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cAMP-Dependent Protein Kinase Inhibits the Chloride Conductance in Apical Membrane Vesicles of Human Placenta

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Summary. The role of adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase (PK-A) on the Cl^- conductance has been studied in the apical membrane vesicles purified from the chorionic villi of human placenta. In order to phosphorylate the cytosolic side of the membranes, vesicles have been hypotonically lysed, loaded with 100 nM catalytic subunit of PK-A purified from human placenta and 1 mM of the phosphatase resistant adenosine 5′-thiotriphosphate (ATP-gamma-S) and resealed. Cl^- conductance has been measured by the quenching of the fluorescent probe 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) at 23°C with membrane potential clamped at 0 mV. The actual volume of the resealed vesicles was measured in each experiment by trapping an impermeable radioactive molecule ([^{14}C]-sucrose) and included in each Cl^- flux calculation. In 19 independent experiments, the mean Cl^- conductance in placental membranes in the absence of phosphorylation was 3.67 ± 3.18 whereas with the addition of PK-A and ATP-gamma-S it was 1.97 ± 1.75 nmol · sec^{-1} · (mg protein)$^{-1}$ (mean ± SD). PK-A dependent phosphorylation reduced the Cl^- conductance in 14/19 experiments. The same protocol applied to the apical membranes of bovine trachea, where PK-A is known to activate the Cl^- channels, confirmed that the PK-A dependent phosphorylation increased the Cl^- conductance in 11/13 experiments, from 1.01 ± 0.61 to 1.85 ± 0.99 nmol · sec^{-1} · (mg protein)$^{-1}$(mean ± SD). These studies indicate that the PK-A dependent phosphorylation inhibits one or more Cl^- channel(s) of the apical membranes of human placenta.

Key Words chloride conductance · human placenta · bovine trachea · 6-methoxy-N-(3-sulfopropyl)quinolinium · cAMP-dependent protein kinase · ATP-gamma-S

Introduction

Considerable interest has been recently focused on the regulation of Cl^- conductance in apical membranes of epithelial cells. These investigations are important also in relation to the most frequent lethal genetic disease among Caucasians, cystic fibrosis, where the molecular defect seems to be a defective regulation of the Cl^-conductance [34]. Studies performed in epithelial cells from several organs indicate that the Cl^- conductance can be regulated by adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase (PK-A) and by protein kinase C [11, 12, 18, 25]. A Cl^- conductive pathway has been demonstrated also in the apical membranes of the chorionic villi of human term placenta (syncytiotrophoblast) [6, 19], but its regulatory mechanisms are still obscure. The principal models that can be utilized for these studies are the cells originating the syncytiotrophoblast, namely the cytotrophoblast, or the microvillar vesicles (MVV) purified from the syncytiotrophoblast itself. However, optimized culture conditions for the cultivation of the cytotrophoblast from term placenta have not yet been developed [26]. On the other side, the membrane vesicles may lose regulatory components (protein kinases, cyclic nucleotides) during the purification steps.

Recent investigations indicate the possibility of loading protein kinases and cyclic nucleotides in membrane vesicles to regulate the function of membrane transporters or channels [8, 27, 32, 33]. The aim of this work was to investigate the effect of PK-A on the Cl^- conductance of the MVV by adding the catalytic subunit of PK-A and adenosine 5′-thiotriphosphate (ATP-gamma-S) to osmotically lysed and resealed vesicles. Cl^- conductance was studied by adding to the opened vesicles the Cl^--sensitive fluorescent probe 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) together with an impermeable radioactive molecule ([^{14}C]-sucrose) in order to measure the actual volume of the resealed vesicles in parallel. In order to validate the whole experimental protocol, the effect of PK-A on Cl^- conductance was tested in bovine tracheal apical membranes where PK-A is known to stimulate the Cl^- conductance [31]. The results indicate that Cl^-conductance in the placental membranes is inhibited by PK-A dependent phosphorylation.

Materials and Methods

Materials

ATP-gamma-[^{35}S] and [^{14}C] methylated proteins as molecular weight standards, Hyperfilm-MP were from Amersham (Buckinghamshire, UK); [^{14}C]-sucrose, En^{3}Hance from Du Pont de Nemours (Dreieich, FRG); SPQ from Molecular Probes (Eugene, OR), ATP-gamma-S from Serva (Heidelberg, FRG); acrylamide, bisacrylamide, N, N, N', N', tetramethyl-ethylendiamine, ammonium persulfate from LKB (Bromma, Sweden); Sephadex G-25M from Pharmacia (Uppsala, Sweden); DPC from Fluka (Buchs, Switzerland); dithiothreitol, catalytic subunit of PK-A from bovine heart, PK-A inhibitor from bovine heart, from Sigma (St. Louis, MO), all the other reageants from Merck (Darmstadt, FRG).

