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Effects of protein kinase C inhibitors on viral entry and infectivity

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The protein kinase C inhibitor H-7 (2–20 μM) inhibited dose-dependently the infectivity of the vesicular stomatitis virus on cultured human fibroblasts. Electron microscopy showed that H-7 inhibited the viral entry. H-7 also inhibited the infectivity of four other enveloped viruses, herpes simplex I, turkey herpes, vaccinia and Sindbis. Similar results were obtained using staurosporine (2.5 nM), tamoxifen (40 μM), phloretin (140 μM), or W-7 (40 μM). However, the infectivity of non-enveloped viruses (e.g. poliomyelitis I) was not inhibited by H-7. These results show that protein kinase C is critically involved in the infectivity of *enveloped* viruses, most probably at the level of viral entry (receptor-mediated endocytosis).

Protein kinase C; Viral entry; Viral infectivity; Enveloped virus; Endocytosis

1. INTRODUCTION

The specific entry of viruses into their target cells is the key process triggering infection and replication. For enveloped viruses, this process is accomplished by receptor-mediated endocytosis and fusion mechanisms [1,2]. The lack of knowledge regarding the (early) molecular events of virus/cell-receptor translocation across the host-cell membrane ultimately impairs the design of efficient antiviral therapy.

The well-known protein kinase C (PKC) [3,4] was recently found to promote the internalization of several cell-surface receptors (i.e. T-cell surface CD3 complex, epidermal growth factor receptor) (for review see [5]). We have previously shown that PKC is implicated in the Fcγ-receptor mediated endocytosis of human neutrophils [6], and in the internalization of cell-membrane phospholipase C [7]. Therefore we tested the effect of selective PKC inhibitors on viral entry and infectivity.

2. MATERIALS AND METHODS

2.1. *Cells*

Confluent monolayers of human embryo fibroblasts (HEF) or chicken embryo fibroblasts (CEF) were grown in Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and were used 3–4 days after seeding [8,9].

2.2. *Viruses*

Vesicular stomatitis virus (VSV) (Indiana strain), herpes simplex I virus (HSV-I) (MacIntyre strain), poliomyelitis virus I (Mahoney strain), vaccinia virus (Elstree strain) and Sindbis virus (Ar-339 strain) were passaged and assayed on HEF cells, and turkey herpes virus (THV) (Calnek strain) on CEF cells.

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2.3. *Protein kinase C inhibitors*

H-7 (1-(5-isoquinoylsulfonyl)-2-methylpiperazine) [10] was kindly provided by Prof. H. Hidaka, Nagoya University, Japan. Staurosporine [11], tamoxifen [12], phloretin [13] and W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphtalene-sulfonamide) [14] were purchased from Sigma (St. Louis, MO).

2.4. *Antibodies directed to cell-membrane phospholipases*

Inhibitory antibodies directed to cell-membrane phospholipases C (phosphoinositide-preferring, PLC-PI, or phosphatidylcholine-preferring, PLC-PC) and to phospholipase A_2 (PLA_2) were obtained as described [15–17].

2.5. *Viral infectivity assays*

Cells were treated for 1 h with PKC inhibitors or antiphospholipase antibodies, then washed twice in Eagle's medium, and challenged with viruses (0.01 $TCID_{50}$/cell) (TCID, tissue culture infectious dose). Virus samples taken at the indicated postinfection intervals were tenfold diluted and titrated on HEF cells or CEF cells grown on microtest tissue culture flasks (Falcon Plastics, Los Angeles, CA) as previously described [8]. Four assays (10^5 cells/assay) were carried out for each sample and each experiment was repeated at least twice. Toxicity assays for all chemicals or antibodies were done using the Trypan blue exclusion test.

