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Inhibitory Effect of Papaverine on HVJ (Sendai Virus) Replication in Rat Glioma C6 Cells

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SUMMARY

The replication of HVJ (Sendai virus) in C6 rat glial cells was found to be inhibited by treatment of the cells with papaverine, an inhibitor of cAMP phosphodiesterase, but not with cAMP or dibutyryl cAMP. In addition, cyclic GMP which often manifests a reciprocal relationship to cAMP did not counteract the inhibition of HVJ yield by papaverine. Both viral genome replication and transcription were suppressed slightly by treatment of the cells with papaverine. In the cells cultured in the presence of papaverine, the synthesis of viral proteins and their phosphorylation occurred at normal rates. Membrane immunofluorescence and cell surface immunoprecipitation showed that the viral glycoproteins HN and F_0 were expressed on the cell surface of the papaverine-treated cells. Moreover, all the viral structural proteins were associated with plasma membrane isolated from the treated cells. These results indicate that papaverine treatment suppresses some part of the process of virus budding at the plasma membrane.

INTRODUCTION

It is well known that paramyxoviruses, especially measles virus, infect and subsequently persist in the central nervous system (CNS) and cause subacute sclerosing panencephalitis (SSPE). Defective synthesis of viral M protein in the CNS has been confirmed by analyses of isolated brain tissue of SSPE patients (Hall & Choppin, 1979*a*, *b*) and of antibodies in sera of SSPE patients (Hall *et al.*, 1979; Stephenson & ter Meulen, 1979; Wechsler *et al.*, 1979). Recently, Carter *et al.* (1983) revealed that although messenger RNA for M protein was present in a SSPE cell line, M protein synthesis was not detected during translation *in vitro*. However, this impairment of M protein synthesis in the CNS remains to be clarified. Interactions between measles virus and cells of neural tissue origin have therefore been investigated. Since neural cells contain abundant endogenous cAMP with levels closely correlated with terminal differentiation, cessation of cell division and induction of neural specific function (Greengard, 1978; Ruben & Rosen, 1975), and intracellular cAMP may play an important role in the restricted replication of measles virus in neural tissue. Miller & Carrigan (1982) reported specific inhibition of M protein synthesis in measles virus-infected neural cells following addition of cAMP-related agents to culture media. Reduced synthesis of the P and M proteins following cAMP exposure was observed in non-neural cells accompanied by reduction of measles virus yield (Robbins & Rapp, 1980). We recently demonstrated that M protein synthesis was selectively restricted at a high incubation temperature in C6 cells infected with wild-type HVJ (Sendai virus; Ogura *et al.*, 1984). The purpose of this study was to investigate the possible relationship between cAMP and the above phenomenon using papaverine, an inhibitor of cAMP phosphodiesterase, and other cAMP-related agents.

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METHODS

Cells and viruses. A rat glioma cell line (C6) was grown in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum and 100 µg/ml kanamycin. A rhesus monkey kidney cell line (LLCMK2) was cultured with DMEM containing 10% calf serum and 10% tryptose phosphate broth. Wild-type HVJ (Nagoya 1-60 strain) was propagated in the allantoic cavity of embryonated chicken eggs. Virus purification was carried out according to the method described by Kimura *et al.* (1976).

Infectivity and haemagglutinin assay. Infectivity of HVJ was determined by plaque counts in LLCMK2 cells and expressed in p.f.u./ml (Sugita *et al.*, 1974). Haemagglutinin was titrated by a microtitre technique according to the procedure of Sever (1962).

Immunofluorescence assay. The procedure of membrane immunofluorescence was performed as described previously (Ogura *et al.*, 1980); antiserum against the glycoproteins of HVJ which has been described previously (Ogura *et al.*, 1981) was used.

Estimation of nucleocapsid formation in infected cells. Isolation and quantification of the nucleocapsid were carried out according to the method of Roux & Waldvogel (1981).

