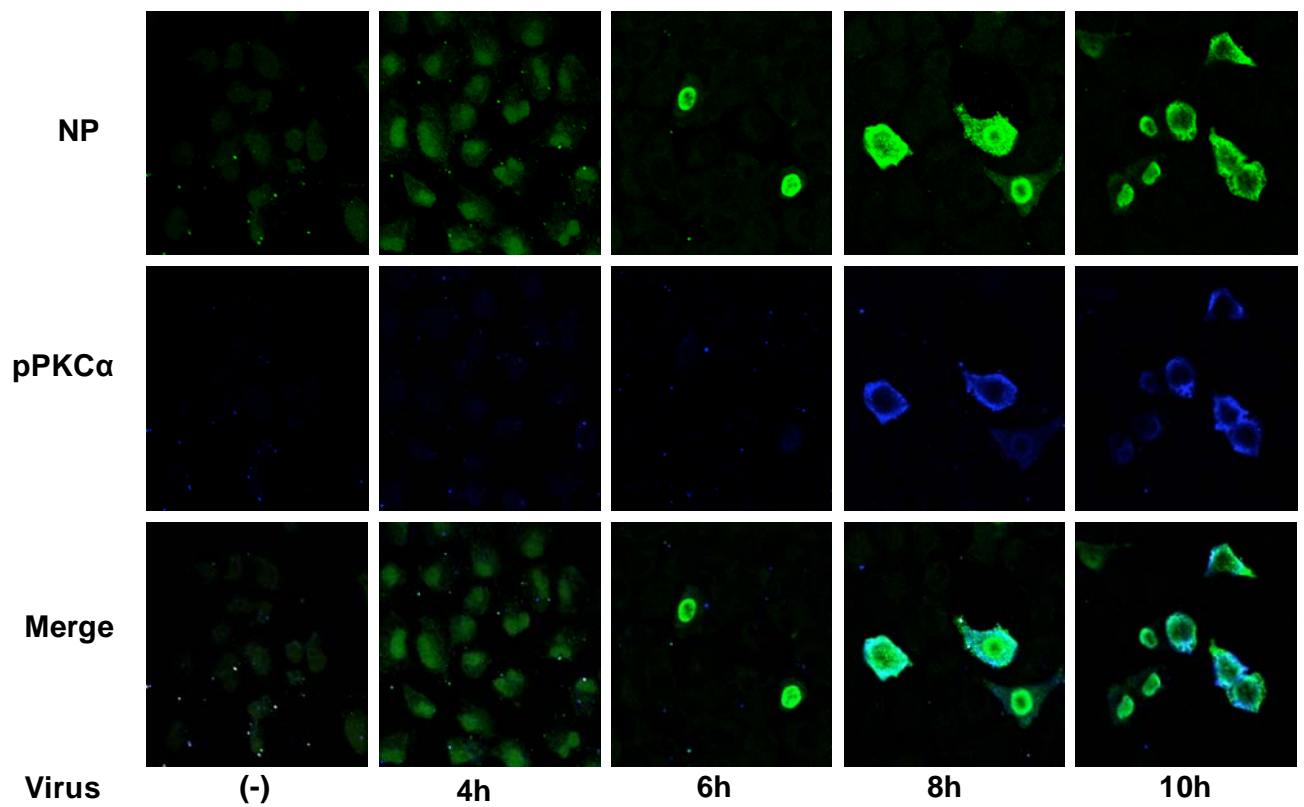**Fig 3.10: Confocal laser scanning microscopic analaysis of the RNP-export and activation of PKCα (pPKCα) in IV (MOI=1) infected A549-cells:** A549 cells were infected with IV (MOI=1). At indicated time point's p.i cells were fixed permeabilized and stained with primary mouse anti-NP and goat anti-pPKCα antibodies followed by secondary FITC conjugated anti-mouse and CY5 conjugated anti-goat antibodies.

NP					
pPKCα					
Merge					
Virus	(-)	4h	6h	8h	10h
Vera	(-)	(+)	(+)	(+)	(+)

Fig 3.11: Confocal laser scanning microscopic analysis of the effect of Verapamil on RNP-export: A549 cells were infected with IV (MOI=1) and after 2 h p.i cells were treated with Verapamil at non toxic concentration (100μM). At indicated time point's p.i cells were fixed permeabilized and stained with primary mouse anti-NP and goat anti-pPKCα antibodies followed by secondary FITC conjugated anti-mouse and CY5 conjugated anti-goat antibodies.
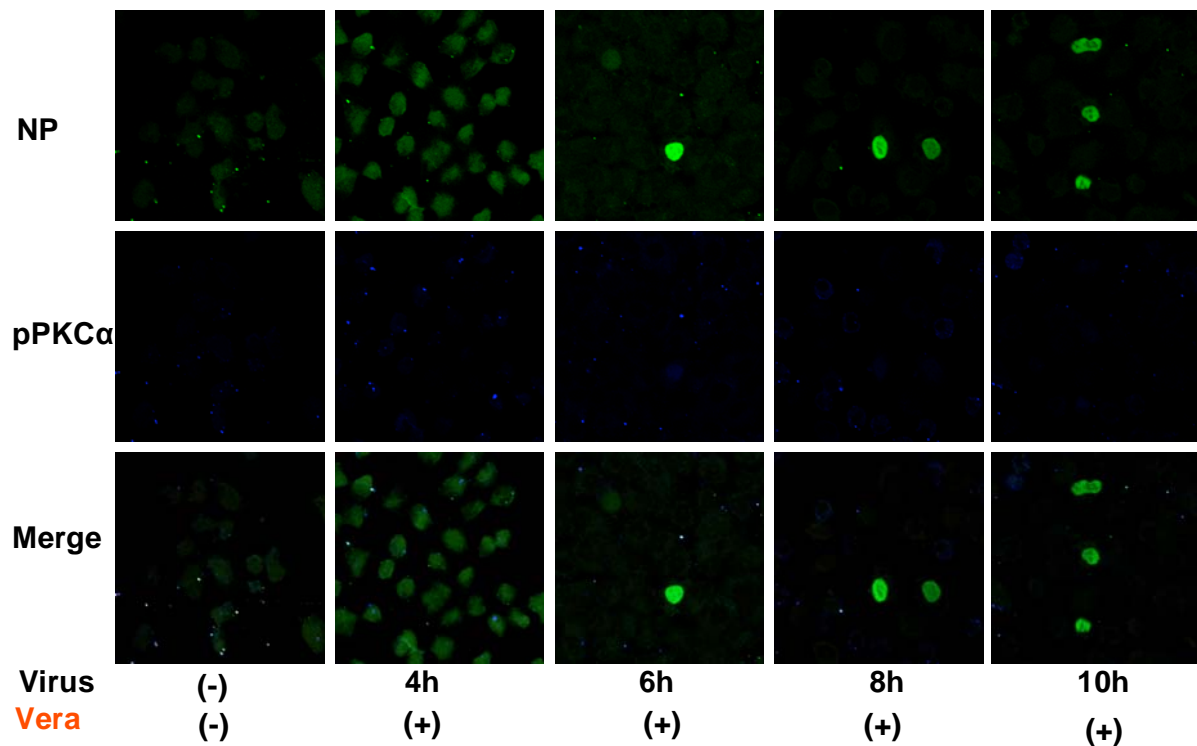
Fig 3.12: Confocal laser scanning microscopic analysis of PKCα- activation in IV infected MDCK cells: MDCK cells were infected with IV (MOI=1). 2 h p.i cells were either left untreated or treated with Verapamil at non toxic concentration (100μM). At indicated time point's cells were fixed, permeabilization and stained with primary mouse anti-NP antibodies and goat anti-pPKCα antibodies followed by secondary FITC conjugated anti-mouse and Cy5 conjugated anti-goat antibodies. (265)
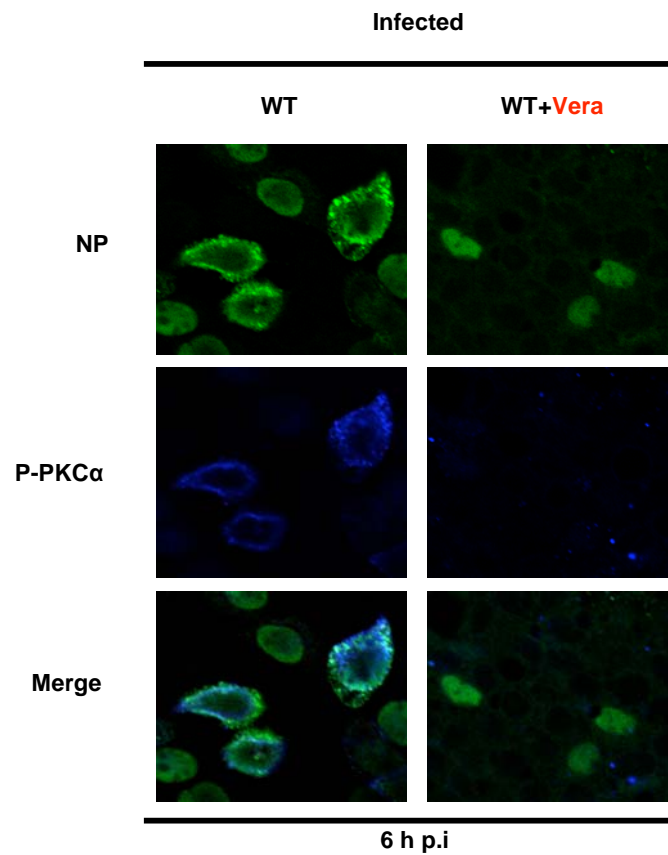
3.13.1& 3.13.2 (A-D) Analysis of viral protein production in virus infected A549 cells (+/-) Verapamil treatment

