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Factors Affecting Chloride Conductance in Apical Membrane Vesicles from Human Placenta

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Summary. Apical membrane vesicles from human term placenta were isolated using a magnesium precipitation technique, and the purity of the vesicles was assessed morphologically using scanning and transmission electron microscopy, and biochemically, using marker enzymes. The vesicles were found to be morphologically intact and significantly enriched in enzymes associated with apical membranes.

$^{36}Cl^-$ uptake into these vesicles was studied in the presence of an outwardly directed Cl^- gradient. This uptake was found to be time dependent, with an initial rapid uptake tending to peak between 10 and 20 min and thereafter decline. Uptake was found to be voltage dependent since 5 μM valinomycin caused a decrease in uptake. The effects of N-phenylanthranilic acid (NPA) and 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) and bumetanide on the initial rate of Cl^- were examined in the presence and absence of 5 μM valinomycin. NPA and DIDS inhibited isotope uptake strongly with IC_{50} values of 0.83 ± 0.35 μM and 3.43 ± 0.37 μM, respectively, in the absence of valinomycin. Although valinomycin reduced $^{36}Cl^-$ uptake by about 80% when added before the isotope, DIDS reduced the uptake which remained in a concentration-dependent fashion with an IC_{50} of 5.6 ± 2.1 μM. Under these conditions, NPA was without effect at concentrations below 100 μM. Bumetanide was without effect at the concentrations used in the absence of valinomycin. However, following valinomycin pretreatment, bumetanide reduced $^{36}Cl^-$ uptake significantly at 100 μM concentration. Vesicle diameter, as assessed by flow cytometry, did not change under the conditions employed.

The effects of some fatty acids were also investigated. Arachidonic acid and linoleic acid inhibited Cl^- uptake with IC_{50} values of 37.6 ± 14.9 μM and 4.59 ± 0.51 μM, respectively. Arachidonyl alcohol and elaidic acid were found to be without effect. These studies show that human placental brush border membrane vesicles possess a chloride conductance channel, the activity of which can be measured in the presence of an outwardly directed Cl^- gradient and this channel is sensitive to Cl^- channel inhibitors, especially N-phenylanthranilic acid, and can be inhibited by unsaturated fatty acids such as arachidonic acid and linoleic acid.

Key Words chloride channel · human placenta · cystic fibrosis · DIDS · N-phenylanthranilic acid · fatty acids

Introduction

Brush border membrane vesicles isolated from epithelial cells have been used extensively in the study of mechanisms of solute transport. They have the advantage of being free from the complex regulatory and compensatory mechanisms which are present in the intact cell and can also be used to study transport phenomena occurring at one particular side of an epithelial cell, i.e., the apical or the basolateral membrane. The ionic composition of the internal and external buffers can also be exactly defined by preloading or resuspending the vesicles in solutions of exact composition.

In this study, brush border membrane vesicles isolated from human placenta were used to investigate Cl^- transport and its pharmacological modulation under certain conditions. The study of Cl^- transport in epithelia is relevant since it is an important process in fluid and electrolyte homeostasis. Chloride enters the cell by the basolateral Na^+ K^+ $2Cl^-$ electroneutral cotransporter, thus increasing the intracellular concentrations of these ions. Na^+ and K^+ leave the cell via the Na^+ pump and basolaterally located K^+ channels, respectively. Chloride can then leave the cell, down its electrochemical gradient, by passing through apical chloride channels which are regulated by cAMP-dependent protein kinase (A-kinase) and protein kinase C (Placchi et al., 1991). Agents such as beta agonists, prostaglandins and bradykinin can activate this channel by increasing cAMP and Ca^{2+} levels within cells (Cuthbert, 1991). The major counter ion for Cl^- is Na^+ which can leave the cell through apical Na^+ channels or by paracellular routes.

The disease cystic fibrosis is believed to be mainly due to abnormal regulation of chloride transport in epithelia such as airways and gastrointestinal

tract (Welsh, 1990). Human placenta is known to express the CF gene (Riordan et al., 1989), and is a readily available source of epithelial tissue. Since transport of chloride across cells is composed of a number of components, the study of mechanisms of such transport can be facilitated by the use of membrane vesicles. In the transport studies reported in this paper, we have made use of the principle that the generation of an outwardly directed ion gradient in membrane vesicles will result in the formation of a membrane potential and this membrane potential provides the driving force for isotope uptake (Garty & Karlish, 1989). Part of this work has been previously presented in preliminary form (Faller, Higgins & Ryan, 1990).

Materials and Methods

Preparation of Placental Brush Border Membrane Vesicles

Human placental brush border membrane vesicles (BBMV) were prepared using the method of Ganapathy et al. (1985). This involved differential centrifugation and magnesium precipitation. Placentae were obtained within 30 min of delivery. They were transported from the maternity hospital in 1 liter of a solution containing 500 ml of DMEM and 500 ml of 2× PBS at 4°C. The maternal decidua was removed and the central portion between the maternal and fetal surfaces of the placenta was used in the preparation of the BBMV. The maternal villous tissue was chopped into small pieces and was washed in a buffer containing 300 mM mannitol, 10 mM HEPES/Tris pH 7.0. The tissue was then chopped very finely and placed in 300 ml of the same buffer and was agitated with a magnetic stirring bar for 1 h at 4°C. The solid tissue was then removed by filtration through gauze and the filtrate was centrifuged at 1,000 × *g* for 10 min. The resulting supernatant was centrifuged for a further 15 min at 10,000 × *g*. This resulted in a supernatant which was ultracentrifuged at 86,000 × *g* for 35 min. The pellets resulting from this centrifugation were collected in 50 ml of ice-cold mannitol HEPES/Tris buffer and homogenized using a Dounce homogenizer. This homogenate was made 10 mM with respect to $MgCl_2$, stirred at 4°C for 15 min and allowed to stand for an additional 15 min. The homogenate was then centrifuged at 5,000 × *g* for 15 min and the resulting supernatant was ultracentrifuged at 86,000 × *g* for 35 min. The pellets were resuspended in the appropriate buffers by five passages through a 25-gauge needle. Aliquots were snap frozen and stored in liquid nitrogen before use.

Characterization of BBMV

Marker Enzyme Assays

Alkaline Phosphatase. Alkaline phosphatase activity was measured in placental BBMV and homogenate using the method of Lansing et al. (1967). *p*-Nitrophenylphosphate was used as a substrate for the enzyme. The amount of *p*-nitrophenol produced was measured spectrophotometrically at 420 nm and was used as an index of alkaline phosphatase activity. Enzyme activity was expressed as μmoles *p*-nitrophenol formed per mg protein per minute.

Gamma-glutamyltranspeptidase. Gamma-glutamyltranspeptidase was measured in placental BBMV and homogenate using a modification of the method of Szasz (1969). This enzyme hydrolyzes peptide bonds which have a terminal glutamic acid residue linked by its carboxyl group to an amino group. Enzyme activity was expressed as international units of enzyme activity per mg protein per minute.

Electron Microscopy

BBMV samples were fixed in phosphate buffer containing 2.5% gluteraldehyde pH 7.4. Transmission electron micrographs were produced using a Philips 201 electron microscope. Scanning electron micrographs were produced using a JEOL JEM-200FX electron microscope.