[³H]Uridine labelling of viral RNAs, estimation of viral genome replication, viral transcription and viral primary transcription. For labelling with [³H]uridine (60 Ci/mmol; Amersham), mock-infected and HVJ-infected C6 cells were treated with 5 µg/ml actinomycin D (Pharmacia P-L) in DMEM for 30 min before radiolabelling. The cultures were labelled at 21 h after infection with 20 µCi/ml of [³H]uridine for 3 h in the presence of actinomycin D and then harvested. Estimation of genome replication and of viral transcription were performed as described by Roux & Waldvogel (1981). Viral primary transcription was measured as described by Nagai *et al.* (1983).

Extraction and electrophoresis of ³H-labelled viral RNAs. Viral RNAs labelled with 100 µCi/ml [³H]uridine (60 Ci/mmol; Amersham) in the presence of 10 µg/ml actinomycin D were extracted essentially by the method described by Chirgwin *et al.* (1979). Briefly, the labelled cells were mixed with 4 M-guanidine thiocyanate and gently homogenized in a glass Dounce homogenizer. The homogenate was overlaid onto 1·5 ml of CsCl (1·7 g/ml) and centrifuged at 36000 r.p.m. for 12 h at 20 °C in an SW50 Ti rotor. The pelleted RNA was dissolved in water, extracted once with phenol–chloroform (phenol–chloroform–isoamyl alcohol, 50:50:1), precipitated with ethanol, dried, dissolved in water and stored at −70 °C.

Electrophoresis of viral RNAs was done in horizontal 1·25% agarose gels in 10 mM-sodium phosphate buffer (pH 7·0) after the RNAs had been denatured by glyoxal treatment at 50 °C for 15 min as described previously (Maniatis *et al.*, 1982). After electrophoresis, the gels were fixed, fluorographed, dried and exposed to Kodak film at −70 °C.

Immunoprecipitation and SDS–polyacrylamide gel electrophoresis (PAGE). To examine intracellular viral protein synthesis mock-infected or HVJ-infected cell monolayers were labelled with 50 µCi/ml [³⁵S]methionine (1000 to 1300 Ci/mmol; Amersham) for 1 h in methionine-free DMEM and then solubilized in lysing buffer (0·15 M-NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0·1% SDS, 0·01 M-Tris-HCl pH 7·4) containing 100 µg/ml PMSF. The cell lysates were immunoprecipitated with anti-HVJ hamster serum, prepared by immunizing inbred hamsters with syngeneic hamster cells infected with HVJ, and then analysed by SDS–PAGE according to the method of Laemmli (1970) followed by fluorography.

Cell surface immunoprecipitation. Mock-infected and HVJ-infected cells were labelled with 50 µCi/ml [³⁵S]methionine for 4 h and washed with Hanks' solution. Anti-HVJ hamster serum was added and the cultures were incubated at 4 °C for 30 min. The cultures were washed with ice-cold phosphate-buffered saline (PBS) five times to remove excess antibody and solubilized in lysing buffer. The lysates were precipitated by Protein A–Sepharose CL-4B, and analysed by SDS–PAGE.

Phosphorylation of viral proteins. In phosphorylation studies infected cells were labelled for 4 h with 100 µCi/ml [³²P]orthophosphate (Amersham) in phosphate-free medium at 20 h after infection. The labelled cells were lysed, immunoprecipitated and analysed by SDS–PAGE as described above.

Isolation of plasma membrane. Infected cells were labelled with 50 µCi/ml of [³⁵S]methionine for 4 h. Plasma membranes from the labelled cells were isolated by a modification of the fluorescein mercuric acetate method of Warren *et al.* (1966), as described by Yoshida *et al.* (1979). Isolated plasma membranes were solubilized and subjected to immunoprecipitation and SDS–PAGE as described above.

Other drugs. Dibutyryl cAMP, cAMP and cGMP were purchased from Yamasa Shoyu Co., Chosi, Japan. Papaverine, theophylline and caffeine were obtained from Wako, Osaka, Japan.

