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Chloride transport by human placental microvillous membrane vesicles

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Unidirectional uptake of chloride by microvillous membrane vesicles prepared from human term placentas was studied over a range of membrane potentials in the presence and absence of chloride transport inhibitors alone and in combination at maximally effective concentrations. At 0 mV, inhibition of chloride uptake by 0.1 mM DIDS, 0.5 mM DPC, and 0.5 mM DPC plus 0.1 mM DIDS was similar, suggesting a common action upon an anion exchanger; neither 0.1 mM furosemide nor 0.1 mM bumetanide alone had any effect. An inside-positive membrane potential was created by imposing an inwardly directed potassium ion gradient in the presence of valinomycin. Total chloride uptake increased with increasing membrane potential (0, 4.6, 17.3, 25.8 and 32.0 mV). The inhibition of uptake by DPC and DPC/DIDS increased with the membrane potential. The effect of DPC compared to DPC/DIDS was significantly different at 4.6, 17.3 and 25.8 mV, suggesting a degree of additivity of inhibition. Neither furosemide nor bumetanide had any effect at any potential. There was a significant increase in inhibition due to DIDS alone until the membrane potential reached 25.8 mV. But there was no significant difference between the level of inhibition at 32 mV as compared to 0 mV, providing evidence of a DIDS-sensitive conductance similar to that previously seen in patch clamp studies. We suggest that uptake of chloride across the microvillous membrane of the human placenta may be by at least three different pathways; an electroneutral, DIDS-sensitive anion exchanger, a DPC-sensitive chloride conductance and a DIDS-sensitive chloride conductance.

Introduction

Chloride is the bulk anion of extracellular fluid in the fetus, as it is in the adult, but the mechanisms by which it crosses the placenta are still uncertain.

The human placenta has two complete cellular layers separating maternal and fetal blood, the syncytiotrophoblast and the fetal capillary endothelium, but due to its syncytial nature it is the former which is believed to constitute the major barrier to maternofetal exchange of small solutes [1]. Transcellular transport across the syncytiotrophoblast must involve movement across both its microvillous (maternal facing) and basal (fetal facing) plasma membranes.

Studies of chloride uptake into microvillous membrane vesicles (MMV) prepared from human placentas have provided evidence for a diisothiocyano-2–2′-disulfonic stilbene (DIDS) sensitive anion exchanger (probably Cl^-/HCO_3) which accounts for around 40% of uptake [3–5], and a chloride conductance which is blocked by diphenylamine 2-carboxylate (DPC) but is insensitive to DIDS [2,4]. This conductance was demonstrated as the chloride influx in response to an inside positive potential between +35 and +70 mV [2,4]. In patch clamp studies designed to reveal the channel(s) which might be responsible for the conductance, we identified a maxi chloride channel at the microvillous membrane of intact villi [6]. In contrast to what was expected from the vesicle conductance data, this maxi chloride channel was blocked by DIDS but unaffected by DPC [6]. However, the channel exhibited a marked voltage dependency, having a higher open probability between +30 and −30 mV but quickly inactivating and remaining closed at more extreme potentials. It is therefore unlikely that the maxi chloride channel would have contributed to the DPC sensitive chloride conductance at the potentials imposed in previous vesicle studies [2,4].

The aim of this study was to assess whether the DIDS and DPC sensitive channels do indeed co-exist and contribute to the chloride conductance in MMV

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from human term placentas. Chloride influx into the MMV was therefore measured in the presence and absence of DIDS and DPC alone and in combination at a range of (inside-positive) membrane potentials over which the maxi chloride channel has a high open probability [6].

As furosemide has been demonstrated to inhibit vesicle chloride influx in one study [5] but to have no effect in another [4], we took the opportunity to investigate its effect on chloride influx into the vesicles over a range of membrane potentials. We also hoped to confirm recent work [7] that showed chloride uptake to be inhibited by 0.1 mM bumetanide.

A preliminary report of this work has been published previously [8].

