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Chloride conductance in membrane vesicles from human placenta using a fluorescent probe. Implications for cystic fibrosis *

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Previous evidence suggests that the molecular defect in cystic fibrosis (CF) could reside in an altered chloride conductance of epithelial tissues. Since the brush border of the syncytiotrophoblast of the chorionic villi of human placenta is an abundant source of epithelial membranes and it is unaltered by secondary pathology or treatment we chose to characterize its chloride conductance and to compare it in normal and CF membranes. Chloride transport was studied in microvillar vesicles (MVV) by the quenching of the fluorescent probe 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ). Chloride conductance at 23°C: (a) increased by 39% under a membrane potential change of 70 mV; (b) was inhibited by diphenylamine 2-carboxylate (K_i = 150 μM); (c) displayed an activation energy of 3.5 kcal · mol^{-1}. The comparison of the chloride conductance for an inwardly directed gradient of 150 mM Cl^- at 23°C (membrane potential set at 0 mV) between CF and control membranes was not significantly different. These findings demonstrate the presence of a chloride conductive pathway in microvillar vesicles from human placenta and preliminary results exclude major differences in the conductance of CF derived material in the absence of neurohormonal stimuli.

Introduction

Cystic Fibrosis (CF), the most common lethal genetic disease among Caucasians, seems to be related to defective transport of chloride across epithelia. The original observation of chloride impermeability in the collecting tubule of sweat glands by Quinton [1] was confirmed in respiratory epithelia by Knowles et al. [2]. Further evidences obtained either by electrophysiological [3] and patch-clamp techniques [4,5] put forward the possibility of a defective activation of the chloride conductance in the apical membranes of CF tracheal cells after the stimulation with β-agonists. So far no clear understanding is available on the molecular step involved in this defective activation. Questions arise whether a regulatory protein or an altered protein channel is the target of the molecular defect. In this respect, the major disadvantage of the present approach concerns the lack of material for purification of a protein channel.

Microvillar vesicles (MVV) obtained from the apical membranes of the syncytiotrophoblast of the chorionic villi of the human placenta seem an interesting model since they are (a) very abundant; (b) of epithelial origin; (c) derived from the

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same genetic material as the fetus itself; (d) the membranes specialized in the exchange of all the necessary molecules between mother and fetus [6,7]. A chloride exchange transport in these membranes has been observed by Shennan et al. [8] while a conductive pathway has been only suggested by Illsley and Verkman [9]. The aim of this work was to investigate the presence of a chloride conductive pathway in MVV and verify whether the chloride defect described in CF respiratory epithelia and sweat glands is expressed also in these membranes. Chloride transport has been studied by the quenching of the fluorescent probe 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) which has been recently described as a sensitive and noninvasive method to measure the instantaneous chloride concentration in cells and vesicles [9].

Materials and Methods

Vesicle preparation. Normal human full term placentas were collected in ice within 30 min from the delivery. Placentas from CF offspring were kept in ice and carried to the lab within a maximum of 3 h by a fast delivery system. CF MVV were compared with normal MVV obtained under the same storage conditions. MVV were isolated according to Illsley and Verkman [10] by shearing, magnesium precipitation and sucrose density gradient. At the end of the preparation, MVV were resuspended in 250 mM sucrose, 10 mM Hepes-Tris (pH 7.4) and stored in liquid nitrogen.

Characterization of the vesicles. Membrane purification was tested by measuring marker enzyme activities. All the enzyme assays were performed at 30°C. Alkaline phosphatase was measured according to Bowers and McComb [11] as a marker of the brush border. NADH-cytochrome-*c* reductase was assayed by the method of Sottocasa et al. [12] in the absence and presence of rotenone. The rotenone insensitive fraction was measured for the presence of endoplasmic reticulum while the rotenone sensitive activity was assayed to determine mitochondrial contamination of MVV.

