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Biochimica et Biophysica Acta 1763 (2006) 1344–1362
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Review

Cytobiological consequences of calcium-signaling alterations induced by human viral proteins

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Received 21 August 2006; received in revised form 13 September 2006; accepted 15 September 2006
Available online 23 September 2006

Abstract

Since calcium-signaling regulates specific and fundamental cellular processes, it represents the ideal target of viral proteins, in order for the virus to control cellular functions and favour its persistence, multiplication and spread. A detailed analysis of reports focused on the impact of viral proteins on calcium-signaling has shown that virus-related elevations of cytosolic calcium levels allow increased viral protein expression (HIV-1, HSV-1/2), viral replication (HBx, enterovirus 2B, HTLV-1 $p12^{I}$, HHV-8, EBV), viral maturation (rotavirus), viral release (enterovirus 2B) and cell immortalization (EBV). Interestingly, virus-induced decreased cytosolic calcium levels have been found to be associated with inhibition of immune cells functions (HIV-1 Tat, HHV-8 K15, EBV LMP2A). Finally, several viral proteins are able to modulate intracellular calcium-signaling to control cell viability (HIV-1 Tat, HTLV-1 $p13^{II}$, HCV core, HBx, enterovirus 2B, HHV-8 K7). These data point out calcium-signaling as a key cellular target for viral infection and should stimulate further studies exploring new calcium-related therapeutic strategies.

Keywords: Calcium-signaling; Human virus; Viral protein; Signal transduction; Viral infection; Calcium measurement

1. Introduction

It is well known that viruses need the cell machinery for their multiplication and survival. Since calcium-signaling is a common regulatory system able to control fundamental cellular processes, it is not surprising that several viruses encode proteins which modify calcium-signaling allowing them to highjack cellular functions to serve one or several steps of infection.

During the last decades, new methods to explore intracellular calcium-signaling have brought a revolution in our understanding of the rules of the intracellular calcium "cosmos" and provided the tools to better understand how viruses and cells interact and how this interaction may generate diseases.

This review, which is not meant to be exhaustive, is focused on calcium-signaling and/or calcium-dependent pathways alterations related to the intracellular presence of human viruses or their viral proteins. Its aim is to point out the calcium-dependent mechanisms of viral infection and stimulate further studies applying modern calcium-signaling approaches to gain new insight on viral strategies to drive cellular processes.

It has been observed that "almost everything we do is controlled by Ca^{2+} [1]. At the intracellular level, this control is mediated by elevations in the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_{cyt}$) [1,2] which are known to regulate a wide variety of elementary cellular processes, such as signal transduction pathways and gene expression, and of global

Abbreviations: aa, amino acids; $[Ca^{2+}]_{cyt}$, cytosolic calcium-concentration; $[Ca^{2+}]_{er}$, endoplasmic reticulum calcium-concentration; $[Ca^{2+}]_{Golgi}$, Golgi apparatus calcium-concentration; $[Ca^{2+}]_{mt}$, mitochondrial calcium-concentration; Ca^{2+}, calcium; EBV, Epstein–Barr virus; ER, endoplasmic reticulum; HBV, hepatitis B virus; HCV, hepatitis C virus; HHV-8, human herpesvirus type-8; HIV-1, human immunodeficiency virus type-1; HPV, human papillomavirus; HSV-1/2, herpes simplex virus type-1 and type-2; HTLV-1, human T lymphotropic virus type-1; IP_3, inositol 1,4,5-triphosphate; IP_3R, inositol triphosphate receptor; kb, kilobase; kDa, kilodalton; NCX/HCX, Na^+/Ca^{2+} exchanger and H^+/Ca^{2+} exchanger; NFAT, nuclear factor of activated T cell; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco-Endoplasmic reticulum Ca^{2+}-ATPase; ROC, receptor operated channel; RYR, ryanodine receptor; SERCA, sarco-Endoplasmic reticulum Ca^{2+}-ATPase; SOC, store operated channel; SPCA, secretory pathway Ca^{2+}-ATPase; TRPC, members of the transient receptor potential ion-channel family

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doi:10.1016/j.bbamcr.2006.09.025

cellular processes including fertilisation, contraction, secretion, learning and memory, cell growth and apoptosis [3–5]. To control these processes, Ca^{2+} spikes and waves have evolved to be highly versatile not only in terms of amplitude, frequency and spatio-temporal patterning [1] but also in terms of shape, number, direction, velocity and route [2], achieving differential modulation of Ca^{2+}-binding proteins and Ca^{2+}-dependent effectors [6]. Ca^{2+}-signals can be spatially restricted into nano- and microdomains, such as those located beneath the plasma membrane and those located between ER and mitochondria [7]. Recent findings had highlighted the primordial role of calcium cross-talk between the ER and the mitochondria in the fine regulation of intracellular calcium-signaling [7]. Key cellular calcium-handling proteins allow to finely regulate intracellular, including intra-organelles calcium-signaling (Fig. 1).

The ability to record changes in the free Ca^{2+}-concentration in living cells is fundamental to study Ca^{2+}-signaling. Fluorescent Ca^{2+} indicators such as quin-2 and Fura2-AM enabled the first quantitative measurements of the changes in the free Ca^{2+}-concentration occurring inside cells. Today, dozens of different chemical probes have been generated using this strategy (reviewed in [8]). Even if fluorescent dyes have a number of advantages over genetically encoded indicators (they are cheap, easy to use, have a high dynamic range, and are easy to calibrate), they cannot be selectively targeted to specific cellular organelles or compartments. An alternative approach to the use of fluorescent dyes was introduced in 1992 by the development of recombinant targeted aequorin calcium probes, allowing selective measurements of $[Ca^{2+}]$ changes in virtually all intracellular compartments of the transfected or transduced cells [9]. The mechanism of these luminescent probes is distinct from that of the fluorescent Ca^{2+} dyes. The conversion of measured luminescence to $[Ca^{2+}]$ values is based on the rate of consumption of the probe, rather than on the binding equilibrium between the probe and Ca^{2+} as in the case of fluorescent probes [9].

This review reports about calcium-signaling alterations related to the intracellular presence of complete virions or viral proteins. In addition to calcium dyes and probes (direct

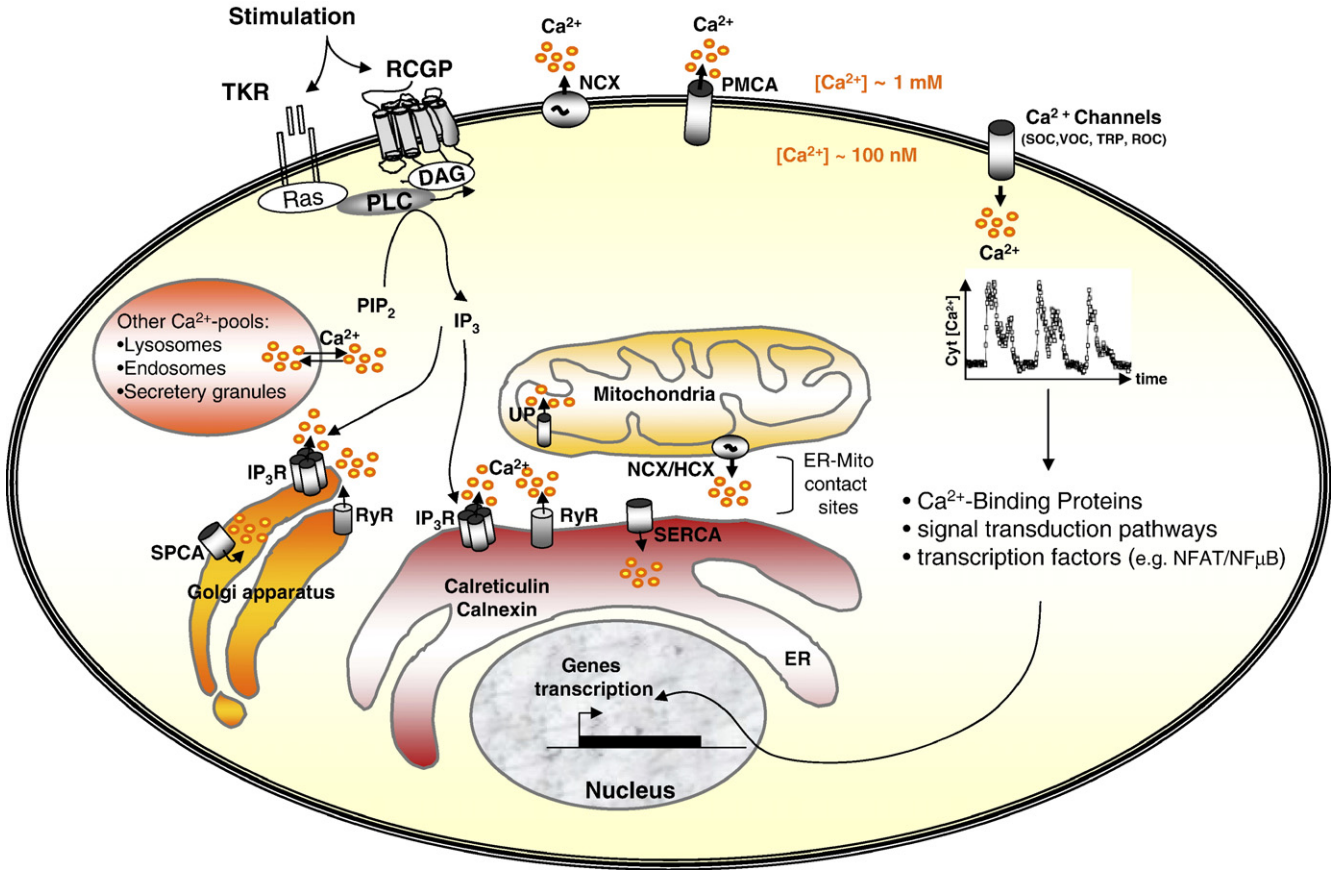

Fig. 1. Schematic representation of intracellular calcium-signaling. In the resting cell, free $[Ca^{2+}]$cyt is kept very low (100 nM) primarily by active Ca^{2+} transporters that either extrude Ca^{2+} across the plasma membrane (PM) to the extracellular environment (PMCA and Na^+/Ca^{2+} exchanger (NCX)) or uptake Ca^{2+} into intracellular organelles (mainly SERCA) [5]. Free extracellular Ca^{2+}-concentration is around 1–2 mM [5]. Cytosolic Ca^{2+} elevations are due to the entry of extracellular Ca^{2+} via Ca^{2+} channels in the plasma membrane (VOC, ROC, TRPC and SOC) or the release of stored Ca^{2+} through ER Ca^{2+} channels (inositol-1,4,5-trisphosphate receptors (IP^3R) and ryanodine receptors (RyR). Calcium release through IP^3R occurs upon stimulation of plasma membrane receptors, the receptors coupled to G proteins (RCGP) and the tyrosine kinase receptors (TKR), and subsequent synthesis of IP^3 by PLC [3–5]. In addition to ER Ca^{2+} store, other intracellular Ca^{2+}-pools are implicated in the control of intracellular calcium-signaling such as Golgi apparatus, lysosomes, endosomes and secretory granules. Mitochondria accumulate calcium via the uniporter (UP) following physiological stimulation [210]. This accumulation depends on the high calcium-concentration which occurs close to the plasma membrane and ER calcium-channels (microdomains) [161]. Mitochondrial calcium extrusion is achieved by the Na^+/Ca^{2+} exchanger and H^+/Ca^{2+} exchanger (NCX/HCX).

