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INFLUENCE OF CHLORPROMAZINE ON THE REPLICATION OF INFLUENZA VIRUS IN CHICK EMBRYO CELLS

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Summary. — Influence of chlorpromazine (CPZ) on the production of influenza virus was followed in chick embryo cell (CEC) monolayers. CPZ — which binds specifically to calmodulin (CaM) — inhibited in concentration of 20-100 μmol/l the activity of CaM-stimulated 3′, 5′-c′AMP phosphodiesterase (PDE). When administered together with the virus, CPZ in concentrations of 20-50 μmol/l reduced virus yields by 2-3 log PFU. Addition of CPZ 15 min before or 1 hr after influenza virus adsorption had no effect. The inhibitory action of CPZ was reversed by purified CaM. The adsorption of influenza virus to CEC or to erythrocytes was not affected. The participation of CaM on the mechanism of influenza virus penetration is discussed.

Key words: *influenza virus*; *calmodulin*; *chlorpromazine*

Introduction

In recent years data have accumulated that calmodulin (CaM) may participate in many Ca^{2+}-dependent functions (Vincenzi and Farrance, 1977; Kakiuchi *et al.*, 1978; Dedman *et al.*, 1979; Vincenzi, 1979; Brewer, 1980; Klee *et al.*, 1980) and that phenothiazines inhibit Ca^{2+}-dependent enzyme activities (Weiss and Levin, 1978; Levin and Weiss, 1979; Weiss *et al.*, 1980). This effect of phenothiazines can be explained by their selective binding to the Ca^{2+}-CaM complex which, thereafter, can not react with the given enzyme. The specificity of trifluoperazine binding to CaM and the Ca^{2+}--dependence of this reaction has already been confirmed (Weiss and Levin, 1978; Weiss *et al.*, 1980). For such reason, phenothiazines are often used to elucidate the physiological functions of CaM.

In CEC as well as in membranes of these cells the activity of CaM-dependent PDE decreases early after influenza virus adsorption (Križanová *et al.*, 1977; Križanová, 1979). As postulated, CaM may participate in the early stage of virus-cell interaction. To elucidate the role of CaM in the penetration of influenza virus, the effect of CPZ on adsorption, penetration and reproduction of the virus was followed.

Materials and Methods

Cells. Chick embryo cells (CEC) were prepared from 11 days old chick embryos (Dulbecco, 1952; Dulbecco and Vogt, 1954).

Virus. Influenza virus strain A/WSN was passaged 4 times in CEC and once in the allantoic fluid of 9 days old embryos. The harvested allantoic fluid containing 10^7-10^8 PFU/ml virus was stored at $-70°$ C in 1 ml aliquots.

Plaque assay. CEC were resuspended in Earle's medium containing 0.3% lactalbumin hydrolysate and 10% inactivated calf serum. Plastic Petri dishes 6 cm in diameter (Koh-i-Noor, Hardtmuth, ČSSR) were seeded with 6×10^6 cells and incubated for 72 hr. Monolayers were washed for 15 min at room temperature with phosphate buffered saline (PBS) as described by Dulbecco and Vogt (1954) and then infected with 1 ml stock virus diluted to $10^{-4}-10^{-8}$ in PBS. One hr after adsorption, the rest of virus was washed off, cells were overlayed with medium containing agar (Dulbecco, 1952). After 72 hr incubation at 37°C, plaques were detected by staining with neutral red and counted.

Chlorpromazine (CPZ) and virus adsorption. CEC were washed in PBS and mixed with the stock virus in a proportion of 1×10^7 cells to 1 ml of undiluted virus (1024–2048 HA units) in the presence (20 μmol/l) or absence of CPZ. Chlorpromazine hydrochloride was kindly supplied by Dr. Z. Vejdelka from Research Institute for Pharmacy and Biochemistry, Prague. The stock solution of CPZ had pH 6.9 and concentration 10 mmol/l. The mixtures were incubated for 1 hr either at 0°C or at 37°C. The unadsorbed fractions in the supernate were tested by haemagglutination (HA). Uninfected (control) cells were incubated in the presence or absence of the drug.

Inactivation of influenza virus particles in the presence of CPZ was tested by incubation of the virus (diluted 1 : 1 in PBS) with the CPZ solution (20 μmol/l) for 1 hr at room temperature. The control sample was kept in the absence of CPZ. After incubation, the virus was sedimented at 20 000 rev/min for 45 min and resuspended in the original volume of PBS; titrations were performed either in CEC or in 11 days old chick embryos.