In the first experiment I analyzed the inhibitory effect of Verapamil on PKCα mediated Raf/MEK/ERK signalling, RNP-export and virus replication. In addition to this effect, analysis of additional inhibitory effect of Verapamil on viral protein production was an interesting question for me. Therefore I wanted to investigate the effect of Verapamil on different viral proteins (PB1, NP, M1 and NS1) production level. A549 cells were infected with IV at (MOI=1) and 2 h.p.i cells were treated with Verapamil at non-toxic concentration (100µM). After indicated time points p.i and treatment, cell lysates were made and were used for western blot for viral protein detection. Blots were incubated with primary antibodies (goat anti-PB1-, 1:200; mouse anti-NP-, 1:200; mouse anti-M1-, 1:500; and goat anti- NS1-1:500) and subsequently with HRP-conjugated secondary antibodies. Equal amounts of ERK2 protein were loaded. Western blot result shows that Verapamil strongly inhibits the amount of viral proteins (PB1, NP, M1 and NS1) production, which suggests that Verapamil might also effect viral replication and/or transcription.

Fig 3.13.1 belongs to one set of experiment where triplicate samples were pooled together at indicated time points post infection (+/-) Verapamil and analyzed for the detection of viral proteins (PB1, NP and NS1).

Fig 3.13.2 (A-D) belongs to another set of experiment where also triplicate samples were pooled together at indicated time points post infection (+/-) Verapamil and were analyzed for viral proteins (PB1, NP, M1 and NS1).

Both results (Fig 3.13.1 & Fig 3.13.2 (A-D) indicate that Verapamil strongly reduces viral protein production that might be one of the potent causal factor for the inhibition of virus replication.
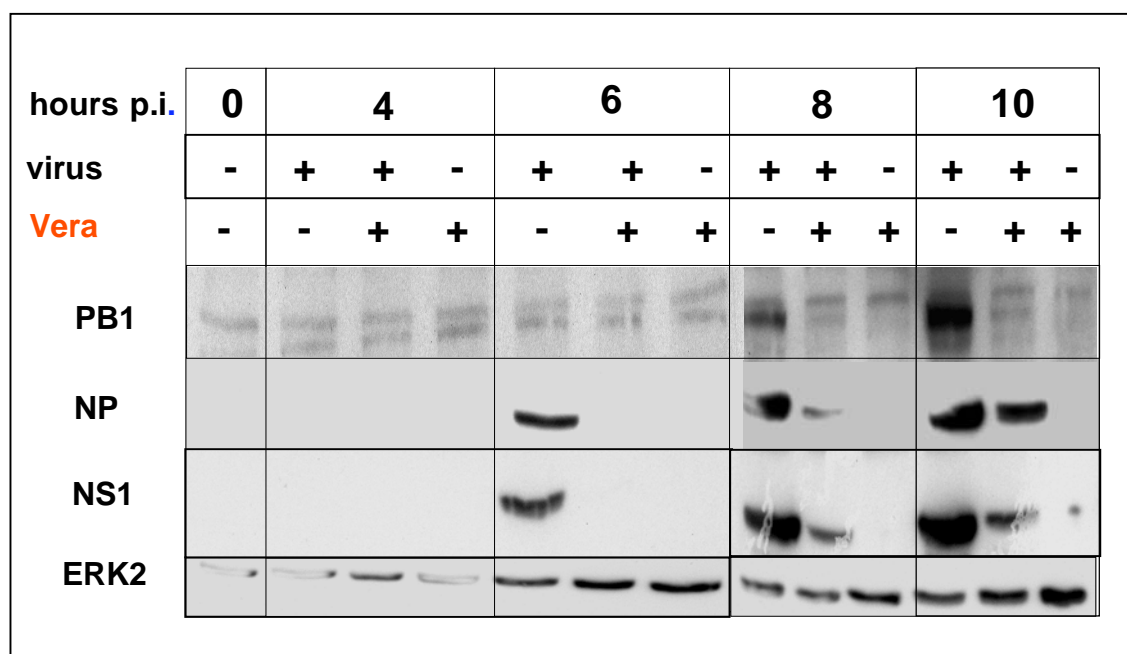
Fig 3.13.1: Western blot analysis of the inhibitory effect of Verapamil on viral protein production: A549 cells were seeded in 3.5 cm dishes and were infected with IV at (MOI=1) and 2 h.p.i cells were treated with Verapamil at non-toxic concentration (100μM). After indicated time points cell lysates were made and were used for viral protein detection. Blots were incubated with primary antibodies (anti-goat PB1-, 1:500; anti-mouse NP-, 1:500; and anti-goat NS1-1: 500) and subsequently with HRP-conjugated secondary antibodies. Equal amounts of protein were loaded (ERK2).