RESULTS

Effect of cAMP-related agents on HVJ yield in C6 cells

Monolayers of C6 or LLCMK2 cells were infected at a multiplicity of 5 p.f.u./cell. After 1 h adsorption at room temperature, the cultures were incubated at 32 °C in the presence or absence of the drugs for 35 h. The culture media were harvested and assayed for infectivity.
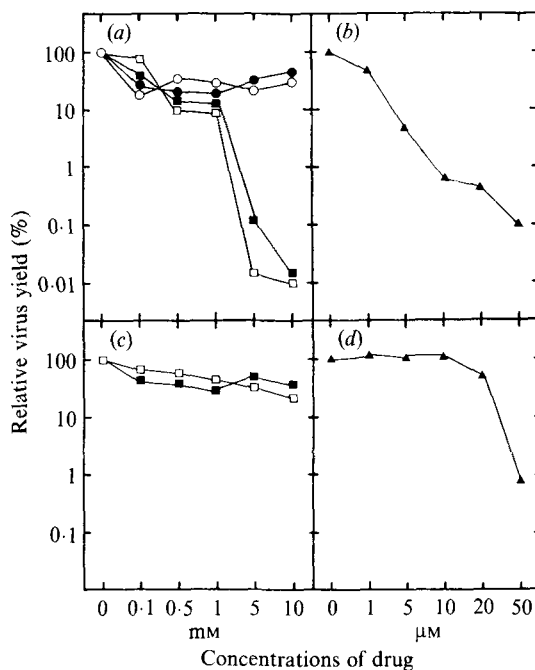
Fig. 1. Effect of cAMP-related agents on the growth of HVJ in C6 (*a* and *b*) and LLCMK2 (*c* and *d*) cells. Cultures were infected at a multiplicity of 5 p.f.u./cell. Various concentrations of dibutyryl cAMP (○), cAMP (●), theophylline (□), caffeine (■) and papaverine (▲) were added after 1 h adsorption at room temperature. The cultures were incubated at 32 °C for 35 h, harvested and then assayed for infectivity.

As shown in Fig. 1, additions of exogenous cAMP and dibutyryl cAMP did not affect HVJ growth in C6 cells. In contrast, HVJ yield was reduced to about 1/130, 1/4000 and 1/500 by 10 μM-papaverine, 5 mM-theophylline and 5 mM-caffeine respectively, which all increase endogenous cAMP, whereas in papaverine-treated LLCMK2 cells the HVJ yield was suppressed at a high concentration (50 μM); theophylline and caffeine showed no inhibitory effect, even at higher concentrations. Thus, HVJ seems to be more susceptible to papaverine, theophylline and caffeine in C6 cells than in LLCMK2 cells.

In order to examine whether or not this inhibition was accompanied by release of incomplete virions, infected cells were labelled with [^{35}S]methionine in the presence of papaverine and the infectivity of culture media and c.p.m. of purified virions in the media were measured. These values decreased proportionally as the drug concentration increased (data not shown), suggesting little production of incomplete virions.

In general, cAMP-controlled reactions are counteracted by cGMP and the effect of cGMP on inhibition by papaverine was therefore examined. Papaverine and cGMP were added simultaneously to the infected cultures and virus titres were assayed 36 h after virus infection (Table 1). There was no significant increase or decrease of the titres in infectious virus when compared with that in infected cultures in the presence of papaverine alone.

Next, 10 μM-papaverine was added to the infected cultures at various times after infection and was found to inhibit HVJ production up to 18 h (Fig. 2). When papaverine was removed from the culture media, infectivity began to reappear within 1 h after the removal, although it was not so rapid as in the control culture in the absence of the drug (Fig. 3). Thus, the suppressive effect of papaverine on the growth of HVJ was reversible.