Flow Cytometry: Measurement of BBMV Diameter

To assess possible changes in BBMV size during the course of the experiments, the diameter of the membrane vesicles was measured using a Beckton Dickinson Facstar Plus Flow Cytometer. Forward light scatter of BBMV was measured using a 488 nm argon ion UV laser at 100 mW. Initially, the diameter of untreated BBMV was determined relative to size marker beads of diameter 4.81 μm and 6.6 μm. To investigate if size changes of the BBMV were a factor to be considered as a consequence of the removal of extravesicular Cl^-, or the addition of valinomycin, the mean forward scatter of BBMV was measured under these conditions. Initially, a 500 μl aliquot of BBMV in 130 mM KCl, 6 mM $MgCl_2$ and 10 mM imidazole acetate pH 7.0 was added to 3.5 ml of a solution containing 74.29 mM potassium gluconate, 3.43 mM magnesium gluconate, 107.14 mM sucrose and 10 mM imidazole acetate. 0.9 mM unlabeled KCl was added at this point. The concentrations of the components of this solution were the same as in a typical $^{36}Cl^-$ uptake experiment. After 2 min, this suspension was analyzed in the flow cytometer and the forward scatter was measured. This procedure was repeated for BBMV which were passed through a gluconate exchange column using a procedure similar to that described in the section concerned with $^{36}Cl^-$ uptake. After 2 min, this suspension was added to the flow cytometer and forward scatter was measured. A third sample of BBMV was treated in a similar fashion except 5 μM valinomycin was added following vesicle elution from the gluconate exchange column, and the forward scatter of this sample was also measured.

Measurement of Chloride Transport

Chloride transport into BBMV was measured using the method of Landry et al. (1987) with the modification that the isotope taken up by the membrane vesicles was removed from isotope remaining in the medium by passing samples through gluconate exchange columns rather than by rapid filtration.

Preparation of Ion Exchange Resin

Amberlite IRN-78L tetraalkylammonium hydroxide resin was washed in deionized water and was titrated with a 50% (w/v) gluconic acid solution to pH 1-2. The resin was washed extensively with deionized water to pH 5-6 and was stored at 4°C until use. Disposable glass pasteur pipettes were plugged with Dacron wool and filled with the amberlite resin to a height of 5 cm. Just before use, the columns were washed with 2 ml of a solution containing 250 mM sucrose, 10 mM imidazole acetate pH 7.0.

Chloride Uptake Assay

A BBMV suspension (500 μl) containing 130 mM KCl, 6 mM $MgCl_2$, 10 mM imidazole acetate pH 7.0 was applied to a gluconate exchange column and eluted with 2 × 750 μl of 250 mM sucrose, 10 mM imidazole acetate pH 7.0. The eluted membrane suspension was collected in an equal volume of a solution containing 130 mM potassium gluconate, 6 mM magnesium gluconate, 10 mM imidazole acetate pH 7.0. $^{36}Cl^-$uptake was initiated by addition of 0.7 μCi/ml $^{36}Cl^-$ which corresponded to 0.9 mM Cl^-. The reaction mixture was incubated at 25°C. The uptake reaction was terminated at appropriate times by placing 250 μl samples in duplicate of the reaction mixture onto gluconate exchange columns followed by elution with a 250 mM sucrose solution. This step served to remove any extravesicular isotope. Each eluant (1.8 ml) was placed in a scintillation vial and radioactivity was counted in a Wallac LKB 1217 Rackbeta liquid scintillation counter. A 400 μl sample of each eluant was retained for subsequent protein assay. Results are expressed as nmol $^{36}Cl^-$ per mg BBMV protein.

Effect of Valinomycin

Valinomycin was dissolved in ethanol and was added to the Cl^- uptake reaction at a 1 in 100 dilution to give a final concentration of 5 μM. Ethanol alone was added to control tubes to give a final concentration of 1% (v/v).

Efflux of ^{36}Cl from BBMV

Two 500 μl samples from the same BBMV preparation were passed through gluconate exchange columns in a similar fashion as in the uptake reaction and the eluants were collected in gluconate-containing medium as described above. $^{36}Cl^-$ was added and both tubes were incubated at 25°C. After 35 min, two 250 μl samples were removed from each tube and were passed through a gluconate exchange column to remove extravesicular isotope as in the uptake reaction. Once this sample had been removed, valinomycin was added to one tube; an equivalent volume of ethanol was added to the other; 250 μl samples were subsequently removed from each tube in duplicate at various times and passed through gluconate exchange columns as before.

Effect of Putative Inhibitors

Cl^- uptake was measured over 2 min in the presence of increasing concentrations (0–100 μM) of N-phenylanthranilic acid (NPA), 4-4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) and bumetanide. BBMV (100 μl) in 130 mM KCl, 6 mM $MgCl_2$, 10 mM imidazole acetate pH 7.0 was added to a disposable pasteur pipette containing 3.5 cm of amberlite gluconate exchange resin which had been washed with 250 mM sucrose. BBMV protein was eluted from these columns with 2 × 400 μl aliquots of a solution containing (in mM): 93.75 sucrose, 81.25 potassium gluconate, 3.75 magnesium gluconate and 10 imidazole acetate pH 7.0. Inhibitors were added at 1 in 100 dilutions. NPA and bumetanide were dissolved in dimethylsulphoxide; DIDS was dissolved in water. Control tubes contained vehicle instead of drug. $^{36}Cl^-$ was added to give the same final concentration as in the time course study. The reaction was terminated by placing 250 μl samples in duplicate onto gluconate exchange columns and BBMV protein was eluted with 2 × 1 ml washes of 250 mM sucrose and processed as in the time course.

The effects of increasing concentrations of NPA, DIDS and bumetanide on BBMV Cl^- uptake in the presence of valinomycin were also investigated. The above protocol was followed throughout with the exception that following elution of BBMV from the gluconate exchange column, valinomycin was added to give a final concentration of 5 μM. After 2 min, $^{36}Cl^-$ was added and the assay was carried out as above. Results were expressed as % inhibition of control. IC_{50} values were calculated using Apple Macintosh Cricket Graph software.

Effects of Fatty Acids

Lineolic, arachidonic and elaidic acids, and arachidonyl alcohol were dissolved in ethanol and their effects on Cl^- uptake at increasing concentrations (0–100 μM) were examined. The protocol used was the same as that of the inhibitor studies and the results were expressed as before.

Protein Determination

Protein was measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Materials

$H^{36}Cl$ was obtained from Amersham (specific activity 0.8 mCi/mmol). Amberlite IRN-78L (in the tetra-alkyammonium hydroxide form) was purchased from Rohm and Haas, Paris, France. N-phenylanthranilic acid was obtained from Aldrich Chemicals, bumetanide was a gift from Leo Laboratories. Calibration beads of 4.81 μm were obtained from Polysciences, Warrington, PA. Calibration beads of 6.6 μm were purchased from Becton Dickinson, Oxford, England. All other chemicals were of the highest grade commercially available.

Results

Marker Enzyme Assays

Alkaline phosphatase and gamma-glutamyltranspeptidase activities were measured in placental homogenate and in the BBMV. Both of these enzymes are localized on the apical membrane of epithelial

cells, and we found that they were highly enriched in the BBMV samples when compared to the homogenate samples. Alkaline phosphatase activity in BBMV was found to be 7.56 ± 0.72 μmol *p*-nitrophenol phosphate formed per mg protein per minute compared to the activity in the homogenate which was 0.32 ± 0.03. This corresponds to an enrichment factor of 25.3 ± 4.8 (mean ± SEM; $n = 4$). Gamma-glutamyltranspeptidase activity in BBMV was found to be 1160.6 ± 280 IU/mg protein/min and the activity in the homogenate was found to be 22.4 ± 4. This corresponds to an enrichment factor of 53.4 ± 8.9 (mean ± SEM; $n = 5$).

