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Inhibition of arenavirus multiplication in vitro by phenotiazines

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Abstract

Trifluoperazine (TFP) and chlorpromazine (CPZ), two pharmacologically active phenotiazine derivatives, were evaluated for their inhibitory activity on the replication of the arenaviruses Junin (JV), the etiological agent of Argentine hemorrhagic fever, Tacaribe virus and Pichinde virus. Both compounds achieved a concentration-dependent inhibition of viral multiplication at concentrations not affecting cell viability. The 50% inhibitory concentration (IC_{50}) values determined by a virus yield inhibition assay for several strains of JV, including a human pathogenic strain, were in the range of 7.7–23.0 μM and the 90% inhibitory concentration (IC_{90}) fluctuated between 16.6 and 35.2 μM. From time of addition and removal experiments, it can be concluded that CPZ inhibited an early stage in the replicative cycle of JV, probably viral entry. TFP also affected JV penetration when present soon after virus adsorption, and also interfered with a later step of viral maturation when added after 7 h of infection. The expression of viral antigens in the cytoplasm of infected cells was highly reduced in the presence of the compounds, as revealed by immunofluorescence staining, whereas no JV proteins were detected at the cell membrane. The distribution pattern of viral proteins was altered in the few cells exhibiting positive fluorescence after treatment with the phenotiazines. The TFP-induced inhibitory effect on JV multiplication was significantly reversed in the presence of 5 μM calmodulin. These data indicate that TFP and CPZ inhibit JV replication in vitro. Our findings suggest that the integrity of the actin microfilaments may be required for optimal arenavirus multiplication.

Keywords: Arenaviruses; Argentine hemorrhagic fever; Calmodulin antagonists; Junin virus; Phenotiazines

1. Introduction

Junin virus (JV), a member of the Arenaviridae family, is the etiologic agent of Argentine hemorrhagic fever (AHF), an endemo-epidemic disease affecting the population of the most fertile zone of Argentina (Weissenbacher et al., 1987). The disease is sometimes characterized by predominantly hemorrhagic signs, whereas other cases exhibit neurological manifestations. Four other arenaviruses also cause severe hemorrhagic disease in man: Lassa virus, Lassa fever in West Africa; Machupo virus, Bolivian hemorrhagic fever; and the two recently emerging viruses Guanarito virus, from Venezuela, and Sabia virus from Brazil (Weissenbacher et al., 1987; Salas et al., 1991; Coimbra et al., 1994). Besides, almost all arenaviruses cause persistent infections in rodents, which represent their reservoir host in nature.

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Different approaches have been pursued to obtain successful prevention and treatment of AHF. Current therapy is the early administration of standardized doses of convalescent plasma (Maiztegui et al., 1979; Enria et al., 1984). However, this therapy is not efficient when it is initiated after 8 days of illness and a late neurological syndrome is observed in 10% of the treated patients (Enria and Maiztegui, 1994). With respect to drug therapy, ribavirin is the only compound that has shown partial efficacy against arenavirus infections in studies performed in experimental animals and humans (Jahrling et al., 1980; McCormick et al., 1986; Smee et al., 1993), but several disadvantages have been recorded for ribavirin treatment such as the lack of efficacy in patients with advanced disease and the development of side effects including thrombocytosis and anemia (Enria et al., 1987; Kenyon et al., 1986; McKee et al., 1988; Weissenbacher et al., 1986). More recently, various nucleoside analogues and sulfated polysaccharides were found to be selective inhibitors of the in vitro replication of JV and Tacaribe virus, a non-pathogenic arenavirus closely related to JV. These compounds represent promising drugs for the treatment of arenavirus infections (Andrei and De Clercq, 1990). Finally, an attenuated live vaccine named 'Candid' 1 has been developed for AHF and is currently being evaluated in the human population of the endemic area (Maiztegui et al., 1991). Even with an effective vaccine, occasional outbreaks of AHF are expected to occur due to changes in the habits of the natural reservoir, the cricetid *Calomys musculinus*. All these considerations provide the basis for the search for antiviral compounds effective against arenavirus multiplication.

