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EFFECT OF INFLUENZA VIRUS ON PROTEIN PHOSPHORYLATION IN ISOLATED MEMBRANES OF CHICK EMBRYO CELLS

O. KRIŽANOVÁ, J. MATIS, E. ZÁVODSKÁ, *D. STANČEK

Institute of Virology, Slovak Academy of Sciences, 817 03 Bratislava, and *Research Institute of Preventive Medicine, Bratislava, Czechoslovakia

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Summary. — Membranes isolated from chick embryo cells (CEC) were found to contain an endogenous proteinkinase that phosphorylated endogenous proteins. ^{32}P incorporation into membrane proteins was analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography. Membrane phosphorylation in the presence of physiological saline (PM-phys) and in the presence of influenza virus (PM-V) were compared. Under short-time incubation (<1 min) with γ-^{32}P ATP there was practically no difference between PM-phys and PM-V in ^{32}P incorporation. Under prolonged incubation (3—15 min), gradual dephosphorylation of the phosphoprotein moving in SDS-PAGE in the zone of relative molecular mass (M_r) of 60 kDa (phosphoprotein P60) was observed in PM-phys whereas in PM-V, phosphorylation of P60 increased with the time of incubation with γ-^{32}P ATP. Dephosphorylation of P60 in PM-phys was inhibited by 2.5 mmol/l EGTA as well as by 100 μmol/l chlorpromazine (CPZ) and stimulated by calmodulin (CaM) in the presence of Ca^{2+}. Responsible for the dephosphorylation was probably the endogenous Ca^{2+} and/or CaM-dependent membrane proteinphosphatase. It was inhibited by influenza virus (even in the presence of Ca^{2+} and CaM). The possible role of P60 phosphorylation in the first step of virus infection is discussed.

Key words: membrane protein phosphorylation; membranes; influenza virus; calmodulin; chlorpromazine; proteinkinase; phosphoproteinphosphatase

Introduction

Alteration of normal metabolism of the cell in the course of virus infection is well known. The available data on changes induced in cell membranes by virus adsorbtion have been reviewed by Fuchs and Kohn (1983). Although these authors adduce various alternatives to explain the possible mechanism of virus-induced changes, the precise mechanism has not been elucidated yet.

Ohki *et al.* (1975) were the first to report that doubling of the intracellular concentration of c'AMP shortly after interaction between Sendai virus (HVJ) and Ehrlich ascites tumour cells. They suggest that this increase in c'AMP concentration is part of a control mechanism of HVJ virus-induced fusion of eukaryotic cells. The fusion process as a whole also requires ATP and Ca^{2+}. Similar results were obtained by Križanová *et al.* (1976), who showed that c'AMP in the presence of Ca^{2+} enhances the fusion of viral envelope with membranes isolated from chick embryo cells (CEC) and the nucleoprotein release from the virion. Later Križanová *et al.* (1982) found that CPZ, an antagonist of CaM, when added to cells together with virus, i.e. during its adsorption, inhibits influenza virus replication in monolayer CEC cultures and that CaM reverts this inhibition. Under similar conditions, inhibition of virus reproduction by EGTA (1 mmol/l) was observed (Závodská, Križanová, unpublished data).

On the basis of the above observations one may assume that c'AMP and Ca^{2+}/CaM are needed for or participate in the regulation of the first steps of cell infection by virus. It is a generally accepted view that the action of c'AMP and Ca^{2+} in cell regulation is mediated via phosphorylation-dephosphorylation of cell proteins by protein-kinases and proteinphosphatases. Many data suggest that membrane-assosiated proteinkinases may play some physiological role in the process of transmembrane signaling (Zick *et al.*, 1983). For instance, Schubart *et al.* (1982) found that induction of Ca^{2+} influx and insulin release stimulates phosphorylation of 60 000 dalton protein (P60) and that the neurotransmitter release correlated with the phosphorylation of synaptic vesicle tubulin (De Lorenzo, 1981; Burke and De Lorenzo, 1982).

Our interest of this study was devoted to the question, whether the action of c'AMP or Ca^{2+} at the first step of infection (i.e. during the interaction of virions with the cell membrane) can also be mediated by phosphorylation of a specific membrane protein.