Viral RNA synthesis in HVJ-infected C6 cells in the presence of papaverine

The infected monolayers were cultured in the presence or absence of papaverine. At 21 h after infection, the cultures which had been pretreated with 5 μg/ml actinomycin D for 30 min were
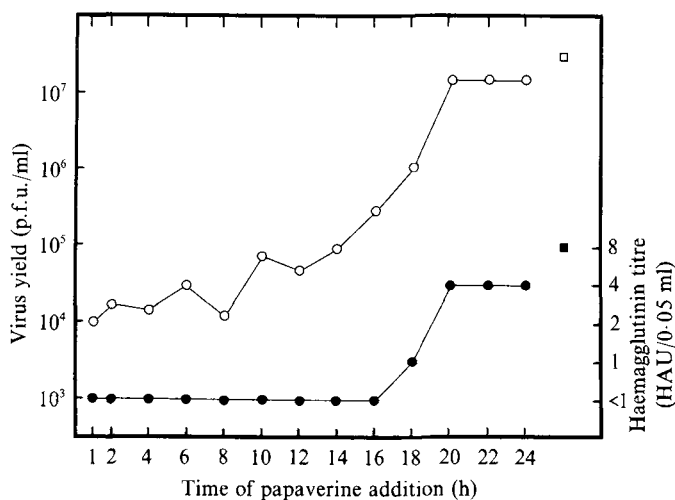
Fig. 2. Effect of papaverine on the growth of HVJ in C6 cells. Cultures were infected at a multiplicity of 5 p.f.u./cell. Papaverine was added at different times after infection to give a final concentration of 10 μM and remained present throughout the experiment. The cultures were incubated at 32 °C for 35 h, harvested and then assayed for infectivity (O) and haemagglutinin (●). Infectivity (□) and haemagglutinin (■) of control cells without papaverine are also shown.

Table 1. *Effect of cyclic GMP on papaverine inhibition of HVJ replication in C6 cells**

| | Time of drug addition after infection | |
Treatment	1 h	14 h
Control	2.58×10^7†	
Papaverine 2 μM	1.24×10^7	1.10×10^7
Papaverine 10 μM	9.50×10^4	1.78×10^5
Papaverine 50 μM	1.38×10^4	1.20×10^4
cGMP 10 mM	2.30×10^7	2.42×10^7
Papaverine 2 μM + cGMP 10 mM	1.58×10^7	1.42×10^7
Papaverine 10 μM + cGMP 10 mM	3.53×10^4	3.25×10^5
Papaverine 50 μM + cGMP 10 mM	5.03×10^3	1.25×10^4

* Cultures were infected at a multiplicity of 5 p.f.u./cell. Papaverine or cGMP was added simultaneously to the cultures at 1 h or 14 h after infection and the cultures were incubated at 32 °C. The culture media were harvested at 36 h after infection and assayed for infectivity.

† Values given as infectivity (p.f.u./ml).

labelled with 20 μCi/ml [³H]uridine for 3 h in the presence or absence of papaverine. The cultures were then harvested and assayed for viral genome replication and transcription as described in Methods.

Both viral genome replication and transcription rates (Table 2) were similarly suppressed to 60% by 10 μM-papaverine, although the virus yield at this concentration was over 100-fold lower than that in the absence of the drug (Fig. 1). Fifty μM-papaverine inhibited viral genome replication and transcription more than 90%. On the other hand, viral primary transcription rate was not reduced to the same extent by papaverine (data not show). There was no detectable difference between the electrophoretic pattern of RNA from untreated cells and that of treated cells at the lower concentrations of papaverine (Fig. 3). Although the synthesis of various viral RNAs was suppressed by the addition of 50 μM-papaverine there was no specific defect in the
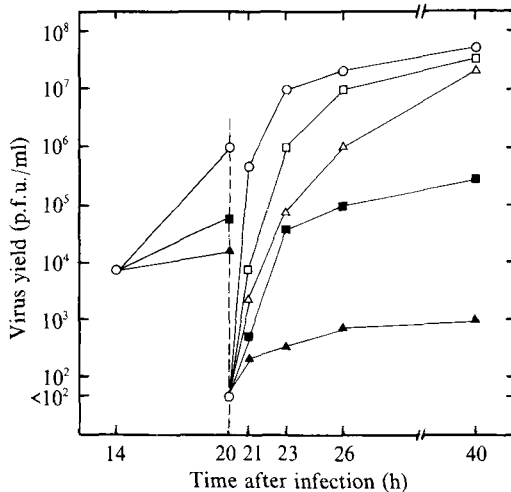
Fig. 3. Reversibility of the inhibitory effect of papaverine on HVJ replication in C6 cells. Cultures were infected with HVJ at a multiplicity of 5 p.f.u./cell and after 1 h adsorption were incubated at 32 °C. Papaverine was added at 14 h after infection. The cultures were washed with Hanks' solution five times at 20 h after infection and then incubated in the presence or absence of papaverine. The culture media were harvested at various times and assayed for infectivity: ○, infectivity in the absence of papaverine throughout the incubation; ■ and ▲, infectivity in the presence of 10 or 50 μM-papaverine, respectively; □ and △, infectivity after removal of 10 or 50 μM-papaverine, respectively.