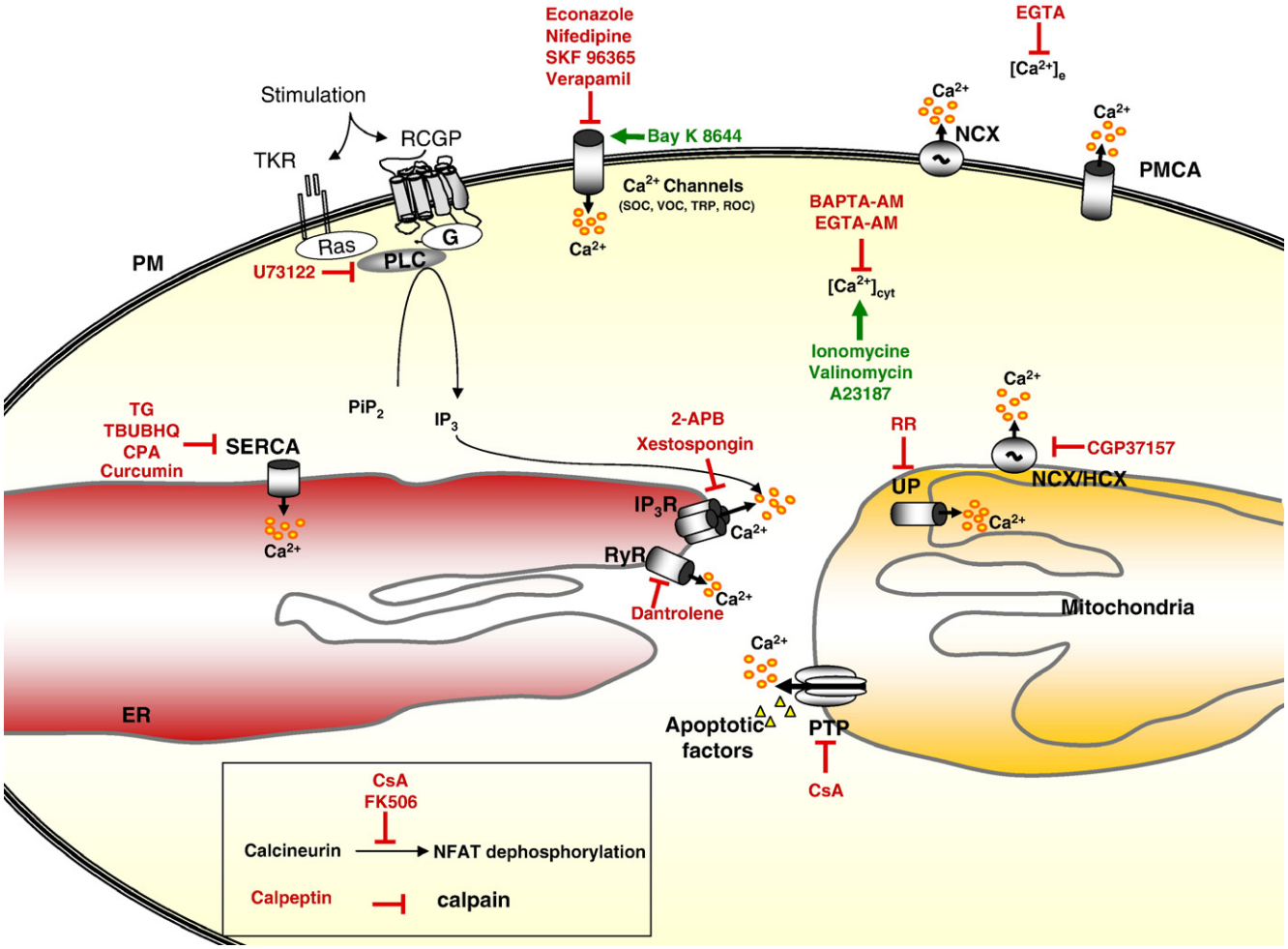

Fig. 2. Cellular targets of "calcium" drugs. Inhibitory drugs are shown in red and activating drugs are shown in green. Intracellular [Ca^{2+}] chelation is obtained by using BAPTA-AM (1,2-Bis (2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakisacetoxymethyl ester) or EGTA-AM (Ethylene-bis (oxyethylenenitrilo) tetraacetic acid Glycol ether diamine tetraacetic acid-acetoxymethyl ester). Increase of intracellular [Ca^{2+}] is obtained by using different ionophores: ionomycine, valinomycin and A23187. EGTA chelates extracellular Ca^{2+} ($[Ca^{2+}]_e$) thus reducing Ca^{2+} entry. Inhibition of Ca^{2+} entry is also obtained through inhibition of Ca^{2+} channels: Nifedipine and Verapamil inhibit L-type Ca^{2+} channels, SKF 96365 is a selective inhibitor of receptor-mediated Ca^{2+} entry (ROC) and voltage-gated Ca^{2+} entry (VOC). Econazole blocks all Ca^{2+} channels. Activation of Ca^{2+} entry is obtained by Ca^{2+} channel agonist BayK8644. TG: thapsigargin, CPA: cyclopiazonic acid, TBUBHQ and curcumin inhibit SERCA pump activity thus discharging ER Ca^{2+}-pool. Inhibition of ER Ca^{2+}-release is obtained by blocking IP^3 receptor (IP^3R) by 2-APB and xestospongin or Ryanodin Receptor (RyR) by dantrolene. Inhibition of Ca^{2+}-release through IP^3R is obtained by inhibition of IP^3 production by phospholipase C (PLC). Cyclosporin A (CsA) binds to cyclophilin D thus blocking the permeability transition pore (PTP) opening and mitochondrial Ca^{2+}-release. Mitochondrial Ca^{2+}-release is prevented by the Na^+/Ca^{2+} exchanger (NCX) inhibitor, CGP37157. Inhibition of mitochondrial uniporter (UP) by ruthenium red (RR) blocks mitochondrial Ca^{2+}-accumulation. CsA binding to calcineurin blocks NFAT (Nuclear Factor of Activated T cells) dephosphorylation and activation. Calpeptin blocks the calcium-dependent protease calpain.

measurements), "calcium" drugs, having different degrees of specificity and inhibiting calcium handling and binding proteins (indirect measurements), are used to identify mechanisms and/or partners implicated in calcium dependent regulations of cellular processes (Fig. 2). Tables 1 and 2 report results according to the type of measurement used.

2. RNA viruses

2.1. Human immunodeficiency virus type-1

Human Immunodeficiency Virus (HIV) is a retrovirus targeting mainly T CD4+ cells and is the causative agent of Acquired Immuno-Deficiency Syndrome (AIDS). AIDS is the most advanced stage of HIV infection, characterized by a gradual depletion of CD4+, and thus a higher sensitivity to opportunistic infections, and also associated with heart, nervous system or kidney diseases. The worldwide development of HIV-related disease is alarming, with more than 36 million existing infections, and about 20 million deaths [10]. The HIV-1 9.2 Kb single-stranded RNA genome encodes nine open reading frames, giving birth to structural proteins: Gag, Env and Pol polyproteins proteolysed into MA (matrix), CA (capsid), NC (nucleocapsid), p6, SU (surface, also known as gp120), TM (transmembrane or gp41), PR (protease), RT (reverse transcriptase), and IN (integrase). HIV-1 encodes also six additional or accessory proteins: Vif, Vpr, Nef, Tat, Rev and Vpu. After HIV-1 infection of resting memory or naive CD4+ T cells, macrophages

Table 1
Effect of viruses/viral proteins on intracellular calcium-signaling: measurements with calcium dyes and probes

