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# Changes in Paracellular and Cellular Ionic Permeabilities of Monolayers of MDCK Cells Infected with Influenza or Vesicular Stomatitis Viruses

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**Summary.** MDCK cells (epithelioid line derived from the kidney of a normal dog) form monolayers which retain the properties of transporting epithelia. In these cells viruses bud asymmetrically: influenza from the apical, and vesicular stomatitis (VSV) from the basolateral membrane (E. Rodríguez-Boulán and D. D. Sabatini, *Proc. Natl. Acad. Sci. USA* **75:** 5071–5075, 1978; E. Rodríguez-Boulán and M. Pendergast, *Cell* **20:** 45–54, 1980). In the present study, we analyzed whether these viruses affect specific ion-translocating mechanisms located in the plasma membrane. We studied the effect of infection on membrane and transepithelial conductance, passive and active unidirectional fluxes of $Na^+$ and $K^+$, intracellular potentials, cellular content of $Na^+$ and $K^+$, and formation of blisters which, in these preparations, are due to the vectorial transport of fluid. Two main observations are derived from these studies. First, infection with VSV caused an increase in transepithelial electrical conductance, due to the opening of tight junctions, 5 to 6 hr after the start of infection, coincident with the accumulation of envelope protein in the cell surface and with the rise in the curve of virus budding. Infection with influenza, on the other hand, increased the transepithelial conductance only late in the infection (12 to 14 hr) when virus production has already stopped. Second, viruses did affect membrane permeability. Yet, the changes observed may not be ascribed to a perturbation of the specific translocating mechanisms for $Na^+$ and $K^+$ which operate in the same region of the plasma membrane that the viruses use to penetrate and leave MDCK cells. The methods used in the present study are not suitable to decide whether the nonspecific changes in permeability elicited by the viruses occur over the whole cell membrane or are restricted to a given region.

**Key Words** cultured epithelia · MDCK cells · ionic fluxes · intracellular potentials · viral infection · virus production

## Introduction

Permeability changes produced by viruses in animal cells have been known for more than two decades (Klemperer, 1960). The specific alterations of water, ion, sugar and amino acid fluxes that they introduce are now well-documented (Hatanaka, Huebner & Gilden, 1969; Isselbacher, 1972; Fuchs & Gilberman, 1973; Kalckar et al., 1973; Pasternak & Micklem, 1973; Venuta & Rubin, 1973; Weber, 1973; Negreanu, Reinhertz & Kohn, 1974; Pasternak & Micklem, 1974*a*,*b*; Okada et al., 1975; Imprain et al., 1980). It was even proposed that through these permeability changes, and the resulting modifications of the ion composition of the cytoplasm, viruses can re-direct the machinery of the cells to synthetize only viral components (Carrasco & Smith, 1976; Carrasco, 1978, 1980; Fernández-Puentes & Carrasco, 1980). In this study, we analyze the effect of influenza (FLU) and vesicular stomatitis (VSV) viruses on $Na^+$ and $K^+$ unidirectional fluxes and on electrical parameters in MDCK cells.

MDCK cells (epithelioid of renal origin) can be cultured as monolayers which retain many of the properties of the tissue from which they derive (Misfeldt, Hamamoto & Pitelka, 1976; Cereijido et al., 1978). Unidirectional ion fluxes through the cellular membranes of these cells, as well as through the monolayers they form, have been well characterized (Rindler et al., 1979*a*,*b*, 1982; Simmons, 1981; Cereijido et al., 1980*a*,*b*, 1981a,b; Aiton et al., 1982; McRoberts et al., 1982). The budding of influenza and VSV from MDCK cells has the remarkable property of being polarized: influenza buds from the apical, and VSV from the basolateral surfaces, respectively (Rodríguez-Boulán & Sabatini, 1978). Polarized budding appears to depend on the asymmetric insertion of viral envelope glycoproteins in the respective surface domain (Rodríguez-Boulán & Pendergast, 1980). Therefore, the question arises of whether influenza and VSV would affect ion permeability and electrical phenomena in MDCK cells, and of whether these changes would

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be specific for the ion-translocating mechanisms assumed to be located at the surface used for budding.

In order to study this problem, we measured unidirectional fluxes of $K^+$ and $Na^+$ using tracers, under conditions that allow a discrimination between active and passive components (Cereijido et al., 1980*b*). Yet because of the time taken by these determinations, tracers are not suitable to study early membrane events in viral penetration. Therefore, we also used intracellular microelectrodes that permit a recording of the electrical potential across the membranes being traversed by the viruses, as well as its resistance and its capacity (Stefani & Cereijido, 1983). Furthermore, monolayers of MDCK cells prepared on permeable supports (disks of a nylon cloth coated with collagen), which resemble natural epithelia (Misfeldt et al., 1976; Cereijido et al., 1978), were also used to investigate the effect of the viral infection on the transepithelial resistance. These data, together with the intracellular recordings, allowed us to discriminate whether the observed effects were due to permeability changes in the plasma membrane itself, or in the sealing capacity of the occluding junctions.

## Materials and Methods

### Cell Culture

Starter MDCK cultures were obtained from the American Type Culture Collection (MDCK, CCL-34; Madin & Darby, 1958). In most experiments, cells were between the 75th to the 79th passage. They were grown at 36°C in roller bottles with an air-10% $CO_2$ atmosphere or T 150 flasks (Costar, Cambridge, Mass.) with air-5% $CO_2$ humid atmosphere and Complete Dulbecco's Minimal Essential Medium (CMEM) with Earle's salts (GIBCO F-11, Grand Island, N.Y.), 100 U/ml of penicillin, 100 $\mu$g/ml of streptomycin, and 10% calf serum (GIBCO 617). Cells were harvested with trypsin-EDTA (GIBCO 540), and plated at confluency on 9.6 $cm^2$ plastic Petri dishes (Linbro Chemical, New Haven, Conn.) or on glass cover slips coated with rat-tail collagen.