Preparation of Human Placental Vesicles

Brush border from the syncytiotrophoblast of the chorionic villi of human placenta has been purified according to Illsley and Verkman [20] as previously reported [6] by shearing, magnesium precipitation and sucrose density gradient. Vesicles were resuspended in 200 mM mannitol, 10 mM $MgSO_4$, 100 mM HEPES, 25 mM Tris (pH 7.1), and processed immediately or stored frozen in liquid nitrogen.

Preparation of Bovine Tracheal Vesicles

Vesicles from the apical membranes of bovine trachea were prepared according to Langridge-Smith et al. [24].

Briefly, bovine tracheas collected from the local slaughterhouse immediately after the killing of the animals were placed in ice-cold Ringer's solution. Strips of mucosa were scraped with glass microscope slides. The scraped material was homogenized and the cytoplasmic membranes were enriched by differential centrifugations. Apical membrane vesicles were obtained after magnesium precipitation and resuspended in 200 mM mannitol, 10 mM $MgSO_4$, 100 mM HEPES, 25 mM Tris (pH 7.1) and processed immediately after purification or stored frozen in liquid nitrogen. Alkaline phosphatase has been chosen as a marker of apical membrane purification. The enrichment values in the basolateral and apical membranes in comparison to the homogenate were 0.51 ± 0.07 and 17.17 ± 3.79, respectively (mean $\pm$ SD of three preparations), whereas the percentage yield value for the apical membrane was 1.06 ± 0.26.

Opening, Phosphorylation and Resealing of the Membrane Vesicles

Bovine tracheal or human placental vesicles were opened and resealed by hypotonic lysis by a partial modification of the method of Weinman et al. [33] in order to phosphorylate membrane proteins from the cytosolic side with PK-A.

Lysis was obtained by adding membrane vesicles (40 μl, 4 mg membrane protein/ml) to 160 μl of the lysis buffer: 10 mM $MgSO_4$, 3.75 mM dithiothreitol (DTT), 12 mM HEPES, 6 mM Tris (pH 6.8) containing 1.25 mM ATP-gamma-S, the catalytic subunit of PK-A from bovine heart (50 nM) or human placenta (125 nM). The final concentration in the incubation mixture (corresponding to the solution of the external side of the membrane vesicles) was: 40 mM mannitol, 10 mM $MgSO_4$, 9.8 mM Tris, 29.6 mM HEPES, 3 mM DTT, with or without 1 mM ATP-gamma-S and 40–100 nM catalytic subunit of PK-A at the final pH of 6.9.

Lysis buffer was pre-equilibrated at room temperature for 10 min before the addition of the membrane vesicles. Membrane lysis and phosphorylation were performed by incubation at 30°C for 15 min. In control experiments (lysis and resealing without phosphorylation) ATP-gamma-S and the catalytic subunit of the PK-A were omitted. In the experiments of endogenous phosphorylation ATP-gamma-S was present in the incubation mixture.

Resealing of the membrane vesicles was obtained at 20–25°C for 45 min by the addition of 50 μl of resealing solution (840 mM mannitol, 250 mM K-gluconate) in order to obtain the following solution inside the resealed vesicles: 200 mM mannitol, 8 mM $MgSO_4$, 50 mM K-gluconate, 23.7 mM HEPES, 7.8 mM Tris, ($\pm$0.8 mM ATP-gamma-S, $\pm$32–80 nM catalytic subunit of PK-A) at pH 7.0.

The opened and resealed vesicles were finally processed according to the proper protocols (control of opening and resealing, chloride flux measurement, autoradiography of phosphorylated membrane proteins).

Control of Resealing Procedure and Active Volume Measurement

The efficacy of the opening and resealing procedure and volume measurement in parallel with the protein phosphorylation and chloride flux was performed by entrapping [^{14}C]-sucrose, which is considered an impermeable molecule in the time span of these experiments.