Virus	Protein	Ca^{2+}-signals	Mechanisms	Cytobiological effects	Model	Method	References
HIV-1	Viral particle	↗$[Ca^{2+}]_{cyt}$	↗IP^3 pathway	Signal transduction	H9		[13]
	Nef	↗$[Ca^{2+}]_{cyt}$ basal	↗Ca^{2+} influx	T cell activation (NFAT)	Jurkat T	Fluo-3 + Fura-Red, aequorin	[16]
		↗$[Ca^{2+}]_{cyt}$	↗Non-ER Ca^{2+} stores		CEM and differentiated HL60	$^{45}Ca^{2+}$ and Fura2-AM	[17]
			↘Ca^{2+} influx		differentiated HL60	Fura2-AM	[18]
	Tat	↗$[Ca^{2+}]_{cyt}$	↗ ER Ca^{2+}-release (IP^3R)	TNFα production	macrophages	Fura2-AM	[37]
		↘$[Ca^{2+}]_{cyt}$	L-type Ca^{2+} channel inhibition	Inhibition of NK/DC functions	NK, DC	Fura2-AM	[38–40]
		↗$[Ca^{2+}]_{cyt}$, ↗$[Ca^{2+}]_{mt}$	↗ER Ca^{2+}-release, ↗Ca^{2+}-influx	Apoptosis induction	neurons, astrocytes, microglial cells	Fura2-AM, Indo-1, Calcium-Orange, Rhod-2	[45–49]
	gp120	↗$[Ca^{2+}]_{cyt}$	↗Ca^{2+}-influx (L-type Ca^{2+} channels)	Apoptosis induction	neurons, astrocytes	Fura2-AM	[51]
		↗$[Ca^{2+}]_{cyt}$	↗ER Ca^{2+}-release (IP_3R)		HT-29-D4	Fura2-AM	[54]
HTLV-1	$p12^I$	↗$[Ca^{2+}]_{cyt}$ basal	↗ER Ca^{2+}-release (IP_3R) ↗Ca^{2+}-influx	T cell activation (NFAT) Viral replication, Adhesion	Jurkat T	Fura2-AM	[31,58,59,61] [62]
	$p13^{II}$	↘$[Ca^{2+}]_{mt}$	Mitochondrial Ca^{2+}-leak	Apoptosis induction	isolated mitochondria	Calcium Green 5N	[63]
HCV	Core	↘$[Ca^{2+}]$er	ER stress, ↗calreticulin, ↘SERCA2 activity	Apoptosis induction	HuH7	erAEQ	[77]
		↗ $[Ca^{2+}]_{cyt}$, ↗$[Ca^{2+}]_{er}$	ER Ca^{2+}-leak	T cell activation (NFAT)	Jurkat T	Fura2-AM	[83]
Enterovirus	Viral particle, 2B	↗ $[Ca^{2+}]_{cyt}$	↘ ER Ca^{2+}-store, ↗ Ca^{2+}-influx	Viral release	HeLa	Fura2-AM	[103]
	2B	↗ $[Ca^{2+}]_{cyt}$, ↘ $[Ca^{2+}]$mt	↗ Ca^{2+}-influx	Apoptosis inhibition	HeLa, CHO	Aequorin probes, Fura2-AM	[106]
		↘ $[Ca^{2+}]$er, ↘ $[Ca^{2+}]$Golgi	ER and Golgi Ca^{2+}-leak (pore formation)				
Rotavirus	Viral particle	↗ $[Ca^{2+}]_{cyt}$	L-type Ca^{2+} channel activation	Viral maturation	MA-104, Caco-2, HT29	Fura2-AM, quin-2-AM	[116,119,120]
	NSP4	↗ $[Ca^{2+}]_{cyt}$	↗ ER Ca^{2+}-release (IP^3R), ↗ Ca^{2+}-influx		HT29, Sf9, HEK293	Fura2-AM	[124–126]
HBV	HBx	↗ $[Ca^{2+}]_{cyt}$	PMCA inactivation, mitochondrial Ca^{2+}-leak	Apoptosis induction	HepG2, HeLa	Aequorin probes	[139]
		↗ $[Ca^{2+}]_{cyt}$		Signal transduction	CHL-X	Fura2-AM	[140]
HHV-8	K15	↘ $[Ca^{2+}]_{cyt}$	Blocks BCR	Immunity down-regulation	BJAB	Indo-1	[143]
	K1	↗ $[Ca^{2+}]_{cyt}$	Interactions with SH2 proteins	T cell activation (NFAT)	BJAB, 293T	Indo-1	[158]
	v-MIP I/II	↗ $[Ca^{2+}]_{cyt}$	↗ Ca^{2+}-influx (CCR5/8)	Signal transduction	BCBL-1, PMBC	Indo-1	[162,163]
	K7	↗ $[Ca^{2+}]_{cyt}$	↗ ER Ca^{2+}-release	Apoptosis inhibition	BJAB	Indo-1	[159]
HSV-1/2	Viral particle	↗ $[Ca^{2+}]_{cyt}$	↗ ER Ca^{2+}-release (IP^3R)	Viral protein expression and transport Signal transduction	Vero, Caski	Fura2-AM	[166]
EBV	Viral particle	↗ $[Ca^{2+}]_{cyt}$	↗ Ca^{2+}-influx (L-type Ca^{2+} channels)	Cellular transformation	B cells, Ramos	Quin-2	[173]
	LMP2A	↘ $[Ca^{2+}]_{cyt}$	Blocks BCR	Immunity down-regulation	BJAB, PMBC	Fluo-3	[176–178]
Influenza A virus	Viral particle	↗ $[Ca^{2+}]_{cyt}$	↗ ER Ca^{2+}-release (IP^3R)	Neurophysiological changes	Neurons	Fluo-4-AM	[189]
		↗ $[Ca^{2+}]_{cyt}$	↗ ER Ca^{2+}-release (IP^3R)	Neutrophil deactivation	Neutrophils	Fura2-AM, $^{45}Ca^{2+}$	[191]

Abbreviations: $[Ca^{2+}]_{cyt}$, cytosolic calcium-concentration; $[Ca^{2+}]$er, endoplasmic reticulum calcium-concentration; $[Ca^{2+}]$Golgi, Golgi apparatus calcium-concentration; $[Ca^{2+}]$mt, mitochondrial calcium-concentration; BCR, B cell receptor; Ca^{2+}, calcium; CCR5/8, chemokine receptor type-5 and type-8; DC, dendritic cell; EBV, Epstein–Barr virus; ER, endoplasmic reticulum; HBV, hepatitis B virus; HCV, hepatitis C virus; HHV-8, human herpesvirus type-8; HIV-1, human immunodeficiency virus type-1; HSV-1/2, herpes simplex virus type-1 and type-2; HTLV-1, human T lymphotropic virus type-1; IP_3, inositol 1,4,5-triphosphate; IP_3R, inositol triphosphate receptor; NFAT, nuclear factor of activated T cells; NK, natural killer cell; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco-Endoplasmic reticulum Ca^{2+}-ATPase; TNFα tumor necrosis factor α.

Table 2
Calcium-dependent effects of viruses/viral proteins: assessment by calcium drugs

Virus	Protein	Drug	Effects of viruses/viral proteins	Model	References
H1V-1	Viral particle	Ionomycin, TG, TBUBHQ, CPA	Viral protein expression	ACH-2, J1.1	[14]
		econazole	Viral protein expression		
	Nef	EGTA, SKF 96365, CsA, 2-APB	NFAT activation	Jurkat T	[16]
	Tat	xestospongin, BAPTA-AM, U73122	TNFα production	macrophages	[37]
		BayK 8644	IL-12 secretion	NK, DC	[38,39]
		BAPTA-AM, RR, CsA	Neuronal cell death	Hippocampal neurons	[46]
HTLV-1	p12I	BAPTA-AM, CsA	NFAT activation	Jurkat T	[58,59]
		BAPTA-AM, SKF 96365, calpeptin	LFA-1 expression	Jurkat T	[60]
		TG	LFA-1 expression		
HCV	Core	CsA, EGTA	NFAT activation	Jurkat T	[85]
		SERCA2 overexpression	Mitochondrial depolarisation and apoptosis	HuH7	[77]
	NS5A	EGTA-AM, RR	NFκB and STAT3 activation	HuH7	[94]
Enterovirus	Viral particle, 2B	Medium without Ca^{2+}	Viral replication	HeLa	[103]
Rotavirus	Viral particle	TG, A23187	Viral maturation	MA-104	[116,117]
		BAPTA-AM, verapamil	Viral maturation and cell oncosis	MA-104, HT29	[119,121]
HBV	HBx	BAPTA-AM, CsA, EGTA	Apoptosis	HepG2, HeLa	[139]
		BAPTA-AM, CsA	AP-1 transactivating activity	CHL-X	[140]
		BAPTA-AM, EGTA	FAK activity and viral replication	NIH3T3, HepG2	[146]
		BAPTA-AM, CsA, CGP37157, CPA, U73122	Pyk2 activation and viral replication	HepG2	[141,142,144]
		TG, valinomycin	Pyk2 activation and viral replication	HepG2	[141,144]
		TG	HBV capsid formation	HepG2	[143]
HHV-8	Viral particle	Ionomycin, TG	Viral reactivation	BCBL-1	[153]
		BAPTA-AM, CsA	Viral reactivation	BCBL-1, DMVEC	
HSV-1/2	Viral particle	BAPTA-AM, 2-APB	Viral protein expression and transport	Vero, Caski	[166]
		EGTA-AM, CGP37157, RR	NFκB activation	J774A.1	[168]
		Ionomycin	Viral release and cell death	A431	[169]
EBV	Viral particle	Verapamil	Viral entry and B cells immortalization	B cells, Ramos	[173]
		CsA, FK506	Viral reactivation	Akata	[179]
		Curcumin	Viral reactivation	RajiDR-Luc	[184]
		Ionomycine	Viral reactivation	Akata	[178]
HPV	Viral particle	$CaCl^2$	Cellular transformation	keratinocytes	[195]

Abbreviations: AP-1, activator protein-1; 2-APB, 2-Aminoethyl diphenyl borate; BAPTAAM, 1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis-acetoxymethyl ester; Ca^{2+}, calcium; $CaCl_2$, calcium chloride; CPA, cyclopiazonic acid; CsA, cyclosporin A; EBV, Epstein–Barr virus; EGTA(-AM), Ethylene-bis (oxyethylenenitrilo) tetraacetic acid Glycol ether diamine tetraacetic acid (-acetoxymethyl ester); ER, endoplasmic reticulum; FAK, focal adhesion kinase; HBV, hepatitis B virus; HCV, hepatitis C virus; HHV-8, human herpesvirus type-8; HIV-1, human immunodeficiency virus type-1; HPV, human papillomavirus; HSV-1/2, herpes simplex virus type-1 and type-2; HTLV-1, human T lymphotropic virus type-1; IL-12, interleukine-12; NFκB, nuclear factor κB; Pyk2, proline-rich tyrosine kinase 2; NFAT, nuclear factor of activated T cells; RR, ruthenium red; SERCA, sarco-Endoplasmic reticulum Ca^{2+}-ATPase; STAT3, signal transducer and activator of transcription 3; TBUBHQ, 2,5-di-(t-butyl)-1,4-benzohydroquinone; TG, thapsigargin; TNFα, tumor necrosis factor α.

or mononuclear cells, infected cells enter a non-productive latent infection phase. The molecular mechanisms that determine whether a virus is going to interrupt or continue its life cycle are still unclear [11,12].

In the past, a chronic activation of inositol phosphate pathway and a rise in intracellular free Ca^{2+}-concentration have been observed in HIV-infected H9 lymphoblastoid CD4+ cells [13]. Papp et al. had also shown that HIV protein expression is enhanced by drugs increasing $[Ca^{2+}]_{cyt}$ and blocked by drugs decreasing $[Ca^{2+}]_{cyt}$ in HIV-1 infected T lymphoblastoid cells. This study indicates that viral-induced mobilization of Ca^{2+} from intracellular storage pools and through the CCE pathway may represent a key component of HIV replication [14].

Four HIV-1 proteins have been shown to modify calcium-signaling: Nef, Vpr, Tat and gp120.

HIV-1 Nef protein is a small cytoplasmic myristoylated protein which plays an important role in the pathogenesis of AIDS as demonstrated in vivo by Nef-expressing transgenic mice which develop an AIDS-like syndrome [15]. Cell culture studies have revealed that Nef induces enhancement of HIV replication and particle infectivity, down regulation of cell surface expression of CD4 and major histocompatibility complex I (MHC I), and modulation of intracellular signaling. Nef acts as an adaptor, which binds cellular protein kinases, like Src, resulting in interference of normal cellular signaling processes and enhanced HIV-1 infectivity.