Infectious JV particles are enveloped virions containing two segments of single-stranded RNA. After adsorption to the cell surface, JV internalize by an endocytic mechanism (Castilla et al., 1994) and the replicative cycle proceeds until progeny viruses are released by budding at the plasma membrane. Several investigations have shown that events at the plasma membrane such as endocytosis, exocytosis, capping of ligand–receptor complexes and cell morphology can be influenced by phenothiazines, neuroleptic drugs with a wide clinical use (Osborn and Weber, 1980; Horwitz et al., 1981; Merrit et al., 1981; Salisbury et al., 1981). These events are involved in virus–cell interactions occuring at different stages during the multiplication of enveloped viruses. We have now evaluated the effect of two phenotiazine derivatives, trifluoperazine (TFP) and chlorpromazine (CPZ), on the multiplication of JV in Vero cells.

2. Materials and methods

2.1. Drugs

Trifluoperazine dihydrochloride (TFP), chlorpromazine hydrochloride (CPZ) and purified calmodulin from bovine brain (CAM) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions at a concentration of 10 mM were prepared in dimethylsulfoxide (DMSO) and sterilized by filtration.

2.2. Cells

Vero cells were grown in Eagle's minimum essential medium (MEM) containing 5% inactivated calf serum and 50 μg/ml gentamycin. Maintenance medium (MM) consisted of MEM supplemented with 2% calf serum and gentamycin.

2.3. Viruses

The following arenaviruses were used: the naturally attenuated IV4454 strain of JV obtained from a mild human case (Contigiani and Sabattini, 1977), the prototype pathogenic XJ strain of JV isolated from a severe human case of AHF (Parodi et al., 1958), its avirulent derivative XJCl3 (Guerrero et al., 1969), the TRLV 11573 strain of Tacaribe virus and the An 3739 strain of Pichinde virus. Virus stocks were prepared in Vero cell cultures and titrated by plaque formation.

2.4. Cytotoxicity assay

To evaluate cytotoxicity, Vero cells were seeded in 24-well microplates (2.0×10^4 cells/well) and after 24 h incubation, the cells were refed with

MM containing 2-fold serial dilutions of the compounds. After 48 h of incubation at 37°C, the medium was removed and the cells were trypsinized. The number of viable cells was determined by the trypan blue exclusion method and the concentration required to reduce cell viability by 50% (50% cytotoxic concentration, CC_{50}) was determined.

2.5. Inhibition of virus multiplication

Antiviral activity was determined by a virus yield inhibition assay. Vero cell monolayers grown in 24-well microplates were infected at a multiplicity of infection (m.o.i.) of 0.1. After 1 h adsorption at 37°C, cells were washed and refed with MM containing various concentrations of the compounds. Two replicates per dilution of each compound were tested. After 24 h of incubation at 37°C, supernatant cultures were harvested and extracellular virus yield was determined by the plaque assay. The 50% inhibitory concentration (IC_{50}) and the 90% inhibitory concentration (IC_{90}) were calculated as the drug concentrations that reduced virus yield by 50 and 90%, respectively, in the compound-treated cultures compared with the untreated ones. To determine cell-associated virus, the cells remaining after harvesting supernatant cultures were washed, frozen and thawed twice, centrifuged at $1000 \times g$ and the supernatant was assayed by plaque formation.

To examine the effect of CAM on TFP-induced inhibition of JV replication, Vero cells were infected with JV strain IV4454 (m.o.i. 0.1) and then incubated in the presence of either 30 μM TFP or a mixture of 30 μM TFP and 0.1–5 μM CAM. As a control, infected cells treated with CAM alone were also processed. At 24 h postinfection (p.i.) virus yields in the cell supernatants were assayed.

2.6. Effect of time of drug addition or removal on JV replication

Monolayers of Vero cells were allowed to adsorb JV strain IV4454 at a m.o.i. of 0.1 for 60 min at 4°C. After removal of the inocula, the cells were washed twice with PBS and then MM containing 30 μM CPZ or TFP was added to infected cells at 1, 3, 5, 7, 9 and 16 h p.i. and further incubated at 37°C. Another set of infected cultures were incubated with medium containing CPZ or TFP immediately after adsorption and the drug was removed by medium change at 3 and 6 h p.i.. In all cases, extracellular virus yields were measured at 24 h postinfection.