Electron Microscopy

The transmission and scanning electron micrographs (Fig. 1*A* and *B*) showed the BBMV to be intact spherical structures of varying sizes with the characteristic brush border facing outwards.

Measurement of BBMV Diameter

Using the technique of flow cytometry, we investigated any possible changes in BBMV size during the course of uptake experiments. Vesicle suspensions with ionic compositions similar to those which would be present during the course of $^{36}Cl^-$ uptake experiments were added to a flow cytometer in the presence of size calibration beads and the forward light scatter was measured. The diameters of the calibration beads were 4.81 μm and 6.6 μm. From the histograms in Fig. 2, it is apparent that the diameter of the BBMV is less than 4.81 μm. Measurement of the forward light scatter and analysis of this data on the computer showed that under the conditions employed in the $^{36}Cl^-$ uptake experiments, there were no significant changes in BBMV size.

Chloride Transport

Time Course

Chloride transport was measured over a 40-min time course. In Fig. 3, isotope uptake was shown to be rapid between 30 sec and 12 min rising to a maximum value of 18.0 ± 1.9 nmol Cl^-/mg protein. Between 20 and 40 min the amount of accumulated isotope tended to diminish, reaching a value of 12.8 ± 1.8 nmol Cl^-/mg protein at 40 min (mean ± SEM; $n = 12$). In a separate series of experiments, we investigated the extent of $^{36}Cl^-$ accumulation into the BBMV at the later times of 60, 90 and 120 min. We found that $^{36}Cl^-$ levels within the vesicles were reduced from the maximum levels at 12 min to 5.41 ± 0.59 nmol Cl^-/mg protein by 60 min, 4.04 ± 0.53 nmol Cl^-/mg protein by 90 min and 3.03 ± 0.49 nmol Cl^-/mg protein by 120 min. The driving force for $^{36}Cl^-$ uptake was a transient positive charge within vesicles that contained a chloride conductance, this positive charge was generated by the removal of extravesicular Cl^- by the ion exchange resin. Therefore, in vesicles that contained a chloride conductance, and assuming that this Cl^- permeability was greater than the total potassium permeability of the vesicles, the removal of extravesicular Cl^- led to the generation of an outwardly directed Cl^- gradient resulting in the formation of a potential difference across the membrane (positive inside). Addition of tracer amounts of $^{36}Cl^-$ to vesicles with this positive charge led to membrane potential-dependent isotope uptake.

Effect of Valinomycin

Valinomycin is a K ionophore which serves to increase membrane permeability to potassium ions. In an uptake system which is dependent on membrane impermeability to these ions, addition of valinomycin should abolish the driving force for isotope uptake. We have shown (Fig. 3) that addition of 5 μM valinomycin to the medium interfered with $^{36}Cl^-$ uptake. When this antibiotic was added after allowing the reaction to proceed for 2 min, the result was a discharge of isotope that had accumulated up to that point. Valinomycin, added before the addition of $^{36}Cl^-$ to the reaction, prevented the high levels of isotope accumulation into the BBMV which were observed in the absence of the ionophore. For example, uptake at 2 min in the presence of valinomycin was 3.2 ± 0.6 nmol Cl^-/mg protein, whereas in the absence of valinomycin, uptake at 2 min was 16.1 ± 1.6 nmol Cl^-/mg protein corresponding to a reduction of approximately 80%. These effects of valinomycin clearly demonstrate the involvement of a membrane potential as the driving force for isotope accumulation. The effect of valinomycin on the uptake process also showed that $^{36}Cl^-$ uptake into the placental BBMV under the conditions employed, was dependent on these vesicles not being greatly more permeable to K^+ ions than to Cl^- ions since vesicles in the presence of valinomycin, which are freely permeable to K^+, did not accumulate $^{36}Cl^-$ to any great extent.

Efflux of ^{36}Cl from BBMV

To further examine the effect of valinomycin on $^{36}Cl^-$ transport in the presence of an outwardly di-

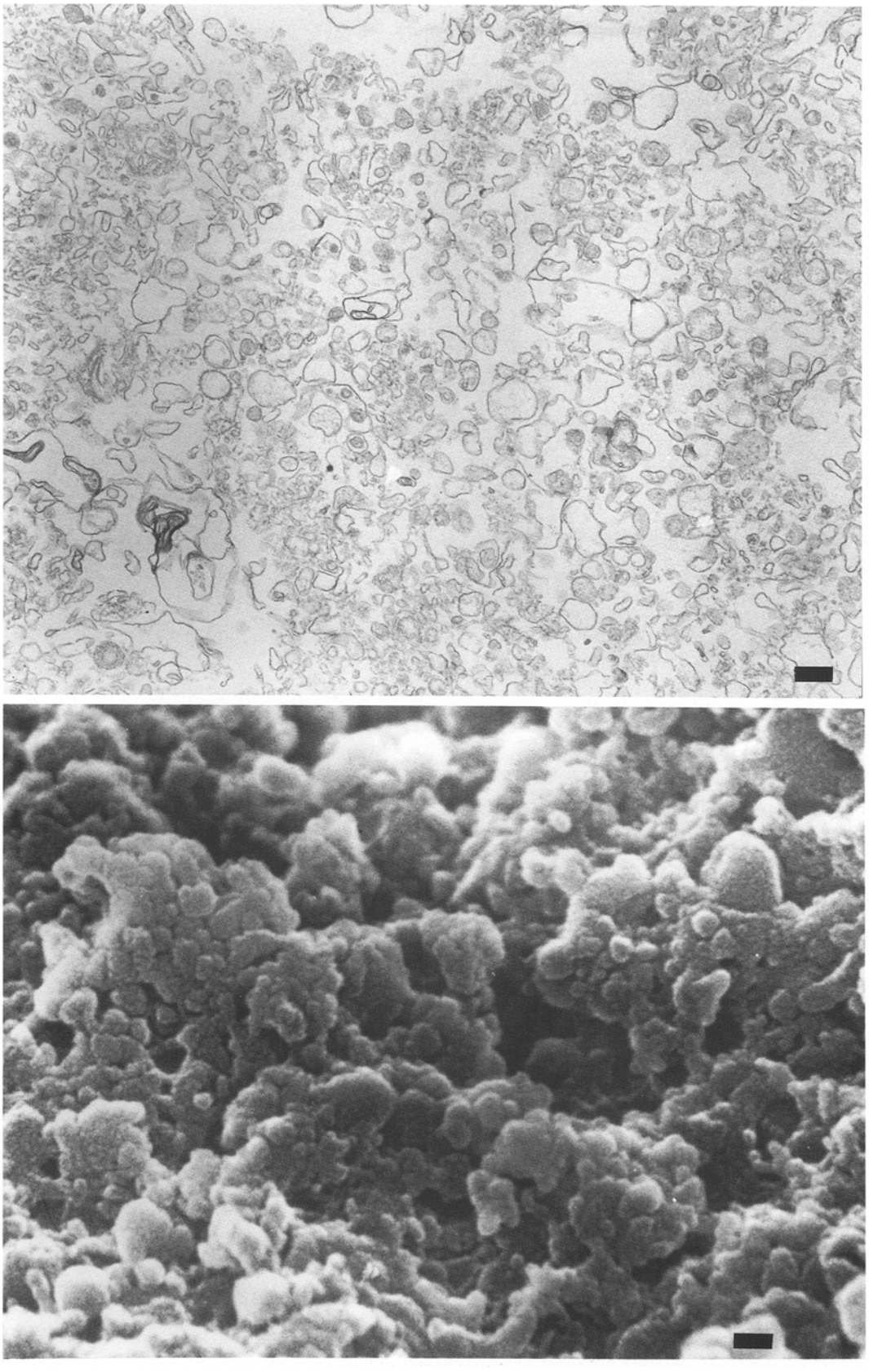

Fig. 1. Transmission (*A*) and scanning (*B*) electron micrographs of placental BBMV. Samples were fixed in 2.5% gluteraldehyde (pH 7.4) and were post fixed in 1% osmium tetroxide. In the transmission electron micrograph, the calibration bar corresponds to 0.3 μm; in the scanning electron micrograph, the calibration bar corresponds to 0.1 μm.