Nef has been demonstrated to increase cytosolic calcium basal levels (in Jurkat T cells [16]) and after ionomicin treatment (in lymphoblastoid CEM cells [17] and promyelocytic HL60 differentiated cell lines [18]). However, the mechanism potentially involved in this effect was shown to be different in the different cellular models. In Jurkat T cells, extracellular calcium influx was shown to be activated by Nef, while analysis using "calcium" drugs did not show calcium release from any intracellular calcium store despite evidence of Nef binding to IP_3R1 [16]. These data suggest that Nef/ IP_3R1 interaction may promote cytosolic Ca^{2+}-increase via enhanced physical coupling between IP^3R and plasma membrane channels [19]. In Nef transfected lymphoblastoid CEM cells, extracellular calcium influx was not analysed. However, studies using $^{45}Ca^{2+}$ showed

higher increase of $[Ca^{2+}]_{cyt}$ in Nef cells treated with ionomycin suggesting that Nef expression is associated with a higher filling of non-ER calcium stores [17]. In promyelocytic HL60 differentiated cell lines, studies using Fura2-AM showed a decreased extracellular calcium influx associated with an increase of non-ER calcium stores [18].

Overall, these studies provide direct evidence that Nef expression induces a cytosolic Ca^{2+} increase. This Nef-induced increase in cytosolic Ca^{2+} was shown to activate Nuclear Factor of Activated T cells (NFAT) [16,20] (a transcription factor responsive to low-amplitude $[Ca^{2+}]_{cyt}$ oscillations [6]), which activates HIV-1 transcription (by activation of HIV-1 LTR) and viral replication [21].

Actually, gene expression profiling analyses have shown that Nef triggers a nearly identical transcriptional program to that of T Cell Receptor (TCR) activation in Jurkat T cells [22]. This activation, which has been debated [23–25], is known to be calcium-dependent and characterized by NFAT induction and IL-2 production [22]. It would support HIV replication which is known to be favoured by T cell activation, which regulates both pre and post-integration steps in HIV life cycle [26,27].

HIV-1 viral protein R (Vpr), a 14 kDa accessory protein, which expression is associated with progressive infection, is mainly localized to the nucleus and mitochondria and implicated in various functions including nuclear translocation of HIV pre-integration complex, cell cycle arrest and apoptosis [28].

Vpr has been shown to cooperate with the adenine nucleotide translocator (ANT) (which forms with the voltage-dependent anion channel (VDAC) the permeability transition pore (PTP)) and forms ion channels in lipid bilayers. In fact Vpr has been shown to induce mitochondrial membrane permeabilization presumably leading to Ca^{2+}-leakage from mitochondria and apoptosis [29,30].

Vpr was shown to interact with Ca^{2+}-related proteins and be involved in Ca^{2+}-regulated processes. Indeed, Vpr directly binds to and activates the transcriptional co-activators p300, a Ca^{2+}-responsive activator [31], and CREB-binding protein (CBP) [32,33] cooperating with Nef in NFAT-directed T cell activation. Since p300/CBP is in limited amount, its activation by Vpr could favour viral activation [32–35].

The HIV-1 transactivator of transcription (Tat) is the most important regulator of viral gene expression and replication. Tat is a 72–86 aa protein, acting in the nucleus to activate HIV-1 LTR [11]. Tat is also actively released from HIV-1 infected cells into the extracellular environment, and thus may exert many biological effects on bystander cells. Accumulating evidences suggest that Tat is, by this way, an important mediator of neurotoxicity related to HIV-associated dementia (HAD) [36]. Tat-mediated Ca^{2+} deregulations were reported in two different cellular models: in immune cells (dendritic cells (DC), natural killer cells (NK), macrophages and T cells), and in neuronal cells. Primary macrophage cultured cells exposed to Tat show an increase of $[Ca^{2+}]_{cyt}$ level in a dose-dependent manner, as demonstrated by using Fura2-AM. Actually, the increase in $[Ca^{2+}]_{cyt}$ was shown to originate from IP_3-regulated pools, since xestospongin, a specific inhibitor of IP_3-dependent Ca^{2+}-release, almost abrogated release of Ca^{2+} induced by Tat application [37]. The increase of $[Ca^{2+}]_{cyt}$ has been shown to induce the production of the pro inflammatory cytokine TNFα [37].

On the contrary, in primary NK cells and DC, Tat application inhibits cytosolic Ca^{2+}-signal following physiological stimulation (cross-linking of CD11a or CD16 and apoptotic body-DC interaction) thus blocking IL-12 secretion by DC [38] and cytolytic activity in NK cells [39]. Tat has been shown to act by blocking L-type Ca^{2+} channels (without affecting calcium stores), as shown by using the calcium channel agonist Bay K 8644 [38,39]. Inhibition of calcium entry by Tat has been shown to inhibit NK cells activation by DC [40]. This activation is known to be dependent on interferon gamma via Calmodulin kinase II (CAMKII) activation by extracellular Ca^{2+} entry, which can be blocked by Tat [40].

In Jurkat T cells, Tat has been proved to mediate transcriptional events in HIV-infected cells, by interacting with the Ca^{2+}-sensitive p300 cofactor, thus enhancing Tat-dependent HIV-1 gene expression [31,34]. Other studies have reported that Tat affects several Ca^{2+} mediated events in immune cells, including T cell activation, apoptosis and cell proliferation [41–44].

Tat application on cultured human fetal neurons, microglial cells and astrocytes increases intracellular calcium level in a dose-dependent manner, as demonstrated by Fura2-AM, Indo-1 or Calcium-Orange experiments [45–49]. Calcium responses to Tat were characterized by an initial transient increase and a prolonged secondary increase. The first calcium rise corresponds to intracellular Ca^{2+}-release through IP_3R, as demonstrated by reduction of Ca^{2+}-transients by the specific IP^3R inhibitor xestospongin. The secondary Ca^{2+} increase was due to Ca^{2+}-influx through plasma membrane channels and was dependant on an *N*-methyl-D-aspartate (NMDA) receptor, as shown with specific antagonists [47,49]. Mitochondrial Ca^{2+}-overload was also observed after Tat application on hippocampal cell cultures as measured with Rhod-2 dye [46]. The main consequence of Ca^{2+}-alterations induced by Tat in neurons and astrocytes is the induction of apoptosis [46]. Indeed, Tat is neurotoxic, mediates neuroexcitatory processes, and finally promotes Caspases and oxidative stress-dependent apoptosis [45,46]. Tat-induced apoptosis was demonstrated to be dependent on cytosolic and mitochondrial calcium-levels since it was abrogated by the intracellular calcium chelator BAPTA-AM, and the inhibitor of mitochondrial calcium-uptake ruthenium red [46].

Gp120 is a structural protein, which forms a complex with gp41, corresponding to HIV-1 surface spikes, proved to be essential for viral entry into target cells. Thus, gp120 is a cell-surface attachment glycoprotein, which binds mainly to CD4, a member of the immunoglobulin superfamily, and thereby determines the first steps of the infection [50]. Gp120 is like Tat protein, neurotoxic and implicated in HAD [36]. Similarly to Tat, gp120 application was demonstrated to promote intracellular Ca^{2+}-rise in human fetal astrocytes and neurons, by using Fura2-AM. The increases in $[Ca^{2+}]_{cyt}$ observed in neurons appear to be due to gp120 activation of Ca^{2+}-influx implicating L-type calcium channels, Na^+/H^+ exchanger and

NMDA-type excitatory amino acid receptor, as demonstrated by using selective blockers [51]. However, a proposed mechanism for gp120-induced neuronal injury/death includes increases of $[Ca^{2+}]_{cyt}$, interplaying with increased levels of extracellular glutamate (that activate excitatory amino acid receptors), and increased accumulation of reactive oxygen intermediates [52]. Gp120 is thought to participate in HIV-1 pathogenesis of HAD through neurotoxic effect related to Caspase-dependent cell death [53].

Finally, gp120-mediated increase of $[Ca^{2+}]_{cyt}$ was described in human colonic adenocarcinoma cell line HT-29-D4 suggesting its role in HIV-related enteropathy [54].

2.2. Human T-Lymphotropic Virus type-1

Human T-Lymphotropic Virus type-1 (HTLV-1) is the etiologic agent of adult T-cell leukaemia/lymphoma (ATLL) and of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [55]. Its 8.5 kb single-stranded RNA genome encodes the retroviral genes gag, pol, pro and env. In addition, several open reading frames located in the genomic 3′ (pX) region encode regulatory proteins (Tax and Rex) and accessory proteins ($p12^{I}$, $p27^{I}$, $p13^{II}$, $p30^{II}$ and $p21^{Rex}$) [56].

Two HTLV-1 proteins, $p12^{I}$ and $p13^{II}$, were demonstrated to be implicated in Ca^{2+}-signaling regulation.

HTLV-1 $p12^{I}$ is a small hydrophobic protein, which contains four proline-rich SH3 domain binding motifs associated to regulation of signal transduction. The protein associates with the 16 kDa subunit of the vacuolar H^{+}-ATPase, binds to IL-2 receptor β and γ chain and has been shown to enhance papillomavirus E5 transforming ability (for a review see [56]).

Located to the membrane of ER and of cis-Golgi, $p12^{I}$ was first demonstrated to associate with two ER Ca^{2+}-binding chaperones calreticulin and calnexin, suggesting a role of $p12^{I}$ in Ca^{2+}-mediated signals [57]. Ding et al. reported later that the expression of $p12^{I}$ slightly increases basal cytosolic calcium, by increasing both calcium-outflow from the ER, through IP_3R, and capacitative calcium entry. Acting on calcium-signaling, $p12^{I}$ enhances T lymphocytes activation, and thus proviral DNA integration, through the activation of NFAT [58,59]. In agreement with these data, inhibition of Ca^{2+}-dependent signals by cyclosporin A (CsA), or BAPTA-AM, abolishes $p12^{I}$-mediated activation of NFAT. Overexpression of calreticulin was shown to block $p12^{I}$-dependent activation by preventing calcium-release from the ER and calcium entry through the plasma membrane [59]. It has been reported that $p12^{I}$ could modulate NFAT activation, through competitive binding to the phosphatase calcineurin (which activates NFAT) [60].

$p12^{I}$-dependent increase of $[Ca^{2+}]_{cyt}$ has been shown to have an impact on several Ca^{2+} regulated proteins, including the transcriptional co-activator, p300 [31,61] which can modulate viral genes transcription from the HTLV-1 LTR [31].

$p12^{I}$ has been recently demonstrated to promote cell-to-cell viral spread by inducing LFA1 clustering on T cells via a calcium-dependent mechanism [62]. In fact, expression of $p12^{I}$ in Jurkat T cells and mobilization of intracellular calcium by thapsigargin (TG) both enhanced the expression of adhesion molecule LFA-1, which was inhibited by BAPTA-AM, SKF 96365 (a calcium channel blocker), and calpeptin (an inhibitor of the calcium-dependent protease calpain) [62].