[^{14}C]-sucrose (2.7 μM, 671 mCi/mmol specific activity) was added in the lysis buffer. At the end of the resealing procedure the extravesicular [^{14}C]-sucrose was removed by gel filtration with Sephadex G-25M. 250 μl vesicles suspension was placed on top of columns (13×0.7 cm) pre-equilibrated and eluted with 200 mM mannitol, 8 mM $MgSO_4$, 50 mM KCl, 3.8 mM HEPES, 1.3 mM Tris, pH 7.1, at room temperature. Eluted fractions containing proteins were counted in a Packard Tri-carb 4000 (Downers Grove, IL) scintillation counter. Nonspecific binding of [^{14}C]-sucrose to the membranes was measured by incubating vesicles in an isotonic buffer at the same temperature and for the same time as for the osmotically lysed vesicles. These vesicles were finally subjected to gel filtration as described above. The [^{14}C]-sucrose associated to the intact vesicles was assumed as unspecific binding to the membranes and subtracted.

Intravesicular volume of intact vesicles was determined by long term (18–24 hr) incubation at 4°C in isotonic buffer containing [^{14}C]-sucrose, which is supposed to equilibrate in the internal volume in these conditions.

Volume of bovine tracheal vesicles was 2.18 ± 0.48 μl $\cdot$ (mg protein)$^{-1}$ (mean $\pm$ SEM), which is very similar to the reported one of 2.14 ± 0.13 [11], and volume of human placental vesicles was 1.40 ± 0.13 μl $\cdot$ (mg protein)$^{-1}$, which is comparable to the previously reported value of 1.0 μl $\cdot$ (mg protein)$^{-1}$ [6].

Chloride Flux by Fluorimetric Assay

Cl^- flux was studied by the quenching of the fluorescent probe SPQ according to Illsley and Verkman [21] as previously reported [6]. A representative time course is shown in Fig. 1.

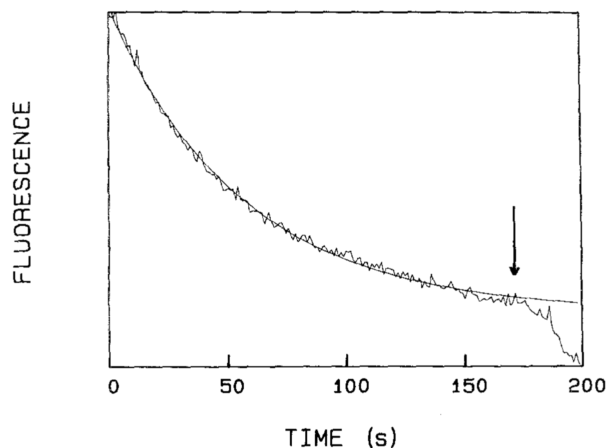

Fig. 1. Representative time course of Cl^- influx by the quenching of the fluorescent probe SPQ in placental vesicles. The smoothed line through the trace is the fitted single exponential function. The arrow indicates the time of addition of Triton X-100 (0.01% final concentration)

Loading of 10 mM (final concentration) SPQ inside the membrane vesicles was performed by addition of the probe in the resealing buffer. The external SPQ was removed at the end of the resealing procedure by two centrifugations (46,000 × *g* for 10 min at 4°C) and resuspensions of the vesicles in approximately 75 volumes of ice-cold SPQ-free buffer (200 mM mannitol, 8 mM $MgSO_4$, 50 mM K-gluconate, 3.8 mM HEPES, 1.3 mM Tris at pH 7.1).

The final pellet, approximately 300 μg membrane protein, was resuspended and added in the acrylic cuvette containing 2 ml of the same buffer with the exception that K-gluconate was replaced with KCl, in order to obtain a 50 mM inwardly directed chloride gradient. Membrane vesicles were continuously stirred and thermostated at 23°C. All solutions were filtered through Millipore filters with 0.22 μm pore diameter in order to minimize light scattering. Membrane potential was set by K-valinomycin (25 mg/ml) from an ethanolic stock in order to obtain a final concentration of 25 μg/mg membrane protein.

SPQ fluorescence was excited at 350 nm (8 nm band pass) and measured with a 420 nm high pass filter in an SLM 8000C spectrofluorometer (Urbana, IL) interfaced with an IBM-XT computer for data acquisition and analysis. Cl^- flux was calculated according to Illsley and Verkman [21] as described with the Methods.

Autoradiography of Phosphorylated Proteins

Membrane vesicles were opened and resealed as previously described and phosphorylated in the presence of adenosine-5′-(gamma-[^{35}S]-thio)-triphosphate (ATP-gamma-[^{35}S]).

At the end of the resealing procedure membrane vesicles were solubilized by the addition of a fivefold concentrated sample containing 62.5 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol (vol/vol) (pH 6.8) (final concentration) and boiled for 5 min. 50 μg membrane proteins were loaded on 10% polyacrylamide gels and electrophoresis was performed for 4 hr at 40 mA/gel according to Laemmli [22].

