JOURNAL OF VIROLOGY, Aug. 1997, p. 6214–6217 Vol. 71, No. 8
0022-538X/97/$04.00+0

Poliovirus Protein 2BC Increases Cytosolic Free Calcium Concentrations

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Received 18 December 1996/Accepted 5 May 1997

Poliovirus-infected cells undergo an increase in cytoplasmic calcium concentrations from the 4th h postinfection. The protein responsible for this effect was identified by the expression of different poliovirus nonstructural proteins in HeLa cells by using a recombinant vaccinia virus system. Synthesis of protein 2BC enhances cytoplasmic calcium concentrations in a manner similar to that observed in poliovirus-infected cells. To identify the regions in 2BC involved in modifying cytoplasmic calcium levels, several 2BC variants were generated. Regions present in both 2B and 2C are necessary to augment cellular free calcium levels. Therefore, in addition to inducing proliferation of membranous vesicles, poliovirus protein 2BC also alters cellular calcium homeostasis.

Intracellular calcium concentrations play a key role in the regulation of a number of cell functions. In addition, calcium modulates several steps during the animal virus life cycle, such as virus replication (9, 24), virus morphogenesis (34), the cytopathic effect (CPE) (43), interaction of viral particles with the cell membrane by fusion of membranes (13), and capsid penetration (30, 37). Cells infected by different plant and animal viruses undergo modifications in the cytosolic free calcium concentration ($[Ca^{2+}]_i$) (7, 12). Thus, cells infected by Semliki Forest virus (33), cytomegalovirus (31), rotaviruses (26, 27, 43), influenza virus (20), human immunodeficiency virus (25), or poliovirus (21, 23) show variations in $[Ca^{2+}]_i$ at early and/or late times during infection. One of the best studied systems in this respect is the infection of cells by human rotavirus. Both cytoplasmic and stored calcium levels increase during the virus life cycle (27). These changes influence the transcriptase activation (9, 24), the maturation of rotavirus particles, and the development of CPE (27, 43). The use of thapsigargin, an inhibitor of the endoplasmic reticulum (ER) Ca^{2+}-ATPase which depletes the ER of free Ca^{2+}, has helped to elucidate the role of calcium modifications in different cellular compartments in those processes. Thapsigargin blocks rotavirus glycosylation and morphogenesis, suggesting that these processes depend on a high $[Ca^{2+}]$ in the ER cisternae, while the CPE is not affected by the inhibitor (26). Therefore, the induction of CPE by rotavirus, but not the formation of mature viral particles, requires a high cytosolic free $[Ca^{2+}]_i$ (26, 27). In fact, elevation of $[Ca^{2+}]_i$ was achieved by the individual expression of the rotavirus nonstructural glycoprotein NSP4, a transmembrane ER-specific protein, in insect cells (46). This was the first demonstration of the capacity of an individual animal virus protein to alter the intracellular Ca^{2+} homeostasis by mobilizing this cation from the ER (45). More recently it was found that overexpression of an integral membrane protein, A38L, encoded by vaccinia virus promoted the influx of extracellular Ca^{2+} into cells (39). This effect is mediated by the ability of A38L to form pores in membranes that allow Ca^{2+} to enter the cells. The resulting increased $[Ca^{2+}]_i$ induces cell necrosis and lysis (40). Human immunodeficiency virus type 1-induced fusion requires calcium ions (10), and it is down-modulated by phorbol myristate acetate (17), while infection by this virus increases the cells' permeability to divalent cations (25). Moreover, synthetic peptides of the human immunodeficiency virus gp41 protein also form pores which allow Ca^{2+} influx (28). Finally, poliovirus-infected human cells also undergo an increase in $[Ca^{2+}]_i$ at a stage during infection when a generalized permeabilization of membranes is observed (7, 23). This increase seems to be a consequence of the entry of extracellular calcium (21). However, virtually nothing is known about the poliovirus protein responsible for this effect and its mode of action. The individual expression of poliovirus nonstructural proteins in cultured cells helped to identify poliovirus proteins 2B and 2BC as responsible for the enhanced permeability of membranes to hygromycin B (1, 11).