2.6. *Electron microscopy*

HEF cells were processed in the plastic dishes in which they were grown (5×10^5 cells/sample). After treatment for 1 h with 20 μM H-7 or culture medium at 37°C (for control), cells were twice washed in Eagle's medium, and challenged with VSV (1 $TCID_{50}$/cell) for 1 h, at 37°C. HEF monolayers were then washed, equilibrated at room temperature (22±2°C), washed for 30 s in 0.075 M cacodilate buffer containing 3% sucrose, prefixed 5 min in 2.5% buffered glutaraldehyde, briefly washed and postfixed in 2% OsO_4 solution for 10 min, at 4°C. Specimens were dehydrated in a graded series of ethanols and embedded in Epon. Ultra-thin sections were cut with an American Optical ultramicrotome, stained with uranyl acetate and lead citrate, and examined under a Philips 301 electron microscope.

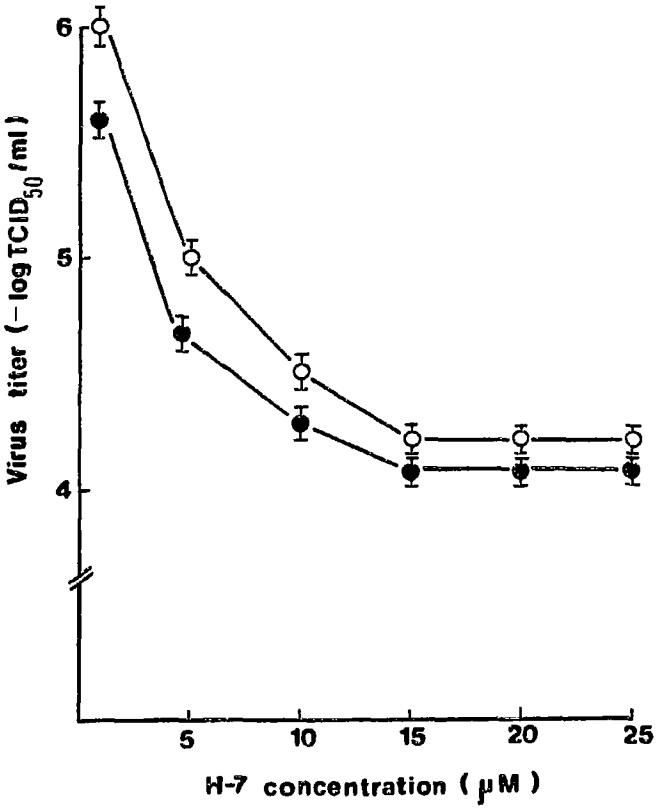

Fig. 1. The dose-dependent inhibitory effect of H-7 (1 h pretreatment of fibroblasts) on the infectivity of VSV (empty circle) or HSV-1 (solid circle). Viral titers ($-\log TCID_{50}$/ml) are shown for the 24 h postinfection interval. Each point is the mean of four determinations ±SE.

3. RESULTS

Fig. 1 shows that 1 h pretreatment with H-7 (2–20 μM) inhibited in a dose-dependent manner the infectivity of VSV and of HSV-1 on cultured fibroblasts. The K_i value of H-7 against PKC is 6.0 μM [10]. Addition of H-7 1 h prior to the viral challenge exerted the maximal antiviral effects. The addition of H-7 3–24 h before the viral challenge resulted in significantly lower antiviral activities, while maintaining the inhibitor in culture medium after virus challenge enhanced the antiviral effect (not shown).

Electron microscopy showed that 1 h pretreatment of HEF cells with 20 μM H-7 impaired the VSV entry (Table I). The number of internalized VSV particles was significantly decreased ($P<0.05$). Fig. 2 shows a typical electron microscope image of VSV particles bound to the cell surface.

Several PKC inhibitors, staurosporine (2.5 nM), tamoxifen (40 μM), phloretin (140 μM) and (W-7) (40 μM) inhibited in a similar manner (1–1.5 log viral titer) the infectivity of the other enveloped viruses (vaccinia, Sindbis or THV).

To investigate the possible mechanism by which PKC inhibitors inhibited the infectivity of the enveloped viruses, we used antibodies directed to cell-membrane phospholipases, shown to inhibit DAG production in living cells, including fibroblasts [15–17]. But the inhibitory antibodies against cell-membrane PLC-PI, PLC-PC, PLA_2 (10–100 μg/ml) or rabbit non-immune IgG (640 μg/ml) (for control) did not mimick the effect of PKC inhibitors on VSV infectivity in HEF cells or on THV in CEF cells.