Fluorography was performed by equilibrating the gels with En^{3}Hance after protein fixing. Dried gels were exposed to Hyperfilm-MP X-ray films using cassettes containing tungsten phosphate intensifier screens for 2–7 days at −70°C.

The autoradiograms were scanned with a 2202 Ultroscan-laser densitometer (LKB, Bromma, Sweden).

ATP-gamma-[^{35}S] in the phosphorylation mixture was 5 μCi/lane (sp act 600 Ci/mmol).

The following [^{14}C]-methylated proteins were run as molecular weight standards: myosin (200 kDa), phosphorylase B (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), lysozyme (14.3 kDa).

Chloride Flux Calculations

Data points were fitted to single exponential functions by a computer program. The rate of Cl^- flux was calculated from the derivative of the fitted exponential at the initial time, where the $[Cl^-]$ inside is known. A two-point calibration curve of SPQ fluorescence *vs.* chloride concentration was obtained by exposing the intravesicular SPQ to the external Cl^- after adding 0.01% Triton X-100 (final concentration) at the end of the time course. The final fluorescence value was collected after complete vesicle lysis and standardized 5 min after the end of time course. The variation of fluorescence as a function of time $(dF/dt)_{t=0}$ was converted into $(d[Cl^-]/dt)_{t=0}$ by deconvoluting the Stern-Volmer equation according to Illsley and Verkman [21]. In our experimental conditions, chloride quenched the internal SPQ with Stern-Volmer constant of 85 M^{-1} (which is the result of the multiple collisional interactions of SPQ with either chloride and gluconate). The actual intravesicular volume (V_a), which is variable from experiment to experiment depending on the efficacy of the lysis and resealing procedure, has been measured by [^{14}C]-sucrose trapping, as described in the Method section, and expressed in μl/mg protein as $V_a = ([S]_{in}/M)/[S]_b$, where $[S]_{in}$ is the [^{14}C]-sucrose trapped inside the opened and resealed vesicles (in pmol), $[S]_b$ is the [^{14}C]-sucrose concentration in the lysis and resealing buffer (in pmol/μl) and M is the amount of membrane protein. The percentage of opened and resealed vesicles (R) has been calculated from V_a of each experiment and the average volume of intact vesicles (V_i) (1.40 μl/mg placental membrane protein and 2.14 μl/mg tracheal membrane protein) as $R = (V_a/V_i) \cdot 100$. Flux rates (J_{Cl}) in nmol · sec^{-1} · (mg protein)$^{-1}$ of the opened and resealed vesicles were calculated by multiplying $(d[Cl^-]/dt)_{t=0}$ by V_a. Membrane protein was measured at the end of the time course in each cuvette.

Miscellaneous Methods

Catalytic subunit of PK-A from human placenta was purifid by DEAE cellulose anion exchange chromatography, cAMP elution and hydroxylapatite adsorption chromatography according to Sudgen et al. [29]. Purification of a single 40 kDa mol wt protein was checked by SDS polycrylamide electrophoresis. Specific activity was 3.64 × 10^5 pmol phosphate transferred on histone IIA · min^{-1} · (mg protein)$^{-1}$ at 30°C (pH 6.8).

Alkaline phosphatase was determined by the method of Bowers and McComb [1].

Proteins were determined by the method of Bradford [2].

Results

Characteristics of the Lysed and Resealed Vesicles

The average Cl$^-$ initial rate of the resealed placental vesicles as $(d[Cl^-]/dt)_{t=0}$ in mM · sec^{-1} at 0 mV membrane potential was 0.104 (SD = 0.002), which is very similar to that previously reported in intact vesicles (mean = 0.138, SD = 0.050) [6]. 100 μM diphenylamine-2-carboxylate (DPC), a Cl$^-$ conductance inhibitor, reduced the flux of the resealed vesicles to 0.054 ± 0.014 (mean ± SD). At this concentration we observed the same percentage of inhibition (about 50%) in intact vesicles [6]. These similarities between intact and resealed vesicles seem to exclude that the procedure used caused major undesirable artifacts (pore formation, Cl$^-$ channels modifications).

Since different lysis and resealing protocols might have variable efficiency, we calculated the percentage of opened and resealed vesicles by measuring the entrapping of [^{14}C]-sucrose (*see* Method section for details). In several independent experiments, we calculated that 46 ± 29 (mean ± SD) of human placental (n = 19) and 76 ± 20 in bovine tracheal membranes (n = 13), respectively, actually resealed. Due to the high variability of the resealing protocol in different experiments, the percentage of lysis and resealing was measured in parallel in all the flux experiments and included in the final flux calculations as described in the Method section.