Materials and Methods

Vesicle preparation

MMV were prepared from normal term human placentas (37–42 weeks) obtained within 20 min of delivery by the method of Glazier et al. [9] which involved precipitation of non-microvillous membranes with magnesium ions and differential centrifugation. MMV were then suspended in intravesicular buffer (IVB) containing 160 mM sucrose, 10 mM Cl (as HCl), 6.6 mM Hepes, pH 7.5 with KOH (25 mM).

Vesicle properties

Vesicle purity was determined by assaying for alkaline phosphatase, a marker of the microvillous membrane, by the method of McComb and Bowers [10], succinate dehydrogenase, a marker of mitochondria, by the method of Green et al. [11], and NADH-dehydrogenase, a marker of smooth endoplasmic reticulum, by the method of Sottocasa et el., [12]. Dihydroalprenolol (DHA) binding, as a marker of basal membrane contamination, was determined by the method of Whitsett et al., [13]. Anion exchange column recovery was determined from the ratio of alkaline phosphatase activity of effluent to that in the reaction mixture. Vesicle protein content was assayed by the method of Lowry et al. [14].

Chloride uptake assay

The assay was performed using the method described by Shennan et al. [5], except that all experiments were conducted at room temperature rather than at 4°C.

Vesicles were diluted to a protein concentration of 10 mg/ml and preincubated with the potassium ionophore valinomycin (20 nmol/mg protein) for at least 1 h at room temperature.

Uptake was initiated by adding 952 μl extravesicular buffer (EVB) containing 10 mM $H^{36}Cl$, χmM potassium gluconate, (160–2χ)mM sucrose, 6.6 mM Hepes, pH 7.5 with KOH, to a reaction vial containing 420 μl of the membrane sample and inhibitors and/or IVB (2×14 μl), the total volume being 1400 μl. The concentration of potassium gluconate in the EVB was varied from 25 to 80 mM so as to induce K^+ diffusion potentials across the membranes ranging from 0 mV to 32.0 mV inside-positive (as calculated by the Nernst equation, assuming $E_m = 58 \log [K]_o/[K]_i$). Inhibitors were used alone and in combination at similar concentrations to those previously shown to be maximally inhibitory [4,5] at the same or higher chloride concentrations (confirmed for DIDS in preliminary experiments, data not shown). Concentrations used were 0.1 mM diisothiocyano-2–2′-disulfonic stilbene (DIDS), 0.5 mM diphenylamine-2-carboxylate (DPC), 0.1 mM furosemide, 0.1 mM bumetanide and a combination of DPC/DIDS. DIDS was dissolved in IVB, DPC in dimethylsulphoxide and furosemide and bumetanide in 70% methanol; in preliminary experiments we found that neither dimethylsulphoxide nor methanol altered chloride uptake at the concentrations used.

Uptakes were determined at various time intervals up to 60 min by removal of a 200 μl aliquot of reaction mixture (0.6 mg of protein) which was then placed onto an anion exchange column. Columns had been previously prepared by filling disposable Pasteur pipettes with Dowex anion exchange resin [15] and washed with 5 ml of ice-cold stop buffer containing 180 mM sucrose, 6.6 mM Hepes, pH 7.5 with KOH at 4°C. Following addition of the sample to the top of a column, it was immediately washed through with 2 ml of stop buffer. The eluents were collected into scintillation vials and mixed with 12 ml of scintillant (Optiphase HiSafe II) before counting on a Packard Tricarb 2000A β-counter.

Concurrent no-protein controls were run with each uptake and standards counted with each batch of samples. If sufficient protein was available assays were routinely performed in duplicate. In order to estimate the degree of binding of ^{36}Cl to MMV equilibrium uptake was determined in the presence of a range of extravesicular buffers in which the sucrose concentration was varied to give a range of osmolalities (70–699 mosmol). Binding was then determined by plotting equilibrium uptake against 1/osmolality and extrapolation to infinite osmolality when there would be theoretically no intravesicular space.

Statistics

Data are expressed as mean ± S.E. with n being the number of different vesicle preparations. Statistical analyses were performed using one-way ANOVA followed by t-tests with Bonferroni correction or by paired Student's t-test.