Materials and Methods

Purification of cell membranes. CEC were prepared from 11-day-old chick embryos (Dulbecco, 1952). Their membranes were isolated at 4 °C by the modified method of Joos and Anderer's (1979). Harvested CEC were homogenized in 10 mmol/l Tris-HCl pH 7.5 + 1 mmol/l $MgCl_2$ (Tris-Mg buffer). Nuclei were spun at 2000 rev/min (600 g) for 10 min and the supernate was sedimented on 35% sucrose at 13 000 g for 1 hr. The crude membrane fraction which was found at the interface of 35% sucrose and buffer was carefully removed, diluted with 4 vol of Tris-Mg buffer and sedimented at 30 000 g for 1 hr. The pellet was resuspended in a small amount of Tris-Mg buffer and centrifuged at 24 000 rev/min in SW 25 rotor (Spinco) in a discontinuous sucrose gradient (45, 35, 30, 25% w/v sucrose in Tris-Mg buffer). The zone formed at the interface of 30 and 35% sucrose was removed, diluted with buffer and the membranes were sedimented at 30 000 g for 1 hr. The pellet further referred to as purified membranes (PM) was resuspended in water and directly used in the experiments. PM inhibited the virus-induced agglutination of avian erythrocytes. The heamegglutination-inhibition titre of 1 mg PM proteins when competing with 4 haemagglutination units (HAU) of virus varied in different preparations from 512 to 2048, which implied that 1 mg of membranes bound 2048—8196 HAU of virus.

Preparation of the virus. Influenza virus A/WSN/H1N1 was adapted to CEC as described (Križanová *et al.*, 1982) and purified by differential centrifugation. The purified virus was resuspended in physiological saline (buffered with 10 mmol/l Tris-HCl pH 7.2 or 10 mmol/l phosphate

Table 1. Effect of incubation time with γ-^{32}P ATP on ^{32}P incorporation into P60 in CEC membranes

Incubation conditions	Incubation in min				
	<1	3	5	10	15
PM-phys	5.2	2.0—2.5	3.0	2.4—2.6	1.7
PM-V	5.156	4.0—6.1	8.0	8.8—9.3	10.6

Phosphorylation of membranes was done in F buffer containing 1 μmol/l c′AMP and 1 μmol/l CaM with γ-^{32}P ATP.
The values in the table represent the percentage of P 60 area after densitometry of autorafuograms. (See Fig. 5 for illustration.)

buffer pH 7.2) to a concentration of 64 000—128 000 HAU/ml. Such virus preparation was used for further work not later than within 1 week.

Inactivated virus was obtained by incubating purified virus at 56 °C for 30 min.

Virus adsorption to PM. Purified virus mixed with PM usually in an amount of 5 000 to 10 000 HAU/mg membrane proteins was incubated on ice for 30 min.

Membrane-protein (MP) phosphorylation was performed at pH 7.0 and 30 °C for 1—15 min according to Schulman and Greengard (1978) in a total volume of 50 μl. The phosphorylation mixture contained 40 mmol/l Pipes buffer pH 7.0, 10 mmol/l $MgCl_2$, 0.1 mmol/l DTT, 0.1 mmol/l $CaCl_2$ (= F buffer), 40—47 μg PM either with adsorbed virus (PM-V) or analogously treated with physiological saline (PM-phys), and γ-^{32}P ATP (1—3 × 10^7 c.p.m.). The effect of c′AMP, CaM, CPZ and EGTA was tested at the following final concentrations: 10^{-6} mol/l, 0.75 and 2 μmol/l, 50 and 100 μmol/l, 1 and 2.5 mmol/l.

The reaction was stopped by adding 20 μl of 4-fold concentrated solubilization solution (0.2 mol/l Tris-HCl pH 6.9, 8% SDS, 10% MCE, 40% sucrose). Following heating to 100 °C for 2 min, MP were analysed in SDS-PAGE according to Laemmli (1970) upon 8% gel (Matis and Rajčáni, 1980). Phosphorylated proteins were detected by autoradiography using intensifying Perlux foils (G.D.R.) and Medik Rapid film (Czechoslovakia), the interval being usually 48—72 hr. Quantitative evaluation of ^{32}P incorporation into specific bands was done by scanning radioautograms in a Laser densitometer (LKB-Bromma 2202 ultroscan) with a connected integrator (3390 A Hewlett-Packard) to measure peak areas. Proteins were determined by the method of Lowry *et al.* (1951).