2.7. Immunofluorescence assay

Vero cells grown in coverslips were infected with JV strain IV4454 (m.o.i. 1), and 30 μM TFP or CPZ was added to the culture medium after adsorption. For cytoplasmic immunofluorescence, cell monolayers were washed with cold PBS and fixed in methanol for 15 min at -20°C. Indirect staining was carried out by using anti-JV immunoglobulins purified from hyperimmune rabbit serum reactive against all JV proteins (Candurra et al., 1990) and fluorescein-labeled goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO, USA). For surface immunofluorescence, infected cells were fixed with 3% paraformaldehyde for 15 min at room temperature and then processed as previously described.

3. Results

3.1. Cytotoxicity and antiviral activity of phenotiazines against arenaviruses

TFP and CPZ were evaluated for antiviral activity against JV strain IV4454 by a virus yield inhibition assay. As can be seen in Fig. 1, virus replication was inhibited in a concentration-dependent manner. The efficacy to reduce extracellular virus yields was similar for both compounds and the IC_{50} values determined from these data were 10.0 and 12.5 μM for TFP and CPZ, respectively, whereas the IC_{90} values (i.e. the drug concentration that reduces virus yield by 1 log) were 28.7 μM for TFP and 28.8 μM for CPZ (Table 1). The production of cell-associated virus was as sensitive to phenotiazines as extracellular virus formation (data not shown), with an IC_{50} value for TFP of 7.8 μM.

The lack of toxicity of the compounds for Vero cells was determined by assessing their effects on cell growth. No alterations either in the number of viable cells or in cell morphology were observed at the antivirally effective concentrations (Fig. 1). The CC_{50} in Vero cells was 81.4 and 61.9 μM for CPZ and TFP, respectively.

The inhibitory action of phenotiazines on JV multiplication was observed with several viral strains, including attenuated isolates such as the above-mentioned IV4454 and the XJCl3 strains as well as the highly pathogenic XJ strain (Table 1). When other species of arenaviruses were tested, the values of IC_{50} and IC_{90} for Tacaribe virus were similar to those obtained for JV whereas the arenavirus Pichinde was less susceptible to the inhibitory effects of CPZ and TFP (Table 1).

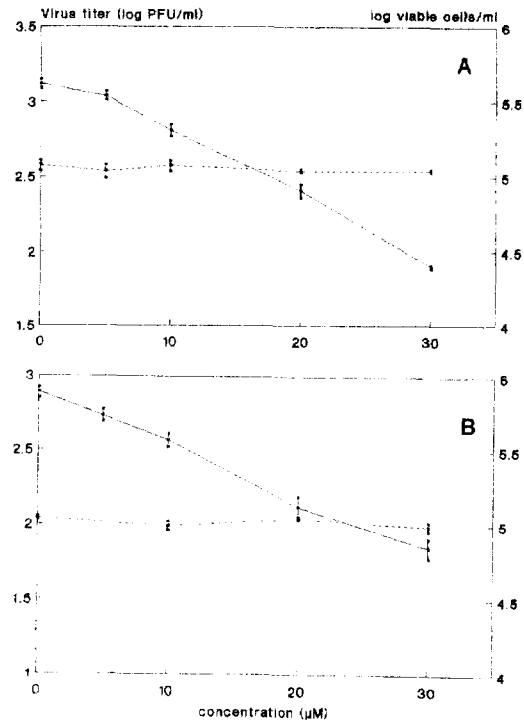

Fig. 1. Concentration–response curves of antiviral activity and cytotoxicity of phenotiazines. Vero cells were incubated for 48 h in the presence of different concentrations of TFP (A) or CPZ (B), and then the number of viable cells was determined by the trypan blue exclusion method (- - -). Other sets of cultures were infected with JV and, after 24 h of infection in the presence of the compounds, extracellular virus yields were determined (——). Each point is the mean value of duplicate determinations ± standard deviation.

Table 1
Antiviral activity of phenotiazines against arenaviruses as determined by the virus yield assay

Virus	IC_{50} (μM)		IC_{90} (μM)	
	TFP	CPZ	TFP	CPZ
Junin virus				
IV4454 strain	10.1 ± 0.8	12.5 ± 0.8	28.7 ± 0.1	28.8 ± 1.7
XJCl3 strain	7.7 ± 0.1	17.5 ± 0.2	16.6 ± 1.4	35.2 ± 2.7
XJ strain	8.9 ± 0.6	23.0 ± 0.6	19.5 ± 0.1	30.0 ± 1.1
Tacaribe virus	18.0 ± 0.9	13.5 ± 0.4	28.6 ± 1.1	27.3 ± 0.8
Pichinde virus	31.8 ± 0.6	28.7 ± 1.0	> 50	> 50

Data are mean values from two separate experiments ± standard deviation.
The CC_{50} values for CPZ and TFP in Vero cells were 81.4 ± 0.5 and 61.9 ± 0.3 μM, respectively.