Virus	-	+	+	+	+	+	+	+	+
Vera	-	-	-	-	-	+	+	+	+
Hour	0	4	6	8	10	4	6	8	10
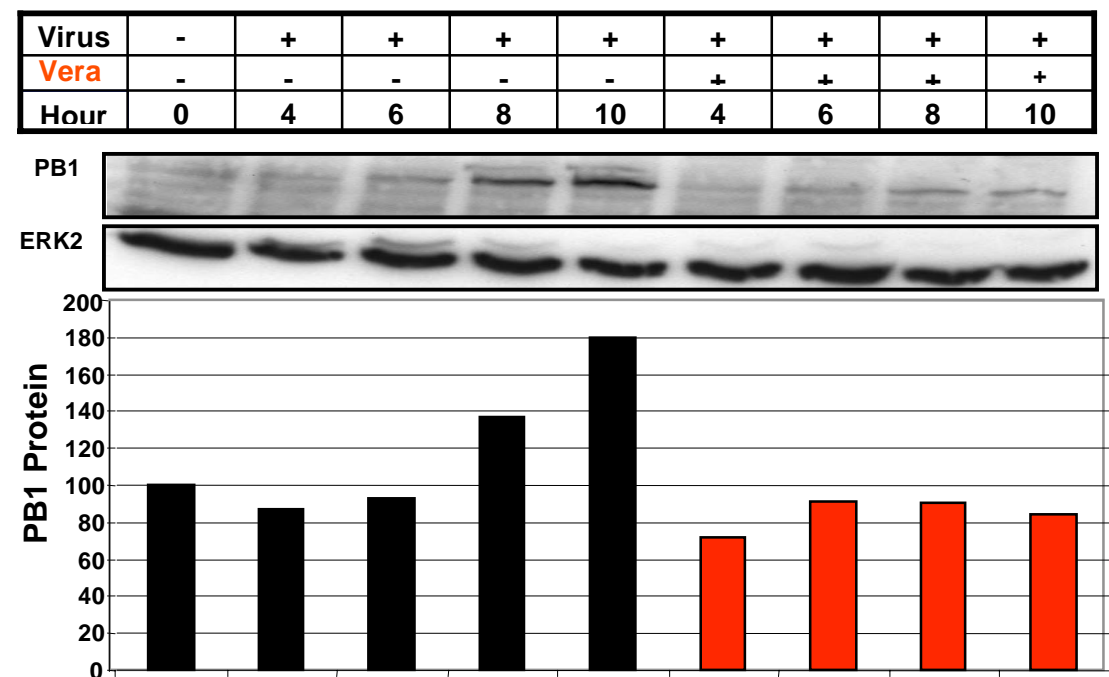
Fig 3.13.2 (A) Production of PB1 protein of influenza virus.

Virus	-	+	+	+	+	+	+	+	+
Vera	-	-	-	-	-	+	+	+	+
Hour	0	4	6	8	10	4	6	8	10
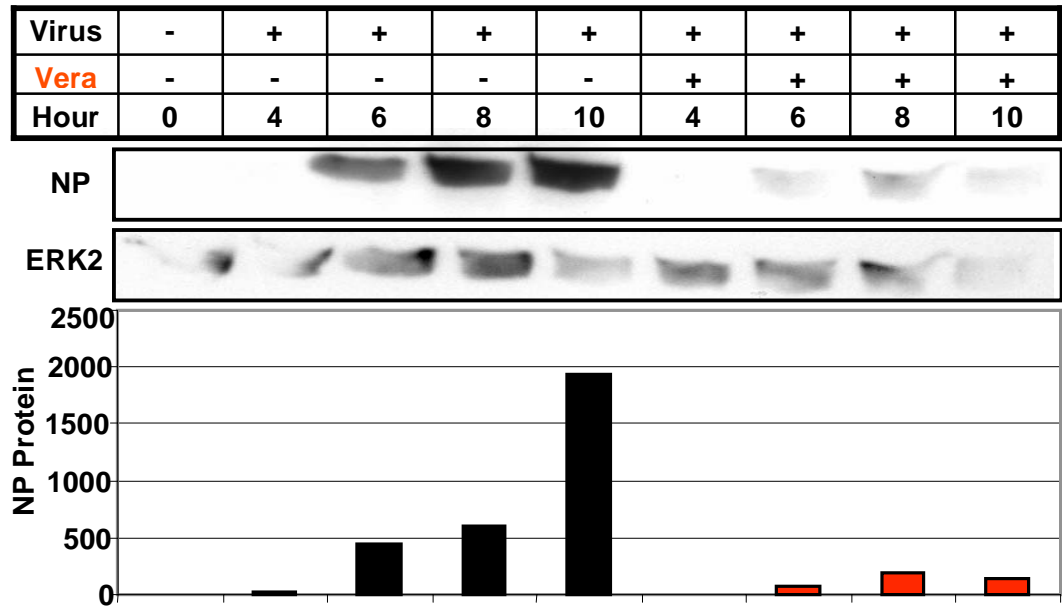
Fig 3.13.2 (B) Production of NP protein of influenza virus.

Virus	-	+	+	+	+	+	+	+	+
Vera	-	-	-	-	-	+	+	+	+
Hour	0	4	6	8	10	4	6	8	10
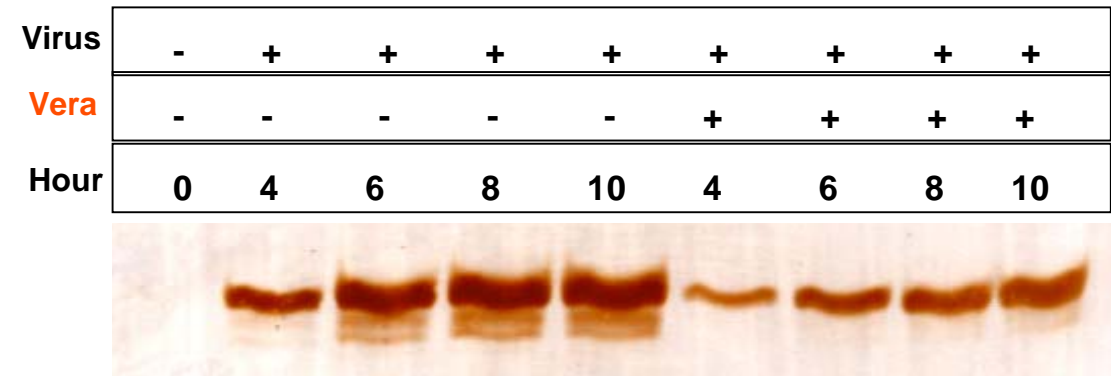
Fig 3.13.2 (C) Production of M1 protein of influenza virus. (DAB/Urea staining)