This system was utilized in the present work to characterize the poliovirus proteins responsible for the enhanced $[Ca^{2+}]_i$ that occurs after infection of HeLa cells. To achieve this end, the poliovirus nonstructural genes were cloned in the pTM1 vector under the control of a T7 promoter. Expression of each poliovirus nonstructural protein was achieved by Lipofectin-mediated transfection of the recombinant vectors into cells previously infected with a vaccinia virus bearing the T7 RNA polymerase gene (VT7). To avoid potential modifications in $[Ca^{2+}]_i$ caused by recombinant vaccinia virus infection, virus replication was inhibited by treatment with 1-β-D-arabinofuranosylcytosine (ara-C). Under these circumstances no vaccinia virus late proteins are detected by [^{35}S]methionine labeling, while the recombinant poliovirus gene is efficiently expressed from the pTM1 vector (16). Measurement of the $[Ca^{2+}]_i$ was accomplished by cytofluorimetric analyses using the fluorescence probe fluo-3, developed by Minta et al. (22, 29). The acetoxymethylester form of fluo-3 (fluo-3 AM) is nonfluorescent in the presence of Ca^{2+} until it is hydrolyzed inside the cell. The intensity of fluorescence of the fluo-3 generated depends on the amount of calcium bound. Fluo-3 was obtained from Molecular Probes Inc. (Eugene, Oreg.), dissolved in dry dimethyl sulfoxide, and stored at −20°C until used.

The expression plasmids pTM1-2A, -2B, -2BC, -2C, -3A, -3AB, and -3C, and mutant forms of 2BC, were constructed by standard PCR techniques (1, 2). Oligonucleotides were designed to hybridize with the corresponding regions of poliovirus type 1 cDNA cloned in vector pT7XLD. Amplified prod-

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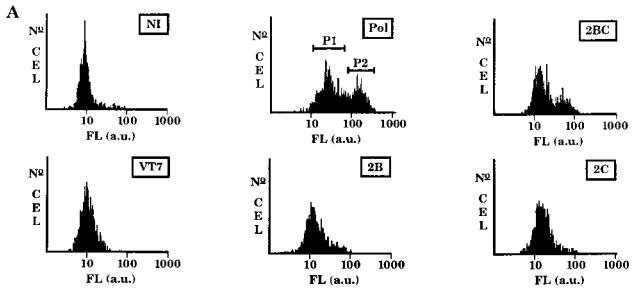
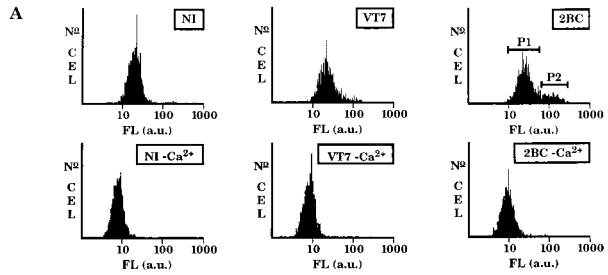


B

		Ni	VT7	2A	2B	2BC	2C	3A	3AB	3C	Polio
%	P1	93	98	93	90	75	91	92	93	91	60
	P2	6	—	6	8	24	8	7	6	7	38
Mean	P1	9	10	6	13	14	15	15	16	13	25
	P2	46	—	37	53	58	59	53	50	58	126