### Infection

Viruses were grown and titrated as described elsewhere (Rodríguez-Boulán, 1983). Confluent monolayers ($2 \times 10^5$ cells/$cm^2$) were inoculated with VSV with a multiplicity of infection (MOI) of 10 PFU/cell in Dulbecco's MEM (GIBCO F-11) complemented with 100 $\mu$g/ml of DEAE-Dextran (Sigma Chemical Co., St. Louis, Mo.). After one hour at 36°C, the inoculum was removed and substituted by Dulbecco's MEM with 2% fetal calf serum (GIBCO G-29). Inoculation with influenza virus was carried out with a MOI of 10 PFU/cell in Dulbecco's MEM supplemented with 0.2% bovine serum albumin (Sigma). After one hour at 36°C, the inoculum was removed and substituted by the same medium.

### Na Washout

Confluent monolayers in Petri dishes were washed twice with a K-free Ringer's solution containing (mM): 140 NaCl, 22 $NaHCO_3$, 1.8 $CaCl_2$, 5.0 glucose, at pH 7.4. This solution was then replaced by 1.0 ml of fresh Ringer's containing 1 $\mu$Ci of $^{22}Na$ and incubated at 37°C for 1 to 2 hr. After this loading period, the monolayers were washed twice with ice-cold 0.1 M $MgCl_2$, and left in contact with this solution for 2 min. The washout was then followed by incubating the monolayers at 37°C with 1.0 ml of Ringer's with 5 mM KCl. After 1 min the Ringer's was collected for $^{22}Na$ counting and the Petri dish refilled with fresh Ringer's. The procedure was repeated four times. At the end of the sampling period, 1.0 ml of 1.0 N $NO_3H$ was added, and left 1 hr at room temperature. This fluid was then analyzed for total and tracer sodium using an atomic absorption spectrophotometer (Unicam S.P. 90) and an Auto Gamma Counter (Nuclear Chicago, Chicago, Ill.). Studies of Rindler et al. (1979*b*) and Cereijido et al. (1980*a*) have shown that the washing with 0.1 M $MgCl_2$ is sufficient to eliminate $Na^+$ from the extracellular but not from the intracellular compartment, so this procedure is adequate to measure unidirectional Na fluxes from monolayers of MDCK cells.

### K Uptake

The monolayers were washed at 37°C as described above with the same Ringer's solution, except for the addition of 5 mM KCL. This solution was then replaced by a fresh one containing 1.0 $\mu$Ci of $^{86}Rb$. After 1 min, the loading solution was discarded, and the monolayer was quickly washed three times with ice-cold 0.1 M $MgCl_2$. The fourth time $MgCl_2$ was left for 2.0 min. At the end of this period, the $MgCl_2$ solution was removed and $^{86}Rb$ was extracted with 1.0 ml of 0.5 N NaOH for 2.0 hr. A sample of 500 $\mu$l was added to 10 ml of Aquasol (New England Nuclear) and counted in a Beta Counter (Packard TRI-CARB).

### Intracellular Ion Content

Monolayers of 2.2 $cm^2$, plated in Linbro 24-well chambers (Flow Lab., McLean, Virginia), were inoculated with virus for 1 hr as detailed above, and then incubated four additional hours with medium containing $^{22}Na$ or $^{86}Rb$. This period is long enough to equilibrate the specific activity of sodium and potassium in the cells with those in the bathing solutions. The monolayers were then washed five times with ice-cold 0.1 M $MgCl_2$, the last washing lasting 2 min, extracted as described above, and $^{22}Na$ and $^{86}Rb$ radioactivities were determined in aliquots of the extracts and incubating media. Using the specific activities of these tracers in the bathing solutions, the cellular Na and K contents were calculated.

### Measurements of the Electrical Resistance across the Monolayer of MDCK Cells

Disks of nylon cloth, 1 cm in diameter, were coated with rat-tail collagen, sterilized and plated with MDCK cells at high densities ($5 \times 10^5$ cells per square centimeter in one milliliter) as described by Cereijido et al. (1978). Ninety minutes after plating, the disks were transferred to fresh CMEM without cells, and the only MDCK cells remaining were those forming the monolayer. The disk was mounted as a flat sheet between two Lucite® chambers filled with CMEM, and the voltage deflection produced by passing 100 $\mu A\ cm^{-2}$ was used to calculate the transepithelial resistance. The contribution of the empty disk, solutions and electrodes was substrated. Further details are given in Cereijido et al. (1978). Each disk is used for a single measurement and discarded.