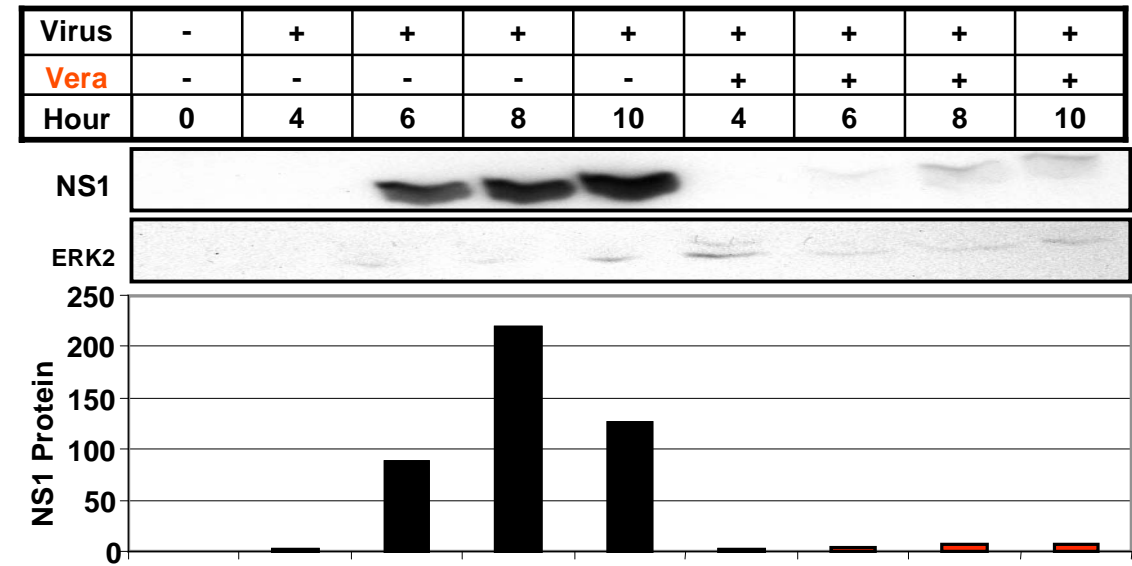| Virus	-	+	+	+	+	+	+	+	+
Vera	-	-	-	-	-	+	+	+	+
Hour	0	4	6	8	10	4	6	8	10

NS1

ERK2

Fig: 3.13.2 (D) Production of NS1 protein of influenza virus.

3.14 (A & B) Primer extension analysis of the effect of Verapamil on viral mRNA production

The above result of the analysis of the effect of Verapamil on viral protein production provided the formal proof that inhibition of viral protein production by Verapamil could be due to the impairment of viral replication and/or transcription. The above result also shows that Verapamil strongly inhibits PB1 protein which is one of the subunits of the viral polymerase. Therefore I wanted to analyze the effect of Verapamil on viral polymerase activity by quantifying different RNA (cRNA, vRNA and mRNA) species production using Primer Extension Assay.

293T cells were seeded in 3.5 cm dishes and were either transfected with 5 plasmids for the expression of the viral polymerase subunits, the NP and a virus-like vRNA (pHMG-PB1, -PB2, -PA, -NP, and pPOL1-CAT-RT) (1, 2) or with the pPOL1-CAT-RT plasmid alone and then super infected with IV (MOI=1) 24h p.t. (3, 4). 32 h p.t. (1, 2) or 8 h p.i. (3, 4) total RNA was isolated and used for primer extension analysis. The relative amount of virus-like mRNA normalized to a loading control (5SrRNA) was calculated. Result indicates that Verapamil seems to have a negative effect on viral mRNA production in virus infected cells, while it seems not to effect mRNA production in a plasmid based replication system. This indicates that the effect of Verapamil on viral transcription depends on virus infection.

Fig 3.14 (B) shows the original 8% sequencing gel picture for the quantification of mRNA production. Fig 3.14 (A) is the derivatives of Fig 3.14 (B)
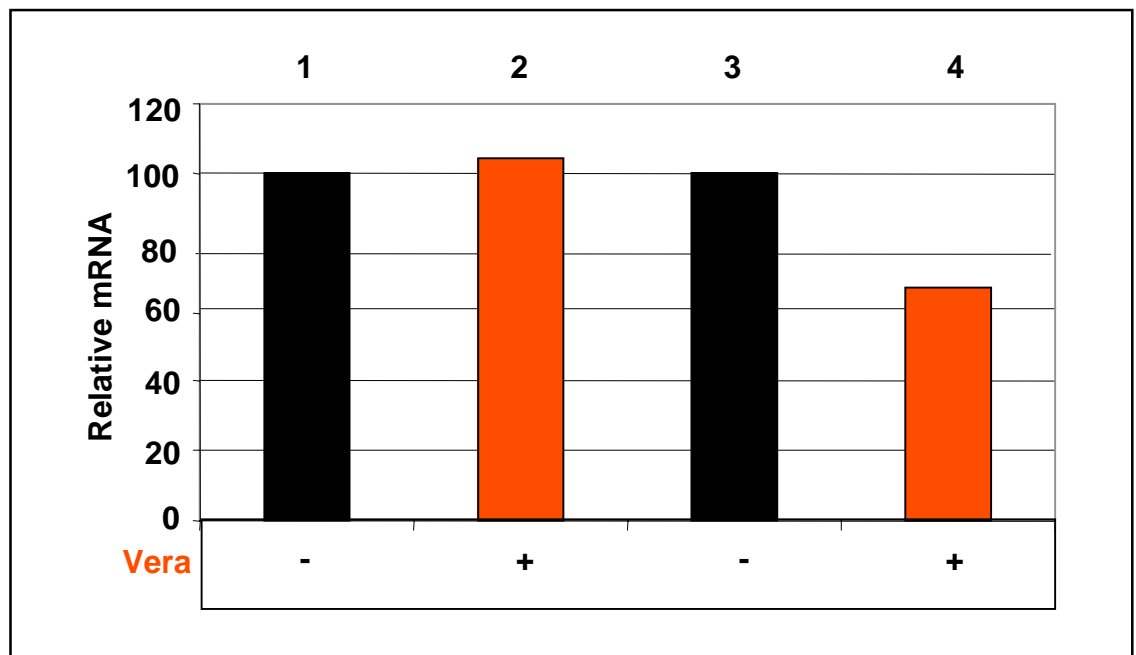
Fig 3.14 (A): Primer extension analysis of the effect of Verapamil on viral polymerase activity (n=2): 293T cells were seeded in 3.5 cm dishes and were either transfected with 5 plasmids for the expression of the viral polymerase subunits, the NP and a virus-like vRNA (pHMG-PB1, -PB2, -PA, -NP, pPOL1-CAT-RT) (1, 2) or with the pPOL1-CAT-RT plasmid alone and then super infected with IV (MOI=1) 24h p.t. (3, 4). 32 h p.t. (1, 2) or 8 h p.i. (3, 4) total RNA was isolated and used for primer extension analysis. The relative amount of virus-like mRNA normalized to a loading control (5SrRNA) was calculated.
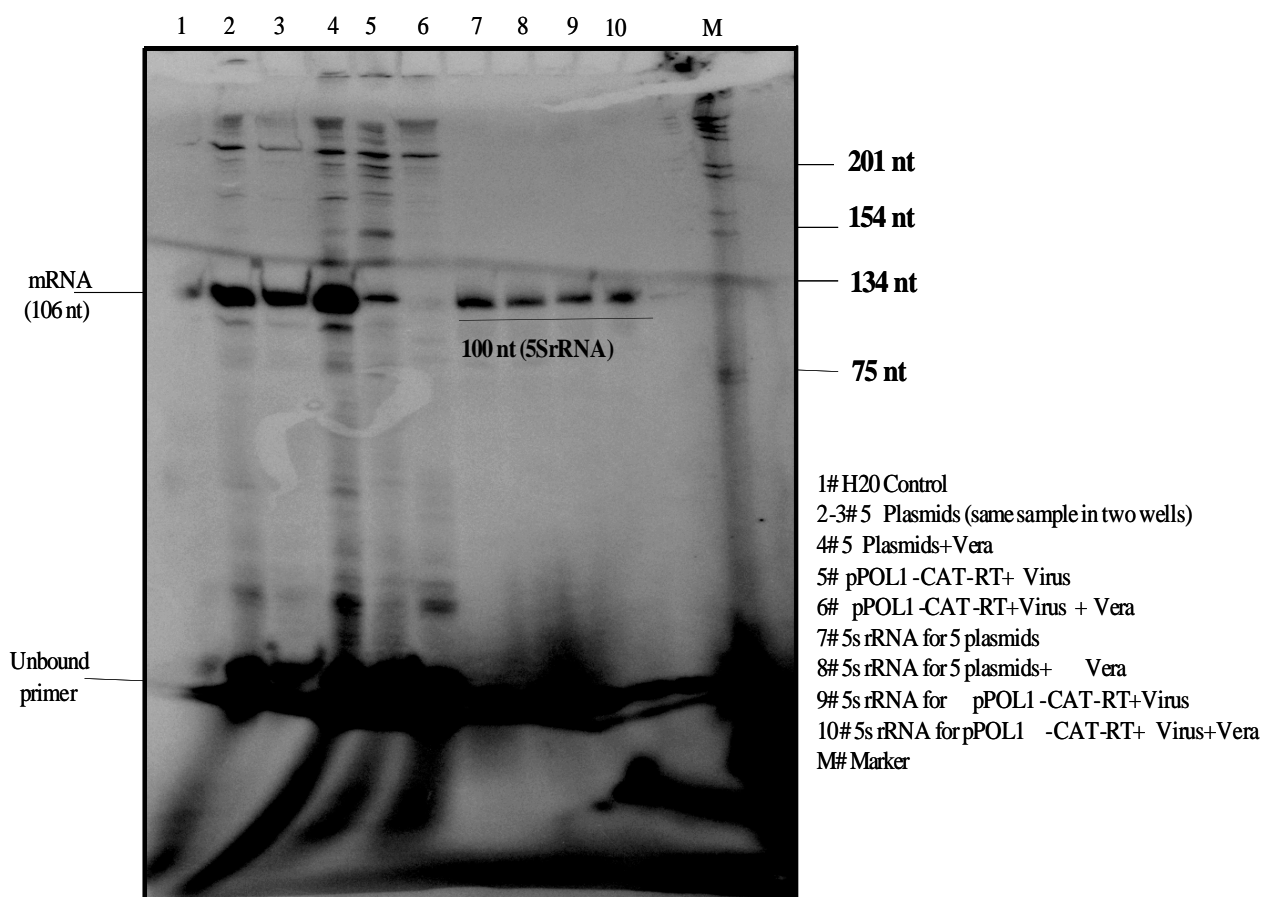
Fig 3.14 (B): 8% sequencing gel picture of the primer extension analysis of the effect of Verapamil on viral mRNA production.