To detect the presence of phosphoprotein phosphatase activity in CEC membranes, 1 mg of PM was labelled with γ-^{32}P ATP in F buffer containing 10^{-6} mol/l c′AMP and 0.75 μmol/l CaM

Table 2. Effect of different virus concentrations on incorporation of ^{32}P into P60 in CEC membranes

	Virus concentration					
HAU/mg PM	0	2000	4000	6000	8000	10000
area % of P60	1.9—3.3	9.7	8.3	9.2	9.8	9.2

Incubation of membranes with γ-^{32}P ATP was 10 min in F buffer containing 1 μmol/l c′AMP and 1 μmol/l CaM.

Table 3. Effect of virus on membrane phosphorylation and on membrane proteinphosphatase activity

	Reaction conditions			
	PM-phys		PM-V**	
^{32}P incorporation into PM	139 370*		330 839*	
	phys	V**	phys	V**
^{32}P release	11 268*	0*	12 888*	0*

Membrane labelling (^{32}P incorporation) was done with γ-^{32}P ATP at 30 °C for 10 min in the presence of physiological saline (PM-phys) or virus (PM-V) under conditions stated in Materials and Methods.
^{32}P release (proteinphosphatase activity) = (c.p.m. in supernate after 25 min incubation — c.p.m. in supernate after 0 min incubation) at 30 °C of labelled and washed membranes mixed with physiological saline (phys) or virus (V) (see Materials and Methods).
** = Amount of virus/mg PM = 10 000 HAU.
* c.p.m./0.5 mg of PM

at 30 °C for 10 min. Labelled membranes were spun down in a microcentrifuge (type 320a, Poland) at 0 °C, washed twice with F buffer containing 10^{-6} mol/l c'AMP and 1 mmol/l EGTA, then once with F buffer containing c'AMP and resuspended in F buffer containing c'AMP and CaM to the original volume. Fifty microlitres of PM contained <100 000—300 000 c.p.m. Washed PM were then incubated at 30 °C in F-c'AMP-CaM buffer either with virus or with physiological saline for 0 and 25 min. The reaction was stopped by adding two vol of 15% TCA containing 1% active carbon, the samples were spun down and the radioactivity was counted in the supernate with a liquid scintilation spectrometer (Packard Fricard Model 3390). The amount of released ^{32}P was computed from the radioactivity difference in supernate at 0 and 25 min.

Reagents. c'AMP, Sigma; EGTA, Fluka; Pipes, Calbiochem; γ-^{32}P ATP (37TBq/mmol), U.S.S.R.; CPZ was obtained from Dr. Z. Vejdelka, Institute for Pharmacy and Biochemistry, Prague. Other reagents were analytical grade.

Results

Analysis in SDS-PAGE (following autoradiographic detection) of membranes incubated with γ-^{32}P ATP showed that membranes isolated from CEC contained endogenous proteinkinase(s) that phosphorylated endogenous proteins. Phosphorylation was the highest in high-molecular-weight proteins that remained at the start; these were followed by proteins of M_r > 100 000, 60 000 and 50 000 daltons. The last one formed a double zone in some samples. There was practically no difference in label intensity between phosphoproteins phosphorylated in the presence of influenza virus (PM-V) or physiological saline (PM-phys) if the reaction lasted less than 1 min (Fig. 1, lanes 1—4). Under prolonged incubation of the membranes with γ-^{32}P ATP the phosphoprotein zone of M_r 60 kDa (P60) in PM-phys decreased in intensity, whereas in PM-V its intensity increased (Fig. 1, lanes 6—8; Fig. 2, lanes 1—6). A difference between P60 phosphorylation in the presence of PM-phys and PM-V appeared already at 3-min incubation, increased with the time of incubation with γ-^{32}P ATP and was highest at 15 min (Fig. 2 and Table 1).

Table 4. Effect of CPZ (100μmol/l) and EGTA (2.5 mmol/l) on ^{32}P incorporation into P60 in CEC membranes

Incubation conditions	Buffer F with		
	(c'AMP + CaM)	(c'AMP + CPZ)	(c'AMP + EGTA)
PM-phys	3.8	5.2—7.9	6.4
PM-V	9.3	8.6	9.3

Incubation of membranes with γ-^{32}P ATP was for 10 min. For legend see Table 1.

The degree of P60 phosphorylation did not depend on virus concentration (2 000—10 000 HAU/mg PM; Table 2, Fig. 6).

