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External Anions Regulate Stilbene-Sensitive Proton Transport in Placental Brush Border Vesicles†

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Received March 13, 1986; Revised Manuscript Received May 27, 1986

ABSTRACT: The mechanism for HCO_3^--independent proton permeability in microvillus membrane vesicles (MVV) isolated from human placenta was examined by using the entrapped pH indicator 6-carboxyfluorescein (6CF). Proton fluxes (J_H) across MVV were determined in response to induced pH and anion gradients from the time course of 6CF fluorescence, the MVV buffer capacity, and the 6CF vs. pH calibration. In the absence of anions, J_H was 12 ± 2 nequiv s^{-1} (mg of protein)$^{-1}$ (pH_{in} 7.4, pH_{out} 6.0, MVV voltage-clamped with K^+/valinomycin, 23 °C), corresponding to a proton permeability coefficient of 0.02 cm/s, with an activation energy of 9.1 ± 0.3 kcal/mol. J_H was inhibited 20% by dihydro-4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (H_2DIDS) with K_I = 8 μM ($[Cl^-]_{out}$ = 0 mM). For a 0.5-unit pH gradient J_H increased from 1.5 to 4.6 nequiv s^{-1} (mg of protein)$^{-1}$ as the internal MVV pH was increased (5.5-7.5). External Cl^-, Br^-, and I^- (but not SO_4^{2-} and PO_4^-) increased J_H 1.3-2.5-fold for both inwardly and outwardly directed pH gradients with K_D = 1.0 ± 0.4 mM (Br^-) and >100 mM (Cl^-). This increase was blocked by 100 μM H_2DIDS but not by amiloride or furosemide. Internal Cl^- did not alter J_H induced by pH gradients nor were proton fluxes induced by anion gradients in the absence of a pH gradient. Experiments in which J_H was driven by membrane potentials (induced by valinomycin and K^+ gradients) indicated that proton transport was voltage-sensitive. These experiments demonstrate a stilbene-sensitive electrogenic proton transport mechanism in MVV that is regulated allosterically by anions at an external binding site.

Passive and ion-coupled proton permeabilities in biological membranes are important for regulation of cell pH and for the net transepithelial transport of ions and proton equivalents. In pure lipid bilayers the mechanism of electrogenic proton transport is not well understood; models involving proton transport along strands of hydrogen-bonded water and proton transport facilitated by endogenous mobile carriers have been proposed to explain the anomalously high permeabilities for protons as compared to monovalent ions and the lack of significant dependence of proton conductance on pH (Cafiso & Hubbell, 1983; Guknecht, 1984; Nichols & Deamer, 1980). In biological membranes, passive proton transport in HCO_3^--free media occurs primarily by electrogenic passive

† Supported by NIH Grant AM35124 and grants from the UCSF Academic Senate, the MSC Clough Fund, and the National Cystic Fibrosis Foundation. G.C. was supported by Associazione Veneta Fibrosi Cistica and USL 25 Regione Venete of Italy.

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0006-2960/86/0425-6300$01.50/0

diffusion and electroneutral Na^+/H^+ and Cl^-/OH^- countertransport (Jennings, 1978; Krishnamoorthy & Hinkle, 1984; Reenstra et al., 1981; Verkman & Ives, 1986). Although protein-mediated mechanisms for countertransport have been established in systems such as the erythrocyte and the renal proximal tubule, the mechanisms for passive proton conductance are less clear.

We have examined the mechanism of passive proton permeability in purified microvillus membrane vesicles (MVV) isolated from human placenta by measuring the rate of collapse of preformed pH gradients using the entrapped pH-sensitive fluorescent dye 6-carboxyfluorescein (6CF).[1] Recently, an amiloride-sensitive electroneutral Na^+/H^+ countertransporter was identified in the MVV (N. P. Illsley and A. S. Verkman, unpublished data). We report here the existence of a voltage-sensitive stilbene-inhibitable passive proton permeability in MVV with an activation energy of 9.1 kcal/mol, much lower than the 18–25 kcal/mol activation energy for proton permeability in lipid bilayers (Rossignol et al., 1982). Proton fluxes induced by both inwardly and outwardly directed pH gradients were increased in the presence of external, but not internal, Cl^-; the increase was blocked by stilbene transport inhibitors. These findings suggest that HCO_3^--independent proton transport in MVV is mediated in part by a membrane protein and that the protein contains an external anion binding site which regulates proton permeability.