CPZ and cell growth. CEC were grown in Müller flasks. When $4-5\times10^6$ cells were seeded per bottle, about 1×10^6 cells became attached within 20 hr. By 48–72 hr, cells were replenished with medium containing either 20, 50 or 100 μmol/l CPZ or in CPZ-free medium and further incubated for 24–72 hr at 37°C. Thereafter the monolayers were washed with PBS, cells were trypsinized and their number counted. In addition, the protein concentration in cell monolayers hydrolysed with 1 N NaOH was determined by the method of Lowry (1956).

Preparation of 3′, 5′-c′AMP-phosphodiesterase and calmodulin. The purification of 3′, 5′-c′AMP--phosphodiesterase (PDE) and CaM from CEC was performed as described (Križanová *et al.*, 1979). CaM from CEC was further purified by hydroxylapatite chromatography according to Wolff *et al.* (1977).

Electron microscopy. Purified influenza virus (strain A/WSN) treated and untreated with CPZ were contrasted with 2% phosphowolframic acid, pH 7.5 on formvar carbon coated grids. CEC infected with influenza virus in the presence or absence of CPZ were incubated for 10–30 min at different temperatures and then scraped off by a rubber policeman. After centrifugation for 10 min at 2000 rev/min, the pellet was fixed with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.2 for 30 min at 4°C and postfixed in 1% OsO_4 in the same buffer for 60 min at room temperature. Fixed cells were dehydrated in increasing concentrations of acetone and embedded into Araldite CY 212 (Serva, Heidelberg). Ultrathin sections were prepared on Ultrotome III/ /LKB, stained with 2% uranyl acetate and lead citrate and observed in electron microscope Philips EM 300 at 80 kV.

Results

Effect of CPZ on the activity of purified CaM-dependent PDE was first tested in vitro. As shown on Fig. 1, the inhibition of the CaM-stimulated PDE activity was dependent on the drug concentration. The CaM stimulated PDE activity was calculated by substracting the PDE activity in the absence

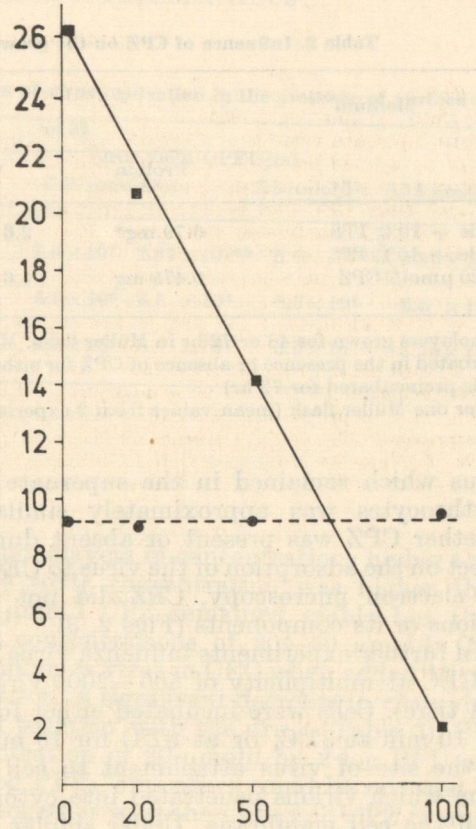

Fig. 1.

Relationship between the activity of 3′, 5′-c′AMP phosphodiesterase and CPZ PDE and CaM (calmodulin, calcium-dependent regulator protein) were isolated by DEAE cellulose chromatography. The basal activity of PDE was determined in 0.5 ml of following reaction mixture: 40 mmol/l imidazol-HCl buffer pH 7.5 containing 0.2 mmol/l Ca^{2+}, 2.0 mmol/l Mg^{2+}, 1 mmol/l 3′, 5′-cAMP and purified PDE. The total activity of the PDE-CaM complex, in addition, was determined in the same mixture containing 0.3 μg/ml isolated CaM
Abscissa: μmol/l of CPZ; ordinate: per cent of 3′, 5′-c′ AMP hydrolysis

of CaM from the activity in its presence. The basal PDE activity was not influenced by the CPZ concentration.

At concentration of 20 μmol/l. CPZ did not change the HA titre of influenza virus. The infectious titre of the drug treated virus (20—50 μmol/l) remained unaltered as compared to the untreated one (Table 1). The HA titre of the

Table 1. Influence of CPZ on the infectivity of influenza virus

Test system	Virus infectivity	
	Control (untreated)	CPZ treated
Chick embryos (11 days old)	5×10^{8}*	1×10^{9}
CEC	1.2×10^{7}**	8×10^{6}

* ID/ml (infectious dose).
** PFU/ml.