### Measurements with Intracellular Microelectrodes

Microelectrode studies using monolayers of MDCK cells cultured on collagen-coated glass coverslips were carried out on the stage of a Leitz phase-contrast microscope. The temperature of the recording solution, CMEM, was electronically set at 37°C and monitored with a small tip telethermometer (Yellow Spring Instruments Co.), located close to the culture coverslip. To avoid evaporation, the saline was covered with a thin layer of oil (Cannon Instruments Co.). Microelectrodes were pulled with a two-stage puller (Brown Flaming, Sutter Inst. Co.), from microfiber borosilicate glass capillary tubes (Hilgenberg, OD 1.0 mm; ID 0.5 mm), and filled with 4 M K-acetate. In most cases, we used microelectrodes with resistance of 100 to 150 MΩ which gave stable impalements and showed little rectification. Microelectrodes were connected to standard high impedance amplifiers (WPI KS 700) which allow to record the membrane potential and inject current simultaneously via a subtracting circuit. The injected current was recorded via a current-to-voltage converter (Analog Devices 52) with 100 MΩ in the negative feedback loop, thus 1 mV = 0.01 nA. The bath was grounded via a silver chloride wire connected to the negative input of the current-to-voltage converter.

Prior to penetration, the voltage-subtracting circuit was carefully adjusted by delivering current steps (0.1 to 1 nA). Micropipettes which showed prominent rectification were discarded at this stage. Once the cell was impaled, slight adjustments of the balance were occasionally needed, and the null point could be accurately determined because of the relative long time constant of the membrane in comparison with the time constant of the recording circuit. This procedure could be optimally carried out with the negative capacity compensation set close before oscillation. Furthermore, the null point was checked again after withdrawal of the micropipette from the cells. Square pulses of current of increasing amplitudes were delivered to obtain the current/voltage relationship. The membrane time constant was measured at 63% of the steady-state level of the voltage response.

The microelectrodes were mounted on piezoelectric step drive (Burleigh, Inchworm Controller, PZ-550) mounted on Huxley type micromanipulator. To impale the cells 4-$\mu$m steps were used. Occasionally, brief oscillations produced by the capacity compensation circuit helped the penetrations. Further details on these electrophysiological procedures, as well as the values of basic electrical parameters of MDCK cells were given by Stefani and Cereijido (1983).

Results are expressed as mean ± standard error (number of observations).

## Results

### Blisters and Ion Contents

MDCK monolayers cultured on impermeable support form blisters because of transepithelial transport and accumulation of fluid under the monolayer (Leighton, et al., 1969). A blister results from the compromise of several factors (Cereijido et al., 1981*a*): 1) the vectorial transport and accumulation of ions and fluid under the basal side; 2) the sealing of the occluding junctions; 3) the detachment of the monolayer from the substrate as a result of the accumulation. Three-day-old monolayers were infected with influenza or VSV. All of them (168 monolayers) had blisters. Blisters were still present 5 hr after the infection with both types of viruses. By the 4th to 5th hour, the VSV-infected monolayers had a large number of new little blisters. No changes in blister size, number or distribution were observed during this period in the monolayers left as control.

**Table 1.** Electrolyte content of monolayers of MDCK cells 5 hr post-infection with VSV or FLU

| | $Na^+$ | $K^+$ |
|---|---|---|
| | ($nmol \cdot cm^{-2}$) | |
| Control | 7.09 ± 0.30 | 56.4 ± 1.9 |
| VSV | 8.26 ± 0.13 | 47.1 ± 2.0 |
| FLU | 9.98 ± 0.17 | 56.7 ± 3.0 |

$n = 8$

Infection with VSV or influenza produce only minor modifications in the $Na^+$ and $K^+$ content of the cells (Table 1), but the normal high-K/low-Na composition of the cell is preserved.

### Electrical Resistance Across the Monolayer

MDCK cells make contact with each other to form occluding junctions which confer the epithelial monolayer with the properties of an effective permeability barrier, including the generation of transepithelial electrical resistance (TER) (Cereijido et al., 1978). Figure 1 shows that neither VSV nor influenza produce an effect on the TER in the first hours of infection. After 5 to 6 hr, however, monolayers infected with VSV experience a sharp drop in TER, which coincides with the rise in the virus production curve (Fig. 1) and the accumulation of envelope G protein in the plasma membrane (Rodríguez-Boulán & Pendergast, 1980). In spite of displaying similar kinetics as VSV in production (Fig. 1), and in shutting off cell protein synthesis (*not shown*), influenza virus does not affect the TER until very late in the infection (12 to 14 hr) when virus budding has already stopped. Since monolayers of MDCK cells have a transcellular and a paracellular route of permeation (Cereijido et al., 1980*b*, 1981*b*), it is not possible to assert, on the basis of these transepithelial recordings alone, whether the drop in the electrical resistance is due to an effect of the viruses on the cellular membrane of the cells, or to an opening of the occluding junctions (Martínez-Palomo et al., 1980; Cereijido et al.,