FIG. 1. Flow cytometry of calcium ions in HeLa cells expressing poliovirus proteins. HeLa cells were infected with VT7 (at an MOI of 5) and transfected with the plasmids pTM1-2A, -2B, -2BC, -2C, -3A, -3AB, and -3C or were infected with poliovirus (at an MOI of 50). Vaccinia virus infection and transfections were carried out in the presence of ara-C (40 mg/ml) to prevent the CPE of vaccinia virus. After 14 h of vaccinia virus infection or 5 h of poliovirus infection, the cells were detached from plates, incubated with 6 μM fluo-3 AM, and analyzed in a FACScan cytometer. (A) The intensities of fluorescence for noninfected cells (NI), poliovirus-infected cells (Pol), and vaccinia virus-infected cells (VT7), as well as for HeLa cells in which poliovirus proteins 2BC, 2B, and 2C were expressed by using the recombinant vaccinia virus system, are plotted on a logarithmic scale against the number of cells. FL (a.u.), arbitrary fluorescence units; P1, population of cells with the lower fluorescence value; P2, population of cells with the higher fluorescence value. (B) Relative mean fluorescence value (Mean) and percentage of cells of each population (P1 and P2).



B

		Ni	Ni-Ca^{2+}	VT7	VT7-Ca^{2+}	2BC	2BC-Ca^{2+}
%	P1	97	98	91	98	80	96
	P2	—	—	7	—	19	—
Mean	P1	19	7	21	7	24	8
	P2	—	—	90	—	111	—

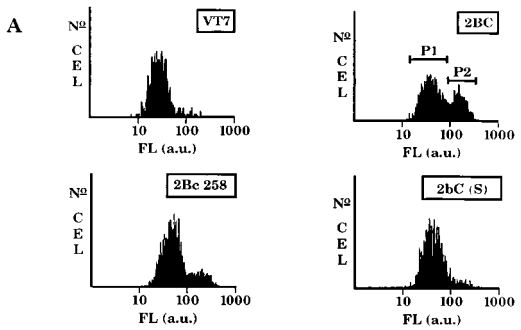
FIG. 2. Effect of Ca^{2+}-free medium on $[Ca^{2+}]_i$. (A) HeLa cells were mock infected or infected with VT7 or were infected and transfected with pTM1-2BC as described in the text in the presence of ara-C, in the presence or absence ($-Ca^{2+}$) of extracellular calcium. After 14 h of infection and transfection, the cells were recovered and analyzed as described in the legend to Fig. 1. (B) Relative mean fluorescence value and percentage of cells of each population. Abbreviations are as explained in the Fig. 1 legend.

ucts were purified with the Gene Clean kit, digested with appropriate enzymes, and ligated to previously digested pTM1 vector (15). Recombinant VT7 (kindly given by B. Moss, National Institutes of Health, Bethesda, Md.) was propagated and grown in HeLa cells and titrated by plaque assay in Vero cells. HeLa cells were grown as monolayers in 35-mm-diameter dishes at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. The recombinant VT7 was used to infect HeLa cells at a multiplicity of infection (MOI) of 6 PFU per cell, in the presence of ara-C (40 mg/ml). After 45 min of virus adsorption in DMEM–2% calf serum, a mixture of DNA (2.5 μg/dish) and Lipofectin (5 μg/dish) in DMEM was added to the cells according to the manufacturer's instructions (GIBCO-BRL), in the presence of ara-C. The VT7-infected and transfected cells were harvested for analysis 14 h after transfection. Poliovirus type I (Mahoney strain) was propagated, grown, and titrated by plaque assay in HeLa cells. Cells were infected with poliovirus at an MOI of 50 in DMEM–2% calf serum. After 1 h of adsorption, the medium was removed and cells were placed in fresh DMEM–2% calf serum (hour zero postinfection). In all cases, HeLa cells were detached at the times indicated as described previously (21), resuspended in DMEM–2% calf serum, and incubated for 10 min at room temperature with 6 μM fluo-3 AM. Samples containing 10^6 cells were observed in a Coulter cytometer (Epics Profile II), and data were analyzed with the software provided with the instrument. Cells not loaded with the dye were also analyzed to test for autofluorescence.