PK-A Dependent Phosphorylation of Human Placental Membranes

The possibility that a PK-A could be still associated to the MVV, even at the end of the purification step, was tested by opening the vesicles and adding ATP-gamma-[^{35}S] in the absence and in the presence of 10 mM cAMP as shown in Fig. 2. ATP-gamma-[^{35}S] was chosen instead of [gamma-^{32}P]ATP since placental vesicles contain a very high phosphatase activity that cannot be inhibited by usual inhibitors [10]. Preliminary experiments (*data not shown*) confirmed that placental membranes cannot be effectively phosphorylated with [gamma-^{32}P]ATP because of the high dephosphorylation rate, although the use of ATP-gamma-[^{35}S] eliminates this inconvenience (*data not shown*) since thiophosphate is resistant to phosphatase action [14]. As shown in Fig. 2*A*, the addition of ATP-gamma-[^{35}S] to lysed and resealed vesicles caused the phosphorylation of different proteins. The pattern of phosphorylation did not change by adding 10 μM cAMP (Fig. 2*B*), thus

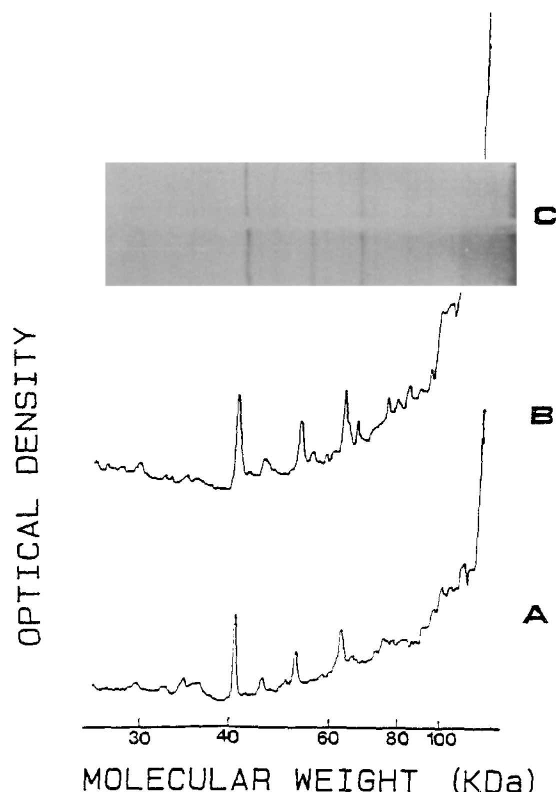

Fig. 2. Effect of cAMP on placental membrane phosphorylation. Laser densitometry tracings of autoradiograms of SDS-polycrylamide gel electrophoresis. Osmotically lysed vesicles (50 μg/lane) were phosphorylated with ATP-gamma-[^{35}S] (5 μCi/lane) and then subjected to SDS-polyacrylamide gel electrophoresis, fluorography, autoradiography and densitometry. (*A*) Basal phosphorylation (membranes + ATP-gamma-[^{35}S]). (*B*) Same as *A* with the addition of 10 μM cAMP. (*C*) Autoradiography of basal phosphorylation (lower lane) and after the addition of 10 μM cAMP (upper lane)

indicating that membrane vesicles have endogenous kinase activities but not a cAMP-dependent protein kinase.

As shown in Fig. 3*A*, the addition of the catalytic subunit of PK-A together with ATP-gamma-S caused the phosphorylation of six major bands with apparent mol wt of 143, 66, 51, 45, 40, and 26 kDa. The dependence of the phosphorylation of these bands by PK-A was demonstrated by the evidence that the selective PK-A inhibitor described by Walsh [5] prevented protein phosphorylation (Fig. 3*B*). A quantitative evaluation of the PK-A dependent phosphorylation is reported in Table 1.