The infectivity of a non-enveloped virus, (poliomyelitis I) was not modified by 1 h pretreatment of HEF cells with 20 μM H-7.

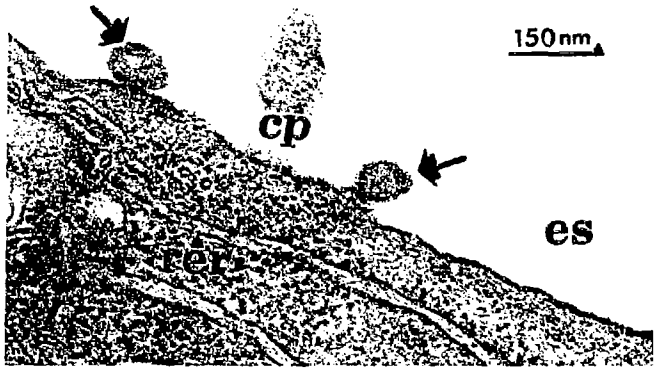

Fig. 2. Electron microscope image of a human fibroblast in culture pretreated 1 h with H-7 (20 μM) and then challenged with VSV. Note two VSV virions attached to the cell-membrane (arrows). No VSV virions penetrated into the cell. cp, cytoplasmic protrusion; rer, rough endoplasmic reticulum; X, microtubules (cross section); es, extracellular space.

4. DISCUSSION

Our results show that 1 h pretreatment of fibroblasts with five different drugs, which share the quality of being PKC inhibitors, inhibited the infectivity of several enveloped viruses belonging to different families: rhabdoviridae (VSV), herpesviridae (HSV-I and THV), poxviridae (vaccinia) and togaviridae (Sindbis). Electron microscopy showed that, in fact, the H-7 pretreatment of fibroblasts inhibited the entry process. Similar results

Table I

Inhibitory effect of H-7 (20 μM) on VSV entry into human fibroblasts in culture

	Virus particles*	
	Bound to the cell surface	Inside the cell
VSV**	2.3 ± 0.4	6.1 ± 1.2
H-7 + VSV***	5.4 ± 1.3	1.1 ± 0.3

*The results are given per 3 cell profiles (the whole contour of the cell visible on a given section).
**30 cell profiles counted
***33 cell profiles counted

were reported by Fields et al. [18] on the entry of another enveloped virus, the human immunodeficiency I virus, into CD4 positive cells.

The effect of PKC inhibitors was not mimicked by inhibitory antibodies directed to cell-membrane phospholipases (PLC-PI, PLC-PC or PLA_2), although these antibodies decreased basal and agonist-induced production of DAG in living cells [15–17]. Noteworthy, PKC inhibitors also inhibited the Fcγ-receptor mediated endocytosis in neutrophils, but in that case the antibodies were also inhibitory [6], suggesting that the routes for PKC activation are different for these two processes. Anyway, the cellular receptor for VSV was shown to contain phosphatidylserine [19], which is a cofactor of PKC [3,4], while vaccinia virus was found to bind the epidermal growth factor receptor [20], which is internalized by PKC [5].

Since the calmodulin antagonist W-7, which inhibits PKC indirectly via phospholipid interactions [14], mimicked the antiviral effects of PKC inhibitors, it is tempting to speculate that, in addition to PKC, some homologous proteins might also be involved in the entry process of enveloped viruses. Indeed, it was recently shown that synaptic vesicle exocytosis for neurotransmitter release is mediated by a vesicle protein homologous to the regulatory domain of PKC [21]. Interestingly, the lack of inhibitory effect of H-7 on the infectivity of non-enveloped viruses (e.g. poliomyelitis I) suggests major differences between the entry pathways of enveloped versus non-enveloped viruses.

Finally, our results showed that PKC inhibitors inhibited the infectivity of different enveloped viruses, most probably at the level of viral entry, although viral attachment to the cell membrane was not impaired.

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