MVV volume was measured according to Bissonette et al. [13] by D-[^{3}H]glucose distribution and filtration with rapid suction through 0.45 μm pore diameter filters (type HAWP 02500, Millipore Corporation, Bedford, MA).

Chloride transport by fluorimetric assay. Cl^- flux was studied by the quenching of the fluorescent probe SPQ according to Illsley and Verkman [9] with 10 mM SPQ in the inside buffer. The inside and outside buffers were: 150 mM K-anion, 100 mM sucrose, 10 mM Hepes-Tris (pH 7.4). For an efflux experiment the inside anion was chloride and the outside was gluconate; the opposite composition was chosen for the influx. The external SPQ was removed by two centrifugation (46 000 × *g*, 10 min, 4°C) and resuspensions of the MVV in ice-cold SPQ free buffer just before each time course. In the experimental conditions reported in the results, the leakage of SPQ was < 5% per hour. Taking into account that the fluxes were calculated from the derivative of the fitted exponential function at the initial time, this leakage can be considered negligible. Membrane potential was set by K-valinomycin added from an ethanolic stock (25 mg/ml) in order to obtain a final concentration of 10 μg/mg membrane protein.

The overnight preincubation of the MVV at 4°C with SPQ does not affect the Cl^- transport systems as demonstrated by Illsley and Verkman [9] and validated in different membrane vesicles (see Ref. 14). The effect of the freezing-thawing procedure on Cl^- fluxes was checked by $^{36}Cl^-$ uptake (see method below). $^{36}Cl^-$ uptake is perfectly equal both in freshly prepared and frozen-thawed membrane vesicles (data not shown). The same result was presented by Shennan et al. [15].

SPQ fluorescence was excited at 350 nm (8 nm band-pass) and measured at 420 nm (4 nm band-pass) in an SLM 8000C spectrofluorometer (Urbana, IL) interfaced with an IBM-XT computer for data acquisition and analysis. Samples were placed in acrylic cuvettes and continuously stirred in a thermostated cuvette holder. Actual temperature was checked with an electronic thermistor. Fluorescent signal was averaged over 2 s time intervals. All solutions used in fluorescence experiments were filtered through 0.22 μm pore diameter Millipore filters in order to minimize light scattering.

Chloride transport by ^{36}Cl uptake. ^{36}Cl transport into MVV was carried out using the ion exchange

column assay of Gasko et al. [16]. The separation of the extravesicular tracer was obtained by eluting the incubation mixture through columns packed with a Dowex 1-X8 (50–100 mesh, formate form) anion exchange resin. The external, anions were rapidly bound to the resin, while MVV were eluted in 2–4 s by rapid suction.

Membrane potential measurement. Membrane potential was measured with the fluorescent probe 3,3'-dipropylthiodicarbocyanine iodide (diS-C_3-(5)) according to Cabrini and Verkman [17]. A calibration curve was obtained by varying the outside to inside K^+ ratio in MVV. Membrane potential was induced by the addition of valinomycin. The calibration points were fitted to a quadratic equation and the experimental data were consequently corrected by computer program in order to give the direct value of the membrane potential as a function of time.

Chloride flux calculation. Data points of the SPQ fluorescent signal were fitted to an exponential function by the non-linear Newton's method. The rate of Cl^- flux was calculated from the derivative of the fitted exponential function at the initial time, where the $[Cl^-]_{in}$ is known. The resulting variation of fluorescence as a function of time $(dF/dt)_{t=0}$ was converted into $(d[Cl^-]/dt)_{t=0}$ expressed as $mM \cdot s^{-1}$ by deconvoluting the Stern-Volmer equation, according to the original method of Illsley and Verkman [9].

Chemicals. SPQ and diS-C_3-(5) were from Molecular Probes (Junction City, OR), diphenylamine 2-carboxylate (DPC) and Dowex 1-X8 from Aldrich (Steinheim, F.R.G.), $H^{36}Cl$ and D-[^{3}H]glucose from Amersham (U.K.), all the other reagents were from Sigma Chemical Co. (St. Louis, MO). Acrylic cuvettes were from Sarstedt (F.R.G.).