5 μl trizole isolated RNA was used for making cDNA. cDNA was further used for primer extension with the primer designed for the amplification of the product length of the viral mRNA which is about 106 nuclotide in length. I also used 5SrRNA as a loading control for the respective samples to be sure that the samples were loaded equally. Lower intense signals are radioactive labeled primers which did not bind with the target sequence. H20 was also used as a negative control where we see no amplification.

3.15 Analysis of the expression of CAT-protein by transfecting 293T cells with pPOL1-CAT-RT along with four helper plasmids (pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP)

pPOL1-CAT-RT plasmid transfection leads to the synthesis of the viral RNA like transcript and it is under the control of cellular promoter. The newly synthesized vRNA like transcript is dependent on the viral polymerase for its transcription and replication leading to the expression of CAT-protein. To demonstrate the effect of Verapamil on viral polymerase that might lead to reduced expression of CAT-protein, therefore I was interested to perform CAT-assay. This experiment is just a positive control experiment for the further analysis of the effect of Verapamil on viral polymerase activity by analyzing CAT-protein expression either in Verapamil treated or untreated samples.

My preliminary result shows that chloramphenicol is acetylated by CAT enzyme that suggests that CAT-enzyme expression is efficient for minireplicon system. On the basis of this result further analysis of the effect of Verapamil on CAT-protein expression, could be exercised. Unacytylated form of chloramphenicol was observed in the maximum dilution (10^{-3}) of the extracted enzyme that suggests that acetylation of chloramphenicol is inversely proportional to the dilution factor.
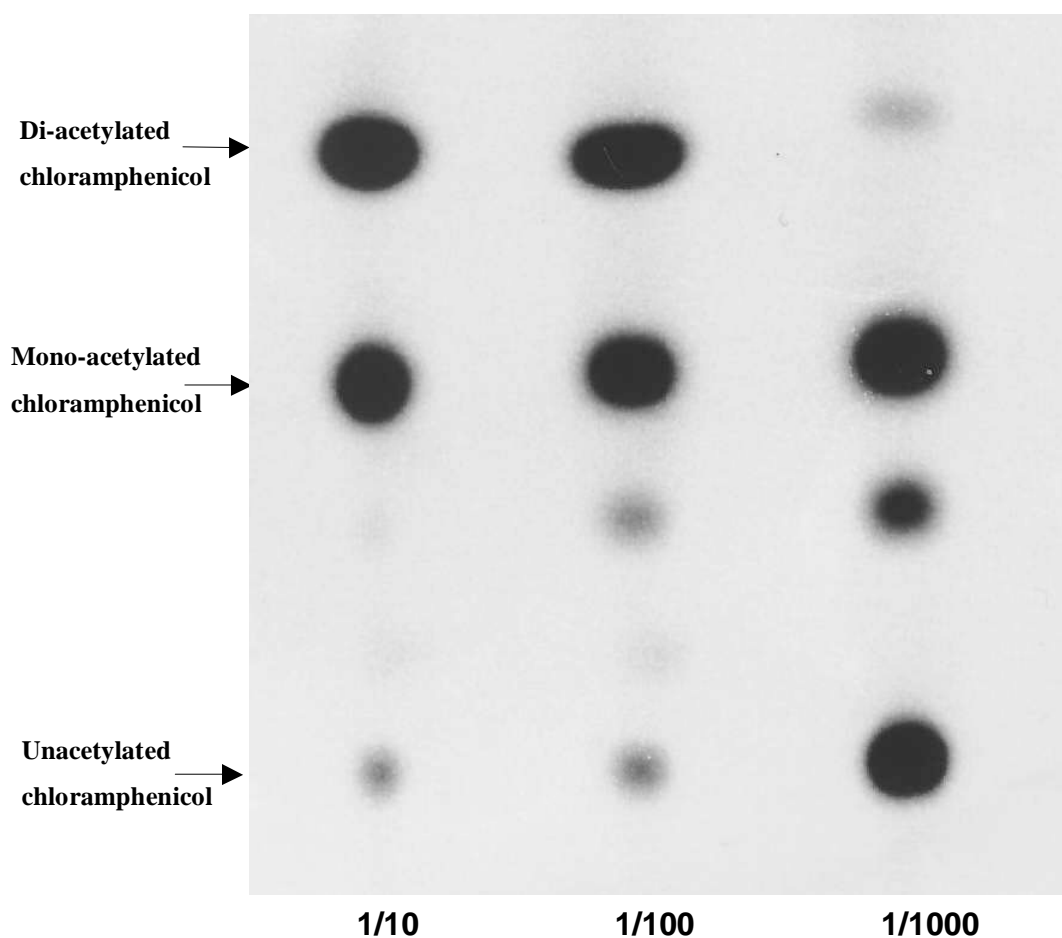
Fig: 3.15 Chloramphenicol acetyl transferase analysis of the CAT protein expression: 293T cells were transfected with pPOL1-CAT-RT. After 48 h p.t. cell lysates were made and used for CAT assay. Enzyme extracts were diluted as 10^{-1}, 10^{-2}, and 10^{-3}. The reaction mixture was placed on TLC sheet and lowered into the TLC chamber with solvent in the bottom. Spotted reaction mixture on TLC plate was allowed to migrate up the TLC sheet. When the solvent reaches the top of the TLC sheet, it was stopped and allowed to dry. Later it was to be quantified by typhoon but I have not quantified the visible spots on the sheet. The amount of acetylation is directly proportional to the amount of CAT enzyme present.