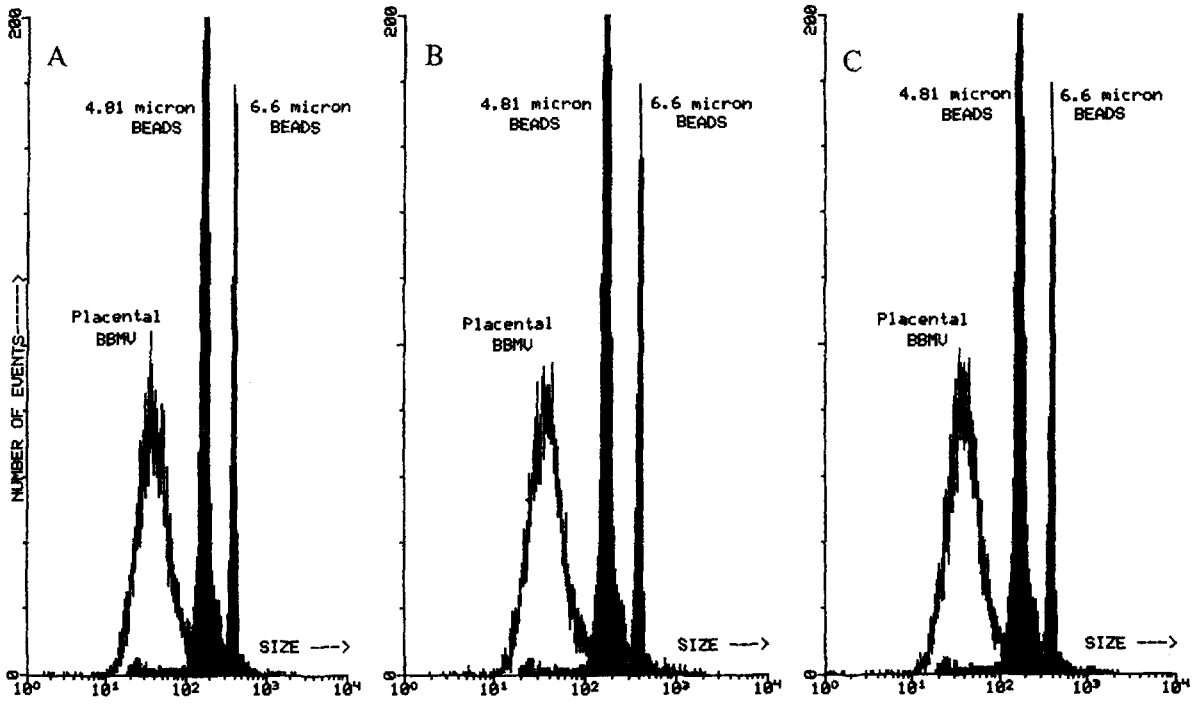

Fig. 2. Measurement of BBMV diameter using flow cytometry. (*A*) represents diameter of control BBMV which was determined with reference to density marker beads of 6.6 and 4.81 μm. Analysis of forward light scatter showed the BBMV to be less than 4.81 μm in diameter. (*B*) represents BBMV which had been passed through an amberlite gluconate exchange column to remove extravesicular Cl^-. (*C*) represents BBMV which were subjected to similar conditions as in *B*, except 5 μM valinomycin was added after 2 min.

rected Cl^- gradient, the effect of this ionophore on BBMV which had accumulated $^{36}Cl^-$ for 35 min was studied. Figure 4 shows that valinomycin had the effect of increasing the rate of isotope discharge from the BBMV. Two samples from the same placental BBMV preparation were used in each experiment. The amount of $^{36}Cl^-$ accumulated by 35 min in the sample which was designated "control" was 8.1 ± 0.3 nmol/mg protein and in the sample designated "valinomycin", the value was 8.3 ± 0.2 nmol/mg protein. Following the addition of 5 μM valinomycin to the test sample and an equivalent volume of ethanol to the control sample, the amount of accumulated $^{36}Cl^-$ tended to fall at a faster rate in the sample to which valinomycin was added. By 120 min, the control BBMV sample contained 3.7 ± 0.3 nmol $^{36}Cl^-$/mg protein, whereas the corresponding sample (BBMV with valinomycin) contained 1.8 ± 0.1 nmol $^{36}Cl^-$/mg protein.

Effect of Putative Inhibitors

These were investigated in both the absence and presence of valinomycin. Valinomycin clamps the membrane potential to zero and is therefore useful in distinguishing between conductive or electrogenic transport and other forms of Cl^- transport under the conditions employed in our experiments. N-phenylanthranilic acid (NPA) is an anthranilic acid derivative and many of these derivatives have previously been shown to be effective Cl^- conductance channel inhibitors (Landry et al. 1987). In the absence of valinomycin, we added increasing amounts of NPA (0–100 μM) to the chloride transport system and found that it inhibited isotope uptake in a concentration dependent fashion (Fig. 5*A*). Uptake was reduced to approximately 10% of control values in the presence of 100 μM NPA. The IC_{50} value was calculated to be 0.83 ± 0.35 μM (mean ± SEM; n = 3), considerably lower than any of the anthranilic acid derivatives screened by Landry et al. (1987) and also much lower than the value of 154 μM reported by Bayliss, Reeves and Andreoli (1990) for NPA inhibition of Cl^- uptake into basolateral membrane vesicles prepared from rabbit kidney outer medulla. However, in the presence of valinomycin (Fig. 5*B*), NPA was much less effective and did not significantly inhibit $^{36}Cl^-$ uptake at concentrations below 100 μM. The maximum inhibition was to approximately 60% of control values.

We also examined the effects of the stilbene derivitive 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) on conductive Cl^- transport (Fig.