Experimental Procedures

Materials. 6CF, H_2DIDS, and diS-C_3-(5) were obtained from Molecular Probes Inc. (Junction City, OR). All other chemicals and reagents were purchased from Sigma (St. Louis, MO). DBDS was a gift of Dr. James A. Dix. Stilbenes and amiloride were stored as 5 mM aqueous stock solutions at 0 °C in the dark. Furosemide was stored as a 10 mM stock solution in methanol. Acrylic cuvettes (Starstedt, West Germany) were used in diS-C_3-(5) experiments to minimize dye binding.

Membrane Preparation. MVV were isolated from human placenta by a modification of methods described previously (Booth et al., 1980; Bissonnette et al., 1981). Fresh term placental tissue was diced and washed in 150 mM NaCl and 100 mM $CaCl_2$ sequentially and then minced and stirred at low speed in 250 mM sucrose and 10 mM HEPES/Tris, pH 7.0 (buffer A), for 60 min to release microvilli. Tissue fragments and cellular debris were removed by filtration through coarse gauze and centrifugation at 2500*g* for 10 min. $MgCl_2$ (12 mM) was added, and the mixture was stirred slowly for 60 min to aggregate nonmicrovillus membranes. Aggregates were removed by centrifugation at 2500*g* for 10 min, and MVV were pelleted by a 50000*g* spin for 30 min. MVV resuspended in buffer A were layered on 35% (w/v) sucrose and 10 mM HEPES/Tris, pH 7.0, in a SW28 rotor and centrifuged at 140000*g* for 60 min. The interfacial layer was collected and repelleted. All preparative procedures were performed at 4 °C. Alkaline phosphatase activity was purified >25-fold, while ouabain-inhibitable (Na/K)-ATPase was purified 0.3-fold; microsomal and mitochondrial membrane contamination was negligable as judged by NADPH and succinate–cytochrome *c* reductase activities (Illsley & Verkman, 1986b).

Proton Flux Measurements. MVV were loaded with 6CF by incubation for 18 h at 0 °C in 100 μM 6CF in buffers chosen to set intravesicular composition (Verkman & Ives, 1986). External 6CF was removed by three washes in >50-fold volumes of 6CF-free buffer. The final pellet (~15 mg/mL protein) was maintained at 0 °C with less than 20% 6CF leakage in 4 h.

Experiments were initiated by diluting MVV (150 μg of protein) into 2 mL of specified isomotic buffers stirred continuously in a thermostated cuvette. All experiments were performed by using HCO_3^--free buffers which were bubbled with CO_2-free N_2 for at least 30 min prior to experiments. Fluorescence was excited at 490 nm and measured at 520 nm (8-nm bandpass) in an SLM 8225 fluorometer (Urbana, IL) interfaced to an IBM PC/XT computer. Absolute proton flux (J_H) in nanoequivalents per second per milligram of protein was calculated from the initial rate of 6CF fluorescence change (fluorescence units per second), MVV buffer capacity (nanoequivalents per milligram of protein per pH unit), and the 6CF fluorescence vs. pH calibration curve (fluorescence units per pH unit) as described previously (Verkman & Ives, 1986). In nine MVV preparations from separate placentas, J_H for a 1.5-unit inwardly directed proton gradient (pH_{in} 7.4, pH_{out} 6.0) in the absence of Cl^- was 12 ± 3 nequiv s^{-1} (mg of protein)$^{-1}$ (SEM), indicating the biological variability in the MVV preparation.

Buffer Capacity. MVV buffer capacity, B(pH), was measured by titration of MVV lysed in HCO_3^--free, deionized, doubly distilled water containing 0.1% Triton X-100 as described previously. B(pH) [in nequiv of OH^-(pH unit)$^{-1}$ (mg of MVV protein)$^{-1}$] in the pH range 5–7.5 fitted closely to the empirical equation B(pH) = 193(pH) − 770.

Calibration of 6CF Response. The dependence of 6CF fluorescence on MVV pH was determined by measuring the amplitude of the fluorescence signal of suspended MVV containing 6CF in response to serial additions of gluconic acid that lowered the pH by 0.5 unit (Verkman & Ives, 1986). Each acid addition caused a rapid fluorescence decrease (<2 ms in stopped-flow experiments) due to titration of extravesicular 6CF (~50% of signal) followed by a slower single-exponential fluorescence decrease (10–30 s) due to proton influx and titration of intravesicular 6CF. The dependence of 6CF fluorescence on pH was identical for both intravesicular and extravesicular pH over the pH range 5–8 and can be described by the empirical equation 6CF fluorescence (arbitrary units) = $1.8(pH)^3 - 1.04(pH)^2 + 0.18(pH) - 0.0095$.