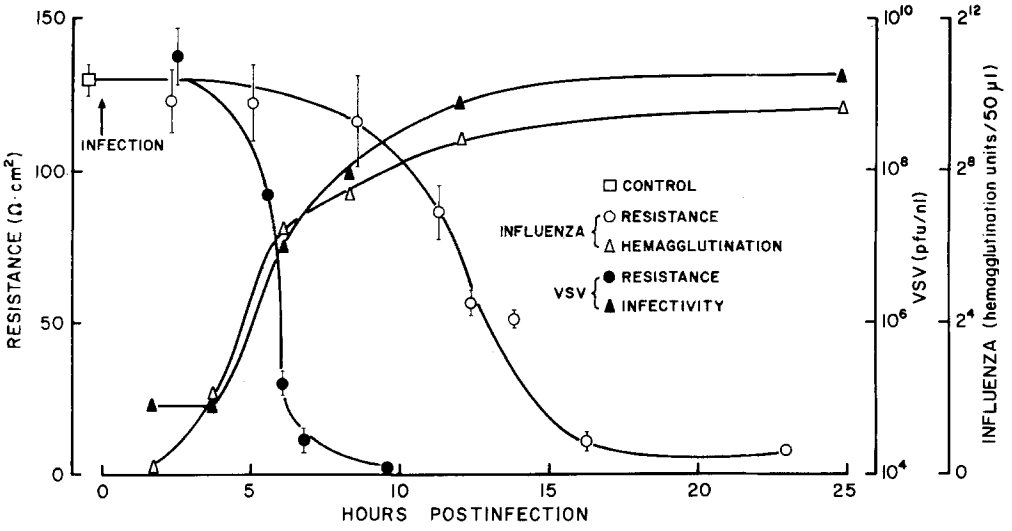


**Fig. 1.** Effect of viruses on the electrical resistance across the monolayers of MDCK cells cultured on disks of nylon cloth coated with collagen. Electrical resistance was calculated from the voltage deflection elicited by a current of 100 $\mu A \cdot cm^{-2}$. The resistance of the support and media were subtracted. Virus production was determined by hemagglutination (influenza) and plaque assay (VSV)

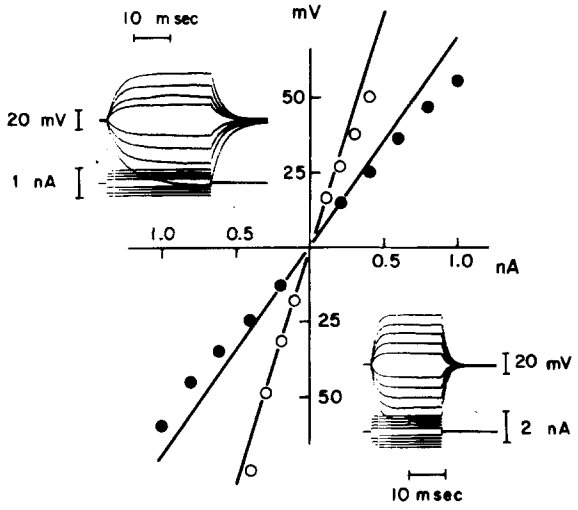


**Fig. 2.** Voltage/current relationship obtained with current pulses of opposite polarity and various intensities in control monolayers (*upper left, and open circles*), and monolayers infected with VSV (*lower right, and full circles*). Notice the asymmetry of the voltage response for large current pulses obtained in the control monolayer. The graph shows the voltage/current relationships measured at the end of the pulse. The increase in resistance for large negative pulses is underestimated because the voltage deflections have not reached a steady-state value. Microelectrode resistance: 122 MΩ. Cells were plated 5 days before

1981*b*). Therefore, in the experiments described in the following sections we measure fluxes and electrical phenomena known to occur across the plasma membrane of the MDCK cells.

## Electrical Phenomena Across the Plasma Membrane

Our first task was to select a current intensity that would permit the determination of the membrane resistance in the linear part of the voltage/current (*V/I*) curve. Figure 2 shows the *V/I* curves obtained with a control monolayer (open circles) and the other with the same monolayer after 10 to 20 min of infection with VSV (full circles). They were drawn with the values obtained at the end of the pulses reproduced in the upper left and lower right of the Figure, respectively. Control recordings show the asymmetry of the voltage response already described by Stefani and Cereijido (1983). Infection with VSV abolishes this asymmetry, and markedly decreases the slope resistance of the *V/I* curve (125 *vs.* 67 MΩ). The curves show that, for current pulses of 0.1 nA in the control cells, or up to 0.5 nA in the infected cells, the voltage/current relationship remains linear, so these current intensities were used to measure the resistance of the membrane during the rest of the experiments.

Figures 3 and 4 show the intracellular potential and membrane electrical resistance before (bar) and after (circles) infection with VSV. Both parameters fall as a consequence of viral infection. Of course, the procedure used does not permit to discriminate whether the increase in membrane permeability occurs as a result of the attachment of the virus to the cell membrane, reflects the process of viral endocytosis, or is due to an effect exerted by the viruses on

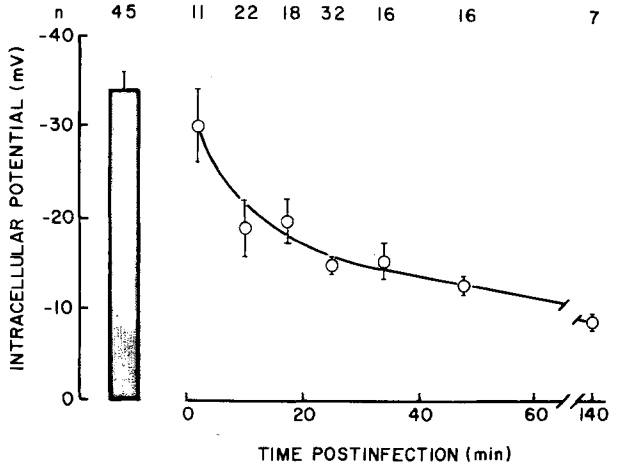


**Fig. 3.** Resting intracellular potential of monolayers of MDCK cells, plated at confluence 5 days before. The column represents control values in the same monolayers before infection. The monolayers were infected with VSV at time zero as described in Materials and Methods. The electrical resistance of the microelectrodes was *ca.* 150 MΩ. The number of observations (*n*) is noted above