Table 2. Influence of CPZ on the growth of CEC in stationary cultures

Medium	Preincubation in culture 48 hr		72 hr	
	Protein	Cells	Protein	Cells
Earle + 10% ITS	0.79 mg*	2.6×10^6	1.1 mg	3.5×10^6
Earle + 10% ITS + 20 μmol/l CPZ	0.475 mg	1.6×10^6	0.9 mg	3×10^6

Monolayers grown for 48 or 72 hr in Müller flaks. Medium was replenished and cells were further incubated in the presence or absence of CPZ for either 24 hr (cells preincubated for 48 hr) or 72 hr (cells preincubated for 72 hr)

* Per one Müller flask (mean values from 2 experiments).

virus which remained in the supernate after adsorption to CEC or avian erythrocytes was approximately similar (64—128 HA units) regardless whether CPZ was present or absent during adsorption. Thus, CPZ had no effect on the adsorption of the virus to CEC or to avian erythrocytes. As shown by electron microscopy, CPZ did not alter the architecture of influenza virions or its components (Figs 2, 3).

In further experiments influenza virus was adsorbed to surface membranes of CEC at multiplicity of 500—2000 virions per cell (as calculated from the HA titre). Cells were incubated either for 30 min by 0°C and subsequently for 10 min at 37°C, or at 37°C for 10 min only. Pinocytic vesicles appeared at the site of virus attachment to cell surface membranes (Figs. 4, 5, 6), from which virions penetrated into cytoplasm by fusion of virion envelopes with the cell membrane. Under similar conditions, the adsorption and the initial stages of pinocytosis were similar in the presence as well as in the absence of the drug. However, in the presence of CPZ no fusion was seen (Figs 7, 8).

Table 3. Influence of CPZ on the replication of influenza virus in CEC

CPZ added	Virus yield* Non	20 μmol/l	50 μmol/l
15 min before	6×10^7	4.4×10^7	—
virus	2.83×10^7	—	1.87×10^7
adsorption	3×10^7	—	2×10^7
Together with	4.1×10^7	2.45×10^5	—
virus	6×10^7	3×10^5	—
adsorption	2.85×10^7	8.7×10^5	6.5×10^4
1 hr after virus	2×10^8	1.2×10^8	—
adsorption	2.26×10^7	—	2.3×10^7

* PFU/ml as detected in CEC.

Table 4. Reversal of CPZ-induced inhibition of virus replication in the presence of purified CaM

CaM added	Virus yield CPFU/ml 0.45 μmol/l[2])		0.56 μmol/l[2])	2.5 μmol/l[2])	5.15 μmol/l[2])
None (virus in PBS)	1.2×10^7	1.5×10^8*	1.6×10^7	2.87×10^7**	5.5×10^7**	2.87×10^7
None (virus in PBS + CPZ[1])	3.7×10^5	2.5×10^6	5.0×10^5	6.5×10^4	2.5×10^4	6.5×10^5
Yes (virus in PBS + CPZ + CaM[2])	2.8×10^6	1.15×10^7*	4.7×10^6	3×10^5	5.6×10^6	3.75×10^7

[1]) 20 μmol/l
[2]) CaM added in given concentrations.
* Average values of 8 experiments.
**50 μ mol/l of CPZ

CPZ was toxic for CEC grown in monolayers in concentrations higher than 20 μmol/l. At short incubation the toxic concentration was higher than 50 μmol/l. Cells cultured with 20 μmol/l CPZ remained viable but their growth was retarded (Table 2). At concentrations of 20—50 μmol/l, CPZ did not influence the replication of influenza virus in CEC when added 15 min before or 1 hr after virus administration. A significant decrease in virus yield was observed when virus was added together with the drug. (Table 3). CPZ inhibited virus replication at concentration of 20 μmol/l by 2 log PFU and at concentration of 50 μmol/l by 3 log PFU. This effect could be reversed by addition of CaM. If CaM was added together with the virus and CPZ or up to 10 min after virus adsorption, the harvests increased as related to CaM concentration (Table 4).

If cells were incubated for 24 hr in the presence of 20 μmol/l CPZ, then washed and infected with influenza virus, the replication was decreased by 50—70%, i. e. the virus yield in pretreated cells was 7×10^6 PFU as compared to 1.5×10^7 PFU in untreated infected ones.