Effect of PK-A on Cl$^-$ Conductance in Human Placental Membranes

Membrane vesicles were phosphorylated by adding the catalytic subunit (C′) of PK-A and ATP-gamma-S in the lysis buffer. Cl$^-$ conductance values in

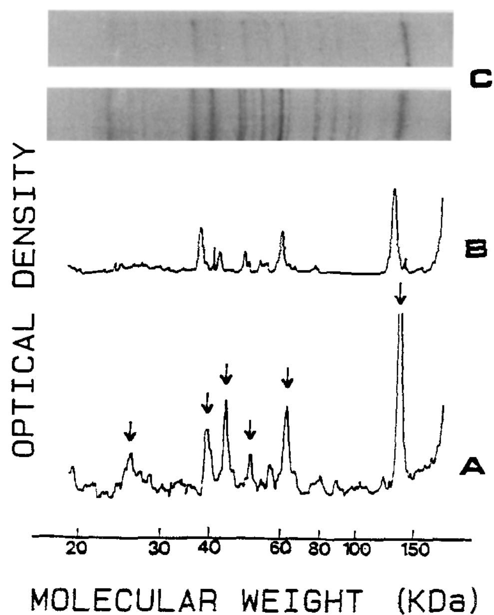

Fig. 3. Effect of the catalytic subunit of PK-A on placental membrane phosphorylation. Laser densitometry tracings of autoradiograms of SDS-polyacrylamide gel electrophoresis phosphorylation has been performed in the same conditions (of buffers, time and temperature) described for Cl^- flux measurement. (*A*) PK-A dependent phosphorylation (membranes + ATP-gamma-[^{35}S] + 100 nM catalytic subunit of PK-A). (*B*) Same as *A* in the presence of the inhibitor of Walsh, 167 μg/ml. (*C*) Autoradiography of PK-A dependent phosphorylation (lower lane) and with the addition of the inhibitor of Walsh (upper lane)

Table 1. Effect of Walsh inhibitor on PK-A dependent phosphorylation of placental membranes

Mol wt (kDa)	Relative densitometric intensity		Residual absorbance (%)
	PK-A	PK-A + Walsh inh.	
26	0.17	0.03	18
40	0.30	0.19	63
45	0.44	0.08	19
51	0.17	0.08	48
66	0.41	0.17	43
143	1.00	0.35	35

Three separate experiments have been normalized, being the band with highest optical density equaled to the value 1.00. The OD of the other bands was consequently transformed. Here reported are the average values of these experiments.

opened and resealed vesicles without the addition of C′ and ATP-gamma-S (control) and after the phosphorylation by the C′ purified from human placenta and ATP-gamma-S are shown in Table 2. It is apparent that PK-A reduced the Cl^- conductance in these membranes. In order to have a clear picture of this

Table 2. Effect of PK-A dependent phosphorylation on Cl^- conductance in human placental membranes

	Chloride conductance	
	nmol · sec^{-1} · (mg protein)$^{-1}$	mM · sec^{-1}
Control	3.67 ± 3.18	2.61 ± 2.33
PK-A, ATP-gamma-S	1.97 ± 1.75	1.35 ± 1.26

Values are mean ± SD of 19 separate paired experiments.

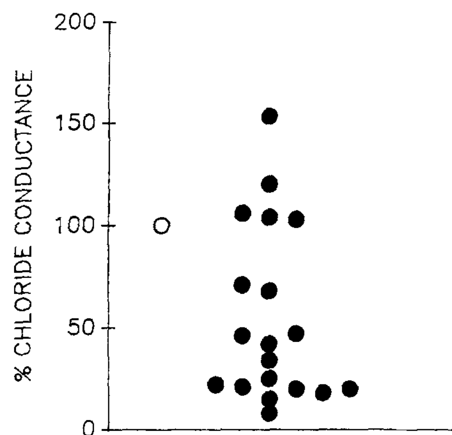

Fig. 4. Effect of PK-A on Cl^- conductance in human placental membranes. Cl^- conductance was measured in 19 pairs of experiments in five different days at 0 mV membrane potential and 23°C by the fluorescent probe SPQ. Vesicles (160 μg membrane protein) were lysed and resealed in the absence (open circle) or presence of 100 nM catalytic subunit of PK-A purified from human placenta and 1 mM ATP-gamma-S (filled circles). In both control and PK-A phosphorylated vesicles half of the membranes were utilized for flux measurement by SPQ and the other half for volume determination by [^{14}C]-sucrose. Data in the presence of PK-A and ATP-gamma-S are reported as percentage of the average Cl^-conductance of the controls of each day

effect, the single experimental data expressed as percentage variation of conductance in comparison to the mean conductance value of the controls in the same experimental day are represented in Fig. 4. It is apparent that Cl^- conductance was reduced in the large majority of the cases (14/19) (minimum = 8%, maximum = 71%, control value = 100%), unchanged in 3/19 and increased in 2/19 experiments. Part of these experiments were performed in freshly isolated membranes, in order to exclude that the PK-A dependent regulation could be different in freshly prepared *vs.*vesicles that were kept frozen in liquid nitrogen. No different behavior was observed in these two kinds of preparations (*data not shown*), suggesting that the substrate for the PK-A dependent phosphorylation was not affected by rapid freezing.