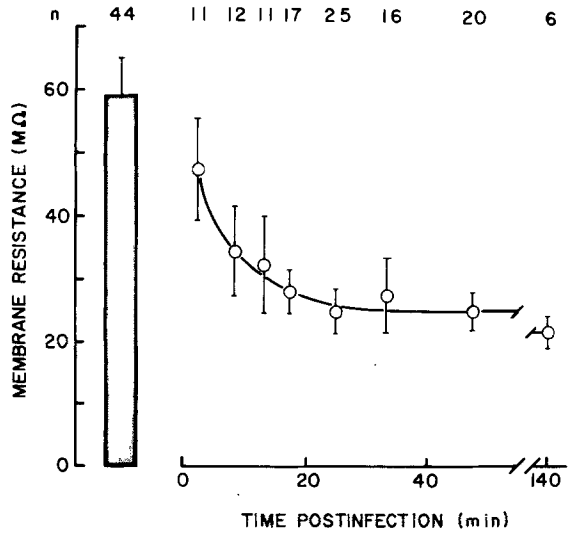


**Fig. 4.** Electrical resistance of the plasma membrane of MDCK cells recorded with microelectrodes using a small negative pulse of 0.1 nA and 5 msec. The monolayers were infected with VSV as in Fig. 3

the cell membrane once they reach the cytoplasm of the infected cell.

Similar studies were performed with influenza virus. Resistance decreases and asymmetry is lost as with VSV virus (Fig. 2). The time course of the potential drop (Fig. 5) is more complicated, however, than in the case of VSV: there seems to be a fast decrease, followed by a transient increase. The effect of influenza on the electrical resistance of the

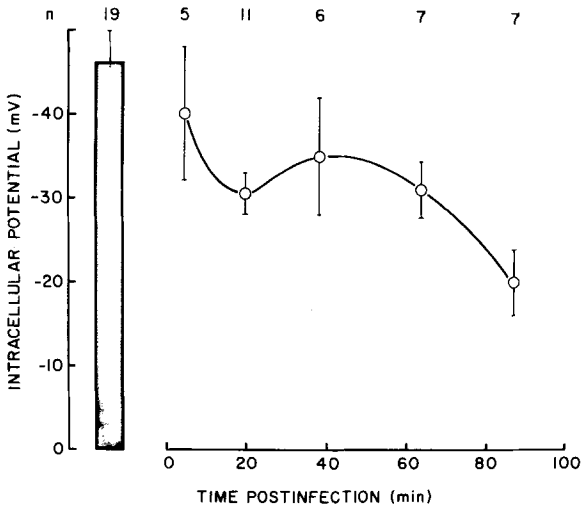


**Fig. 5.** Resting intracellular potential of monolayers of MDCK cells under control (*column*) and FLU-infected condition. Description as in Fig. 3

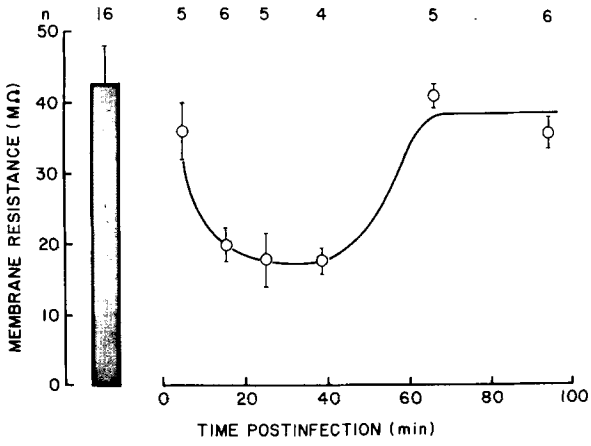


**Fig. 6.** Electrical resistance recorded in control (*column*) and FLU-infected condition. Description as in Fig. 4

MDCK cell (Fig. 6) is also different from the one elicited by VSV, since this parameter recovers after about 60 min.

Table 2 shows the value of the electrical resistance across the plasma membrane of control and infected cells beyond the 5th hour (VSV and FLU) and beyond the 13th hour (FLU). It is interesting to notice that the cells retain a considerably high resistance in spite of the active budding of virus occurring in their membranes at those times.

## Unidirectional Fluxes

We measured the influx of a given ion in the presence and in the absence of ouabain ($10^{-4}$M) in differ-

ent batches of membranes. As shown elsewhere this concentration of ouabain produces a complete inhibition of the pump, and the difference between the flux in noninhibited cells and in those with ouabain may be taken as the active component of the flux (Cereijido et al., 1981*a*). These measurements were made with 140 mM Na and 5 mM K in the washing Ringer's at a very early time of infection (1 hr) and also during virus assembly (5 hr).

**Table 2.** Electrical resistance across the plasma membrane of MDCK cells during budding of VSV or FLU viruses[a]

| | Time post infection (hr) | Membrane resistance (MΩ) |
|---|---|---|
| Control | | 57.6 ± 7.7 (13) |
| VSV | 5–7 | 40.3 ± 12.1 (9) |
| FLU | 5–7 | 49.0 ± 11.5 (10) |
| FLU | 13–15 | 46.3 ± 15.4 (6) |

[a] Resistance was measured by passing a square negative (cell) pulse of current of 1 nA and 40 msec, and recording the voltage deflection with the same microelectrode.