Taken together, these data indicate that $p12^{I}$, by modifying calcium-signaling, can promote viral replication, lymphocyte proliferation and viral spread, thus playing a key role in viral infection.

$p13^{II}$ corresponds to the 87 C-terminal amino acids of $p30^{II}$ and is targeted to the inner mitochondrial membrane. Accumulation of $p13^{II}$ in mitochondrial membrane disrupts the mitochondrial network. Changes in mitochondrial morphology, as swelling and fragmentation of the cristae, are associated to loss of mitochondrial transmembrane potential and leak of Ca^{2+}, thus increasing cell's sensitivity to C_2-ceramide-induced apoptosis. Chemical properties of $p13^{II}$ suggest that its effect on mitochondrial permeability acts through the formation of a channel giving rise to a rapid flux of Ca^{2+} across the inner membrane [63,64]. HeLa cells stably expressing $p13^{II}$ exhibit a marked increase in Ca^{2+}-mediated phosphorylation of the CREB transcription factor, suggesting that $p13^{II}$ might also influence the cellular balance between proliferation and apoptosis [65].

2.3. Hepatitis C virus

Hepatitis C virus (HCV), the major causative agent of non-A non-B hepatitis in humans, is a member of the Flaviviridae family, which contains a positive-stranded RNA virus of about 9.6 kb [66]. Despite the established association of HCV chronic infection with development of liver cirrhosis and hepatocellular carcinoma [67], the molecular mechanisms involved in this process are still unknown. Several reports have shown that, in addition to hepatocytes, HCV can infect T and B lymphocytes.

The HCv genome encodes a single polyprotein of more than 3000 residues which is cleaved by host and viral proteases producing 3 structural proteins (core, E1 and E2) and 7 nonstructural proteins. The non-structural proteins orchestrate viral replication forming a membrane-associated replication complex. All the proteins are anchored to the ER membrane by specific membrane segments: with the exception of E1 and E2, which face the ER lumen, all the other HCV proteins are mainly exposed to the cytosolic space [68]. Two HCV proteins have been demonstrated to interact with calcium-signaling: the HCV core protein and the non-structural protein NS5A.

HCV core has recently emerged as a candidate protein implicated in liver oncogenesis [69]. It has been reported to interfere with cell signaling by modulating mitogen-activated protein kinase (MAPK) signaling, activating nuclear factor κB (NFκB), interacting with signal transducer and activator of transcription 3 (STAT3) and retinoic acid receptor (RXR) and modifying the expression of cellular proto-oncogenes like c-*myc* and tumor suppressor genes (p53, p21 and pRb) (for a review see [70]). In cooperation with H-ras, HCV core has been reported to transform both immortalized and primary rat fibroblasts [71]. Furthermore, in vivo studies have demonstrated that transgenic mice for HCV core [72] or the full-length HCV genome [73] develop HCC. The role of HCV core in the control of cell death

is thought to be potentially involved in its oncogenic activity. In fact, HCV core has been reported to induce or inhibit cell death, upon cell sensitization with anti-Fas antibody, tumor necrosis factor-α (TNFα) or serum starvation (reviewed in [74]) and to determine aggregation of Fas receptors, in lymphoid cells, directly leading to apoptosis [75]. Furthermore, recent reports have shown that in vitro expression of HCV core may directly induce apoptosis in 20–50% of transfected cells [76,77].

According to previous studies, full-length HCV core mainly localizes to the cytosolic side of the ER membrane [78], although HCV core has also been shown to colocalize with lipid droplets [79], mitochondria [72,80], and the intermediate Golgi compartment [81], while C-terminally truncated core localizes to the nucleus [72,82]. HCV core processing and maturation occur in the ER and is strictly dependent on interaction with the ER membrane [78].

HCV core protein has been shown to interfere with calcium-signaling in T cells, where it activates T cell responses [83] and in liver derived cells, where it can induce apoptotic cell death [77].

Bergqvist et al. demonstrated that the expression of HCV core correlates with increased levels of cytosolic Ca^{2+} and spontaneous Ca^{2+} oscillations in transfected Jurkat T cells [83]. Expression of the HCV core protein was shown to activate IL-2 promoter through activation of NFAT [84]. HCV core was shown to accelerate emptying of intracellular calcium stores, inducing Ca^{2+} entry via CRAC channels and favouring high frequency of cytosolic calcium oscillations which specifically activate NFAT [83]. The effect of HCV core on calcium mobilization was not dependent on phospholipase C-γ1 (PLC-γ1) activity or increased IP^3 production and did not require functional IP_3R, leading to hypothesize that insertion of the viral protein in the ER membrane may be sufficient to promote calcium-leakage from the ER. Interestingly, treatment of cells with CsA and depletion of extracellular calcium by EGTA were shown to block NFAT activation confirming a role of calcium mobilization in the activation of T cells upon HCV infection [85].

A decrease in the $[Ca^{2+}]_{er}$ induced by transient and stable expression of HCV core protein in liver-derived cells was reported by our team using recombinant aequorin calcium probes. HCV core was shown to trigger ER stress unfolded protein response, as demonstrated by the induction of Grp78/BiP, Grp94, calreticulin and SERCA2 expression. ER calcium-depletion and ER stress were shown to induce apoptosis in this model through overexpression of the CHOP/GADD153 proapoptotic factor, Bax translocation to the mitochondria, mitochondrial membrane depolarization, cytochrome *c* release, Caspase-3 and PARP cleavage. Reversion of HCV core-induced ER calcium depletion (by transfection of SERCA2) completely abolished mitochondrial membrane depolarization, suggesting that calcium-signaling plays a major role in the HCV core-mediated control of apoptosis. ER stress and apoptosis were also found in a proportion of HCV full-length replicon-expressing cells and in the liver of HCV core transgenic mice. HCV core-induced ER calcium-depletion was shown to follow ER stress and to be related to the impaired function of SERCA pump, possibly due to the HCV core-induced overexpression of calreticulin which has been shown to bind and inhibit SERCA proteins [77,86].

No modification of cytosolic Ca^{2+}-signal was noted upon core expression. This finding could be explained by an increase of Ca^{2+} in the ER-mitochondria microdomains, a process potentially implicated in mitochondrial membrane depolarization and apoptosis [77,87].

Overall, these data show that HCV core determines ER calcium-depletion by inducing ER calcium-leakage (T lymphocytes) and/or ER stress (liver cells), thus leading to T cell activation or modulation of apoptotic death control in epithelial cells. This last effect could be related to mechanisms involved in HCV-induced chronic liver disease and transformation.

NS5A is a serine phosphoprotein, which is localized to the ER membrane [88–90]. NS5A has been shown to interact with double-stranded RNA-dependent kinase (PKR) and inactivate its function, thus modulating the interferon-stimulated antiviral response [91]. NS5A has been shown to function as a transcriptional trans-activator [92,93]. Interaction of NS5A protein with calcium-signaling has been suggested by using "calcium" drugs. In fact, calcium chelating drugs as well as antioxidant reagents were shown to reduce NS5A's ability to activate transcription factors like NFκB and STAT3 [94]. Another calcium-dependent pathway for NS5A-dependent NFκB activation was demonstrated by Waris et al. [95]. This involves NS5A-dependent activation of the calcium-dependent calpain protease which mediates degradation of NFκB inhibitory subunit IκBα [95]. Calpain was also implicated in the cleavage of NS5A [96]. A hypothetic model for the impact of NS5A-induced elevation of cytosolic calcium on signal transduction activation is the following: NS5A could induce ER stress leading to the efflux of calcium from the ER. Calcium release from the ER enhances cytosolic calcium-concentration thus activating calcium dependent proteases such as calpain which in turn cleaves IκBα and NS5A. The calcium released from the ER is taken up by the mitochondria. Elevated $[Ca^{2+}]_{mt}$ directly affects transmembrane potential and increases mitochondrial ROS production, leading to the activation of transcription factors, STAT3 and NFκB [94].

The HCV polypeptide p7, located in the ER membrane was also proposed to form an ion channel in cellular membranes potentially responsible for calcium-flow from the ER [97].

2.4. Enteroviruses

Enteroviruses (e.g. polioviruses, coxsackieviruses, echoviruses, etc.) are a family of nonenveloped, cytolytic viruses containing a 7.5 kb single-stranded RNA genome that encodes four capsid proteins (VP1 to VP4) and ten non-structural proteins (including seven mature proteins ($2A^{pro}$, 2B, 2C, 3A, 3B, $3C^{pro}$, and $3D^{pol}$) and three stable cleavage intermediates with distinct functions from their cleavage products (2BC, 3AB, $3CD^{pro}$). After cell entry and virion uncoating, the RNA molecule acts as an mRNA directing the synthesis of a single polyprotein, which is subsequently processed by virus-encoded proteases to produce the structural capsid proteins and the non-

structural proteins [98]. Replication of the viral RNA takes place in replication complexes at the outer surface of virus-induced membranous vesicles that proliferate and accumulate in the cytoplasm of infected cells [99,100]. Non-structural proteins induce alterations in host-cell functions (inhibition of transcription, disturbance of nucleo-cytoplasmic trafficking, inhibition and re-modeling of the vesicular transport system) which serve to create the proper conditions for efficient viral multiplication by increasing availability of cellular components used by the virus and/or by evading anti-viral host cell responses [101].

The non-structural protein 2B has been shown to modify calcium-signaling. It is a small (97–99 aa) membrane-integral replication protein [102] localized at the surface of the ER- and Golgi-derived membrane vesicles at which viral replication takes place. This protein contains an hydrophobic region which is a cationic amphipathic alpha-helix [103] that can form, by homomultimerization, membrane-integral pores [101,104]. It has been shown that infection of HeLa cells with coxsackievirus results in a reduction of the amount of calcium that can be released from the intracellular stores, associated to a parallel increase in the $[Ca^{2+}]_{cyt}$ due to the influx of extracellular calcium [105]. More recent studies have demonstrated, using organelle-targeted aequorin calcium probes, that 2B reduces $[Ca^{2+}]_{er}$ and $[Ca^{2+}]_{Golgi}$ in HeLa cells [106] without affecting the refilling activity of the SERCA [107]. This leads to a decrease of the amount of calcium that can be released from the stores and of the stimulus-induced rise of $[Ca^{2+}]_{mt}$. These data also confirm that the influx of Ca^{2+} from the extracellular medium is increased, leading to larger cytosolic calcium responses.