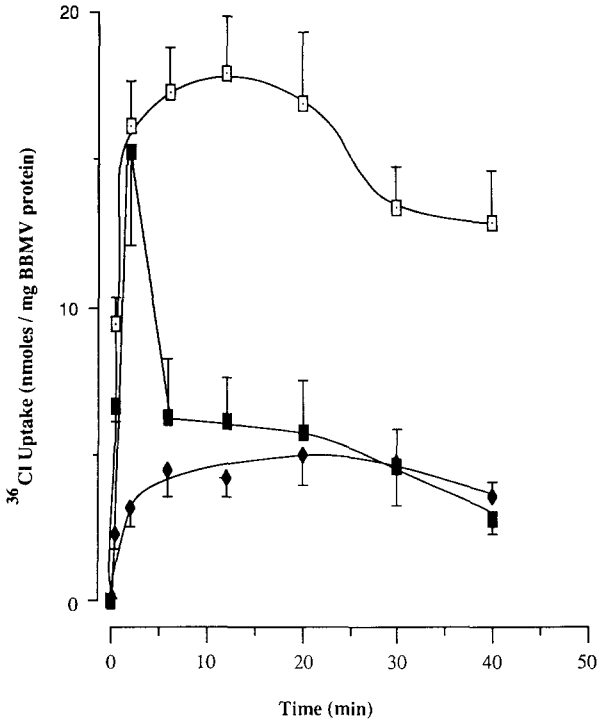

Fig. 3. Time course of $^{36}Cl^-$ uptake into placental brush border membrane vesicles (BBMV). Vesicles were preloaded with 130 mM KCl, 6 mM $MgCl_2$ and 10 mM imidazole acetate pH 7.0. Extravesicular Cl^- was removed by anion exchange chromatography and replaced by gluconate, thus generating an outwardly directed Cl^- gradient. Vesicles were diluted into potassium gluconate medium containing 0.7 μCi/ml $K^{36}Cl$ (0.9 mM Cl^-). (□) Control Cl^- uptake. Reaction mixture contained ethanol at a 1 in 100 dilution (n = 12). (♦) 5 μM valinomycin dissolved in ethanol was added before the uptake reaction began (n = 6). (■) 5 μM valinomycin was added after uptake had commenced for 2 minutes (n = 4).

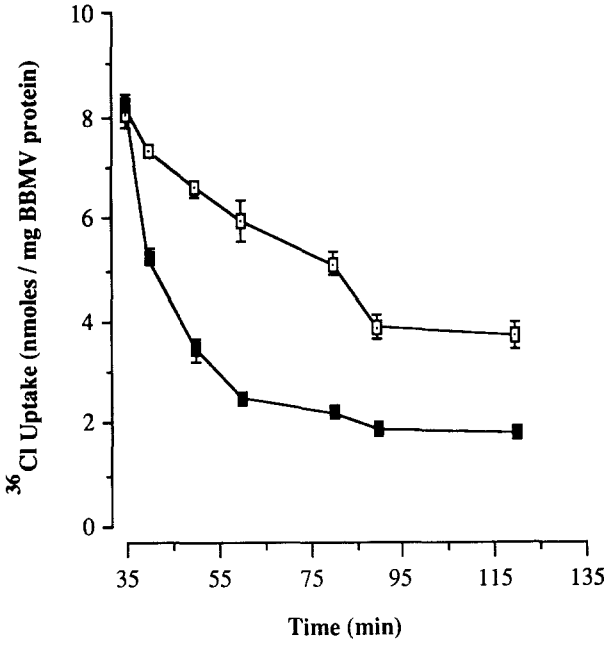

Fig. 4. Efflux of $^{36}Cl^-$ from human placental BBMV. Uptake of isotope was allowed to proceed for 35 min. At 35 min, samples were removed from each tube and ethanol was added to the control tube (□) 5 μM valinomycin was added to the other tube to initiate efflux (■) Data shows the mean ± SEM of three experiments performed in duplicate.

6*A*). Under these conditions, DIDS reduced $^{36}Cl^-$ uptake to approximately 20% of control values at a concentration of 100 μM. We have shown that DIDS inhibited conductive Cl^- uptake in human placental BBMV with an IC_{50} of 3.43 ± 0.37 μM (mean ± SEM; n = 3). In the presence of valinomycin, DIDS also significantly inhibited $^{36}Cl^-$ uptake with maximum inhibition also to approximately 20% of control values at 100 μM (Fig. 6*B*). The IC_{50} for inhibition of ^{36}Cl uptake by DIDS in the presence of valinomycin was 5.6 ± 2.1 μM (mean ± SEM; n = 3). These data clearly show that DIDS inhibits both conductive $^{36}Cl^-$ uptake and other Cl^- transport systems with similar potency, providing additional evidence that DIDS has broad specificity for different Cl^- transport pathways.

Bumetanide was shown to be an ineffective inhibitor of conductive $^{36}Cl^-$ uptake (uptake in the absence of valinomycin) and showed a similar lack of inhibitory effect on $^{36}Cl^-$ uptake in the presence of valinomycin at drug concentrations below 100 μM (Fig. 7*A* and *B*). However, bumetanide at 100 μM, significantly inhibited $^{36}Cl^-$ uptake in the presence of valinomycin. This may indicate lack of specificity of bumetanide at this relatively high concentration or provide some evidence of an electroneutral KCl pathway being involved as a component of net $^{36}Cl^-$ transport under these conditions.

Effect of Fatty Acids

Following reports that arachidonic acid and other *cis*-unsaturated fatty acids inhibited Cl^- transport in certain systems, (Anderson & Welsh, 1990; Hwang, Guggino & Guggino, 1990) we investigated the effect of some of these agents on chloride transport into placental BBMV. We found that linoleic acid and arachidonic acid, both of which are *cis*-unsaturated fatty acids inhibited Cl^- uptake in a concentration dependent fashion (Fig. 8). The IC_{50} values were calculated to be 37.6 ± 14.9 μM for arachidonic acid and 4.59 ± 0.50 μM for lineolic acid (mean ± SEM; n = 4). We found that elaidic acid, which is a *trans*-unsaturated fatty acid, had no effect at concentrations up to 100 μM. We also found that arachidonyl

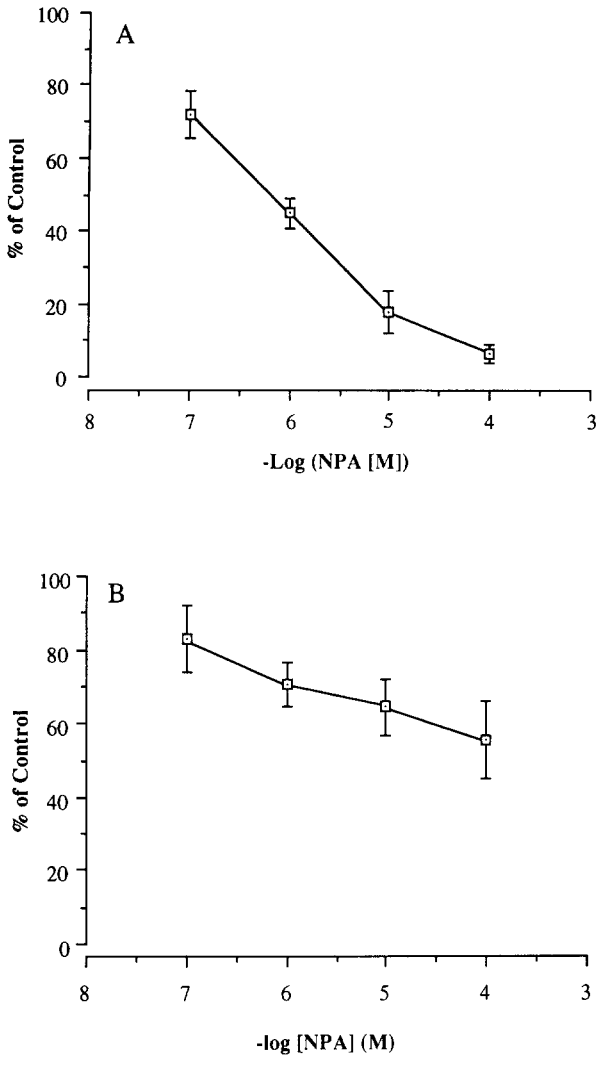

Fig. 5. Effect of N-phenylanthranilic acid (0–100 μM) on $^{36}Cl^-$ uptake into placental BBMV. (*A*) in the absence of valinomycin and (*B*) in the presence of 5 μM valinomycin. Uptake was measured over 2 min in the presence of an outwardly directed Cl^- gradient. The calculated IC_{50} was 0.83 ± 0.35 μM for *A*. An IC_{50} value for *B* was not determined.