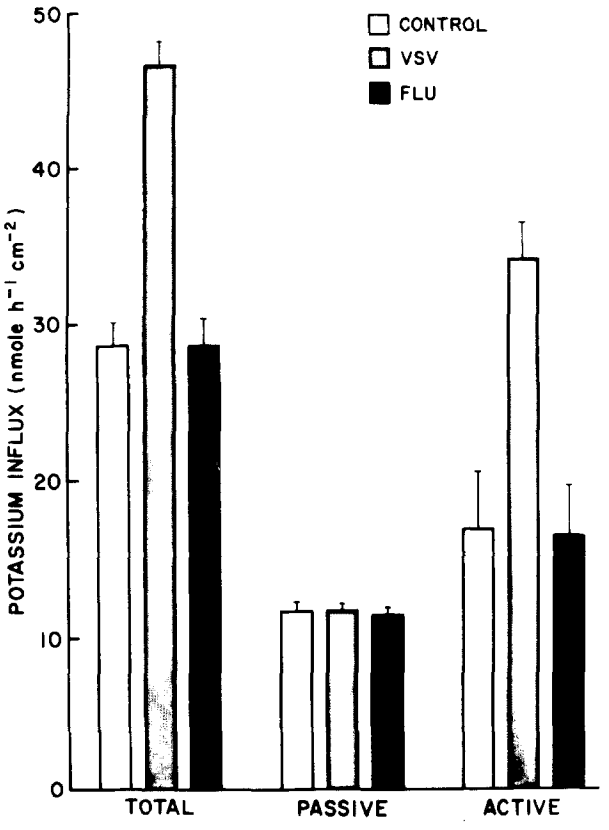


**Fig. 7.** Potassium influx as calculated with the uptake of $^{86}Rb$ in confluent monolayers cultured in 9.6 cm² Petri dishes 3 days after plating. Columns represent values under control, VSV-, and FLU-infected conditions one hour before flux measurements. *Passive* influx is the influx obtained in monolayers treated with 5 × $10^{-4}$ M ouabain 20 min before starting the measurement of the uptake. *Active* influx is the fraction of the total influx inhibited by ouabain

*The Influx of K*

Potassium fluxes were measured using $^{86}Rb$ as tracer. Figure 7 shows the total, passive and active components of the influx after one hour of infection. White bars are controls. Gray bars show that VSV produces a marked increase of the total influx of potassium. This increase is solely due to an increase of the K pumping and no difference exists between the passive components. Influenza virus does not seem to produce a detectable effect on the K fluxes.

K fluxes measured after 5 hr of infection (Fig. 8) show a different situation: 1) the relationship between active and passive K fluxes under control conditions is changed, due perhaps to the longer incubation period. Since these conditions are somewhat different for the two types of viruses, we tested that they do not introduce further differences among themselves (Table 3), so that any change in membrane permeability may be attributed to the virus; 2) the large increase in pumping observed in earlier periods with VSV disappears in absolute value and in relationship with the control; and 3) the operation of passive channels is decreased. As in the earlier period, infection with influenza does not result in an appreciable change in K permeability.

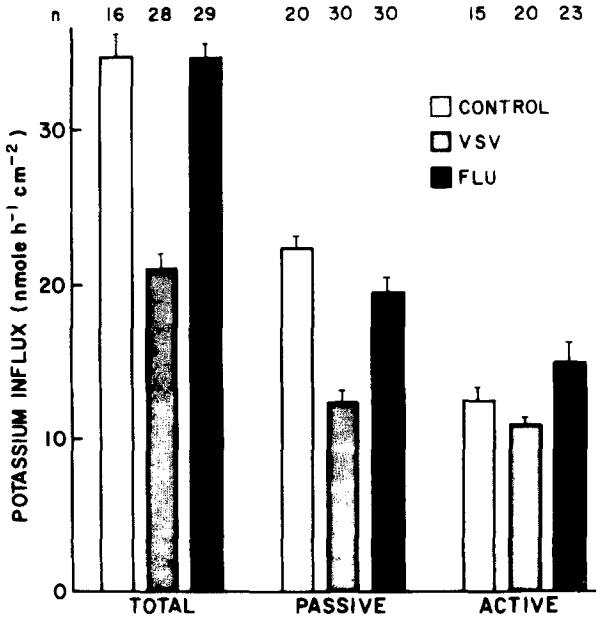


**Fig. 8.** Potassium influx calculated as described in Fig. 7, five hours after infection with VSV or FLU

*Na Efflux*

As described in Materials and Methods, the measurement of Na fluxes in MDCK cells requires a loading period in which the Na content of the cell is increased experimentally. Furthermore, Cereijido et al. (1981*a*) have shown that not only the active and passive rate of Na extrusion vary with the Na content, but they follow different patterns. Therefore, in order to make a valid comparison of the effects of the viruses, it is necessary to determine at which cellular Na content (*S*) were the unidirectional fluxes measured.