Discussion

Influenza A and B viruses are important worldwide pathogens for humans and animals and cause devastating epidemic and pandemic outbreaks and is considered as an annual major public health threat. With the realization that future pandemic of influenza in humans is inevitable and perhaps imminent. One must look at the therapeutic intervention of antiviral agents against influenza.

Previous studies (254) have revealed that both influenza A and B viruses activate the cellular Raf/MEK/ERK (MAPK)-signalling cascade. The virus-induced MAPK signalling is activated late in the viral replication cycle, and RNP nuclear export is dependent on this cellular function and block of this signalling pathway using MEK inhibitors (U0126) at non toxic concentration strongly reduces ERK-activation, RNP-export and virus titers thereby leading to inefficient virus replication without the emergence of resistant variants (266).

However additional information was needed in order to understand the mechanism of MAPK-signalling and for subsequently targeting the virus induced signalling pathway as an anti-viral approach.

MAPK cascades are key regulators of cellular responses such as proliferation, differentiation, and apoptosis. The Raf/MEK/ERK cascade is the prototype of MAPK cascades. Growth factor-induced signals are transmitted by consecutive phosphorylation from the kinase Raf via MEK to ERK, which translocates to the nucleus to phosphorylate a variety of substrates. The MAPK-cascade is normally activated by membrane proximal events through activation of PKCs or through activation of Ras.

The literature mentioned in the introduction part indicates that in virus infected cells there is a significant calcium influx and other literature suggest that a calcium channel blocker (Verapamil) inhibits virus replication. It means that calcium is somehow playing a role in virus induced signalling pathway. As classical PKCs are dependent on calcium therefore the idea came that Verapamil might block calcium influx from calcium storage to the cytoplasm to demonstrate the effect of calcium dependent PKCα on virus induced Raf/MEK/ERK signalling.

As discussed in the introduction part PKCα and PKCβ are classical PKCs and they are activated by Ca^{2+} and DAG (diacyl glycerol). PKC-inhibitors reduce virus entry if added early in infection. PKCβII isoform is activated in early influenza viral replication but not PKCα (263).

These above mentioned data have been the cornerstone for the further investigation of the role of calcium induced PKCs in influenza virus infection and of PKCα as a particular factor of interest for virus-induced Raf/MEK/ERK-signalling, but not of PKCβII because it was shown to be involved during virus entry.

As I introduced in the beginning of this discussion part the inhibitory effect of the MEK inhibitor (U0126) on virus replication, which became one of interesting piece of research to me to further investigate, the upstream cellular factors to be involved in the mechanism of signal transmission to the MAPK-cascade in virus infected cells. Since it was not determined yet that, what could be the upstream kinases for activating Raf/MEK/ERK signalling therefore data from (252) was further elaborated by me to investigate the role of PKCα as a cellular factor which could be responsible for MAPK-cascade activation. There are two possible upstream kinases either PKCα or Ras, which could be involved in the virus induced MAPK activation. Therefore I used Verapamil which is an indirect inhibitor of PKCα to analyze its effect on PKCα mediated Raf/MEK/ERK signalling as well as on viral factors (viral proteins and viral polymerase).

In context to the importance of Ras involvement in virus replication, my first experiment was to investigate the phosphorylation status of tyrosine residues in virus infected cells as Ras is being activated by tyrosine kinases. This could mean that receptor tyrosine kinase in virus infected cells could lead to the phosphorylation of the tyrosine residues of Ras and to its activation. Confocal laser scanning microscope result **(shown in the fig 3.1)** demonstrates that the tyrosine phosphorylations in virus infected cells are weak suggesting that Ras-activity is involved in virus replication to a minor extent.

Therefore I was interested to demonstrate in a comparative analysis of the importance of PKCα versus Ras. To this point I investigated the virus titers from infected cells

either expressing dominant negative PKCα or Ras. The titer result (**shown in the fig 3.2**) indicates that PKCα seems to be more important for virus replication than Ras, nevertheless Ras also plays a role in virus replication but to a minor extent. This result supports the concept that PKCα-activity is important in virus replication. I would also like to critically discuss here that within the two hours interval (6-8 hours) the virus titers result is slightly increased. That indicates that the effect of dominant negative PKCα or Ras is not able to block signalling completely. The fact that cellular PKCα and Ras are also expressed which supports the data of a slight increase of the virus titers within the two hours interval.

Ongoing data interested me for further analysis of the effect of calcium channel blocker Verapamil on virus replication. As I mentioned in the introduction part that, virus infection leads to calcium influx and PKCα which is a calcium dependent kinase might play a role in the activation of Raf/MEK/ERK signalling cascade, which is a prerequisite for virus replication. Therefore I infected the cells and two hours post infection I treated the cells with Verapamil, and at indicated time points virus supernatant was collected and analyzed for virus titration. The result (**shown in the fig 3.4**) shows that Verapamil strongly inhibits virus replication supporting the idea that PKCα induced Raf/MEK/ERK signalling is a prerequisite for virus propagation. Here I would like to mention that I treated the cells with Verapamil after two hours post infection to avoid any effect of Verapamil on virus entry. If Verapamil treatment was done at the time of infection virus entry into the cells might be affected, as it was shown that PKCβII is important for virus entry. My aim was to analyze the effect of Verapamil on the late phase of virus replication, when the MAPK-cascade is activated.

Since Verapamil strongly reduces the virus titers in virus infected cells therefore the interesting question for me was to demonstrate that, Verapamil has no detrimental toxic effect on cell survival. Otherwise that could also be one of the genuine reason to get reduced virus titers, as there are not enough cells available for virus replication if Verapamil kills the cells. Therefore I performed MTT-Formazan test to demonstrate the possible negative effect of Verapamil on cell survival and the result (**shown in the fig 3.3**) shows that Verapamil seems to have no significant toxic effect on cell survival. That means that, the reduced virus titer was not due to killing of the cells by

Verapamil but it was due to the effect of Verapamil on virus replication by yet unknown mechanism.