Similar phosphorylated proteins were obtained with fresh membranes and membranes stored for 1 week at —70 °C. However, P60 dephosphorylation was slowerin the older PM preparations phosphorylated in the presence of physiological saline. Presumably, the phosphoproteinphosphatase (probably also endogenous) was partially inactivated in the older membranes. Incubation of the γ-^{32}P-labelled membranes in F buffer containing 10^{-6} mol/l c'AMP and 1 μmol/l CaM at 30 °C for 25 min released ^{32}P indicating that the membranes contained endogenous phosphoproteinphosphatase. Its activity was inhibited by the virus (Table 3).

Fig. 3 illustrates the influence of EGTA and CPZ on membrane-protein phosphorylation-dephosphorylation. It is evident from Fig. 3 and Table 4 that CPZ in a 100 μmol/l concentration partly inhibited P60 dephosphorylation in PM-phys. They did not influence P60 phosphorylation in PM-V (lower concentrations of CPZ — 50 μM and EGTA — < 1 mmol/l, respectively were only weakly effective). The results suggest that P60 dephosphory-

As evidenced in literature, the phosphorylation of some proteins may be stimulated both by c'AMP and by Ca^{2+}/CaM (Hutner and Greengard, 1979; Burke and De Lorenzo, 1981; Novak-Hoffer and Levitan, 1983; Plank *et al.*, 1983), but probably at different sites; this is also suggested by our results.

At short-time, less than 1-min incubation of the membranes with γ-^{32}P ATP no significant differences in the degree of ^{32}P incorporation into individual MP was observed whether the phosphorylation proceeded in the presence of physiological saline (PM-phys) or virus (PM-V). On prolonged incubation, however, PM-phys samples displayed dephosphorylation of P60 phosphoprotein, whereas in PM-V samples its phosphorylation increased.

It seems that responsible for P60 dephosphorylation was an endogenous phosphoproteinphosphatase which activity was inhibited by virus in membranes isolated from CEC (Table 3). The presence of phosphoproteinphosphatase activity in cell membranes has been demonstrated by several authors (Layne *et al.*, 1973; Tisdale and Phillips, 1976; Layne and Najjar, 1977; Gordon *et al.*, 1979; Makan, 1979; Gergely *et al.*, 1980; Swarup *et al.*, 1982).

and Table 5), which may be explained by the presence of endogenous CaM in the isolated PM. It is also evident from Fig. 4 that in such preparations CaM increased the phosphorylation of the peptide of M_r 50 kDa and less so of P60. c'AMP only weakly influenced (increased) P60 phosphorylation but decreased P50 phosphorylation.

The role of P60 phosphorylation in viral infection has not been elucidated yet, since it has been observed in membranes with adsorbed noninfective

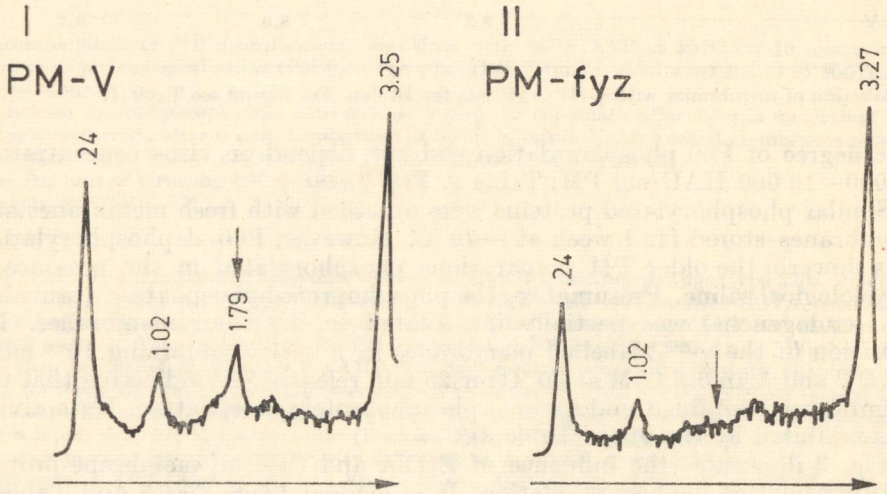

Fig. 5.

Densitometry of autoradiograms showing the virus effect on endogenous phosphorylation of proteins in CEC membranes

I — PM-V; II — PM-phys (= PM-fyz)

Arrow indicates P60 protein.