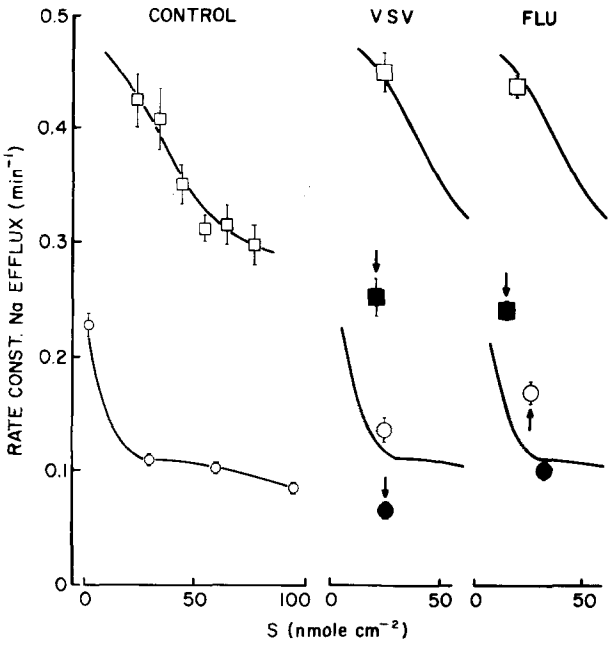


**Fig. 9.** Rate constant for the outflux of sodium as calculated with the washout of $^{22}Na^+$ in monolayers under control, VSV- and FLU-infected conditions. Rate constants are represented as a function of the $Na^+$ pool in the cells (*S*) as it may be noticed that under control condition (*left panel*) they vary with this parameter. The lines fitted by eye to the experimental points under control conditions were represented again in the middle and right panels to permit comparisons with values obtained in FLU- and VSV-infected monolayers. In these panels, open and closed symbols correspond to monolayers infected *one* or *five* hours before, respectively. Square symbols represent rate constants for the total outflux, and circles correspond to those of passive outflux. Arrows were added to remind that the signaled point represents a decrease or an increase from control values

Accordingly, in Fig. 9 we express the rate constants for total (*upper curves*) and passive (*lower curves*) sodium movements. The difference between the two curves corresponds to the active fluxes of Na. In noninfected monolayers, it is clear that the total and passive permeabilities decrease sharply as the Na content increases. This effect was observed to occur in cells from adult epithelia (Cereijido et al., 1964). The curves are represented again in the central and right panels to allow comparisons with the virus-infected monolayers.

MDCK cells infected for 1 hr with VSV do not show a detectable modification of the total (*open square*) nor passive (*open circle*) fluxes, as their values fall on the control curve. Therefore, the infection with this virus does not modify the passive permeabilities to K nor to Na. Yet it might dissociate the pump, because K pumping is stimulated (Fig. 7) but Na pumping is not (Fig. 9).

Five hours after infection, at a time that corresponds to an active budding of VSV, this virus produces a marked inhibition of the total (Fig. 9, *full square*) and passive (*full circle*) permeabilities. Notice that the rate of Na pumping (difference between the total and the passive) is also diminished. This decrease in passive $Na^+$ permeability is similar to the one produced on the $K^+$ permeability (Fig. 8) at this time of infection.

The infection with influenza (Fig. 9, *right panel*) produces no marked change in the total Na permea-

**Table 3.** Ionic fluxes across the plasma membrane of MDCK cells under the incubating conditions used for the different viruses

| Incubating conditions[a] | Potassium influx (nmol $hr^{-1}$ $cm^{-2}$) | Sodium outflux | |
|---|---|---|---|
| | | Na Pool (*S*) (nmol $cm^{-2}$) | Rate const. (*k*) ($min^{-1}$) |
| DMEM + 10% calf serum (1) | 28.6 ± 1.0 | 30.9 ± 1.4 | 0.30 ± 0.03 |
| DMEM + 2% fetal calf serum (2) | 29.0 ± 2.0 | 29.1 ± 3.2 | 0.36 ± 0.02 |
| DMEM + 0.2% bovine serum albumin (3) | 30.6 ± 1.0 | 28.6 ± 1.5 | 0.38 ± 0.01 |

[a] These are the conditions used with control (1), VSV-infected (2), and FLU-infected (3) monolayers. In the experiments of this Table the monolayers were not infected though, and only the possible effects of the incubating conditions were tested. Monolayers were in contact with these media for 1.5 hr, followed by 2.5 hr in the loading K-free Ringer's. After these periods, Na washout or K uptake were measured as described in Materials and Methods.
$n = 5$

bility of MDCK cells (*open square*). However, there is an increase in passive permeability (*open circle*). Since we take as active Na pumping the differences between the total and the ouabain-inhibited (passive) permeability, we must conclude that infection with influenza virus decreases the active efflux of sodium.

During the budding of influenza, the passive efflux of Na is not affected (Fig. 9, *full circle*). Yet the total efflux (*full square*) is markedly depressed at the expense of the inhibition of the active pumping. This virus might also be dissociating the pump, as we observe that the drastic inhibition of Na efflux is not accompanied by a corresponding modification in the active influx of potassium (Fig. 8). The data in Table 3 indicate that the observed decrease of permeabilities are due to the viruses themselves and not to the incubating conditions, as the controls of these conditions would in fact produce a minor increase of the flux.

## Discussion

### Effect of Viral Infection on the Transepithelial Conductance

We observed a dramatic difference in the effects of VSV and influenza on the transepithelial conductance of MDCK monolayers. The relatively early drop in the values of this parameter in monolayers infected with VSV (5 hr of infection) contrasts with the late effects of influenza (12 to 15 hr post infection). Genty and Bussereau (1980) have noted the similarity between the morphologic changes suffered by cells infected with VSV and those found in cells treated with cytochalasin B. Furthermore, they showed that these changes were very slight in cells infected with temperature-sensitive mutants of VSV of complementation groups III and V, at nonpermissive temperatures. Under these conditions, the transport of G protein to the plasma membrane is highly decreased. Therefore, they have proposed an actin depolymerizing effect of G protein. This effect would certainly explain our results, since we have shown (Meza et al., 1980) that cytochalasin B causes a drop in the transepithelial resistance, resulting from the opening of the tight junctions. The presence of actin in enveloped RNA viruses is considerably well documented (Wang et al., 1976; Damsky et al., 1977), and specific interactions between actin and the protein (M) of paramyxoviruses have been described (Giuffre et al., 1982). However, these viruses, like influenza virus, bud from the apical surface of MDCK cells (Rodríguez-Boulán & Sabatini, 1978) and cause relatively little cytopathic effect in MDCK cells as evident from the data presented in this paper. More information on possible interactions between VSV M protein and cytoskeleton is needed to understand a possible disruptive effect of this protein on the cytoskeleton associated with tight junctions.