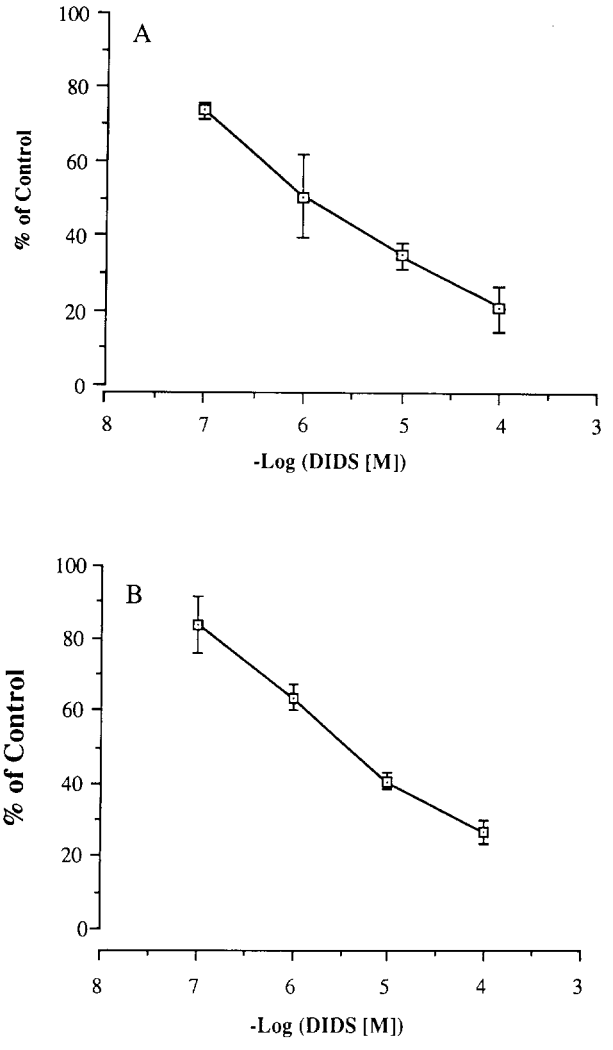

Fig. 6. Effect of 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) (0–100 μM) on $^{36}Cl^-$ uptake into placental BBMV. (*A*) in the absence of valinomycin and (*B*) in the presence of 5 μM valinomycin. Uptake was measured over 2 min in the presence of an outwardly directed Cl^- gradient. The IC_{50} was calculated to be 3.43 ± 0.37 μM for *A* and 5.6 ± 2.1 μM for *B*.

alcohol, an unsaturated fatty alcohol, was without effect at concentrations up to 100 μM.

Discussion

The brush border membrane vesicles (BBMV) used in this study were shown to be highly enriched in the marker enzymes alkaline phosphatase and gamma-glutamyltranspeptidase. The enrichment value of 25.3 for alkaline phosphatase is comparable to a value of 25.4, reported by Grassl (1989) for alkaline phosphatase in placental BBMV. A higher purification factor (53.4) was found for γ-glutamyltranspeptidase. This may relate to interference factors on the assay in placental cytosol. In any case, results with both enzyme assays reflect a high degree of purification in the BBMV preparation. Morphologically, the membrane vesicles appeared to be closed spherical structures with the brush border facing outwards. Flow cytometric analysis also revealed that the BBMV were relatively homogenous in size.

In this study we examined Cl^- transport in the presence of an outwardly directed Cl^- gradient according to the method of Landry et al. (1987) but with the modification that amberlite anion exchange columns were used to terminate the uptake reaction

instead of the rapid filtration technique. The method for studying $^{36}Cl^-$ uptake described in this paper involves setting up an electrical diffusion potential across the membranes using ion gradients so that the ion of interest may selectively accumulate within the vesicles. This technique has been used to study Na and K fluxes across membrane vesicles from a variety of sources, as described by Garty and Karlish (1989). In extensive preliminary investigations, we found that removal of free isotope by ion exchange to be much more reliable and reproducible than rapid filtration for $^{36}Cl^-$ studies in human placental BBMV.

Our initial transport studies were concerned with the time course of $^{36}Cl^-$ uptake into placental BBMV. Uptake was time dependent with initial rapid uptake and later reduction in $^{36}Cl^-$ levels within the BBMV. Therefore, further assays were carried out over 2-min intervals. Addition of 5 μM valinomycin before the reaction began prevented isotope accumulation while addition of this ionophore during the reaction caused a discharge of accumulated isotope. Valinomycin is a pore-forming antibiotic which increases membrane permeability to potassium, causing the collapse of the membrane potential which was generated by the induction of an outwardly directed Cl^- gradient. This collapse of the membrane potential led to the abolition of the driving force for $^{36}Cl^-$ entry and led to isotope discharge when added during the reaction and resulted in the presence of reduced levels of uptake when added before the reaction (Fig. 3). The data obtained from these experiments suggest that, in the absence of valinomycin, $^{36}Cl^-$ was entering the membrane vesicles down its electrochemical gradient. $^{36}Cl^-$ crosses the membrane in a similar fashion as Cl^- leaves the cells via an apically located conductance channel i.e., down its electrochemical gradient. The addition of valinomycin to a vesicle suspension in which there exists an outwardly directed Cl^- gradient generated by the replacement of extravesicular Cl^- with gluconate should have no effect on the outward Cl^- gradient but will clamp the membrane potential to zero by increasing the membrane permeability to K^+. Under these conditions, $^{36}Cl^-$ added to the reaction mixture should enter the BBMV via an electroneutral exchange mechanism rather than by a conductive pathway. Using a method similar to that described by Landry et al. (1987), we also investigated the effect of valinomycin on levels of intravesicular $^{36}Cl^-$ after 35 min. Figure 4 shows that addition of valinomycin leads to an acceleration of the rate of $^{36}Cl^-$ efflux. This finding is consistent with those of Landry et al. (1987) and Garty and Karlish (1989). After a period of time, the electrical driving force for isotope uptake will dissipate and