The main consequences of cell Ca^{2+}-alterations induced by the 2B proteins are:

(1) Impact on viral replication. Infected cells contain both the 2B protein and the 2BC stable precursor, which is responsible for the accumulation of the membrane vesicles at which viral RNA replication takes place [108]. The 2B and 2BC proteins could contribute to the cytoplasmic accumulation of transport vesicles through the reduced Golgi and ER lumenal $[Ca^{2+}]$. It has been shown that mutation of protein 2B causes defects in viral RNA replication [103]. This result has suggested that there is a close correlation between the ability of 2B to alter membrane permeability and to support viral replication [109].

(2) Impact on inhibition of apoptotic host cell response to viral infection. Actually, enterovirus infection leads to the development of the so-called cytopathic effect (CPE), a necrosis-like type of cell death which is the result of a complex interplay between apoptosis-inducing and apoptosis-suppressing functions encoded by the enterovirus genome [110]. Early in infection, the cell apoptotic response is triggered [111]. However, with the onset of viral replication (i.e. about 2 h postinfection), the apoptotic program is abruptly interrupted [106,110]. In fact, at later stages, after development of CPE, some signs of apoptosis are detected [112]. It has been shown that mutants of 2B that were defective in modifying the intracellular Ca^{2+}-fluxes failed to protect cells against apoptosis [106]. The 2B protein, by forming channels in the ER membrane, leads to a decreased $[Ca^{2+}]_{er}$, and to a decreased calcium flow between stores and mitochondria, mimicking a calcium-mediated mechanism of apoptosis suppression described for Bcl-2 [113]. The expression of 2B has been shown to suppress apoptotic cell death induced by C^2-ceramide (which reduces $[Ca^{2+}]_{er}$), but not by etoposide (which acts directly on mitochondria by causing membrane translocation of Bax [114]), suggesting that it specifically targets a Ca^{2+}-sensitive, ER-dependent apoptotic pathway [106]. Thus, it seems that the antiapoptotic activity of 2B protein most likely serves to delay apoptotic responses, providing the virus the time required for genome replication, rather than completely prevent all signs of apoptosis.

(3) Potential impact of 2B-induced inhibition of cell secretory pathway (mediated by the decrease in $[Ca^{2+}]_{er}$ and $[Ca^{2+}]_{Golgi}$) leading to down regulate innate immune responses (secretion of cytokines) as well as adaptive immune responses (exposure of peptide-loaded MHC-I molecules) and to interfere with recycling of death receptors (e.g. tumor necrosis factor receptor) to the cell surface [107].

To summarize, the enterovirus 2B protein forms pores in ER and Golgi membranes leading to a decrease in $[Ca^{2+}]_{er}$ and $[Ca^{2+}]_{Golgi}$ and in calcium-fluxes to mitochondria, thus disturbing intracellular calcium-homeostasis. This activity contributes to create conditions required for replication of the viral RNA genome, to suppress apoptotic host-cell responses which limit viral multiplication and, hypothetically, to evade anti-viral immune response.

2.5. Rotavirus

Rotaviruses, members of Reoviridae family, are recognized as the most important cause of viral gastroenteritis in young children and animals. Rotaviruses are nonenvelopped viruses. Their double-stranded RNA genome (18 to 30 kb), encoding for six structural and five non-structural proteins, is contained within an icosahedral capsid organized in three concentric layers. The internal layer is formed by VP2, the intermediate layer is formed by VP6 and the outer shell consists of glycoproteins VP7 and VP4 organized in dimers to form 60 spikes [115].

The DLP (Double-shelled particles: core plus VP6) sub-viral particles are assembled in viroplasms, electron dense cytoplasmic structures consisting of an accumulation of viral proteins and nucleic acids. It has been well established that the DLP binds to the ER to bud into this compartment. The viral non-structural protein NSP4 acts as a receptor for the DLP. During the budding process, the virus acquires a transitory membrane envelope containing NSP4 and VP7, which are ER membrane-associated proteins, and VP4. Then, the particle matures by a selective retention of the external capsid proteins VP4 and VP7 and the elimination of NSP4 and the membrane lipids [115].

This process has been shown to be strictly calcium-dependent [116,117]. In fact, in the absence of Ca^{2+}, VP7 did not assemble onto virus particles and remained in the cytoplasm outside the ER [118]. ER calcium-depletion impaired the N-glycosylation of VP7 and NSP4 [116,117].

It has been shown that rotavirus infection induces a progressive increase in Ca^{2+} plasma membrane permeability, through activation of an L-type calcium channel, which leads to an elevation of cytosolic and store Ca^{2+}-concentration [116, 119,120].

Several studies indicate that calcium is a critical factor in rotavirus cytopathology (necrosis or oncosis). The cytosolic calcium rise induced by rotavirus infection has been shown to lead to cell death in enterocytes, fetal monkey kidney cells (MA104) [121] and human colon-cancer derived (Caco-2) cells [122]. In fact rotavirus-dependent cell death was reduced by decreasing extracellular Ca^{2+}-concentration, by cell treatment with the BAPTA-AM, and by adding the Ca^{2+} channel inhibitor verapamil [119,121].

The nonstructural rotavirus protein NSP4 has been shown to act as an enterotoxin [123]. In fact, NSP4 is the first characterized viral enterotoxin. Exogenously added NSP4 induces diarrhea in rodent pups and stimulates secretory chloride currents across intestinal segments. This disease response was specific, age and dose-dependent [123].

The enterotoxin effect of NSP4 is related to modification of calcium-signaling. Indeed, NSP4 has been shown to induce an increase of $[Ca^{2+}]_{cyt}$ in Sf9 insect cells, in human intestinal HT-29 cells [124,125] and in HEK 293 cell line [126]. Elevation of $[Ca^{2+}]_{cyt}$ was show to be due to ER calcium depletion and dependent on PLC activation and IP^3 production [124,125].

3. DNA viruses

3.1. Hepatitis B virus

With an estimated 350 million individuals chronically infected worldwide and approximately one million deaths annually, Hepatitis B virus (HBV) is regarded as one of the most fatal human pathogens. Chronically infected people are at risk for developing severe liver cirrhosis that may eventually progress to hepatocellular carcinoma.

HBV is a 3.2 kb partially double-stranded circular DNA virus that undergoes reverse transcription during its replication cycle [127]. The genome includes four open reading frames encoding two structural proteins, the viral envelope and the core, and two non-structural proteins, the reverse transcriptase-polymerase and the X protein.

The hepatitis B X protein (HBx) is a multifunctional protein which has been shown to modify calcium-signaling. HBx is mainly located to the cytoplasm, where it has been shown to target mitochondria [128] and colocalize with VDAC 3 [129]. However, it can also be detectable in the nucleus [130]. HBx exhibits pleiotropic effects that modulate cell responses to genotoxic stress, protein degradation and activation of signaling pathways (for review, see [131]).

HBx transactivates a number of cellular promoters and enhancers containing binding sites for NFκB, activator protein-1 (AP-1), activator protein-2 (AP-2), c-EBP, activating transcription factor/c-AMP-responsive element binding protein (CREB), RNA polymerase and NFAT, cellular promoter of genes associated with cell proliferation such as IL-8, TNF, transforming growth factor-β (TGF-β) and early growth response factor and cytosolic signal transduction pathways such as Ras/Raf mitogen-activated protein kinase, Src kinases, c-*jun* N-terminal kinase and Jak1/STAT [131–133]. Overall, HBx has been shown to play a major role in HBV DNA replication and in HBV-related liver cell transformation [134].

Depending on the cell type and experimental procedure, HBx has been reported either to inhibit [135] or promote [136,137] cell death. Actually, an integrated view of the role of HBx on apoptosis has been proposed. In this model, high levels of HBx, which are present during the acute phase of HBV infection, cause cell cycle block and apoptosis, whereas low HBx levels, such as those observed in chronically infected humans, would allow cell liver proliferation [138].

Impact of HBV on calcium-signaling was mostly demonstrated through the action of HBx protein. The main effect reported is the elevation of $[Ca^{2+}]_{cyt}$ as demonstrated by using recombinant aequorin probes and Fura2-AM dye [139,140].

The consequences of the HBx-mediated modifications of Ca^{2+}-signaling are:

(1) Viral replication. Several studies have shown that the impact of HBx on HBV replication is dependent on the HBx-induced modification of $[Ca^{2+}]_{cyt}$ [140–145]. HBx has been shown, in HepG2 cells, to activate cytoplasmic kinases, proline-rich tyrosine kinase 2 (Pyk2) and focal adhesion kinase (FAK), involved in HBV reverse transcription and DNA replication, in a calcium-dependent manner [141,142,146]. The calcium-dependent action of HBx on HBV DNA replication relies to three lines of evidence: (i) Activation of Pyk2 and FAK are mediated by increased levels of $[Ca^{2+}]_{cyt}$. Chelation of cytosolic calcium with BAPTA-AM blocked HBx-dependent activation of Pyk2 and FAK and in turn HBV DNA replication [141,146]. (ii) Inhibition of mitochondrial channels with CGP37157 or CsA blocked HBx activation of HBV DNA replication [141] (iii) Drugs which increase the level of cytoplasmic calcium functionally replace HBx in stimulating viral DNA replication [141,142]. Xia et al. found that both HBV replication and Pyk2 phosphorylation can be inhibited also by blocking ER Ca^{2+}-ATPase or IP_3R, but not RyR, concluding that ER could play an important role in the HBx-mediated HBV replication. Interestingly, a stronger inhibitory effect was observed by blocking mitochondrial PTP and ER Ca^{2+}-ATPase or ER IP_3R [144] (Fig. 3).

(2) Core assembly. It has been shown that increased $[Ca^{2+}]_{cyt}$, mediated by HBx protein, induced HBV core assembly [143]. Treatment by BAPTA-AM and CsA reduced HBV capsids and treatment by TG increased HBV capsids in transfected HepG2 cells [143].