Initially, different plasmids encoding the poliovirus proteins 2A, 2B, 2BC, 2C, 3A, 3AB, and 3C were transfected into HeLa cells, and at 14 h after infection with VT7 the cells were collected and the $[Ca^{2+}]_i$ was estimated. Only two poliovirus proteins, proteins 2A and 2BC, induced changes in the $[Ca^{2+}]_i$ (Fig. 1). For unknown reasons, expression of the poliovirus protease $2A^{pro}$ decreases $[Ca^{2+}]_i$ while protein 2BC increases the level of this cation in a manner similar to that seen during poliovirus infection, e.g., about 30% of the cell population (Fig. 1A) contains a higher $[Ca^{2+}]_i$. It should be noted that the proportion of poliovirus-infected cells with increased $[Ca^{2+}]_i$ augments with the length of the infection (21). The rest of the poliovirus proteins tested, i.e., 2B, 2C, 3A, 3AB, and 3C, did not show any significant change in $[Ca^{2+}]_i$ (Fig. 1B). It should also be noted that while expression of protein 2B alone is capable of modifying membrane permeability to hygromycin B (1), this protein, unlike 2BC, does not affect $[Ca^{2+}]_i$. The expression of these proteins was monitored by Western blot analysis (1). All of them were synthesized at levels comparable to those in poliovirus-infected cells (1). This result indicates that sequences present in both 2B and 2C are necessary to enhance the $[Ca^{2+}]_i$ in poliovirus-infected cells.

An increase of calcium levels in the cytosol of poliovirus-infected cells is due, in large part, to the passage of extracellular calcium to the cell interior (21). To test if this mechanism was also operating in the 2BC-induced $[Ca^{2+}]_i$ increase, the cells were transfected in medium without calcium. The expression of viral proteins, as measured by Western blot analysis, is efficient in this medium lacking Cl_2Ca (data not shown). Figure 2 shows that the increase in $[Ca^{2+}]_i$ observed in 2BC-expressing cells is blocked in calcium-free medium. This result suggests that, as in the infected cells, 2BC alone promotes the entry of extracellular calcium. It is still not clear whether this permeabilization is achieved by a direct effect of 2BC on the plasma membrane or by an indirect effect on a cellular protein, such as a calcium channel. The requirement of calcium increase for poliovirus replication was assayed in calcium-free medium. No effect on virus macromolecular synthesis was observed; rather, the lack of calcium in the medium seems to affect a late stage in the poliovirus life cycle (data not shown).

2B is a rather small hydrophobic protein that interacts with membranes (3, 8). An amphipathic alpha-helix structure, which could form hydrophilic pores upon oligomerization in membranes and is required for viral RNA replication, has been predicted for coxsackie B3 virus protein 2B (47, 48). On the other hand, poliovirus 2C is an enzyme with nucleoside triphosphatase activity that interacts with membranes and with nucleic acids (35, 36). The different domains identified in poliovirus 2BC are depicted in Fig. 3. To identify which regions in



B

		VT7	2BC	2bC(S)	2bC(D)	2Bc 258	2Bc 128	2Bc 110
%	P1	97	68	95	94	85	78	77
	P2	-	31	4	5	14	21	21
Mean	P1	35	46	53	58	53	50	58
	P2	-	115	108	104	120	123	118

C

Protein expressed	Characteristics	Schematic representation
2B	1-97	
2C	98-426	
2BC	1-426	
2bC(S)	V52D	LV
2bC(D)	V52D, I54K	LD LVII LDIK
2Bc(1-258)	1-258	
2Bc(1-128)	1-128	
2Bc(1-110)	1-110	

FIG. 3. Effects of variations in 2BC on $[Ca^{2+}]_i$ increase. HeLa cells were transfected with the plasmids pTM1-2BC, -2bC(S), -2bC(D), -2Bc 258, -2Bc 128, and -2Bc 110 as described in the text. (A) Effects of VT7, 2BC, and two mutants, 2Bc 258 and 2bC(S), on $[Ca^{2+}]_i$. (B) Relative mean fluorescence value and percentage of cells of each population. (C) Schematic representation of the 2BC mutant proteins. Abbreviations are as explained in the legend to Fig. 1.