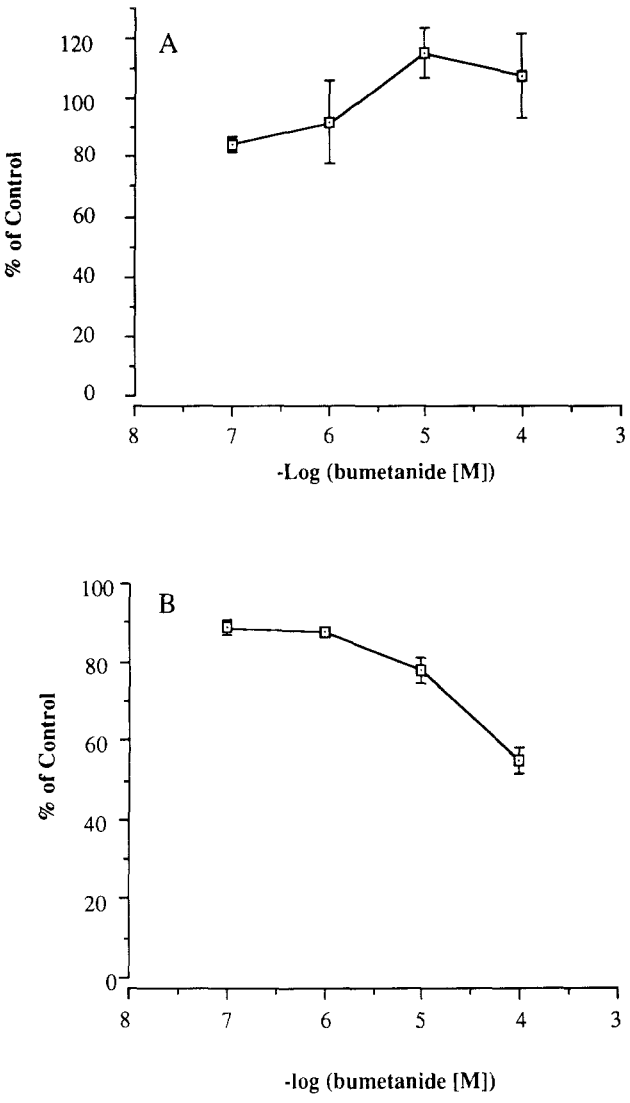

Fig. 7. Effect of bumetanide (0–100 μM) on $^{36}Cl^-$ uptake into placental BBMV. (*A*) in the absence of valinomycin and (*B*) in the presence of 5 μM valinomycin. Uptake was measured over 2 min in the presence of an outwardly directed Cl^- gradient. In *A*, bumetanide had no effect at concentrations up to 100 μM. In *B*, bumetanide caused significant inhibition at concentrations of 100 μM ($P \leq 0.05$).

the accumulated isotope will be released, and this is observed in Fig. 3 under conditions where valinomycin is absent. Since valinomycin increases membrane permeability to K, thereby accelerating the dissipation of the membrane potential, it can be concluded that the observed increase in the rate of $^{36}Cl^-$ efflux in the presence of this ionophore is due to this effect. While Illsley and Verkman (1986) showed using the membrane potential sensitive probe 3,3'-dipropylthiadicarbocyanine (diS-C_3-(5)) that human placental BBMV were more permeable to K^+ than to Cl^-, our observations indicate that this permeabil-

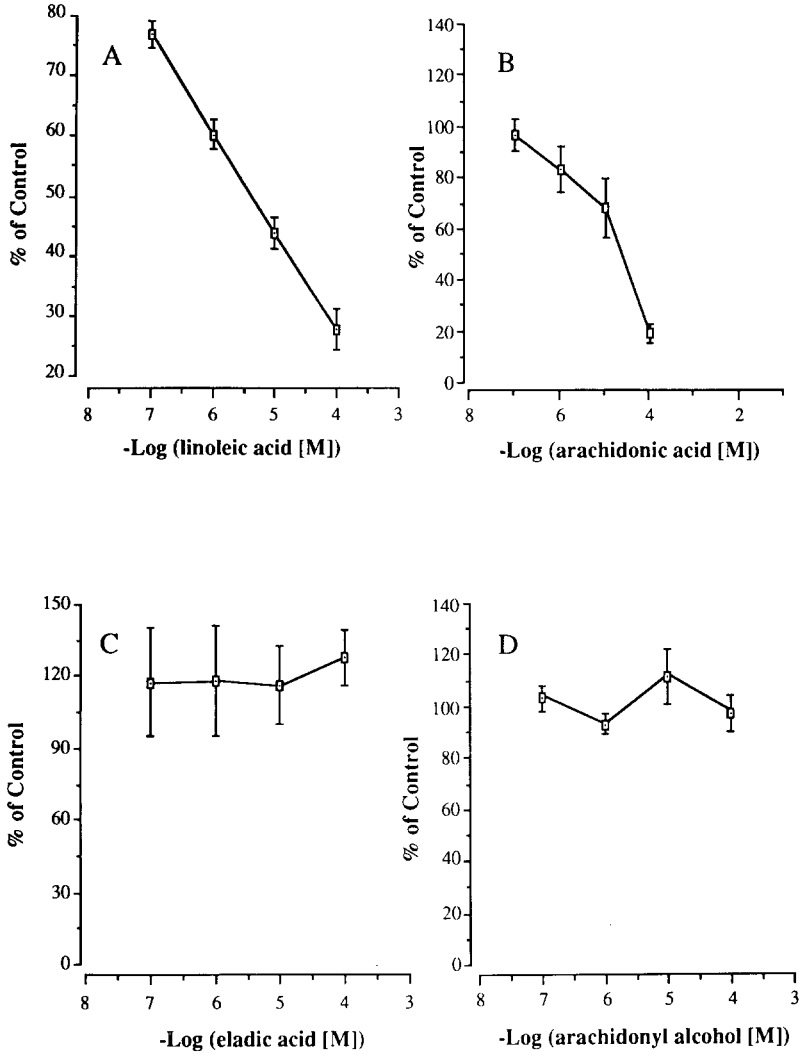

Fig. 8. The effect of increasing concentration of linoleic acid (*A*) arachadonic acid, (*B*) elaidic acid, (*C*) and arachidonyl alcohol (*D*) on Cl^- uptake into placental BBMV in the presence of an outwardly directed Cl^- gradient. Uptake was measured over 2 min in the presence of increasing concentrations of each compound (0–100 μM). The IC_{50} values calculated were 4.6 ± 0.5 μM (n = 3) for linoleic acid and 37.6 ± 14.9 μM (n = 4) for arachidonic acid. Neither elaidic acid nor arachidonyl alcohol had any effect at the concentrations used.

ity to K^+ is not greatly higher than that of Cl^- as demonstrated by prevention of uptake in the presence of valinomycin.

Using flow cytometry, we showed that changes in BBMV size are not a factor to be considered under these conditions as possibly interfering with $^{36}Cl^-$ uptake. We observed no change in BBMV size in the presence of the ionic composition used under the $^{36}Cl^-$ transport conditions imposed. It was also apparent from the associated histograms which show BBMV in the presence of calibration beads, (Fig. 2), that the diameter of the BBMV was less than 4.81 μm.

We investigated the effects of some putative inhibitors of epithelial ion transport on uptake in both the absence of valinomycin and in the presence of valinomycin. N-phenylanthranilic acid (NPA) has been used as a chloride conductance inhibitor in many systems including the shark rectal gland (Di Stefano et al., 1985), basolaterally enriched renal medullary vesicles from rabbit kidney (Bayliss et al., 1990) and in placental BBMV (Dechecchi & Cabrini, 1988). Bayliss et al. (1990) calculated an IC_{50} of 154 μM for uptake inhibition by NPA into membrane vesicles from rabbit kidney, whereas Dechecchi and Cabrini (1988) reported an IC_{50} of 150 μM. We calculated an IC_{50} of 0.83 μM which is considerably lower than values previously reported. However, these differences may reflect both structural and functional differences in the type of membrane vesicles used along with differences in experimental procedures including methods of potential generation, assay temperature, time of drug preincubation and time allowed for drug action. We also found that in the presence of valinomycin, NPA was without effect at concentrations below 100 μM. Under these conditions, $^{36}Cl^-$ enters the BBMV via nonconductive pathways. Therefore, we conclude that NPA, under the conditions employed in this assay, discriminates between conductive and nonconductive pathways.