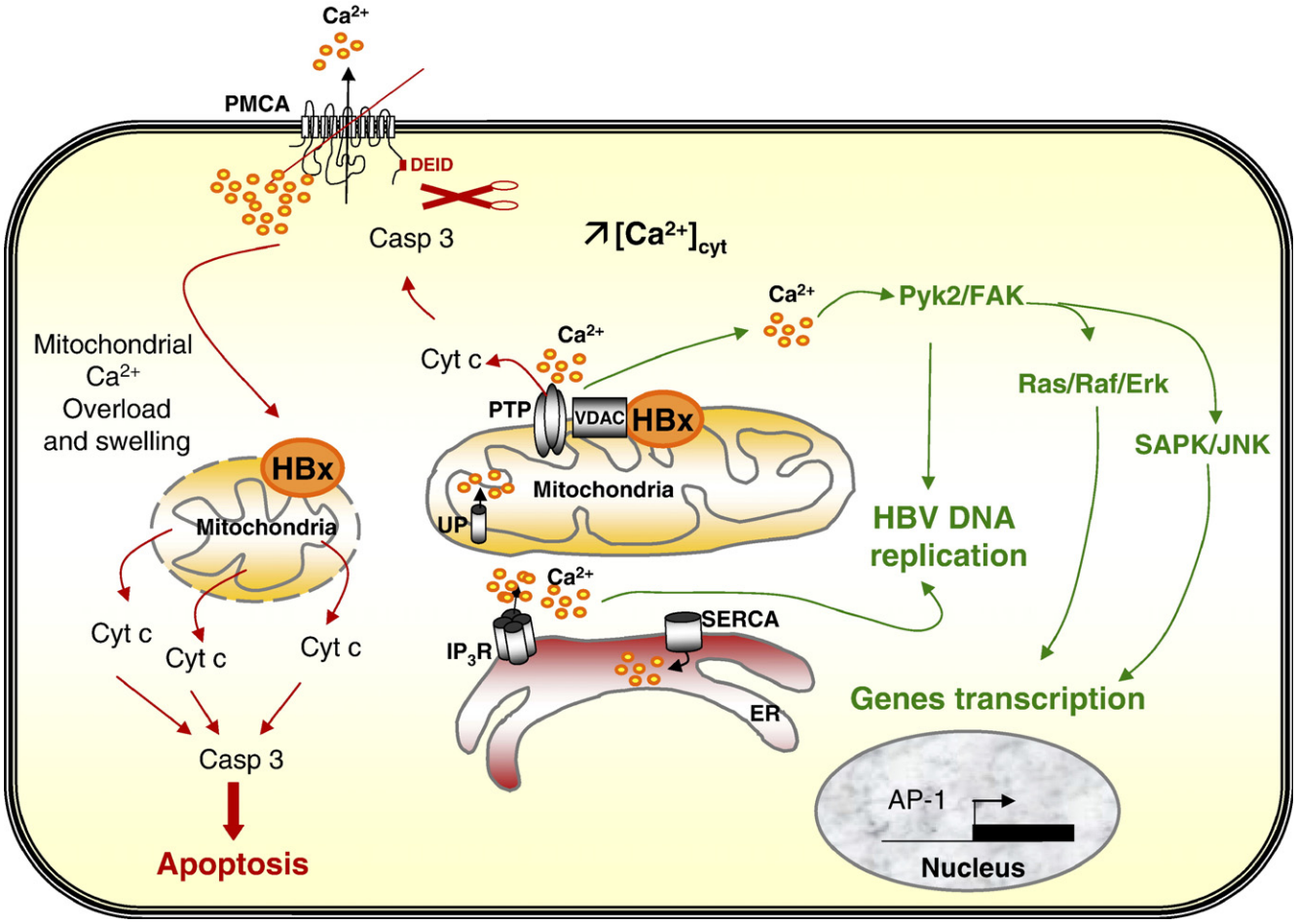

Fig. 3. Schematic model illustrating the effect of HBx on Ca^{2+}-signaling and its possible significance for HBx-induced apoptosis, signal transduction activation and viral DNA replication. Through its mitochondrial localization, HBx has been shown to interact with voltage dependent anion channel. This may lead to opening of the permeability transient pore (PTP) and release of calcium from mitochondria thus increasing cytosolic calcium-concentration ($[Ca^{2+}]_{cyt}$) [139,140]. This activates Pyk2/FAk family kinases [141,142,146], which in turn increase viral replication. ER Ca^{2+} mobilization has also been implicated in virus DNA replication [144]. HBx-mediated elevation of $[Ca^{2+}]_{cyt}$ also induces the activation of both JNK and MAPK signal transduction pathways leading to the activation of cellular genes expression (e.g. through activator protein-1 (AP-1)) (pathways shown in green) [140]. PTP also determines the release of cytochrome *c* (cyt *c*) thus activating Caspase 3 (Casp 3). Caps 3-dependent cleavage of PMCA enhances $[Ca^{2+}]_{cyt}$, leading to mitochondrial Ca^{2+}-overload, mitochondrial structure alteration and further release of Caspase cofactors. This process allows the final commitment of cell death (pathway shown in red) [139].

(3) Intracellular signal transduction pathways. Elevation of $[Ca^{2+}]_{cyt}$ has been demonstrated to play a key role in the HBx-mediated gene transactivation which involves calcium-dependent transcription factors (e.g. NFAT [147]) and calcium-dependent signaling pathways (e.g. JNK and Ras/MAPK/AP-1 [140]). The Pyk-2/FAK kinases family, which is also activated by cytosolic Ca^{2+} elevations, activates Src [148] and Jun [149] kinases leading thus to the regulation of cellular signal transduction cascades, cell proliferation, apoptosis, and cell migration (Fig. 3).

(4) Impact on cell death. HBx has been shown to modulate apoptotic cell death and, in specific cellular models, to have a pro-apoptotic effect [134]. Data obtained by using recombinant aequorin calcium probes have revealed that HBx-induced Ca^{2+}-signaling alterations determine an important potentiation loop in the HBx-induced pro-apoptotic effect [139]. To the molecular mechanisms, our team demonstrated that overexpression of HBx in liver tumor-derived HepG2 cells and in cervix tumor-derived HeLa cells enhanced agonist-evoked cytosolic Ca^{2+}-signals. The HBx protein, which is located at the pore of the mitochondria, was shown to induce the release of cytochrome *c*, which activates the Caspase 3. Caspase 3, in turn, cleaves the PMCA, decreasing its activity and determining the elevation of $[Ca^{2+}]_{cyt}$ in the space beneath the plasma membrane. These calcium-signaling modifications were shown to be associated with mitochondrial Ca^{2+}-overload leading, in our model, to alterations of mitochondrial structure (swelling and permeabilization), followed by reduction of mitochondrial calcium accumulation and cell apoptosis [139] (Fig. 3).

Overall, the studies focused on HBx and calcium-signaling support the following model: HBx localizes to the mitochondria [129] (where it can alter mitochondrial function and physiology [150]): it activates PTP opening and releasing of

mitochondrial calcium to the cytoplasm. Possibly, calcium could also transiently accumulate into the ER through Ca^{2+}-ATPase, and be released through the IP^3R. According to the induced level of calcium elevation in the cytosol, HBx could activate HBV DNA replication and/or modify intracellular signaling pathways leading to cell proliferation or apoptosis. If the level of released calcium in the cytosol is elevated and/or Caspase 3 activation further increases the cytosolic calcium elevation through PMCA cleavage, calcium accumulates in mitochondria, causing mitochondria matrix calcium overloading, PTP opening, further calcium release to cytoplasm and eventually apoptotic cell death.

3.2. Human Herpesvirus type-8

Human Herpesvirus type-8 (HHV-8), also designated as Kaposi's sarcoma-associated Herpesvirus (KSHV), targets mainly circulating B cells and endothelial cells and is associated with the development of Kaposi's sarcoma, primary effusion lymphoma , and a subset Castleman's disease. HHV-8 genome consists of a double-stranded long unique DNA molecule of approximately 140.5 kb flanked by high-G+C terminal repeat units [151]. The proteins and genes associated with latent infection, clustered at a latency locus within the HHV-8 genome, include Latency Associated Nuclear Antigen-1 (LANA1), viral cyclin (v-Cyclin), and viral Fas-associated death domain-like interleukin 1β-converting enzyme inhibitory protein (v-FLIP). HHV-8 genome displays also a number of genes associated to lytic HHV-8 infection that may be involved in tumour growth, as the viral G-protein-coupled receptor (v-GPCR), a viral bcl-2 homologue, regulatory proteins K1 and K7 and chemokine-like proteins called viral Macrophage Inflammatory Proteins (v-MIP) [152]. Several HHV-8 proteins were demonstrated to mediate intracellular calcium alterations or to promote calcium-dependent processes: K15, v-GPCR, K1, K7, VMIP I and II and v-Bcl2.

Reports of alteration of calcium-signaling mainly concerns HHV-8 proteins expressed during the lytic phase of the infection. Indeed, HHV-8 reactivation, as demonstrated by expression of lytic viral accessory protein PF-8 or the late-lytic viral envelope glycoprotein gpK8.1, was proved to occur upon mobilization of intracellular Ca^{2+} induced by ionomycin or thapsigargin and to act through activation of calcineurin [153].

The K15 protein, expressed during latency in infected tumors, significantly inhibits B-cell Receptor (BCR)-mediated calcium mobilization measured by Indo-1 loading [154].

HHV-8 protein v-GPCR is a constitutively active homologous of IL-8 receptor in the plasma membrane. Transient transfection of v-GPCR in COS cells induces agonist-independent accumulation of IP_3 and activation of gene transcription through the PKC-responsive promoter [155]. Interestingly, this protein was found to have a pro-oncogenic function and to promote angiogenesis in vitro and in vivo [156]. The presence of a constitutively active GPCR in the viral genome suggests that its pathogenic role could be related to a permanently activated signaling leading to altered cell growth and neoplastic transformation.

The K1 protein is a lymphocyte B cell receptor-like protein involved in signal transduction through plasma membrane which can down regulate BCR expression [157]. K1 determines, through a phosphorylation cascade of downstream elements, a prolonged increase of intracellular $[Ca^{2+}]$, shown by using Indo-1, and activation of NFAT leading to expression of inflammatory cytokines and growth factors, virus dissemination and virus-associated angiogenic proliferation [158].

K7 is located to the mitochondria, where it acts as an anti-apoptotic protein by preventing the loss of $\Delta\psi_m$. K7 was shown, by using Indo-1, to induce calcium mobilization in the cytoplasm of transiently transfected BJAB cells [159]. Two-hybrid screening analyses have shown a specific interaction of K7 with a T cell modulator of Ca^{2+}-signaling (Calcium-Modulating Cyclophilin Ligand (CAML)) [159,160]. Since CAML is present mainly in the ER and K7 in the mitochondria [159], their interaction may take place in contact sites between the ER and mitochondria [161]. The interaction between K7 and CAML is thought to increase cytosolic Ca^{2+}-concentration through ER Ca^{2+}-release and activation of the CCE [159].

HHV-8 encoded v-MIP I and II cytoplasmic chemokines have been shown, by using Indo-1, to mobilize calcium through chemokine Receptor 5/8 [162,163] suggesting that they may play important roles in the propagation of Kaposi sarcoma and others HHV8-related diseases.