Discussion

Based on presented results we believe that the inhibition of influenza virus replication in CPZ treated CEC was not caused by direct interaction of CPZ with the virus but rather by interaction of the drug with cellular CaM. Electron microscopic examination of CEC immediately after adsorption showed pinocytosis and viropexis in the absence of CPZ as in its presence. The process of fusion between the viral envelope and pinocytic vacuole, however, was more pronounced in the absence of the drug. Several authors (Vincenzi and Farrance, 1977; Kakiuchi *et al.*, 1978; Gnegy and Lau, 1980; Gnegy *et al.*, 1980; Lin *et al.*, 1980; Smoake *et al.*, 1981) showed that CaM is under certain conditions bound to plasma membranes. According to Schubart *et al.* (1980*a*, *b*) the lag phase of trifluoperazin action on hamster inzuloma

cells is 1—2 min. In our experiments, the administration of CPZ was little effective when added 15 min before the virus (Table 3). This indicates that CaM is probably situated on the inner (cytoplasmic) face of the cellular membrane. A significant decrease in sensitivity of cells to the virus was observed only when CPZ was added to monolayers together with the virus. A lower but still clear cut effect was achieved when cells were pretreated for 24 hr despite the absence of the drug during adsorption. This indicates that changes in permeability or reorganization of cell membrane components are important for CPZ action.

As shown, the fusion of influenza virus occurs also with isolated cell membranes (Čiampor, Križanová, 1971; Križanová *et al.* 1971). Polyornitin which binds nonspecifically to CaM (Itano *et al.*, 1980) interfered with its function, in concentration of 0.3 mg/ml it decreased virus yield by 1 log PFU when added together with the virus (unpublished results). Because polyornitin similarly to DEAE cellulose enhances the pinocytic activity of cells (Waksman *et al.*, 1980) this confirms our opinion that the infectious ribonucleoprotein (replication unit) of influenza virus is transferred to the cytoplasm of infected cells by means of fusion between the viral envelope and cellular membrane and that CPZ inhibits this fusion. Poste and Allison (1971) showed that procain hydrochloride and CPZ may prevent virus induced fusion and may impair the release of various secretory granules form cells. Authors suggested that substances blocking the transposition of Ca^{2+} ions from membranes or competing for Ca^{2+} binding sites in the membranes would inhibit fusion.

As recently described, CPZ binds to the hydrophobic domain of CaM in a specific way and that this binding is Ca^{2+} dependent (La Porte *et al.*, 1980; Tanaka and Hidaka, 1980). Action of CPZ for short period (15 min) before or 1 hr after virus adsorption did not influence the harvest of influenza virus in CEC monolayers. Thus CPZ is effective in very early stages of virus penetration when the membrane components are rearranged and certain biochemical changes occur in the membranes (Križanová *et al.*, 1979). A complete reversal of the inhibitory effect of 20 μmol/l CPZ was observed after addition of CaM (in total concentration up to 5 μmol/l); this was in accordance with the data of Klee *et al.* (1980) concerning the balance between CaM and CPZ effects. The latter authors found that 1 mol CaM can bind 2 mols of CPZ in the presence of Ca^{2+} with high affinity.

The presented results strengthen our hypothesis that CaM participates in the control of membrane fusion occurring between the influenza virus envelope and cell membrane although we can not answer the question how it exactly acts. In the case of high CPZ to virus ratio, however, direct inactivation of the virus can not be fully excluded. Inhibition effect of 8.3 μg/ml of CPZ on tick-borne encephalitis virus multiplication in CEC was described by Libíková *et al.* (1977).

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Explanation of Electron Micrographs (Plates XXII—XXIV):

Fig. 2. Particles of influenza virus strain A/WSN, negative staining, 2% PTA pH 7.5 × 100 000.
Fig. 3. The same virus particles treated with CPZ, negative staining, 2% PTA pH 7.5 × 76 000.
Fig. 4. CEC infected with influenza virus strain A/WSN, adsorption at 37°C for 10 min. × 76 000.
Fig. 5. CEC infected with influenza virus strain A/WSN, adsorbed at 0°C for 30 min and at 37°C for 10 min. × 165 000.
Fig. 6. CEC infected with influenza virus strain A/WSN, adsorption at 0°C for 30 min and then at 37°C for 10 min; initial stages of the fusion of viral envelope with the membrane of pinocytic vesicle. × 165 000.
Fig. 7. CEC infected with influenza virus A/WSN in the presence of CPZ, adsorbed at 37°C for 10 min. × 165 000.
Fig. 8. CEC infected with influenza virus A/WSN in the presence of CPZ, adsorbed at 0°C for 30 min and then at 37° C for 10 min. × 165 000.