3.2. *Effect of time of addition or removal of the compounds*

To obtain information on the compound-sensitive step during JV infection, the dependence of the inhibitory effect of TFP and CPZ on the time of drug addition or removal during a growth cycle was next examined. As shown in Fig. 2A, a similar level of inhibition was observed if 30 μM TFP was added very early after virus adsorption or as late as 7 h p.i.. Even when TFP was added at 9 or 16 h p.i., extracellular virus yields at 24 h p.i. were decreased as compared to those of untreated cells, with 63.8 and 60.7% inhibition, respectively. Surprisingly, the time profile of inhibition was different in CPZ-treated infected cells. This compound was much more effective in reducing JV yield when present at early times of infection whereas drug addition after 1 h of infection did not cause any significant reduction in virus release (Fig. 2A). In another set of experi-

ments compounds were added immediately after adsorption and then removed at various times by washing. Both phenotiazines, when present only for a limited period of time after infection (either 3 or 6 h), retained antiviral activity against JV (Fig. 2B). Thus, the results shown in Fig. 2A and B suggest that CPZ inhibits an early step of the replicative cycle, whereas TFP seems to act on two different targets, similarly affecting an early (before 3 h p.i.) and a late stage (after 7 h p.i.).

A variation of this kind of experiment was done next to confirm the length of action and irreversibility of phenotiazines during a more prolonged period of time. Cells were infected and treated with the compounds for 24 h, then culture fluid was replaced with fresh medium without drug, cells were incubated for a further 24 h and supernatant virus yield was assayed. As seen in Table 2, infectivity was not recovered even after 24 h of drug removal and virus titers obtained were similar to those released in the continuous presence of both compounds.

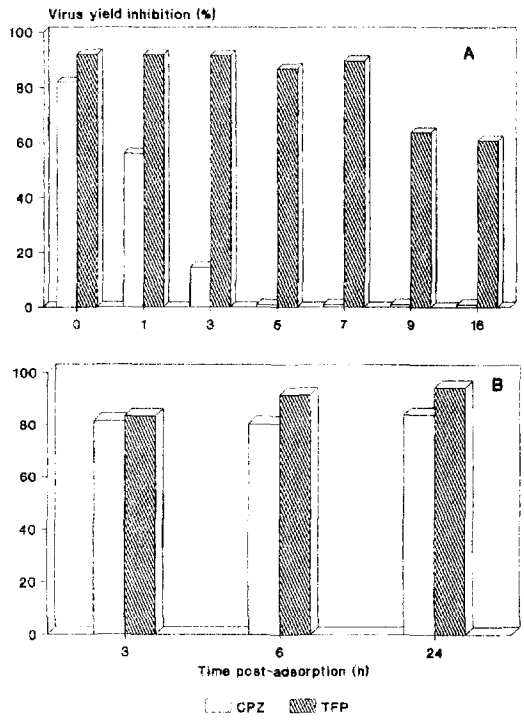

Fig. 2. Effect of the time of addition or removal of phenotiazines on JV replication. Vero cells were infected with JV and after 1 h adsorption at 4°C, cells were washed and MM containing 30 μM TFP or CPZ was added at different times post-adsorption and incubated further at 37°C (A). Another set of infected cultures were incubated with TFP or CPZ immediately after virus adsorption and then drug was removed at 3, 6 or 24 h (B). Extracellular virus yields were determined at 24 h p.i. in all cell cultures and results are expressed as % inhibition with respect to control infected cultures incubated for 24 h without drug. Virus titer in control cultures without compound treatment: 9.0×10^4 plaque forming units (PFU)/ml. Each value is the mean of duplicate determinations.