It is also necessary to discuss here that influenza virus one replication cycle takes about 10 hours so the effect of Verapamil could be studied either for one replication cycle or for multiple replication cycles but at very low multiplication of infeccity (about 0.01 or depends on the aim of the experiments). I have also observed that infecting cells with higher MOI leads to cell death in very short time if cells are incubated for longer time or for multiple replication cycles. Therefore analysis of the effect could be studied either for single replication cycle or multiple cycles but at low MOI. I have observed that Verapamil strongly reduces the virus titers without killing the cells in both of the above cases.

Inhibiting virus replication by Verapamil was a great interest in me to broaden my knowledge by acquiring much information about Verapamil and further analysis of Verapamil action on cellular and viral factors. In order to assure that Verapamil does not inhibit cellular protein synthesis (translation) if Verapamil might be considered as an antiviral drug against influenza, therefore I further investigated the effect of Verapamil on host protein production, which could also be effected by Verapamil and could be one of the potent causal factors for the reduced virus titers. The analysis of the effect of Verapamil on cellular protein production **(shown in the fig 3.5 and 3.6)** indicates that Verapamil seems not to effect cellular protein production. This shows the exclusive inhibitory potency of Verapamil on virus replication. Hence Verapamil could be a potent inhibitor of influenza and might be used as antiviral drug. I would like to mention here that influenza virus replication leads to host cell shut-off **(shown in the fig 3.6 at 8-10 h p.i)** which mean that host protein synthesis (ERK2) is impaired at late time point replication cycle of influenza virus and Verapamil seems to reduce this effect.

From the above study I got considerable information, that Verapamil strongly reduces virus titers suggesting that virus-induced Raf/MEK/ERK signalling is somehow effected. Therefore I investigated the connection between virus-induced calcium dependent PKCα and Raf/MEK/ERK-activation. Therefore, I infected the cells and 2 h p.i. cells were either left untreated or were treated with Verapamil and lysates were

used for western blot to detect the pPKCα and pERK-activation. The result (**shown in the fig 3.7**) so far indicates that in late time point post infection (8-10 h), PKCα and ERK are activated and that Verapamil strongly reduces the activation of ERK. This suggests that virus-induced calcium dependent PKCα transmits the signals to the Raf/MEK/ERK-cascade. Surprisingly the activation of PKCα was not blocked by Verapamil, which suggests that Verapamil might not inhibit activation of PKCα but inhibits its activity. Therefore I further investigated the activity of PKCα and ERK (**shown in the fig 3.8**) by Immune complex kinase assay (ICA) in cooperation with the lab of S. Ludwig, Munster, using MBP as a substrate for PKCα and ERK-activity. The result of ICA suggests that PKCα induced ERK-activity is significantly reduced in Verapamil treated cells while the effect of Verapamil was minor on PKCα-activity. That suggests that virus-induced calcium dependent PKCα-activity leads to the activation of ERK.

In attempt to broaden my knowledge, a strategy for analyzing the PKCα-activation and RNP-export in virus infected and either in untreated or Verapamil treated cells laser scanning confocal microscope was used. The confocal microscopic result (**shown in the fig 3.10, 11 and 12**) shows that at late time points (8-10 h p.i.) of virus replication, PKCα is activated when RNPs of influenza virus was exported out from nucleus to cytoplasm in untreated cells. In contrast to this, PKCα activation and RNP-export was strongly blocked in Verapamil treated cells. That suggests that RNP-export, hence efficient virus replication is dependent on virus induced PKCα-activation. In the introduction part I mentioned that Raf/MEK/ERK is an essential host function for the efficient virus replication, which is activated at late time point post infection and is prerequisite for RNP-export. Therefore confocal or Immunoflorescence results supports the idea that virus induced calcium dependent PKCα-activation transmits the signals to the Raf/MEK/ERK (MAPK)-cascade which leads to the RNP-export for the efficient virus replication.

The above analysis have consistently demonstrated and given significant information of the negative role of Verapamil on PKCα. For a further understanding of the mode of action of Verapamil, I further analyzed the possible adverse effects of Verapamil on viral factors like viral protein production and viral polymerase function. At this point, I investigated the production of viral proteins in cells that were infected and

either left untreated or treated with Verapamil. The western blot results (**shown in the fig 3.13.1 & 3.13.2(A-D)**) shows that at an early time point (4 h.p.i.) the amount of viral proteins (PB1, NP, M1 and NS1) was very low but production levels increased at late time point of virus replication (8-10 h p.i.) in untreated cells. In contrast to this, viral protein production was strongly inhibited in Verapamil treated cells throughout the time course virus replication. This suggests that Verapamil also targets viral protein expression. Therefore reduced viral protein production could be another genuine possible reason for inefficient virus replication.

PB1 protein of influenza virus is subunit of the viral polymerase. Interestingly the western blot analysis shows that, PB1 protein production was strongly reduced in virus infected and Verapamil treated cells. This could mean that the viral polymerase function might be negatively affected in Verapamil treated cells. Therefore more knowledge about the effect of Verapamil on viral polymerase activity was necessary to clarify this point. Therefore Primer Extension Assay to determine the production level of the different viral RNA species (vRNA, cRNA and mRNA) of influenza virus was used. The preliminary result of primer extension (**shown in the fig 3.14 (A) and (B) 8% sequencing gel picture of primer extension**) indicates that Verapamil seems to inhibit mRNA production in wild type virus infected cells while it has no considerable negative effect on mRNA production in a minireplicon system (plasmid based replication system). This suggests that Verapamil negatively effects viral transcription in wild type infection. As the approach of determining the effect of Verapamil on mRNA production in a minireplicon system has not yet been conclusive therefore further research or analysis in this approach is warranted. One possible argument against the non inhibitory action of Verapamil on mRNA production in the minireplicon system could be that, in the minireplicon system, the viral RNA like transcript and the mRNA for the polymerase which are provided by, pPOL1-CAT-RT, pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP are produced by cellular polymerases (POL1 and POL2) and therefore their activity might not be affected by Verapamil. Therefore the effect of Verapamil on viral mRNA production in the minireplicon system is not considerable.

I further wanted to demonstrate the effect of Verapamil on CAT-protein expression by transfecting 293T cells with 5 plasmids (minireplicon system) and my preliminary

CAT-assay result **(shown in the fig 3.15)** without Verapamil treatment shows that transfecting 293T cells with 5 plasmids leads to the expression of CAT-protein. This experiment was a positive control experiment to show the CAT-protein expression.

On the basis of above discussion it can be concluded that influenza virus induces calcium dependent PKCα-activation that transmits the signal to the Raf/MEK/ERK (MAPK)-cascade which is a prerequisite host function for the RNP-export from nucleus to cytoplasm for the efficient virus replication. And Verapamil which is a calcium channel blocker and indirect inhibitor of PKCα reduces the PKCα transmitted Raf/MEK/ERK (MAPK)-cascade activation therefore leading to reduced RNP-export and reduced virus titers. Hence Verapamil acts as potent antiviral agent. Besides, impairment of MAPK-cascade activation, Verapamil also targets on viral translation process thereby reduced viral protein production and impaired RNA-dependent RNA-polymerase activity of influenza virus.