Membrane Potential Measurements. To ensure adequacy of the K^+/valinomycin voltage clamp, MVV membrane potential was measured by using the potential-sensitive carbocyanine probe diS-C_3-(5) (Cabrini & Verkman, 1986). In calibration experiments, the fluorescence (excitation 622 nm, emission 670 nm) of a solution containing MVV (75 μg of protein/mL) and 3 μM diS-C_3-(5) increased 0.4%/mV in the membrane potential range −90 to 0 mV and 0.3% in the range 0–90 mV (Illsley & Verkman, 1986a). Membrane potential was measured under the conditions of the transport experiments with increasing valinomycin as summarized in Table I.

Results

The time course for collapse of a 1.4 pH unit inwardly directed proton gradient in MVV is shown in Figure 1. When MVV with internal pH 7.4 are suspended in buffer at pH 6.0, there is rapid titration of extravesicular 6CF occurring within the fluorometer mixing time (<1 s) which is not observed. This is followed by a slower decline in MVV pH resulting from

[1] Abbreviations: 6CF, 6-carboxyfluorescein; diS-C_3-(5), 3,3′-dipropylthiodicarbocyanine iodide; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; H_2DIDS, dihydro-4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; DBDS, 4,4′-dibenzamido-2,2′-stilbenedisulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

Table I: Effect of Valinomycin on Proton Flux[a]

valinomycin (μg/mg of protein)	$[Cl^-]_{out}$ = 0	$[Cl^-]_{out}$ = 150 mM	
	J_H	J_H	ψ (mV)
0	7.3 ± 0.3	7.7 ± 0.5	−14.2 ± 0.6
12.5	11.5 ± 0.1	12.3 ± 0.2	−2.4 ± 0.4
25	11.9 ± 0.5	13.8 ± 0.6[b]	−1.8 ± 0.6
50	10.9 ± 0.2	13.8 ± 0.5[b]	−0.1 ± 0.6
100	11.4 ± 0.7	14.1 ± 1.0[b]	

[a] MVV containing 6CF and varying valinomycin in 250 mM sucrose, 100 mM HEPES/Tris, and 150 mM potassium gluconate, pH 7.4, were suspended in 250 mM sucrose and 100 mM HEPES/Tris, pH 6.0, 23 °C, containing 150 mM potassium gluconate or 150 mM KCl. J_H [nequiv s^{-1} (mg of protein)$^{-1}$] was measured from the time course of decreasing 6CF fluorescence. In separate experiments, membrane potential (ψ) was measured immediately after MVV were suspended, using the potential-sensitive probe diS-C_3-(5) as described under Experimental Procedures. Measurements were performed in triplicate; errors are 1 SD. [b] Values of J_H for $[Cl^-]_{out}$ = 0 and 150 mM differ significantly at $P < 0.05$ and 0.01, respectively (unpaired t test).

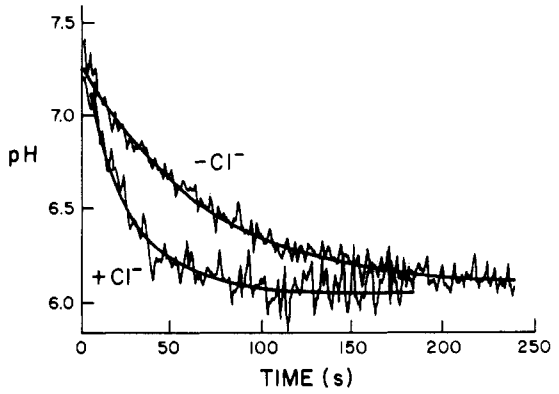

FIGURE 1: Time course of MVV pH following an inwardly directed pH gradient. MVV loaded with 6CF and containing 50 μg of valinomycin/mg of protein in 250 mM sucrose, 50 mM HEPES/Tris, and 150 mM potassium gluconate, pH 7.4, were suspended in 250 mM sucrose and 50 mM HEPES/Tris, pH 6.0, 23 °C, and 150 mM potassium gluconate or 150 mM KCl. The time course of intravesicular pH was determined from the time course of 6CF fluorescence and the fluorescence vs. pH calibration curve measured independently. Fitted single exponentials are shown with time constants of 66 s ($-Cl^-$) and 29 s ($+Cl^-$).

transmembrane proton transport which fits closely to a single-exponential function. In three sets of measurements, proton flux (J_H) in the absence of Cl^- (14.6 ± 1.4 nequiv s^{-1} (mg of protein)$^{-1}$ is increased in the presence of 150 mM external Cl^- (19.6 ± 0.4 nequiv s^{-1} (mg of protein)$^{-1}$, Br^- (26 ± 3 nequiv s^{-1} (mg of protein)$^{-1}$, and I^- (37 ± 3 nequiv s^{-1} (mg of protein)$^{-1}$, but not with 150 mM phosphate (13.5 ± 0.1 nequiv s^{-1} (mg of protein)$^{-1}$ or 75 mM sulfate (15.4 ± 0.2 nequiv s^{-1} (mg of protein)$^{-1}$.