Results

MVV characterization

In order to compare the characteristics of the MVV preparation with those of the original method [10], marker enzyme activities and volume determination were studied.

As shown in Table I the MVV used in the experiments represented the alkaline phosphatase-enriched fraction, thus indicating a high purification by the cellular brush border and minimal contamination by endoplasmic reticulum (rotenone insensitive NADH–cytochrome-*c* reductase) and mitochondria (rotenone sensitive NADH–cytochrome-*c* reductase).

Volume determination as a function of an osmotic gradient was carried out by resuspending MVV into solutions containing increasing concentrations of sucrose and measuring the distribution of radioactive glucose at complete equilibrium (60 min), according to Bissonette et al. [13]. The linear relation between the volume and the gradient of osmolarity illustrated in Fig. 1 indicated that these MVV had a close osmotically active space. The internal space calculated in the absence of an osmotic gradient gives the value of 1 μl/mg membrane protein, which is in agreement with Shennan et al. [8].

TABLE I

MARKER ENZYME ACTIVITIES IN HOMOGENATE AND C2 FRACTION

Enzyme activities are mean ± S.D. of three preparations in $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$ except for alkaline phosphatase which is measured in $U \cdot (mg\ protein)^{-1}$. Recovery is expressed as %. C2 fraction indicates the fraction of membrane vesicles collected on top of a 35% (w/v) sucrose density step gradient according to Illsley and Verkman [10] and Truman et al. [18]. The enrichment and the recovery in the CF membranes were, respectively, 24 and 23 for alkaline phosphatase, 0.2 and 0.3 for rotenone-insensitive NADH–cytochrome-*c* reductase, 1 and 0.01 for rotenone-sensitive NADH–cytochrome-*c* reductase.

Enzyme	Homogenate	C2 fraction	Enrichment	Recovery
Alkaline phosphatase	0.71 ± 0.05	14.20 ± 0.50	21	21
Rotenone-insensitive NADH–cytochrome-*c* reductase	22.2 ± 3.9	8.4 ± 3.1	0.4	0.5
Rotenone-sensitive NADH–cytochrome-*c* reductase	2.8 ± 2.1	0.62 ± 0.44	0.2	0.02

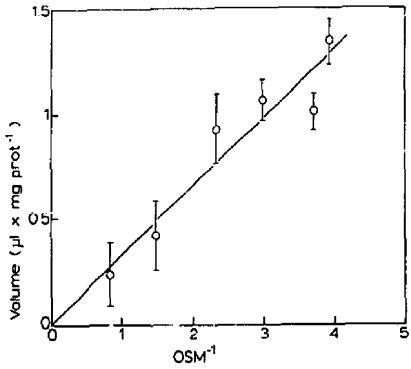

Fig. 1. Volume determination in MVV. 500 µg MVV containing 250 mM sucrose, 10 mM Hepes-Tris (pH 7.4) were incubated for 60 min at 23°C in 10 mM Hepes-Tris (pH 7.4) with sucrose ranging from 0.25 to 1 mol/l and 30 µCi/ml D-[^{3}H]glucose (4.7 µM) in the final volume of 200 µl. At the end of the incubation, triplicate samples of 50 µl were diluted in 2 ml ice-cold solution of 250 mM NaCl, 2 mM phlorizin, 10 mM Hepes-Tris (pH 7.4) and rapidly filtered and washed with two more aliquots of the same solution in a Millipore apparatus. Data are mean ± S.D. of triplicate experiments.