Table 3. Effect of PK-A dependent phosphorylation on Cl^- conductance in bovine tracheal membranes

	Chloride conductance	
	$nmol \cdot sec^{-1} \cdot (mg\ protein)^{-1}$	$mM \cdot sec^{-1}$
Control	1.01 ± 0.61	0.46 ± 0.28
PK-A, ATP-gamma-S	1.85 ± 0.99	0.85 ± 0.45

Values are mean ± SD of 13 separate paired experiments.

Effect of PK-A on Cl^- Conductance in Bovine Tracheal Membranes

In order to ascertain whether the whole experimental design was appropriate for the study of the effect of PK-A on Cl^- conductance, vesicles from tracheal apical membranes were processed as a control model.[1] PK-A is expected to increase the Cl^- conductance in these membranes as deduced from electrophysiological approahces [31]. Table 3 shows the average Cl^-conductance in control conditions and after the addition of C′ from bovine heart and ATP-gamma-S. The average Cl^- conductance was almost doubled after the PK-A dependent phosphorylation. Single experimental data are presented in Fig. 5. Cl^- conductance was markedly increased in 11/13 experiments (minimum = 119%, maximum = 497%, control value = 100%), confirming that the data obtained with the present protocol are consistent with the results of electrophysiological studies [31]. Also for tracheal membranes experiments were performed either in freshly prepared and frozen vesicles, but no significant differences were found between the two conditions (*data not shown*).

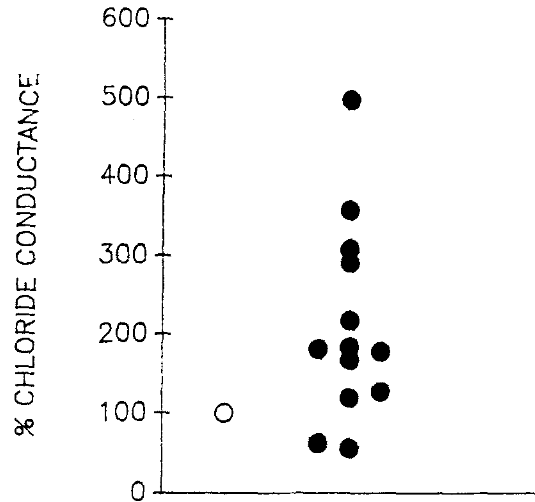

Fig. 5. Effect of PK-A on Cl^- conductance in bovine tracheal membranes. Cl^-conductance was measured in 13 pairs of experiments in four different days in the absence (open circle) or presence of 40 nM catalytic subunit of PK-A from bovine heart and 1 mM ATP-gamma-S (filled circles) as described in Fig. 4

Discussion

The apical membranes of the chorionic villi of human term placenta, due to their abundance, could be a particularly useful human source for the purification of the Cl^-channels which were recently purified from bovine kidney cortex and apical tracheal membrane vesicles [23]. The reconstitution of the integral membrane proteins purified by this method [23] allows the recognition of the function of the purified proteins and of the conservation of their regulatory characteristics. A direct characterization of single Cl^- channel(s) and the elucidation of their mechanisms of regulation in the human term placenta by the patch clamp technique has not been performed, principally due to the difficulties encountered with the cultivation of the cytotrophoblast from term placenta [26, 30]. In order to obtain information on the presence and characteristics of these channels, apical membrane vesicles purified from the chorionic villi of human placenta have been chosen as a suitable model [6, 21]. The characterization of the basic biophysical properties of Cl^- channel(s), analyzed as overall conductance by the fluorimetric method described by Illsley and Verkman [21], which was validated in several vesicle systems [3, 4, 9], has already been reported [6, 19]. In these membranes a Cl^-/HCO_3^- antiporter [13, 19, 28] and a Cl^- conductive pathway [6, 19] have been demonstrated, but a Na^+or K^+-coupled Cl^- transport was not found [21]. However, in the experimental conditions here reported the influence of the antiporter was excluded [6]. The potential disadvantage in the use of the membrane vesicle models for the investigation of the regulation of the ion conductances is the possibility that cytosolic and/or membrane associated molecules that are essential for the conformational modifications of the channels (protein kinases, cyclic nucleotides, second messengers) could be depleted or degraded during the purification of the vesicles. For instance, in studies designed to understand the effect of the PK-A dependent phosphorylation