We have recently shown (Stefani & Cereijido, 1983) that the minimum value of the electrical resistance across the transcellular route of a single epithelial cell ($r_{TC}$) is given by

$$r_{TC} = \frac{R_m}{F_a - F_a^2} \tag{1}$$

where $R_m$ is the electrical resistance across the entire plasma membrane as measured with the microelectrode, and $F_a$ is the fraction of the plasma membrane occupied by the apical. The minimum is given when $F_a$ is 0.5. In this case Eq. (1) reduces to

$$r_{TC} = 4R_m. \tag{2}$$

A confluent monolayer of MDCK cells has less than $5 \times 10^5$ cells per square centimeter, each one acting as a resistance in parallel. Therefore the electrical resistance across the transcellular route of a square centimeter of monolayer has a minimum resistance ($R_{TC}$) given by

$$R_{TC} = \frac{4R_m}{5 \times 10^5}. \tag{3}$$

Since the $R_m$ of a cell infected for 5 to 7 hr with VSV is 40.3 MΩ (Table 2), Eq. (3) predicts that the monolayer will have at least 322 $\Omega cm^2$. A similar calculation for FLU-infected cells for 13 to 15 hr gives 370 $\Omega cm^2$. This indicates that the abolishment of the resistance across the monolayer produced by the viruses is not due to their effect on the plasma membrane of the cells, but to an effect on the paracellular route limited by the occluding junctions. The opening of these junctions, together with the weakening of the attachment to the substrate, result in the rounding up and detachment of the cells at a later stage. However, our information refers to periods when the cells still form a continuous monolayer. The permeability of rounded up or detached cells is not known.

### Effects on Cell Membrane Permeability

We have chosen the unidirectional fluxes of tracers to analyze the possible effect of the viruses. Since these methods do not have a suitable time resolution to follow the early effects of virus penetration, we have also resorted to electrical tests. We have also chosen to study fluxes up to the 5th hour post-infection because, in spite of the fact that by this time infection has already occurred and insertion of

viral envelope proteins in the plasma membrane is well advanced, the monolayer still maintains its electrical resistance across (Fig. 1). These methods are adequate to analyze the mechanism of ion translocation across the cell membrane, yet they give no information on whether the mechanisms are situated on the apical or on the basolateral side. However, the information available on unidirectional fluxes of ions in MDCK cells (*see* Introduction) suggests that: 1) the apical barrier has a passive mechanism for the translocation of $Na^+$ and a second one for the translocation of $Cl^-$; and 2) the basolateral barrier has a common ouabain-sensitive ATPase that pumps $Na^+$ in exchange for $K^+$, and a passive cotransport mechanism of the type called "Na + K = 2 Cl system" (Field, 1978; Frizzell, Field & Schultz, 1979).

During inoculation, the electrical resistance of the plasma membrane and the intracellular potential show a clear and fast decrease. Yet this may not be accounted for by an increased permeability to $Na^+$ nor $K^+$ (although influenza does produce a small increase of Na permeability). Therefore, the penetration of viruses provokes a significant stimulation of the permeability to some ion, other than the ones tested. The penetration of this ion is high enough as to shunt the membrane potential and to abolish the asymmetry of the *I/V* curve. Present studies on the penetration and fate of the viruses inside the cell, indicate that changes in pH are involved (Matlin et al., 1981). It is therefore possible that the modifications of membrane conductance observed in this study were due to movements of $H^+$.

During budding (5th hour post-infection), the cells maintain their $Na^+$ and $K^+$ contents within control values (Table 1), and the monolayer does not lose its blisters. Hence the alterations of the unidirectional fluxes produced by influenza and VSV are not drastic enough as to alter the normal composition of the cells. Therefore, these observations on passive fluxes do not support the notion that the polarized budding of influenza from the apical, and VSV from the basolateral, would affect the specific ion-translocation mechanisms located in these regions of the plasma membrane.

Both VSV and influenza change the active movement of $Na^+$ and $K^+$. Yet, in the case of active movements, it is not certain that the changes observed were due to direct effects on the pump itself, as this mechanism can also be influenced by the supply of ATP, which in turn depends on the modifications that the viruses might be causing to the cellular metabolism. Nevertheless, it is observed that the viruses produce a dissociation of the normal Na/K coupling, as influenza decreases Na pumping without modifying K pumping, and VSV increases the rate of active translocation of $K^+$ without a detectable effect on Na pumping. These effects of the viruses on the stoichiometry of the pump deserve future studies.

In summary: during influenza and VSV infection, the ionic permeability of the cell suffers profound changes, as evidenced mainly by the drastic changes in electrical parameters. Yet the cells do not become leaky as a result of unspecific cellular damage. These viruses are able to penetrate and leave the cell without affecting the $Na^+$ and $K^+$ balance in the cytoplasm, nor the specific mechanisms sitting in the cellular regions where their budding takes place. On the basis of the methods used, one cannot discriminate whether the nonspecific changes in permeability produced by the viruses, take place in the whole plasma membrane, or are restricted to a particular region (e.g. the apical or the basolateral). This elucidation would require the future use of different approaches, such as patch clamping and the use of membrane vesicles obtained from a particular side of the cell only.

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