The amount of chloride entering the vesicles was calculated from their ^{36}Cl content and the specific activity of ^{36}Cl in the EVB. All uptakes are expressed

in nmol/mg protein and have been corrected for column recovery (which averaged 40.4 ± 2.1%, $n = 21$) by multiplying measured results by a factor of 100/(mean% column recovery for each specific experiment)

Materials

Valinomycin, DIDS, Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), furosemide, bumetanide and potassium gluconate were obtained from Sigma Chemicals, Poole, UK. DPC was obtained from Aldrich, UK. All other chemicals were obtained from BDH, Poole, UK, and were of AnalaR grade.

$H^{36}Cl$ was obtained from New England Nuclear and Optiphase HiSafe II was obtained from Pharmacia, UK.

Results

Protein recovery for the vesicles in the study was 0.18 ± 0.01 mg/g placenta ($n = 6$). Table I shows the marker enrichments in the vesicles; only the microvillous membrane marker alkaline phosphatase showed an enrichment significantly greater than unity.

Fig. 1 shows the time-course of chloride uptake with the membrane potential clamped at 0 mV by the absence of a potassium gradient and the presence of valinomycin. It can be seen that uptake was time dependent, being linear over the first minute. Uptake at 120 min was not significantly different to that at 60 min in preliminary experiments implying that equilibrium had been reached (data not shown). Uptake at 60 min in the presence of a range of different extravesicular osmolalities is shown in Fig. 2 and suggests that chloride was entering the intravesicular space and that binding was 23% of equilibrium uptake. Calculated intravesicular volume was 0.82 ± 0.15 μl/mg protein.

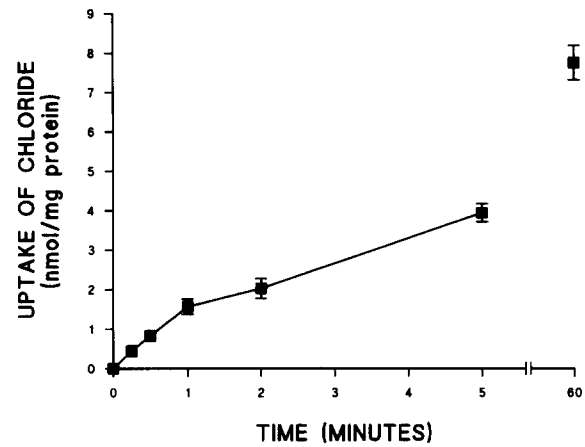

Fig. 1. Time-course of chloride uptake by placental MMV in the absence of inhibitors. Data shown are $n = 6$, mean ± S.E. (Error bars are shown except where these fall within the symbol.)

In subsequent experiments uptake at 30 s was used as a measure of unidirectional chloride influx.

Table II shows the absolute chloride uptake by MMV at five membrane potentials in the absence and presence of the DIDS, DPC and DPC/DIDS. As can be seen, in all three cases chloride uptake was significantly inhibited at all potentials. Neither furosemide nor bumetanide inhibited uptake at any potential (data not shown). Equilibrium (60 min) uptake was not affected by membrane potential or the presence of any inhibitor. In order to determine directly the effect of DIDS, DPC and DPC/DIDS the amount of chloride uptake inhibited ([control uptake]–[uptake in the pres-

TABLE I

Determination of purity of microvillous membrane vesicles by the use of membrane marker enrichment

Enrichment = Activity or binding of vesicles/Activity or binding of homogenate.

Membrane marker	Enrichment (n)
Alkaline phosphatase	22.3 ± 1.7 (15)
Succinate dehydrogenase	0.11 ± 0.02 (6)
NADH dehydrogenase	0.45 ± 0.21 (6)
Dihydroalprenolol binding	0.80 ± 0.35 (4)

TABLE II

Uptake of chloride (nmol / mg protein) at 30 s (initial rate) at a range of imposed membrane potentials ($n = 6$)

Values are mean ± S.E. Neither furosemide nor bumetanide produced significant inhibition at any potential.