Chloride transport by SPQ method

Fig. 2 shows the representative time course of chloride flux in MVV at 23°C with membrane potential set at 0 mV. The upper trace of Fig. 2A demonstrates the increase of fluorescence intensity due to the efflux of chloride from the MVV and, as a consequence, the reduction of the quenching of the intravesicular SPQ. The first part of the trace immediately after the addition of the vesicles (abrupt increase of fluorescence) is related to the sum of the signal of the intravesicular and the residual extravesicular SPQ. This part and the data points acquired during the initial mixing time were not taken into account for the calculation of the rate flux.

It should be noted that no increase of fluorescence occurred in the absence of a chloride gradient, as demonstrated in the lower trace. Fig. 2B shows the decrease of the fluorescent signal due to chloride influx, indicating the symmetry of transport in this vesicular model. Also in this condition the absence of the chloride gradient (upper trace) did not induce fluorescence changes. The addition of Triton X-100 allows the exposure of the intravesicular SPQ to the external chloride for the influx calculations. To assess the accuracy of the

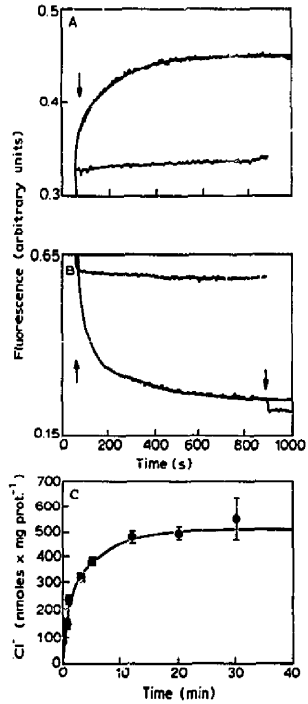

Fig. 2. Time courses of chloride flux at 23°C and with membrane potential set at 0 mV. (A) Efflux by the SPQ method. 250 µg MVV in 50 µl containing 150 mM KCl, 100 mM sucrose, 100 mM Hepes-Tris (pH 7.4) and SPQ were added to 2 ml of 150 mM potassium gluconate, 100 mM sucrose, 10 mM Hepes-Tris (pH 7.4) (upper trace) or 150 mM KCl, 100 mM sucrose, 10 mM Hepes-Tris (pH 7.4). The arrow indicates the addition of MVV. The smooth curve inside the upper trace is the fitted exponential function. (B) Influx by the SPQ method. 250 µg MVV (50 µl) containing 150 mM potassium gluconate, 100 mM sucrose, 10 mM Hepes-Tris (pH 7.4) and SPQ were added to 2 ml of 150 mM KCl, 100 mM sucrose, 10 mM Hepes-Tris (pH 7.4) (lower trace) or to 150 mM potassium gluconate, 100 mM sucrose, 10 mM Hepes-Tris (pH 7.4) (upper trace). The arrows indicate the addition of MVV (left arrow) and of Triton X-100 (0.01% final concentration) (right arrow). (C) Influx by ^{36}Cl uptake. MVV containing 150 mM potassium gluconate, 10 mM Hepes-Tris (pH 7.4) were added to 150 mM KCl, 10 mM H^{36}Cl (0.62 Ci/mol), 10 mM Hepes-Tris (pH 7.4). At the indicated times, 200 µl of this incubation mixture (150 µg membrane protein) were transferred to a Dowex column (0.7×15 cm, 5 ml packed resin) equilibrated with 300 mM sucrose, 10 mM Hepes-Tris (pH 7.4) and immediately eluted with 4 ml of the same buffer. Each point is the mean ± S.D. The curve represents the fitted exponential function.

SPQ measurements, the influx of ^{36}Cl was assayed under similar experimental conditions as shown in Fig. 2C. The exponential function fitted to the experimental data of three different preparations gave similar time constants (τ) (182 ± 44 s (mean ± S.D.)) as for the influx experiments (199 ± 31 s (mean ± S.D.) performed by the SPQ method. These values are also comparable to those calculated in the efflux experiments (203 ± 27 s (mean ± S.D.)) carried out with the fluorescent probe.