3.3. Influence of CAM on the inhibitory effect of TFP against JV multiplication

The influence of CAM in reversing the inhibitory effect of the phenotiazine derivatives on arenaviruses was then examined. As can be seen in Table 3, the simultaneous presence of 5 μM CAM and 30 μM TFP increased the virus yield so that only 44.3% inhibition was achieved in comparison to 98.9% inhibition achieved by TFP alone. The presence of 5 μM CAM alone did not have any significant effect on JV yield. The reversing influence of CAM was concentration-dependent since the effect of 0.1 μM CAM on 30 μM TFP-induced inhibition was negligible.

3.4. Effect of phenotiazines on JV antigen expression

Further studies were conducted to determine the effect of phenotiazines on JV antigen expression by the immunofluorescence technique. As seen in Fig. 3A and B, the amount of cells showing cytoplasmic immunofluorescence was drastically reduced in the presence of 30 μM TFP. In fact, the percentage of infected cells per microscope field showing cytoplasmic immunofluorescence in the presence of TFP with respect to untreated controls was 6.8%. Furthermore, at higher magnification an alteration in the distribution pattern of viral antigen was observed in the few cells exhibiting positive fluorescence in the presence of the compound. Cytoplasmic staining of infected untreated cells revealed a finely dotted regular distribution of viral proteins (Fig. 3C). In the presence of TFP, spots of fluorescence accumulated in the cells and the amount of dotted fluorescence was significantly decreased (Fig. 3D).

Table 2
Length of action and lack of reversibility of the effect of phenotiazines on JV replication

Treatment conditions	TFP		CPZ	
	Virus titer (PFU/ml)	Inhibition (%)	Virus titer (PFU/ml)	Inhibition (%)
Drug-free 48 h	$1.1 \pm 0.1 \times 10^5$	0	$9.5 \pm 0.7 \times 10^5$	0
Drug present 0–48 h	$2.5 \pm 0.1 \times 10^2$	99.76	$5.1 \pm 0.5 \times 10^3$	99.48
Drug present 0–24 h + drug-free 24–48 h	$3.9 \pm 0.3 \times 10^2$	99.62	$9.1 \pm 0.5 \times 10^3$	99.05

Data are mean values from two separate experiments ± standard deviation.

When immunofluorescence staining of viral proteins in the membrane of infected cells was performed, control cells exhibited a linear pattern of fluorescence along the cell surface (Fig. 3E), whereas no viral proteins were detected in TFP-treated cells (Fig. 3F).

Similar results for both cytoplasmic and membrane immunofluorescence were observed in CPZ-treated cells (data not shown).

4. Discussion

In the present study, the phenotiazine derivatives trifluoperazine and chlorpromazine effected a concentration-dependent inhibition of JV multiplication in Vero cells, without affecting cell viability (Fig. 1). The susceptibility of JV and Tacaribe virus to both compounds was similar, whereas the arenavirus Pichinde was less susceptible. The IC_{50} values for several strains of JV, including a human pathogenic strain, were in the range 7.7–23.0 μM and the IC_{90} fluctuated between 16.6 and 35.2 μM. Thus, both phenotiazines can be considered equally active inhibitors of JV replication.

Table 3
Influence of CAM on TFP inhibition of JV replication

Treatment conditions	Virus titer (PFU/ml)	Inhibition (%)
Drug-free 24 h	$8.8 \pm 0.4 \times 10^3$	0
30μM TFP 0–24 h	$9.5 \pm 0.5 \times 10^1$	98.9
5 μM CAM 0–24 h	$9.0 \pm 0.5 \times 10^3$	0
5 μM CAM + 30 μM TFP 0–24 h	$4.9 \pm 0.2 \times 10^3$	44.3
0.1 μM CAM + 30 μM TFP 0–24 h	$2.5 \pm 0.3 \times 10^2$	97.1

Data are mean values from two separate experiments ± standard deviation.