Membrane potential (mV)	No inhibitor	DIDS (0.1 mM)	DPC (0.5 mM)	DPC/DIDS 0.5/0.1 mM)
0	0.589 ± 0.045	0.393 ± 0.032 [a]	0.393 ± 0.031 [a]	0.391 ± 0.036 [a]
+4.6	0.818 ± 0.061	0.526 ± 0.039 [a]	0.408 ± 0.033 [a,b]	0.370 ± 0.028 [a,b,c]
+17.3	0.954 ± 0.049	0.608 ± 0.048 [a]	0.421 ± 0.028 [a,b]	0.380 ± 0.026 [a,b,c]
+25.8	1.150 ± 0.056	0.766 ± 0.019 [a]	0.377 ± 0.055 [a,b]	0.312 ± 0.058 [a,b,c]
+32,0	1.470 ± 0.075	1.269 ± 0.072 [a]	0.358 ± 0.030 [a,b]	0.368 ± 0.025 [a,b]

[a] $P < 0.01$ vs. no inhibitor.
[b] $P < 0.01$ vs. DIDS.
[c] $P < 0.01$ vs. DPC.

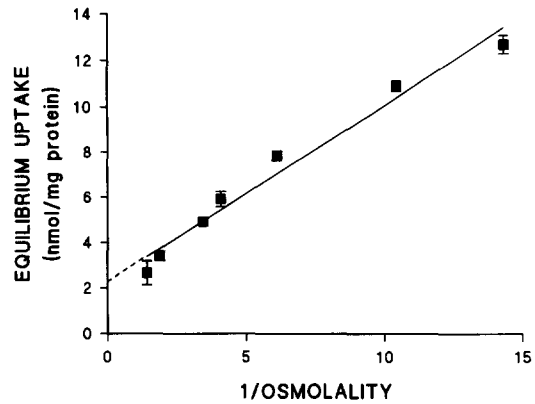

Fig. 2. Effect of osmolarity of the extravesicular buffer on chloride uptake at equilibrium (60 min). Line fitted by linear regression. Data are $n = 3$ (mean ± S.E.) Error bars are shown except where they fall within the symbol. $r = 0.98$; y intercept = 2.2.

ence of inhibitor]) was calculated and is shown in Fig. 3.

At 0 mV there was no significant difference in the inhibition due to DIDS, DPC and DPC/DIDS. At all potentials above 0 mV inhibition due to DPC and DPC/DIDS was significantly higher than that due to DIDS alone. The amount of inhibition appeared to be affected by membrane potential. Thus DPC and DPC/DIDS inhibited chloride influx by an amount which significantly ($P < 0.001$) increased with increasing membrane potential. At 4.6, 17.3 and 25.8 mV, but not at 32 mV, inhibition due to DPC/DIDS was significantly ($P < 0.05$) greater than that for DPC alone. The amount of inhibition due to DIDS also increased significantly ($P < 0.001$) between 0 and 25.8 mV; however it significantly decreased ($P < 0.001$) between 25.8 and 32.0 mV to the level seen at 0 mV.

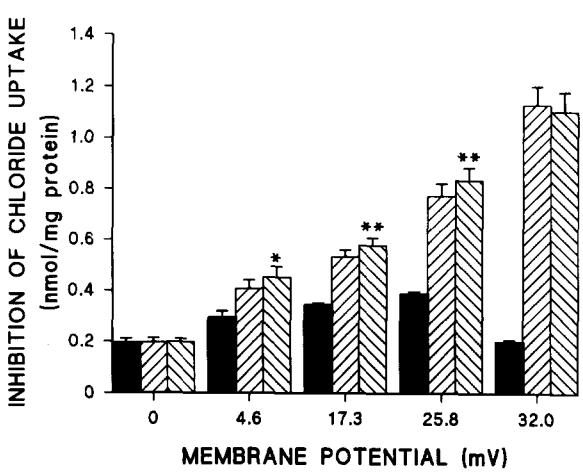

Fig. 3. Effect of inhibitors on chloride uptake at 30 s at differing inside-positive membrane potentials. Data are mean ± S.E., $n = 6$. * $P < 0.05$ vs. DPC; ** $P < 0.01$ vs. DPC. Filled bars: 0.1 mM DIDS. Upward hatched: 0.5 mM DPC. Downward hatched: DPC/DIDS.