Table 2. *Effect of papaverine on viral RNA synthetic activities in HVJ-infected C6 cells**

	Concentration of papaverine (μM)	Genome replication rate (c.p.m./dish)†	Viral transcription rate (c.p.m./dish)†
Mock-infected	0	129	533
	2	153	556
	10	100	301
	50	91	189
HVJ-infected	0	2236 (100)‡	6854 (100)
	2	2663 (119·1)	7375 (107·9)
	10	995 (42·5)	2849 (40·3)
	50	155 (3·0)	578 (6·2)

* Cultures were infected with HVJ at a multiplicity of 5 p.f.u./cell and after 1 h adsorption were incubated at 32 °C. Various concentrations of papaverine were added at 1 h after infection.

† Genome replication rate and viral transcription rate were measured as described by Roux & Waldvogel (1981). The cultures were labelled with [³H]uridine for 3 h and harvested at 24 h after infection.

‡ Percentage of c.p.m. relative to 0 μM-papaverine sample after subtracting each mock infection value.

viral RNA synthesis (Fig. 4). Thus, less than 10 μM-papaverine did not significantly inhibit viral RNA synthesis.

Viral protein synthesis and its phosphorylation in HVJ-infected C6 cells in the presence of papaverine

Viral protein synthesis was analysed by immunoprecipitation and SDS–PAGE. Infected monolayers were cultured in the presence or absence of papaverine. At 23 h after infection, the cultures were labelled with 50 μCi/ml· [³⁵S]methionine for 1 h in the presence or absence of papaverine. The cell lysates were subjected to immunoprecipitation and SDS–PAGE.

As shown in Fig. 5(a), all the structural components were detectable despite the presence of the drug. However, viral protein synthesis was slower when 50 μM-papaverine was added to the culture after 1 h adsorption, possibly because viral RNA synthesis was additionally suppressed.
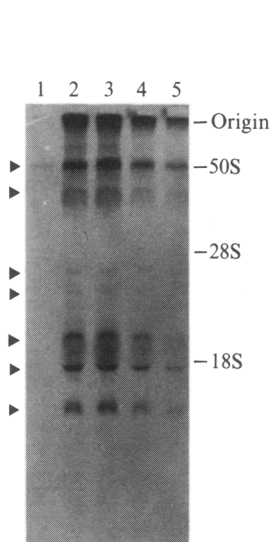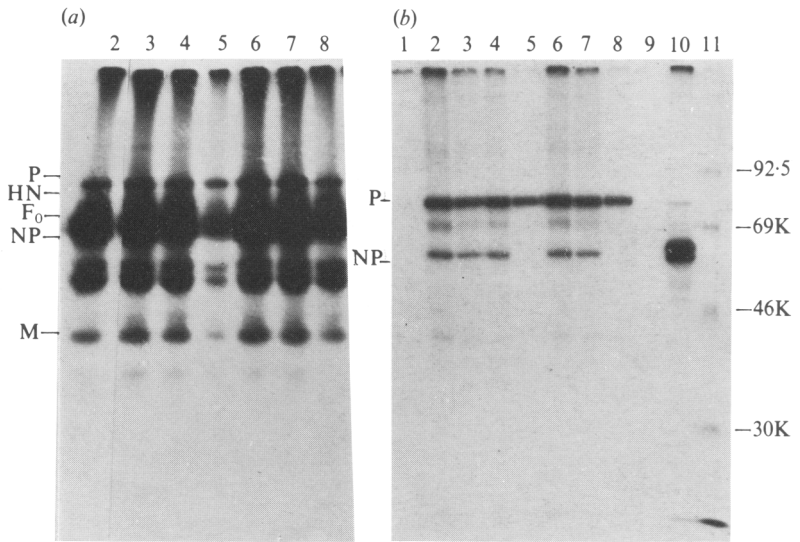
Fig. 4   Fig. 5