From time-related inhibition experiments, it could be deduced that TFP and CPZ interfere at different time points in the replicative cycle of JV. CPZ was effective to reduce JV yields only when added during the early stage of virus multiplication, whereas the inhibitory effect of TFP on JV multiplication was comparable either when present only for a short period after adsorption (0–3 h p.i.) or when added at later times in the replicative cycle (7 h p.i.) (Fig. 2). It has been recognized that phenotiazines act as antagonists of CAM to which they specifically bind in the presence of Ca^{++}, affecting specific events related to cell membranes (Levin and Weiss, 1976; Osborn and Weber, 1980). On this basis, it can be concluded that CPZ would predominantly affect JV entry to the host cell, which occurs by receptor-mediated endocytosis (Castilla et al,, 1994). With respect to TFP, it would also interfere with JV penetration if present immediately after adsorption, but when it is added later during infection it would exert an inhibitory effect on the viral maturation stages, such as morphogenesis or budding. The drug reduced cell-associated virus yield as well as extracellular virus yield, since the IC_{50} for intracellular virus production was similar to that determined when supernatant virus was measured (7.8 and 10.0 μM, respectively). These data

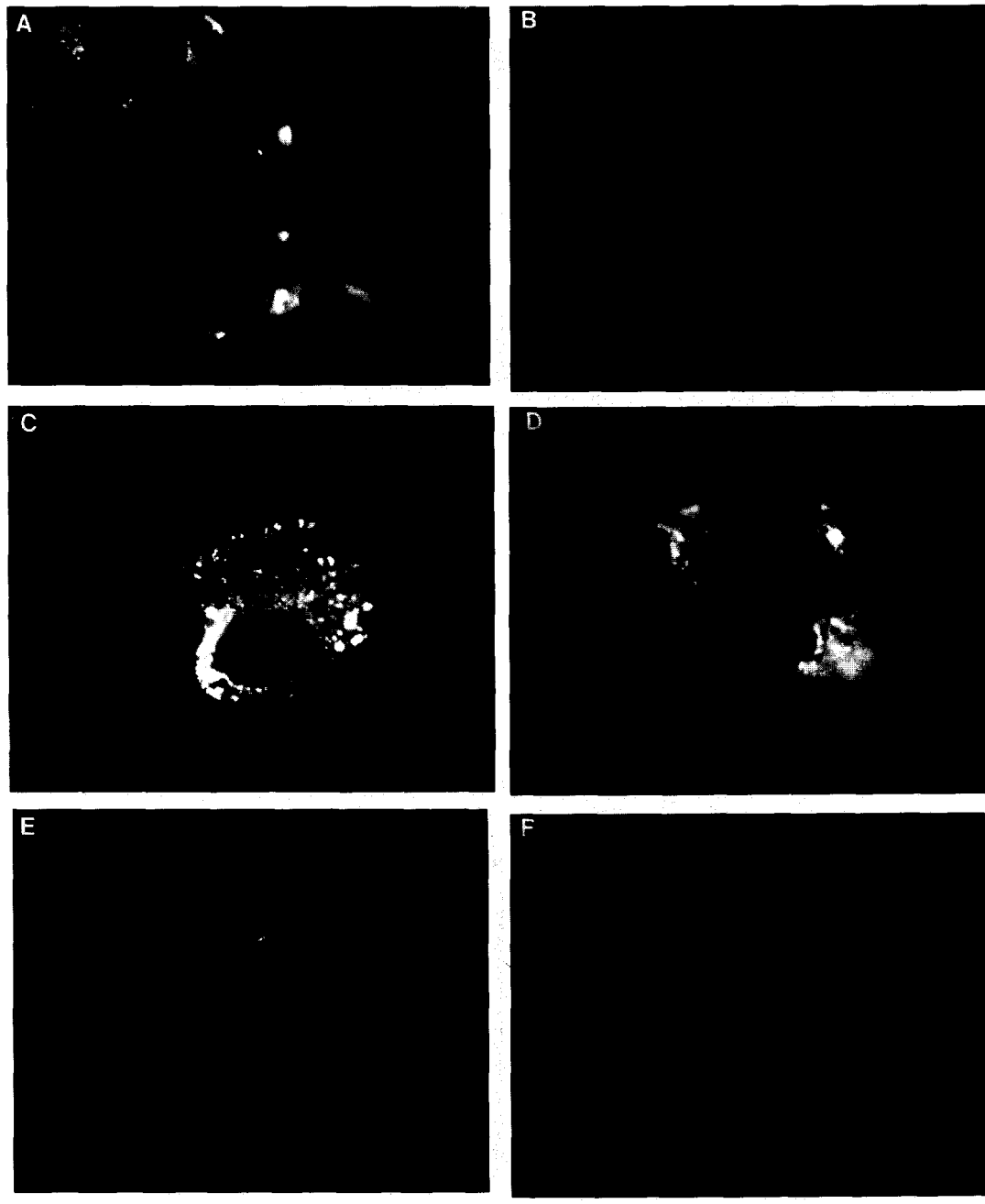

Fig. 3. Action of TFP on expression of JV proteins. Vero cells grown in coverslips were infected with JV and then incubated with MM with (B, D, F) or without (A, C, E) 30 μM TFP during 24 h. Cytoplasmic (A, B, C, D) and surface (E, F) immunofluorescence staining was carried out using anti-JV purified immunoglobulins. Magnification: 400 × (A, B) and 1000 × (C, D, E, F).