the 2BC molecule are involved in modifying $[Ca^{2+}]_i$, a number of 2BC mutants were constructed and expressed in HeLa cells. Figure 3 shows that point mutations in the 2B region, where a potential amphipathic helix is present, abrogate the capacity of 2BC to enhance $[Ca^{2+}]_i$. Thus, mutants 2bC(S) (V52D) and 2bC(D) (V52D I54K), which contain charged amino acids in place of hydrophobic ones, are unable to promote the entry of calcium into cells. Besides, long deletions in 2C, even one that leaves only 12 residues at the N terminus of 2C, are still able to augment $[Ca^{2+}]_i$. This N terminus of 2C seems not only to be crucial for modifying $[Ca^{2+}]_i$ but also to interfere with glycoprotein traffic in yeast cells (3). These two activities are not present in 2B alone. A putative amphipathic helix at the N terminus of 2C has been identified (32). This domain may be involved in the interaction of 2BC or 2C with membranes, either directly with the phospholipid bilayer or indirectly, with a membrane protein (14).

The exact function of poliovirus 2BC during the poliovirus replication cycle remains puzzling (50). Polioviruses with mutations in 2B or 2C are defective in viral RNA synthesis (5, 50), but the exact role that each of these two proteins, or their precursor, 2BC, plays in vesicle proliferation in mammalian cells (3, 8) is still unknown. The generation of these vesicles is necessary for viral RNA synthesis (18). Apart from this effect, 2BC permeabilizes cells to hygromycin B (1, 11) and to calcium ions, as reported in this work. The connections among enhanced membrane permeability, increase of $[Ca^{2+}]_i$, vesicle proliferation, and poliovirus genome replication are still puzzling.

The increased $[Ca^{2+}]_i$ could result from a change in net permeability of either the plasma membrane or the ER membrane in poliovirus-infected cells. The changes in cytoplasmic membrane permeability may not be directly responsible for Ca^{2+} influx, since 2B does not alter $[Ca^{2+}]_i$ (44). However, the increase of cytoplasmic $[Ca^{2+}]_i$ may result from a release of ER and other intracellular Ca^{2+} pools, as documented for both mammalian cells and *Spodoptera frugiperda* Sf9 cells (41, 45). Release of Ca^{2+} from internal stores stimulates Ca^{2+} entry across the plasma membrane (45). Activation of phospholipase C and a subsequent increase of inositol-1,4,5-triphosphate also occurs in poliovirus-infected cells (19) and could account for the observed increase of cytosolic calcium ions. If so, the Ca^{2+} uptake reported in poliovirus-infected HeLa cells (21) may be a secondary rather than a primary event, due to alteration in Ca^{2+} homeostasis at the ER membrane. We do not know if the alteration in Ca^{2+} homeostasis observed upon expression of 2BC during poliovirus infection plays a vital role in the viral replication cycle. Free intracellular calcium is known to activate phospholipases and to act with diacylglycerol to activate protein kinase C (6). In addition, lowering the calcium level in the lumen causes vesiculation of the ER membrane (4, 38), whereas transmembrane Ca^{2+} fluxes per se have been shown to be detrimental to cells (40, 42, 49). The role of Ca^{2+} homeostasis in poliovirus-infected cells requires further investigation, as does the role of 2BC protein in promoting permeability of membranes to this cation.

Future work in this direction will benefit from the construction of viable poliovirus 2BC mutants that could indicate the extent to which the different activities of 2BC can be dissociated and to what extent these activities are necessary for poliovirus viability.

This work was supported by DGICYT project PB 94-0148 and the institutional grant to the CBM of Fundación Ramón Areces.

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