Taken together, PKCα plays an important role in transmitting the influenza virus induced signals to the Raf/MEK/ERK-cascade. As PKCα-inhibition can be achieved at non-toxic Verapamil concentration, leading to strong reduction of virus titers, inhibition of this cellular activity might be a potential anti-viral drug against influenza. Verapamil is already used drug in the treatment of cardiovascular diseases like, hypertension, high blood pressure, angina etc. with no contra-indications or uncommon side effects. Therefore Verapamil could be tested in the treatment of influenza.

Of immediate practical importance is the need to discover potential anti-viral drug in response to the next influenza out break, which is a major and serious concern for World Health Organization (WHO) and Centre for Disease and Control and prevention (CDC).

Influenza is an annual major public health threat. Therefore antiviral agents would be one of the therapeutic options to control the wave of epidemic or pandemic influenza attack globally until adequate supplies of vaccine are available. Currently two classes of antiviral agents are available for the population, the adamantanes (amantadine and rimantadine) and the neuraminidase inhibitors (zanamivir and oseltamivir). All of these drugs have different efficacies and, are licensed for different age groups. These

drugs have certain drawbacks. Viral resistance is often associated with the use of the adamantanes and is also found with neuraminidase inhibitors (267, 268).

Since these influenza antiviral agents are expensive and probably would be in relatively short supply, one can consider cellular factors targeted an antiviral prophylaxis strategy for treating an infected population.

The possible mass use of influenza antiviral agents raises concern about the emergence and spread of drug-resistant influenza viruses. The frequency of resistance is much lower for prophylactic than for therapeutic use of these agents. The frequency of resistance is lower for the prophylactic use of rimantadine or neuraminidase inhibitors than for amantadine. Given that the logistic and theoretical details, cellular factor (PKCα) targeted use of antiviral agents, Verapamil could be an important intervention for epidemic or pandemic influenza. For the potential use of Verapamil as anti-influenza drug, our current data (shown by my lab member Dominique Brenner) shows that Verapamil treatment does not lead to resistant variants of influenza. This suggests that Verapamil could be an interesting and attractive agent for scientific study for the therapeutic intervention than the current anti-influenza drugs. Verapamil might also reduce the viral load in infected cells while the current neuraminidase inhibitors do not reduces the overall viral load in infected cells.

Taking into consideration as a potential anti-influenza drug, Verapamil should be tested in animals and our further ongoing efforts in this direction are under progress.

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Appendices

Abbreviations

AA	amino acids
AcCoA	acetyl co-enzyme A
APS	Ammonium persulfate
AI	Avian influenza
AIV	Avian influenza virus
BODIPY	1-acetyl BODIPY-chloramphenicol
bp	base pairs
BSA	bovine serum albumin
BA	bovine albumin
^{o}C	centigrade
cm	centimeter
CCB	calcium channel blocker
CAT	chloramphenicol acetyltransferase
Ci	curie
CMV	cytomegalovirus
CPSF	cleavage and polyadenylation specificity factor
cRNA	complementary RNA
CTP	cytidine triphosphate
ddH2O	deionized distilled water
DEPC	DiethylenePyrocarbonate
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
Dn-PKCα	dominant negative PKCα
Dn-Ras	dominant negative Ras
DNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EB	elution buffer
ECL	Enhanced Chemiluminescence
EDTA	ethylenediamine tetraacetic acid

eIF2	eukaryotic translation initiation factor 2
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal regulated kinase
et al.	*et alii* (=and others)
FCS	fetal calf serum
FFU	focus forming unit
FPV	fowl plague virus
g	gram
GFP	green fluorescent protein
GTP	guanosine triphosphate
GP	glycoprotein, BDV
h	hour(s)
h.p.i	hours post infection
h.p.t	hours post transfection
HA	hemagglutinin
HEPES	N-2-hydroxyethylpiperazine
HDV	Hepatitis delta virus
His	histamine
HPAIV	highly pathogenic avian influenza virus
HRP	Horse Radish Peroxidase
IFA	indirect immunofluoresces assay
IFN	interferon
IV	Influenza Virus
IAV	Influenza A virus
ICA	Immunocomplex kinase assay
IFA	Immunofluorescence assay
IRF3	IFN regulatory factor 3
Kb	kilobasepairs
KDa	kilodalton
L	RNA-dependent RNA.polymerase, BDV
LB	Luria broth
LPAIV	lowly pathogenic avian influenza virus
M	molar
M	matrixprotein

M1	matrixprotein, Influenza
M2	ion channel protein, Influenza
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MEK	MAPK kinase/ERK kinase
mg	milligram
min	minute(s)
ml	milliliter
mM	millimolar
mm	millimeter
MOI	multiplicity of infection
mRNA	messenger RNA
MTT	3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide
n	number of experiements
NA	neuraminidase
NCR	noncoding region(s)
NEP/NS2	nuclear export factor, non structural protein of Influenza
NES	nuclear export signal
NLS	nuclear location signal
nm	nano meter
NP	nucleoprotein
NPC	nuclear pore complex
NS1	nonstructural protein
NEP	nuclear export protein
ng	nanogram
nt	nucleotide(s)
NTP	ribonucleoside triphosphate
OD	optical density
P	phosphoprotein, BDV
P10/x	10 kilo Dalton protein, BDV
PA	subunit of RDRP, influenza
PABII	poly (A)-binding protein II
PAGE	polyacrylamide gel eletrophoresis
PB1	subunit of RDRP, influenza

PB2	subunit of RDRP, influenza
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit(s)
PFA	paraformaldehyde
PKCα	protein kinase c-alpha
PKR	dsRNA activated protein kinase
Pmol	picomolar
PolyA	polyadenylic acid
Pol I	RNA polymerase I
PNK	polynucleotide kinase
PR8	A/Puerto Rico/8/34 (H1N1)
PVDF	Polyvinylidene fluoride
RDRP	RNA-dependent RNA-polymerase
5S-rRNA	5S-ribosomal RNA
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	RNase inhibitor
RNPs	ribonucleoproteins
RPA	RNase protection assay
rpm	revolution per minute
RT	reverse transcription
s	second(s)
SDS	sodium dodecyl sulfate
TAE	tris-acetate-EDTA
TE	tris- EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TLB	tris lysis buffer
TLC	thin layer chromatography
TPA	12-O-tetradecanoyl-phorbol-13-acetate (TPA
Tris	tris-hydroxymethylaminomethane
ts	temperature sensitive
Tween 20	polyoxyethylenesorbiten monolaurate
U	unit(s)

U	uridine
UTP	uridine triphosphate
UV	ultraviolet
V	volt
Vera	Verapamil
Vol	volume
vRNA	viral RNA
cRNA	complementary RNA
mRNA	messenger RNA
v/v	volume percentage
w/v	weight percentage
WB	western blot
µg	microgram
µl	microliter
µM	micromolar

Acknowledgements

Erklärung

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfe angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Gründsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Giessen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten.

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