Analysis in SDS-PAGE (following autoradiographic detection) of membranes incubated with γ-^{32}P ATP showed that membranes isolated from CEC contained endogenous proteinkinase(s) that phosphorylated endogenous proteins. Phosphorylation was the highest in high-molecular-weight proteins that remained at the start; these were followed by proteins of M_r > 100 000, 60 000 and 50 000 daltons. The last one formed a double zone in some samples. There was practically no difference in label intensity between phosphoproteins phosphorylated in the presence of influenza virus (PM-V) or physiological saline (PM-phys) if the reaction lasted less than 1 min (Fig. 1, lanes 1—4). Under prolonged incubation of the membranes with γ-^{32}P ATP the phosphoprotein zone of M_r 60 kDa (P60) in PM-phys decreased in intensity, whereas in PM-V its intensity increased (Fig. 1, lanes 6—8; Fig. 2, lanes 1—6). A difference between P60 phosphorylation in the presence of PM-phys and PM-V appeared already at 3-min incubation, increased with the time of incubation with γ-^{32}P ATP and was highest at 15 min (Fig. 2 and Table 1).

(heat-inactivated) virus as well as in membranes incubated with γ^{32}P ATP in the presence of 50 μmol/l CPZ or 1 mmol/l EGTA, in which virus reproduction inhibition has been demonstrated in monolayer CEC cultures (Križanová *et al.*, 1982; Závodská, unpublished results). P60 dephosphorylation in PM-phys was inhibited only by higher concentrations of EGTA and CPZ (2.5 mmol/l and 100 μmol/l, respectively). These concentrations were toxic for CEC in monolayer cultures. Densitometric evaluation of autoradiograms showed that CPZ and EGTA decreased P50 phosphorylation (results not shown). Autoradiography did not reveal practically any zone for viruses incubated under identical conditions but in the absence of PM.

Discussion

Endogenous-proteinkinase-catalyzed phosphorylation of membrane proteins has been described for many membrane systems. The membrane proteinkinases from different tissues were in most cases stimulated by c'AMP (Lucid and Cox, 1972; Rubin *et al.*, 1972; Cartens and Weller, 1979; Therien and Mushynski, 1979; Weller, 1979), whereas the proteinkinase of isolated cell membranes were neither dependent on, nor stimulated, or were only weakly stimulated, by c'AMP (Lucid and Griffin, 1977; Ronquist *et al.*, 1979; Gallis *et al.*, 1981; Kübler *et al.*, 1982; Patterson and Brown, 1982*a*, *b*).

Membranes isolated from CEC were also shown to possess endogenous proteinkinase. This proteinkinase catalyzed ^{32}P incorporation into at least five principal membrane proteins (M_r > 200 K, 100—105 K, 60 K, 50 K and low-molecular-weight proteins). One of these proteins (M_r 60 kDa = P60) exhibited significantly higher ^{32}P incorporation in membranes incubated with γ-^{32}P ATP in the presence of virus (PM-V) than in the presence of physiological saline (PM-phys). Densitometry of the autoradiograms showed that P60 phosphorylation was stimulated by 10^{-6} mol/l c'AMP by amount 44% and by 1 μmol/l CaM by about 15%. The stimulatory effect of both agents was additive (about 60%).

As evidenced in literature, the phosphorylation of some proteins may be stimulated both by c'AMP and by Ca^{2+}/CaM (Hutner and Greengard, 1979; Burke and De Lorenzo, 1981; Novak-Hoffer and Levitan, 1983; Plank *et al.*, 1983), but probably at different sites; this is also suggested by our results.

At short-time, less than 1-min incubation of the membranes with γ-^{32}P ATP no significant differences in the degree of ^{32}P incorporation into individual MP was observed whether the phosphorylation proceeded in the presence of physiological saline (PM-phys) or virus (PM-V). On prolonged incubation, however, PM-phys samples displayed dephosphorylation of P60 phosphoprotein, whereas in PM-V samples its phosphorylation increased.

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P60 dephosphorylation in PM-phys was partially inhibited by 100 μmol/l CPZ or 2.4 mmol/l EGTA, which suggests that the phosphoproteinphosphatase of CEC membranes may be Ca^{2+} and/or CaM dependent.