Chloride conductance

Chloride transport in biological membranes is mainly attributable either to an electroneutral anion exchanger or an electroneutral $Na^+/K^+/2Cl^-$ cotransporter or an electrogenic conductive pathway [19]. In order to ascertain whether the observed fluxes were mediated by a conductive pathway, their voltage dependence was verified. Membrane potential was set by varying the external K^+ concentration and adding the K^+ ionophore valinomycin. The increase of the Cl^- efflux in the presence of more negative potentials shown in Table II is strongly consistent with an electrogenic conductive pathway.

An other piece of evidence for a conductive pathway is the variation of the flux after the addition of specific inhibitors. Diphenylamine 2-carboxylate (DPC) has been previously reported as an inhibitor of chloride conductance [20]. Chloride efflux was measured in the presence of increasing concentrations of DPC (Fig. 3); under our experimental conditions the inhibitory constant was 150 μM as calculated by fitting the reported data to a single-site binding model. The possibility of the presence of a leak permeability in parallel with a channel mediated permeability was excluded by the evidence that 500 μM DPC totally inhibits Cl^- flux (data not shown). 100 μM furosemide did not inhibit chloride flux (data not shown), thus weakening the possibility of a transport mediated by a $Na^+/K^+/2Cl^-$ symport. Since the anion exchanger inhibitor dihydro-4,4′-diisothiocyano-2,2′-disulfonic stilbene (H_2DIDS) interferes with the fluorescent signal of the SPQ, 100 μM H_2DIDS was preincubated at 37°C for 60 min with the MVV and the unbound inhibitor in

TABLE II

VOLTAGE DEPENDENCE ON Cl^- EFFLUX IN MVV

Voltage (mV)	J_{Cl^-} (mM·s^{-1}) (Mean (S.D.), $n = 3$)	% activation
0	0.138 (0.05)	0
−35	0.144 (0.05)	4
−70	0.192 (0.04)	39

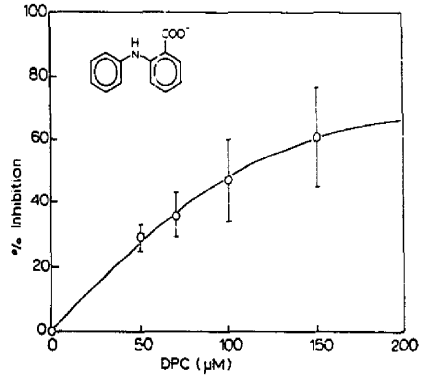

Fig. 3. DPC inhibition of chloride flux. Chloride efflux was measured as described for Fig. 2A in the presence of increasing concentrations of 10 μl DPC dissolved in dimethyl sulfoxide. Data are plotted as percent inhibition of the flux and fitted to a single site binding equation which gave a K_i of 150 μM. The drawing represents the molecular structure of DPC. Data are mean ± S.D. of triplicate assays.

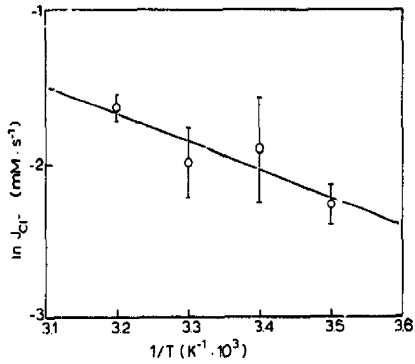

Fig. 4. Arrhenius plot of chloride flux. Efflux experiments as described in Fig. 2A were performed over a range of temperatures with membrane potential set at 0 mV. Data were fitted to a straight line and represent the mean ± S.D. of triplicate assays.