indicate that TFP did not produce accumulation of progeny viruses in the cell. Thus, the most likely event sensitive to the drug would be viral morphogenesis rather than the budding process.

At present, it is difficult to explain the differential response of JV to CPZ and TFP. Both compounds have a similar chemical structure differing only in two substituents on the phenotiazine. The effect of phenotiazines on the multiplication of other animal viruses has been variable. Bohn et al. (1983) have reported that TFP and CPZ inhibit measles virus budding from HeLa cells whereas the maturation of rabies virus in neuronal cultures was not affected (Lockhart and Tsiang, 1991). Coincidentally with our results, contrasting effects of both phenotiazines have been reported for influenza virus. Krizanova et al. (1982) reported that CPZ inhibits influenza virus penetration in chick embryo cells without affecting virus adsorption, whereas Ochiai et al. (1991) demonstrated that TFP acts as a reversible inhibitor of influenza virus morphogenesis. More recently, TFP and other calmodulin antagonists were found to inhibit human immunodeficiency virus-induced cell fusion but not virus replication (Srinivas et al., 1994).

Although it has been reported that phenotiazines might act as non-specific inhibitors against CAM-independent cellular factors, the drug doses effective in inhibiting JV replication (10–30 μM) correspond to those used for their in vitro antagonism of CAM (Levin and Weiss, 1976; Volpi et al., 1981). In addition, the inhibitory effect of TFP was significantly reversed by purified CAM, increasing the virus yield in TFP + CAM-treated cells in comparison to TFP-treated cells (Table 3). Thus, although a non-specific inhibition independent of CAM cannot be totally discarded, our results provide evidence that the interaction of TFP and CPZ with cellular CAM is mainly responsible for the inhibition of arenavirus multiplication.

CAM participates in various cellular processes as a modulator of many Ca^{++}-dependent enzymes and is directly involved as a structural protein in the cytoskeleton of both interphasic and mitotic cells (Osborn and Weber, 1980; Means and Dedman, 1980). Consequently, the treatment with phenotiazines disrupts cytoskeletal organization in different cell types (Adelstein, 1982; Horwitz et al., 1981). Thus, our results about the influence of TFP and CPZ on JV infection would provide evidence that the multiplication of JV, particularly viral penetration and maturation, is dependent on the integrity of the microfilament structures. In previous studies, the identification of actin associated with purified JV preparations (Pasian et al., 1983) led to the proposal that it plays an active role in JV maturation. Furthermore, JV multiplication was also inhibited by other microfilament-disrupting agents such as local anesthetics (Castilla et al., 1994). For other enveloped viruses such as paramyxoviruses and orthomyxovirues, it has also been proposed that microfilaments have an intimate role in the production of infectious virus, notably by the vectorial polymerization of actin at the cell membrane (Bohn et al., 1986). Furthermore, virus-specific proteins and/or infectious virions have been found in close association with the cytoskeletal fraction of extracted cells infected with measles virus (Bohn et al., 1986) and with actin filaments in retrovirus-infected cells (Luftig and Lupo, 1994). Further studies at the biochemical and ultrastructural level will be neccessary to confirm the effective participation of the actin-based cytoskeleton in JV infections.

Among phenotiazines, TFP is one of the most potent neuroleptic drugs usually employed as a psychotropic agent. Furthermore, calmodulin antagonists are being evaluated as antiproliferative compounds and as agents that potentiate the efficacy of chemotherapy (Hait and Lazo, 1986; Stewart and Evans, 1989). Thus, the present findings on the inhibitory action of phenotiazines on JV multiplication raise the possibility that these pharmacologically active substances may be considered as potential antiviral agents for the treatment of arenavirus infections.

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