Fig. 4. Viral RNA synthesis in C6 cells treated with papaverine. Cultures were infected with HVJ at a multiplicity of 5 p.f.u./cell and after 1 h adsorption were incubated at 32 °C. Various concentrations of papaverine were added at 1 h after infection. The cultures were labelled with [^{3}H]uridine and the RNA was extracted as described in Methods. The RNA was analysed on a 1·25% glyoxal gel. Lane 1, RNA from uninfected C6 cells; lane 2, RNA from HVJ-infected C6 cells in the absence of papaverine; lanes 3, 4 and 5, RNA from HVJ-infected C6 cells in the presence of 2, 10 and 50 µM-papaverine, respectively. Arrows indicate viral RNA bands.

Fig. 5. SDS–PAGE of immunoprecipitates of [^{35}S]methionine-labelled (a), or [^{32}P]orthophosphate-labelled (b) C6 cells infected with HVJ in the presence of papaverine. Cultures were infected with HVJ at a multiplicity of 5 p.f.u./cell and after 1 h adsorption were incubated at 32 °C. Various concentrations of papaverine were added to the cultures at 1 h or 14 h after infection. The cultures were labelled with 50 µCi/ml of [^{35}S]methionine for 1 h at 23 h after infection or with 100 µCi/ml of [^{32}P]orthophosphate for 4 h at 20 h after infection in the presence of papaverine. The cell lysates were subjected to immunoprecipitation and SDS–PAGE. Lane 1, ^{32}P-labelled uninfected cells; lane 2, no drug addition; lanes 3 and 6, 2 µM-papaverine; lanes 4 and 7, 10 µM-papaverine; lanes 5 and 8, 50 µM-papaverine; lanes 3, 4 and 5, papaverine was added 1 h after infection; lanes 6, 7 and 8, papaverine was added at 14 h after infection; lane 9, ^{35}S-labelled uninfected cells; lane 10, ^{35}S-labelled infected cells; lane 11, ^{14}C-labelled marker proteins.

While 2 and 10 µM-papaverine did not inhibit phosphorylation of the viral proteins P and NP in infected C6 cells, that of NP protein was selectively blocked in the presence of 50 µM-papaverine (Fig. 5b). These data indicate that neither viral protein synthesis nor viral protein phosphorylation are detectably affected by treatment with the lower concentrations of papaverine.

Expression of viral antigens on the cell surface of HVJ-infected C6 cells in the presence of papaverine

In order to examine the expression of viral antigens on the surface of infected cells in the presence of papaverine, membrane immunofluorescence and cell surface immunoprecipitation tests were carried out. Membrane immunofluorescence showed clear fluorescence of viral glycoproteins on the cell surface of the infected cells in the presence of the drug (data not shown). Cell surface immunoprecipitation and SDS–PAGE confirmed the presence of more HN and F_0 proteins on the surface of infected cells in the presence of 10 µM-papaverine or less, especially when papaverine was added at 14 h after infection, compared with control cells in the absence of the drug (Fig. 6).
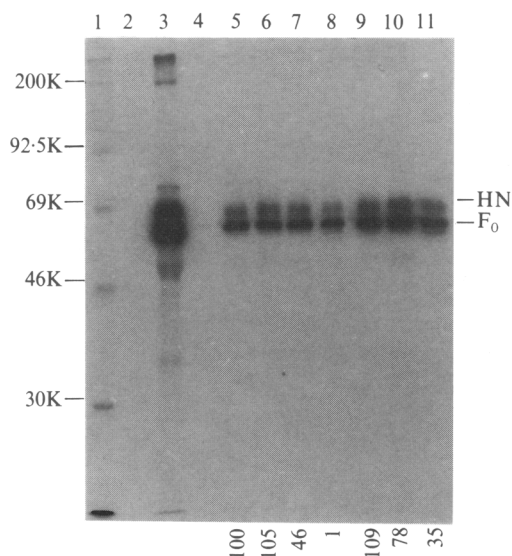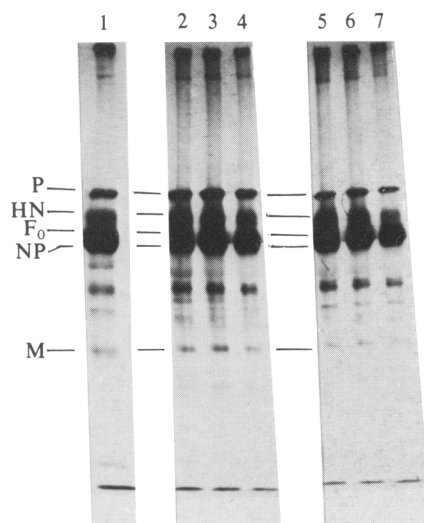
Fig. 6   Fig. 7