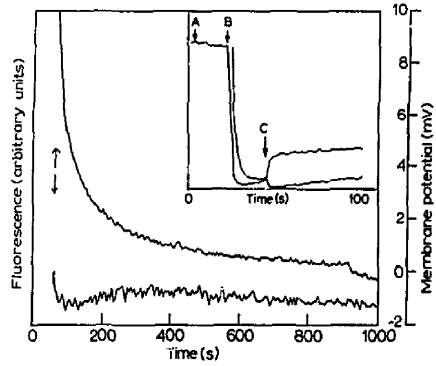

Fig. 5. Membrane potential during chloride flux experiments. The upper trace reprents an influx experiment in the conditions described in Fig. 2B, where the fluorescent signal is due to the SPQ. The lower trace represents the membrane potential calculated by a computer program from the calibration curve of the fluorescent variation of diS-C_3-(5) versus the membrane potential [17]. The arrow indicates the time of vesicle addition. Inset: Representative fluorescence response of dis-C_3-(5) to a membrane potential induced by valinomycin. Upper trace: MVV containing 5 mM KCl were added to 3 μM diS-C_3-(5) in 150 mM KCl. Lower trace: 150 mM KCl inside, 5 mM KCl outside. (A) represents the fluorescent signal of 3 μM diS-C_3-(5), (B) the time of the addition of the membrane vesicles, (C) the time of addition of valinomycin.

excess was removed by two washes and centrifugations (46000 × g, 10 min, 4°C). In these conditions the average J_{Cl^-} was similar in the presence and absence of the inhibitor (0.302 versus 0.269 $mM \cdot s^{-1}$).

Further evidence of a conductive pathway was provided by calculating the activation energy of the flux. Chloride efflux was studied at different temperatures. The activation energy calculated from the slope of the straight line fitted to the data of the Arrhenius plot shown in Fig. 4 gives the value of 3.5 $kcal \cdot mol^{-1}$.

Membrane potential in the flux experiments

The adequacy of the membrane potential clamping by K^+/valinomycin was checked during a typical chloride influx experiment. The fluorescent probe diS-C_3-(5) monitored the membrane potential changes in MVV in real time in the presence of an inwardly directed gradient of 150 mM Cl^- and of 150 mM K^+ on both sides of the membrane. Minor changes are apparent (±2 mV) in the data presented in Fig. 5.

TABLE III

Cl CONDUCTANCE IN CF MVV: INFLUX

Data are mean of triplicate assays.

	J_{Cl^-} ($mM \cdot s^{-1}$)	
	Control	CF
Prepn. 1	0.231	0.205
Prepn. 2	0.179	0.080
Prepn. 3	0.200	0.223
Mean(S.D.)	0.203(0.02)	0.169(0.06)

Comparison between normal and CF membranes

After the characterization of the chloride flux in normal membranes, the possibility of a defective chloride conductance in the MVV derived from CF offspring was investigated. Chloride influx in CF vesicles was compared with daily control material obtained under the same storage conditions. Table III shows that the CF membranes, compared with the daily control, did not have a consistently decreased value of chloride conductance for a flux of 150 mM Cl^- gradient at 23°C with a membrane potential set at 0 mV.

Discussion

Converging data on chloride impermeability in apical membranes of CF sweat glands and respiratory epithelia [1,4,5] led us to look for an apical membrane from an epithelial tissue that would give abundant starting material in order to at-

tempt the purification of the defective protein. The brush border of the syncytiotrophoblast of the human placenta could fit this expectation. Unfortunately, the stem cells (Langhans cells) that generate the overlaying syncytiotrophoblast are almost absent in term placenta [6], thus making unsuccessful the attempt of the in vitro cultivation [21,22] in order to study the chloride conductance by the patch-clamp technique. A consequent choice is to isolate the apical membranes and use purified vesicles for the characterization of the chloride conductance.

The MVV preparations of the present work displayed biochemical properties comparable to those of the original method [10] as indicated by the marker enzyme activities. The determination of the glucose space measured a volume similar to the previously reported one [8] and demonstrated that these vesicles are sealed and have an osmotically active space.