Discussion

The vesicles used in the study were of a purity comparable with that reported previously. Alkaline phosphatase has been histochemically localized to the microvillous membrane [16] and has therefore generally been used as a marker enzyme for this membrane. The enrichment in the study vesicles (21-fold) compares favourably with that reported previously, which ranges between 5.7- [17] and 24.6-fold [18]; an enrichment of 18–19-fold has been previously reported for vesicles prepared in this laboratory by the same method [9]. The lack of enrichment of other membrane markers confirmed the purity of the preparation.

The degree of inhibition of chloride uptake by DIDS at 0 mV membrane potential found here (≈ 30%) was similar to that reported by Shennan et al. [5] and is in agreement with the other studies reporting the presence of an anion exchanger (Cl^-/Cl^- in these experiments) in this membrane [3,4]. However, we also found that DPC inhibited chloride influx to the same extent as DIDS at 0 mV and that there was no additive effect when the two drugs were combined. This suggests that DPC can block the anion exchanger, as suggested by the data of Illsley et al. [4]. Further support for this comes from the report that a derivative of DPC, 192 B, also blocks anion exchange in MMV from the human placenta [19]. Effects of DPC on anion exchange in other epithelia have also been reported [20].

With increasing inside positive membrane potential chloride uptake increased as did the inhibition of uptake by DPC. This is compatible with chloride flux through a DPC sensitive conductance pathway for which others have previously provided evidence [2,4,5].

The amount of chloride uptake inhibited by DIDS increased with increasing membrane potential between 0 and 25.8 mV. At 32.0 mV the amount inhibited fell to that seen at 0 mV. If DIDS only blocked the anion exchanger, which is electroneutral, then it would be expected that as chloride uptake increased with increasing membrane potential due to the conductance pathway, the inhibition by DIDS alone would remain constant. The DIDS-sensitive DPC-insensitive maxi chloride channel at the microvillous membrane found in patch clamp studies has a high open probability at ±30 mV and quickly inactivates and remains closed at more extreme potentials [6]. If it is present in the vesicles it should contribute to the chloride conductance over the range of membrane potentials between 4.6 and 25.8 mV. We therefore suggest that the relationship between membrane potential and DIDS inhibition in our MMV was due to the operation of this DIDS sensitive conductance as well as of the anion exchanger. Once this conductance inactivates at potentials greater than 25 mV, DIDS can only block the anion exchanger and hence the lower amount of inhibi-

tion at 32.0 mV. The finding of significant additivity of inhibition due to DPC and DIDS at 4.6, 17.3 and 25.8 mV but not at 32.0 mV provides further support for two separate conductances, one of which closes at higher potentials.

Furosemide had no effect on chloride uptake in this study under any of the conditions used. This confirms the report of Illsley et al. [4]; it is not clear why Shennan et al. [5] found an effect of this drug in their study, although this was the only one carried out in the cold (4°C); all studies used the same concentration (0.1 mM). In the present study bumetanide also had no effect on chloride influx in contrast to a recent report [7]; differences in vesicle preparation and experimental methods may explain this apparent discrepancy.

In summary, the data from this study demonstrates that there are at least three pathways for chloride flux across the microvillous membrane of the human placenta: via an anion exchanger, via a DPC sensitive conductance (the channel responsible has yet to be identified) and via a DIDS-sensitive conductance (probably the maxi chloride channel). It is impossible to tell from studies with microvillous membrane vesicles whether such pathways are involved in maternofetal exchange or whether they are simply involved with syncytiotrophoblast cellular 'housekeeping'. However, it is interesting to note that DIDS but not DPC markedly reduces unidirectional maternofetal clearance of chloride across the in situ perfused rat placenta [21].

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