Fig. 6. SDS–PAGE of cell surface immunoprecipitates of [^{35}S]methionine-labelled C6 cells infected with HVJ in the presence of papaverine. Cultures were infected with HVJ at a multiplicity of 5 p.f.u./cell and after 1 h adsorption were incubated at 32 °C. Various concentrations of papaverine were added to the cultures at 1 h or 14 h after infection. The cultures were labelled with 50 µCi/ml of [^{35}S]methionine for 4 h at 20 h after infection in the presence of papaverine. After treatment with anti-HVJ hamster serum at 4 °C for 30 min the cultures were washed five times with ice-cold PBS. The lysates were precipitated by Protein A–Sepharose CL-4B and analysed by SDS–PAGE. Lane 1, ^{14}C-labelled marker proteins; lanes 2 and 3, total cell immunoprecipitation; lanes 4 to 11, cell surface immunoprecipitation; lanes 2 and 4, mock infection; lanes 3 and 5 to 11, HVJ infection; lanes 2 to 5, no drug addition; lanes 6 and 9, 2 µM-papaverine; lanes 7 and 10, 10 µM-papaverine; lanes 8 and 11, 50 µM-papaverine; lanes 6, 7 and 8, papaverine was added at 1 h after infection; lanes 9, 10 and 11, papaverine was added at 14 h after infection. Figures at the bottom of lanes 5 to 11 are amounts of intracellular nucleocapsids at 24 h after infection measured as described by Roux & Waldvogel (1981). Amount of NP protein from the cells without papaverine was expressed as 100.

Fig. 7. Association of viral proteins with plasma membrane of HVJ-infected cells in the presence of papaverine. Cells were infected at a multiplicity of 5 p.f.u./cell and after 1 h adsorption were incubated at 32 °C for 19 h. Various concentrations of papaverine were added to the cultures at 14 h after infection. The cultures were labelled with 50 µCi/ml of [^{35}S]methionine for 4 h in the presence of papaverine. Subsequent procedures are described in Methods. Lane 1, purified HVJ virions; lanes 2, 3 and 4, immunoprecipitates of total cell lysates; lanes 5, 6 and 7, immunoprecipitates of plasma membrane fractions; lanes 2 and 5, in the absence of the drug; lanes 3 and 6, in the presence of 10 µM-papaverine; lanes 4 and 7, in the presence of 50 µM-papaverine.

Association of viral proteins with the plasma membrane of HVJ-infected C6 cells in the presence of papaverine

A membrane fraction was purified from ^{35}S-labelled cells according to the method described in Methods, immunoprecipitated and subjected to SDS–PAGE.

As shown in Fig. 7, SDS–PAGE showed that all the structural proteins were detected in the plasma membrane fraction even in the presence of the drug. This indicates that papaverine might inhibit virus budding rather than transport of intracellular viral proteins to the plasma membranes. This would be consistent with the results showing inhibition of viral release when papaverine was added before 18 h after infection and restoration of viral release immediately after removal of the drug.