In a separate series of experiments, we found that 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) inhibited Cl^- uptake with an IC_{50} of 3.43 μM. This value is comparable to the value of 10 μM which Landry et al. (1987) calculated for DIDS inhibition of $^{36}Cl^-$ uptake into renal cortical microsomes. It is generally accepted that DIDS is an anion exchange inhibitor (Grassl, 1989). However, the data presented here along with that of Breuer (1989) and Landry et al. (1987) would suggest that DIDS may inhibit a number of Cl transport systems including a conductance channel and nonconductance systems. Solc and Wine (1991) suggested that stilbenes such as 4,4′-dinitrostilbene-2,2′-disulphonic acid inhibit the outwardly rectifying, depolarization-induced Cl channels (ORDIC channels) in epithelia. The chloride uptake assay described here was set up to measure $^{36}Cl^-$ uptake via chloride channels and not via Cl^-/HCO_3^- exchange. This was achieved by generating an outwardly directed Cl^- gradient in the absence of any pH or HCO_3^- gradient, to drive the influx of isotope. In the report by Grassl (1989) Cl^-/HCO_3^- exchange in placental BBMV was measured in the presence of an outwardly directed HCO_3^- gradient and a transmembrane pH gradient which were optimal conditions for Cl^-/HCO_3^- to occur. In the present work, neither a HCO_3^- gradient nor a pH gradient was imposed, and conditions to stimulate maximal conductive Cl^- uptake were employed. We were satisfied that electroneutral anion exchange did not make a significant contribution to the experiments since valinomycin-induced increases in membrane K^+ permeability and subsequent dissipation of the transient membrane potential resulted in either prevention of high levels of isotope uptake or discharge of accumulated isotope from the BBMV (Fig. 3). Previous work on Cl^- transport into human placental BBMV by Shennan et al. (1986) utilized a transport assay which allowed both anion exchange and Cl^- conductance to be measured simultaneously. These workers reported that there existed a DIDS-sensitive and a DIDS-insensitive pathway for Cl^- entry into the vesicles and they defined the DIDS-sensitive route as being anion exchange and the DIDS-insensitive route as being Cl^- conductance. Under the conditions employed in our experiments, which were different to those employed by Shennan et al. (1986) in terms of temperature, isotope concentration, ionic gradients and preincubation times, we found DIDS to be a potent inhibitor of BBMV conductive Cl^- transport. However, we also found that DIDS inhibits $^{36}Cl^-$ uptake with similar potency under conditions where valinomycin was present. These data suggest that DIDS appears to have a broad specificity for Cl^- transport systems. Dechecchi and Cabrini (1988) investigated the effects of dihydro-DIDS (H_2DIDS), the reduced form of DIDS, on Cl^- fluxes into human placental BBMV under chloride gradient conditions. The authors found that H_2DIDS had no effect on Cl^- fluxes, as assessed by measurement of 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) quenching at 100 μM concentration. However, these workers used the SPQ quenching method rather than the isotope flux method employed here and preincubated the BBMV with H_2DIDS for 1 hr, and followed this with washes and centrifugations to remove unbound inhibitor rather than carry out the flux measurements in the presence of both bound and free inhibitor. Furthermore, these authors measured SPQ quenching and the effect of H_2DIDS in the presence of an inwardly directed Cl^- gradient rather than in the presence of an outwardly directed Cl^- gradient as used in the present study. In addition, Dechecchi and Cabrini (1988) reported an IC_{50} of 150 μM for NPA in their experiments, whereas under our conditions NPA is a much more potent inhibitor with an IC_{50} of 0.83 μM in the absence of valinomycin. It may not be possible, therefore, to compare directly the findings reported here to those reported by Dechecchi and Cabrini (1988). The tritiated form of the DIDS analogue 4,4′-diisothiocyano-ditritio stilbene-2,2′-disulphonic acid ($[^3H]_2$DIDS) has been used to covalently label proteins from TALH basolateral membrane vesicles which appear to be involved in chloride transport (Breuer, 1990). This author showed that $[^3H]_2$DIDS could covalently label more than one membrane protein. This finding, taken along with the evidence reported here, suggests that DIDS and related compounds may bind to regions common to different types of Cl^- transporters.

The absence of an inhibitory effect of bumetanide at concentrations up to 100 μM suggests that Na^+ K^+ $2Cl^-$ transport was not occurring under our conditions. This finding is different to the studies of Shennan et al. (1986) in that they observed Cl^- uptake inhibition by the loop blocker furosemide at concentrations of 100 μM. We have also shown that in the presence of valinomycin, 100 μM bumetanide significantly inhibited $^{36}Cl^-$ uptake into the BBMV. Under the conditions involved in these experiments, a component of the net Cl^- flux may be via electroneutral KCl transport and it is on this component that bumetanide appears to be acting.

We suggest that in the study of conductive chloride transport, it is important to have an assay system which discriminates against other modes of chloride transport including anion exchange and Na^+-K^+-Cl^- cotransport. This is particularly so in studies which attempt to focus on chloride channels in epithelial cell membrane vesicles in relation to

cystic fibrosis, especially in the light of many recent reports which show that the cystic fibrosis transmembrane conductance regulator (CFTR), the protein which is defective in cystic fibrosis, may be a cAMP-dependent protein kinase regulated chloride channel (Frizzell & Cliff, 1991).

Unsaturated fatty acids have been implicated as being inhibitors of Cl^- channels in cultured airway epithelia (Anderson & Welsh, 1990; Hwang et al., 1990). Arachidonic acid is known to affect ion channels in muscle as well as being an important second messenger in cells (Keyser & Alger, 1990). Our data show that linoleic acid and arachidonic acid are potent inhibitors of chloride channels. However, substitution of the carboxyl group of arachidonic acid with an alcohol group (arachidonyl alcohol) leads to a loss of inhibitory activity. Our data also show that the *trans*-unsaturated fatty acid, elaidic acid, does not have any inhibitory properties. Our findings in human placental BBMV are in agreement with Hwang et al. (1990) in that they showed that *cis*-unsaturation and the presence of a carboxyl group on fatty acids were necessary to inhibit Cl^- transport in human fetal tracheal cells using the patch-clamp technique. The double bonds in the hydrocarbon chain of *cis*-unsaturated fatty acids impart to the molecule a kinked rigid structure. The data presented here would lead us to speculate that this kinked structure, along with a carboxyl group, are necessary requirements for Cl^- channel inhibition. A physiological role for this inhibitory effect could be that cellular increases in arachidonic acid may have a function in the regulation of Cl^- channels and, hence, the rate of Cl^- secretion across the cell. It is suggested that abnormal regulation of fatty acid production in the cell may be a possible cause of the chloride channel impermeability in cells affected with the disease cystic fibrosis (Anderson & Welsh, 1990).

In summary, human placental BBMV have been used to measure $^{36}Cl^-$ transport. Uptake was measured in the presence of an outwardly directed Cl^- gradient and was found to be voltage dependent as assessed by valinomycin sensitivity. The effects of some pharmacological agents were investigated and NPA was shown to be an effective inhibitor of conductive Cl^- transport. DIDS appeared to affect both conductive and nonconductive Cl^- transport. Bumetanide was found to be without effect on conductive transport at the concentrations employed. The effects of some fatty acids were also investigated. Unsaturation and the presence of a carboxylic acid group were found to be necessary for transport inhibition. Further work is in progress on Cl^- transport in BBMV from CF placentae.

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