The present work confirms that the recently described technique of the quenching of the fluorescent probe SPQ for the study of chloride transport [9] is reliable and rapid. The experiments of ^{36}Cl influx described in Fig. 2C validate the SPQ method, as suggested by the similar exponential time constants (Fig. 2B) in the presence of the same Cl^- gradient.

Converging pieces of evidence were collected in order to demonstrate the presence of an inhibitable, voltage sensitive chloride channel in the MVV. Cl^- efflux increases in the presence of more negative membrane potentials (Table II). The presence of a non-linear dependence of the flux by membrane potential changes could be explained by the Goldman-Hodgin-Katz flux equation showing that when the ion concentrations inside and outside the membrane are different, the relation between the flux and the variation of the membrane potential is curvilinear [23].

The analysis of the effect of the inhibitors is complex, due to the non-complete specificity of DPC for the Cl^- conductive channel [24]. The calculated value of the inhibitory constant of 150 μM (similarly to that described in renal medulla [20]) and the complete inhibition of the chloride flux in the presence of 500 μM DPC makes unsuitable the contribution of a non-protein mediated 'leaky' permeability. An anion exchange transporter has been already described in these vesicles [8]. In our experimental conditions a Cl^- flux through the described anion exchanger can be reasonably ruled out either because the only anion on the other side of the membrane was gluconate, which is almost impermeable for the span of these time courses, and because of the lack of inhibition by H_2-DIDS. Since a Cl^- modulated proton flux has been described in these membranes [25], these assays were performed in the absence of proton gradients, thus excluding both the possibility of a Cl^-/OH^- exchange pathway and a proton driven Cl^- flux.

The absence of inhibition by furosemide weakens the alternative possibility of a KCl cotransport [26]; moreover, this cotransporter is usually thought to be located in the basolateral membrane of the epithelial cells [27]. It should also be noted that the activation energy value here calculated is lower than that generally described for a carrier mediated transport (3.5 vs. 15–20 $kcal \cdot mol^{-1}$) and in agreement with the figure expected for a channel mediated conductance [28].

So far, the physiological importance of a Cl^- conductive pathway in the brush border of the syncytiotrophoblast of the chorionic villi is not known. The presence of a Na^+/H^+ [29,30] and of a Cl^-/HCO_3^- antiport (Illsley and Verkman, unpublished data) in these MVV could be important for cell pH and volume regulation and implies a NaCl entry through parallel pathways. It could be speculated that the dissipation of the intracellular Na^+ might take place through a Na^+/K^+-ATPase while the intracellular Cl^- might exit through the conductive pathway which has been described in the present work and previously suggested by Illsley and Verkman [9].

In our experimental model, the influx of Cl^- through a conductive pathway is likely to be coupled to a K^+ entry, the latter being mediated by valinomycin, thus opening the possibility of a modification of the membrane potential. However, the experiment reported in Fig. 5 demonstrates the absence of any significant modifications of the membrane potential in the usual experimental conditions of a Cl^- influx.

A defective chloride transport in MVV derived from CF offspring has been reported by Davis et al. [31]. On the contrary, our preliminary data

(Table III) demonstrate for the first time that in the absence of neurohormonal stimuli the average chloride conductance of MVV from CF and control offspring is superimposable. It should be noted that also the apical membranes excised from CF tracheal cells with the patch-clamp technique display the same conductive properties as the control material, while in whole cell recording a defective β-adrenergic stimulation of this conductance has been reported in the CF tracheal cells [4,5]. As a consequence, before excluding that the CF basic defect is expressed in MVV, the stimulation of a transmembrane signal modulating a chloride conductance must be explored. It is important to remember that in studies on membrane vesicles cytoplasmic factors might be absent or much reduced since they could be lost during vesicle preparation (e.g., the ATP concentration in these MVV is zero). Our preliminary experiments demonstrate the presence of an endogenous protein kinase activity in MVV and the attempt of rebuilding the signal transduction machinery in these membranes is in progress in our lab.

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