DISCUSSION

Since cAMP is present in relatively high levels in cells of neural origin, we have examined the effect of cAMP-related agents on HVJ replication in rat glioma C6 cells.

Papaverine, theophylline and caffeine which enhance endogenous cAMP showed a dose-dependent inhibitory effect on HVJ growth in C6 cells, but no significant effect was found in cAMP or dibutyryl cAMP-treated C6 cells. Moreover, simultaneous addition of cGMP did not block the effect of papaverine. Since the differentiation of C6 cells is affected by cAMP or dibutyryl cAMP (Hamprecht et al., 1973), the inhibitory effect of papaverine may not be a consequence of increased endogenous cAMP, but of its direct or other actions. Therefore it should be confirmed whether or not cAMP might be responsible for this inhibitory phenomenon by papaverine, theophylline or caffeine.

Inhibition of virus replication by cAMP-related agents has already been reported in the case of herpes simplex virus (Stanwick et al., 1977) and measles virus (Robbins & Rapp, 1980; Miller & Carrigan, 1982) both of which are responsible for acute and chronic neurological disorders. Robbins & Rapp (1980) demonstrated that exogenous cAMP inhibited the assembly of measles virus in infected non-neural cells by specifically blocking the synthesis of P and M proteins. It has also been reported that M protein was specifically inhibited in measles virus-infected neural cells by the addition of papaverine and that this effect was counteracted by cGMP (Miller & Carrigan, 1982). Moreover, Yoshikawa & Yamanouchi (1984) recently suggested that papaverine reversibly suppresses viral RNA synthesis and phosphorylation of measles virus P and NP proteins in IMR cells, a human neuroblastoma cell line, although the suppressive effect of papaverine treatment differs from cell to cell. In the present study, cell-associated infectivity and release of incomplete virus was not increased by papaverine treatment. The slight decrease in HVJ RNA synthesis, measured by [^{3}H]uridine incorporation into viral RNAs following addition of $10\,\mu M$-papaverine, was not sufficient to explain the reduction of infectivity. In addition, $10\,\mu M$-papaverine did not appear to affect viral protein synthesis or protein phosphorylation. The presence of the HN and F_0 proteins on the cell surface and of all the viral proteins in the plasma membrane fraction strongly suggests that the papaverine inhibition occurs at a stage after virus assembly at the plasma membrane. The result of adding papaverine at different times after infection supports this interpretation, as does the immediate recovery of infectivity in the culture medium after the removal of papaverine. In contrast, $50\,\mu M$-papaverine significantly inhibited viral RNA synthesis, and it is of interest to note that incorporation of [^{32}P]orthophosphate into the NP protein was specifically and completely blocked in the presence of $50\,\mu M$-papaverine, indicating that the NP and P proteins are differently phosphorylated. Although this high concentration of the drug clearly inhibited both the synthesis of viral RNA and protein, there was no selective repression of either.

The assembly of paramyxoviruses at the plasma membrane involves association of the nucleocapsid and M proteins and subsequent incorporation into virions by a budding process (McSharry et al., 1971, 1975; Shimizu & Ishida, 1975; Yoshida et al., 1976). Since all the structural proteins were associated with the plasma membrane in the presence of papaverine, inhibition by the drug seems to occur at some subsequent step(s). However, the mechanism involved in the initiation of the budding process is unclear at present. Previous studies on morphogenesis at the plasma membrane showed that viral antigens or structural proteins were associated with the cytoskeleton (Sundqvist & Ehrnst, 1976; Tyrell & Ehrnst, 1979; Bohn et al., 1986) and indeed cellular actin was incorporated into virions (Lamb et al., 1976). In addition, the M proteins of HVJ and Newcastle disease virus may interact with cellular actin (Giuffre et al., 1982). Papaverine may act on one of these processes, decreasing the yield of HVJ in C6 cells. Further analysis of papaverine inhibition in C6 cells may contribute to the understanding of the mechanism of budding of paramyxoviruses and phosphorylation of HVJ proteins.

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