HHV-8 genome encodes a Bcl-2 homologous protein (even if some structural differences were described). v-Bcl2 could exert its antiapoptotic effect by reducing ER and cellular Ca^{2+}-content, like Bcl-2 [164,165]. Indeed, even if the v-Bcl2 effect on cellular Ca^{2+}-homeostasis has not been studied yet, the viral protein has been shown to promote survival of infected cells thus contributing to the development of Kaposi sarcoma [151].

Thus, HHV-8 encodes Ca^{2+}-regulating proteins which mediate apoptosis inhibition and cell proliferation playing a relevant role in the process of tumorigenesis.

3.3. Human Herpes Simplex viruses

(HSV-1 and HSV-2) are members of the herpes virus family and are known to cause human diseases. Cheshenko et al. [166] demonstrated that exposure of Vero (monkey kidney epithelial) and Caski (human cervical epithelial) cells to Herpes Simplex viruses type-1 and 2 (HSV-1 and HSV-2) results in a rapid and transient increase in $[Ca^{2+}]_{cyt}$ as determined by using Fura2-AM [166]. Pretreatment of cells with pharmacological agents that block release of IP_3-sensitive ER stores abrogates the response. Moreover, release of calcium from IP_3-sensitive stores, but not influx of Ca^{2+} across the plasma membrane, is important for HSV infection [166]. HSV-1 and HSV-2 entry is associated with tyrosine phosphorylation of cellular proteins [167]. In this setting, authors reported that FAK phosphorylation in HSV-infected cells is dependent on cytosolic calcium increase [166]. Phosphorylation of FAK promotes reorganization of the actin cytoskeleton, a process that may be important in nuclear trafficking of internalized virus.

Activation of the transcription factor NFκB in HSV infected macrophages, which participates in proinflammatory response,

was also demonstrated to be mediated by calcium-signaling because specific inhibition of mitochondrial calcium channels prevented NFκB activation [168]. It has also been reported that elevated intracellular [Ca^{2+}] is implicated in cell death and the spread of HSV from infected cells. Indeed, ionomycin was found to increase the release of HSV-1 from epidermoid carcinoma A431 infected cells. The enhanced Ca^{2+}-dependent cell death could promote the virus release which in turn could contribute to the spread of HSV-1 infection [169].

3.4. Epstein–Barr virus

Epstein–Barr virus (EBV) is involved in proliferation of lymphoid and epithelial cells leading to benign and malignant diseases. It has been etiologically linked with infectious mononucleosis and cancers such as Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma or gastric cancer (reviewed in [170]). EBV-related tumors are characterized by the presence of multiple extrachromosomal copies of the viral genome. Expression of EBV latent genes, mainly EBV Nuclear Antigen proteins (EBNA) and Latent Membrane Protein (LMP), also appears to contribute to the viral oncogenic role [171]. The EBV genome is composed of a linear double-stranded DNA, approximately 172 kb in length. Activation of the viral lytic phase results in the expression of two viral transcriptional activators, BZLF1 and BRLF1 and subsequent expression of early and late-activation proteins, such as an IL-10 homologue and a Bcl-2 homologue [170,172].

Early stages of EBV infection, as cell entry and activation of B cells are calcium-dependent. It has been shown that EBV infection of B cells is associated with an increase of intracellular [Ca^{2+}], which can be blocked by a L-type Ca^{2+} channel blocker, verapamil. Verapamil prevented internalization of the virus, polyclonal B cell activation and subsequent transformation [173]. EBV activation of lymphocyte proliferation displays similarities with Ig-induced B cell activation, and favours EBV integration into the host cell genome.

LMP2A and EBNA1 are expressed during latency in long-term virus carriers. [174]. LMP2A acts as a negative regulator on calcium-signaling. Indeed, it has been demonstrated to constitutively aggregate in the plasma membrane mimicking cross-linked BCRs. LMP2A competitively binds cellular phospho-tyrosine kinases (PTKs) thus preventing calcium mobilization upon BCR stimulation [175–177].

As for HHV-8, EBV-induced mobilization of calcium and activation of calcium-related pathways takes place mainly during the lytic phase of the infection. Induction of EBV lytic cycle in EBV-positive Burkitt's lymphoma cells has been achieved by increasing cytosolic calcium through ionophore, phorbol esters or anti-immunoglobulin, and inhibited by CsA, indicating that calcineurin contributes to the lytic process [178,179].

EBV oncoprotein LMP1 was shown to participate in calcium-dependent reactivation of EBV, inducing a Ca^{2+}/calmodulin dependent protein kinase type IV/Gr (CaMKIV/Gr) [172,180]. CaMKIV/Gr is highly expressed in T lymphocytes and determines Ca^{2+}-dependent protein phosphorylation and gene transcription following TCR signaling [181]. Interestingly, transcription of the viral transcriptional activators of the lytic phase is dependent on calcium-responsive sites on the promoters, suggesting a possible involvement of CaMKIV/Gr in calcium-related transcriptional processes. These MEF2 sites are also NFAT-dependent suggesting a synergy between CaMKIV/Gr and calcineurin in latent EBV reactivation [182]. The SERCA inhibitor, curcumin [183], which also inhibits PKC and AP-1 transcription factor, was demonstrated to inhibit transcription and thus prevent EBV reactivation, suggesting a major role of ER calcium-content on EBV switch from latency to replication [184].

4. Interaction of other RNA and DNA viruses with calcium-signaling

Rabies virus infection induces the furious and paralytic form of fetal nervous disorders in humans and a variety of animals. Viral infection preferentially takes place in the brain and spinal cord. It is believed that the pathogenesis of rabies disease is due to functional impairment of virus-infected neurons (reviewed in [185]). Recent findings demonstrated that rabies infection caused loss of the calcium-binding protein calbindin-D28k-immunostaining in the cortical supragranular layers as well as in the striatum of mice infected brains [186]. Calbindin-D-28k regulates the effects of Ca^{2+}-ions on intracellular metabolism [187]. Loss of calbindin-D-28k in the brains of infected mice can disturb Ca^{2+} homeostasis and GABAergic neurotransmission. m-Calpain, another calcium regulated protein was also shown to be upregulated during rabies infection [188].

Influenza viruses infect the respiratory tract (nose, throat, and lungs) in humans. A reduced voltage-dependent Ca^{2+}-current was reported in the neurotropic strain of Influenza A virus infected cells [189] and the upregulation of a novel EF-hand calcium-binding protein, Iba1, was also reported in the central nervous system of infected mice [190]. Neutrophil deactivation related to influenza infection seems to be mediated by a deregulation of calcium responses. In vitro exposure of human neutrophil to influenza virus was reported to increase the generation of IP^3 and determine a rise in intracellular [Ca^{2+}] and efflux of Ca^{2+} from the cell. These data lead to postulate that partial activation of neutrophils by influenza virus leads to impaired availability of intracellular Ca^{2+} stores when subsequent stimuli are applied and offers a biochemical basis for functional deactivation [191].

Human papillomaviruses (HPV) may cause benign epithelial lesions. High-risk genotypes of HPVs, such as HPV-16 or HPV-18 have been recognized as causative agents of high-grade dysplasia, and invasive ano-genital cancer (cervical cancer). Papillomaviruses infect keratinocytes and replicate into their nucleus in a differentiation-dependent manner [192]. Calcium-signaling provides a central control mechanism for growth, differentiation and apoptosis of epidermal keratinocytes, as shown by induction of calcium transients in both dividing and terminally differentiating keratinocytes [193,194].

However, Garrett et al. have suggested that an increase in the concentration of intracellular calcium could also be associated

with progression of HPV-18 immortalized keratinocytes to tumorigenicity [195].

HPV E6 oncoprotein was demonstrated to interact, in HeLa cells, with a Ca^{2+}-binding protein, called E6BP [196], identical to ERC-55 belonging to a new subfamily of the EF-hand superfamily of Ca^{2+}-binding proteins specifically located to the ER [197]. HPV E7 oncoprotein has been shown, in MCF-7 mammary adenocarcinoma cell line, to down-regulate S100P [198], which belongs to the S100 super-family of Ca^{2+}-binding proteins [199–201]. In addition, HPV E7, which induces S-phase entry, is inhibited by S100A8/A9 protein complex. This mechanism could have an important role in modulation of E7 oncogenic activity [202].

Polyomavirus: The human neurotropic JC virus (JCV) and the human BK virus (BKV) in addition to the simian virus 40 (SV40) are members of the Polyomavirus family. Upon reactivation because of immunosuppression, JCV induces the once rare demyelinating disease progressive multifocal leukoencephalopathy most frequently seen in AIDS patients, while BKV induces polyomavirus nephropathy, an increasing common side effect of immunosuppressive therapy in renal transplants recipients.

The virion of polyomavirus is composed of three structural proteins: VP1, VP2 and VP3, as well as a viral minichromosome [203]. VP1 is the major structural protein of the capsid shell protein. The presence of the divalent cation calcium in murine polyomavirus virions was first shown by X-ray fluorometry studies [204] and a structural role for calcium was demonstrated by Brady et al. [204,205] showing that calcium chelation by EGTA (in conjunction with disulfide bond disruption by dithiothreitol), results in the breakdown of the virion into its capsomere subunits and a DNA–protein complex. Interestingly, addition of exogenous $CaCl^2$ to this dissociated mixture permitted reassembly into intact virions which partially regained both infectivity and hemagglutination activity [206–208]. Different studies demonstrated that VP1 protein has calcium-binding capabilities through an amino acid sequence that make up the calcium-binding EF hand structure [209]. Furthermore, recent studies have shown that calcium-binding to VP1 of simian virus 40 is important for virion assembly and for viral cell entry and nuclear entry of the viral genome [209].

Interaction of other viruses such as rhinovirus, mumps virus, HHV-7, cytomegalovirus and adenovirus with calcium-signaling was also reported, however the real impact of this interaction on viral infection and cellular cytopathy remain until today not very well documented.

5. Concluding remarks

This literature analysis highlights recurrent viral strategies targeting calcium-signaling modulation to promote viral infection, cell response to the virus, and escape to the immune response. Calcium dyes and probes allow to define calcium-signaling alterations at the subcellular level helping in understanding the molecular mechanisms responsible for cellular functions perturbations. “Calcium” drugs have been used to explore the cytobiological effects of calcium-signaling deregulations and to block viral-induced effects. These studies will stimulate further analyses paving the way to new therapeutical approaches of viral-related diseases.

Acknowledgements

Work presented in this review has been supported by INSERM (Health and Medical Research Institute), Association pour la Recherche sur le Cancer (ARC), and Agence Nationale de Recherches sur le Sida et les hépatites virales B et C (ANRS). We gratefully acknowledge INSERM for supporting the MD-PhD curriculum (Ecole de l’INSERM) of B. Oulès.

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