[1] We have no evidence that there is such a high protein phosphatase activity in the apical membranes of bovine trachea that needs the use of ATP-gamma-S, since the reported experiments have been done with ATP [31]. However, in order to check this aspect also of our experimental protocol, ATP-gamma-S was also used in the control model of the tracheal membranes.

of membrane channels or transporters in membrane vesicles, a PK-A has been found associated to rabbit and canine cortical renal brush-border vesicles [15, 33] whereas the addition of an exogenous PK-A was necessary in mouse renal modullary membranes [27]. Phosphorylation studies (Fig. 2) indicated that MVV did not contain a cAMP-dependent protein kinase (PK-A). The loading of an exogenous PK-A was therefore mandatory in our experimental conditions.

The balance between the phosphorylating activity mediated by the exogenous PK-A protein phosphatases (PP) activity is crucial for the possibility of phosphorylating target proteins. In principle, if the rate of dephosphorylation by PP is much higher than that of phosphorylation by PK-A the target protein has higher probability of being in the dephosphorylated state than in the phosphorylated one. This problem has already been reported in pig jejunal brush border vesicles [10] and is present in placental membranes, too. Although the usual protein phosphatases inhibitors did not permit a PK-A dependent phosphorylation due to the high PP activity (*data not shown*), the use of ATP-gamma-S allowed an effective membrane protein phosphorylation (Fig. 3). Moreover, the resistance of thiophosphate to the PP could also be a useful means for prolonging and amplifying the biological effect of the phosphorylation.

The exposure of the vesicles to hypotonic solutions [27, 33] or to a single pass of a Potter-Elvejiem homogenizer [10] or the use of a freeze-thawing technique [7, 8] are the most common methods utilized to load proteins and cyclic nucleotides in membrane vesicles. There are no particular preferences between these three possibilities except that freezing-thawing might damage the exogenous PK-A, but the principal problems related to these techniques are the different degrees of effectiveness in both lysis and resealing of the vesicles. In our experience, the hypotoniclysis procedure analyzed by impermeable molecules trapping ([^{14}C]-sucrose) showed variable efficiency, since an average of 46% of the placental and 76% of the tracheal vesicles were actually opened and resealed, with large variability from experiment to experiment. Therefore, only a fraction of the vesicles were presumably phosphorylated by PK-A or, even if phosphorylated, resealed in a closed space (active volume). We consequently decided to calculate the conductance values, taking into account only the active volume, which was measured in each experiment as detailed in the Method section.

The concern that the protocol might have undesired artifacts led us to validate it by the investigation of a membrane in which the characteristics of the Cl^- channels were known from alternative approaches. In fact, the study of Cl^- conductance by SPQ gives useful information on the function of all the channels that could be present in the membranes, but cannot allow distinctions among their different properties. For instance, if a PK-A activated Cl^- channel contributed to a small extent to the overall Cl^- conductance this might not have been detected by the SPQ method. The apical membranes of bovine trachea, which were chosen as control model, have three types of Cl^- channels but more than 80% of the total Cl^- conductance is mediated by a channel that is under strict control of a PK-A dependent phosphorylation [31]. Moreover, the phosphorylation site of the channel is tightly associated to the channel itself since the activation by PK-A is present also after solubilization and reconstitution of the membranes [31]. The application of our protocol to the apical membranes of bovine trachea gave results consistent with those obtained with the electrophysiological approach [31]. We confirmed that Cl^- conductance is largely activated by a PK-A dependent phosphorylation (Fig. 5), which can easily be seen also by the SPQ fluorimetric method.

The PK-A dependent phosphorylation of the human placental vesicles reduces the total Cl^- conductance markedly (Fig. 4), thus suggesting that PK-A inhibits one (or more) Cl^- channel(s). The distinction of which Cl^- channel(s) of the placental membranes is inhibited by PK-A is not possible, due to the limitations inherent in the SPQ method. PK-A dependent phosphorylation of the Cl^- channels of secretory epithelia in general results in the activation of the channels [11, 12]. An interesting exception concerns a nonepithelial membrane like the rat brain synaptoneurosomes, where a gamma-aminobutyric acid-activated chloride channel is inhibited by a PK-A dependent phosphorylation [16]. At this point it is possible to argue that while PK-A dependent phosphorylation could be the molecular mechanism for channel closure, other activatory pathways are involved in the opening of the Cl^- channel(s) of human placenta. A further understanding could be reached by analyzing the role of other means of activation of ion channels like PK-C [18, 25] or G proteins [17].

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