Identification of the P60 membrane phosphoprotein has not been accomplished and the functional role of its phosphorylation-dephosphorylation remains unclear. The incorporporation of ^{32}P into this protein was also enhanced by incubating membranes with γ-^{32}P ATP in the presence of inactivated virus or native virus and agents (CPZ and EGTA, 50 μmol/l and 1 mmol/l, respectively) that, as had previously been found, inhibited virus replication in monolayer CEC cultures. It does not seem likely, therefore, that it should directly condition infection of the cell by virus. Nevertheless, it is possible that its phosphorylation is associated with the early virus-induced changes in the membranes. There is ample evidence that adsorption of virus to cells is accompanied by structural and functional changes in the cell surface membrane: intramembraneous particles aggregate, membrane permeability alters and membrane fluidity is increased (Levanon and Kohn, 1978; Fuchs *et al.*, 1978; Okada *et al.*, 1975*a*, *b*). Some of these changes are induced by infectious as well as noninfectious (UV-inactivated) virus (Levanon *et al.*, 1977; Fuchs and Kohn, 1983). The possibility that the membrane enzymes associated with phosphate metabolism (i.e. proteinkinases and phosphoproteinphosphatases) may play a role in virus-induced cell permeability is admitted by Fuchs and Kohn (1983).

The mechanism of Ca^{2+} and CaM effect in the process of initiation of virus infection in the cell has not been elucidated in this study. According to literature, Ca^{2+} and/or CaM are needed for cell fusion. However, fusion may only take place after a change in membrane structure and function (Okada *et al.*, 1975; Kalderon and Gilula, 1979). Nevertheless, direct evidence for the possibility of a similar mechanism being involved at the first step of cell infection by virus is lacking so far.

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Explanation to Figures (Plates I—V):

Fig. 1. Effect of influenza virus on membrane-protein phosphorylation. Gel autoradiogram illustrating proteins phosphorylated with γ-^{32}P ATP under conditions desribed in Materials and Methods.
The phosphorylation mixture contained 10^{-6} mol/l c′AMP and 2 μmol/l CaM ± 50 μmol/l CPZ in F buffer. Membrane phosphorylation in the presence of physiological saline (PM-phys) (columns 1, 3, 6, 8) or in the presence of influenza virus (PM-V) (2, 4, 7, 9) for less than 1 min at 30 °C (1, 2, 3, 4) or for 3 min at 30 °C (6, 7, 8, 9). Effect of 50 μmol/l CPZ in the absence of CaM is in 3, 4, 7, 8.
Molecular standards (5) in kDa ($M_r \times 10^{-3}$).

Fig. 2. Effect of incubation time on membrane-protein phosphorylation.
Phosphorylation conditions as in Fig. 1.
PM-phys (columns 1, 3, 5) and PM-V (2, 4, 6) incubated with γ-^{32}P ATP at 30 °C for 3 min (1, 2); 10 min (3, 4); 15 min (5, 6).

Fig. 3. Effect of EGTA and CPZ on protein-membrane phosphorylation-dephosphorylation. PM-phys (columns 1, 4, 7), PM-V (2, 5, 8) or inactivated PM-V (3, 6) incubated with γ-^{32}P ATP in the presence of 10^{-6} mol/l c′AMP at 30 °C for 10 min. 1 μmol/l CaM (1, 2, 3), 100 μmol/l CPZ (4, 5, 6) or 2.4 mmol/l EGTA were added as indicated.

Fig. 4. Effect of c′AMP and CaM on membrane-protein phosphorylation.
PM-phys (columns 1, 4, 8), PM-V (2, 5, 9) and PM-V inactivated (3, 6, 10) were incubated with γ-^{32}P ATP for 10 min under conditions described in Materials and Methods.
Addition of 10^{-6} mol/l c′AMP (1, 2, 3) and 2 μmol/l CaM (4, 5, 6) is indicated at the bottom of figure. Lane 7 = virus only, incubated with γ-^{32}P ATP in presence of CaM and c′AMP.

Fig. 6. Effect of different virus concentrations on protein-membrane phosphorylation.
Phosphorylation was done in F buffer containing 10^{-6} mol/l c′AMP and 0.75 μmol/l CaM at °C for 10 min. 1, 7 = PM-phys.
2, 3, 4, 5, 6 = PM-V (10 000, 8 000, 6 000, 4 000, 2 000 HAU/mg proteins, respectively).