There are several possible mechanisms by which external Cl^- could accelerate the rate of MVV acidification. Conductive Cl^- entry could establish a diffusion potential enhancing proton entry by an independent proton conductive pathway if the K^+/valinomycin voltage clamp is not adequate. Electroneutral cotransport/countertransport of Cl^- with H^+/OH^- could occur, or in general, there may be electrogenic coupling between N Cl^- and M H^+/OH^- molecules on a single carrier. Alternately, Cl^- could enhance proton transport by an allosteric mechanism. A series of measurements were performed to distinguish among these possibilities.

To determine the valinomycin concentration at which an adequate voltage clamp is obtained, proton fluxes (J_H) were measured with increasing valinomycin (Table I). In the absence of Cl^-, J_H increases and saturates with increasing valinomycin, indicating that anion-independent proton transport is conductive and is not limited by diffusion potentials when >12.5 μg of valinomycin/mg of protein is used. In the presence of 150 mM external Cl^-, >25 μg of valinomycin/mg of protein produces a voltage clamp adequate to eliminate coupling of proton transport to Cl^- diffusion potentials. Direct measurements of membrane potential with diS-C_3-(5) confirm that membrane potential is voltage-clamped under experimental conditions when 50 μg/mg of protein valinomycin is used. There is small effect of Cl^- on J_H at valinomycin concentrations of 0 and 12.5 μg/mg of protein because of the Cl^- diffusion potential. The −14.2-mV potential induced by external Cl^- would be expected to increase J_H by ~1 nequiv s^{-1} (mg of protein)$^{-1}$ on the basis of the measured effect of a −70-mV induced potential (see below). At higher valinomycin concentrations, where J_H is not restricted by the membrane K^+ conductance, J_H is increased significantly by external Cl^-. Since electroneutral transport of Cl^- with H^+/OH^- should operate at all membrane potentials, these results are not consistent with a coupled electroneutral transport mechanism.

Table II: Dependence of Proton Flux on Cl^- Gradients[a]

$[Cl^-]_{in}$ (mM)	$[Cl^-]_{out}$ (mM)	J_H [nequiv s^{-1} (mg of protein)$^{-1}$]
0	0	12.3 ± 0.2
150	0	12.5 ± 0.2
0	150	15.3 ± 0.1[b]
150	150	14.9 ± 0.3[b]

[a] J_H was measured in vesicles containing 6CF and 50 μg/mg of protein valinomycin for a 1.5 pH unit inwardly directed proton gradient under conditions when internal or external potassium gluconate was replaced by KCl. Measurements were performed in triplicate; errors are 1 SD. [b] Values differ significantly from J_H measured in the absence of Cl^- ($P < 0.01$, unpaired t test).

To confirm that MVV proton transport is voltage-dependent, J_H was measured for a 1.5-unit inwardly directed proton gradient in MVV containing 50 μg/mg of protein valinomycin at 0 mV ($[K_{in}^+] = [K_{out}^+] = 150$ mM) and at −70 mV ($[K_{in}^+] = 150$ mM, $[K_{out}^+] = 10$ mM). J_H increased from 12.3 ± 0.3 nequiv s^{-1} (mg of protein)$^{-1}$ (SD, $n = 3$) to 17.4 ± 0.9 nequiv s^{-1} (mg of protein)$^{-1}$ ($n = 4$) at −70 mV, indicating that transport is voltage-dependent.

To determine whether anion gradients are required to increase J_H, experiments were performed with Cl^- present at either or both sides of the membrane (Table II). J_H increases when Cl^- is present at the external membrane surface, but is unaffected by Cl^- at the internal membrane surface. Since it is the presence of external anions and not internal anions or anion gradients that drives proton transport, it is unlikely protons are transported with Cl^- in a coupled manner on a single protein carrier.

To examine the possibility that Cl^- increases proton permeability by binding to an external site, the effects of external Cl^- on proton efflux were studied. If external Cl^- regulates proton permeability allosterically, it is predicted that external Cl^- would increase the rates of both proton entry and exit. However, if proton transport is coupled to Cl^- transport by diffusion potentials or by a coupled transport mechanism, external Cl^- would increase the rate of proton entry and decrease the rate of proton exit. The data in Figure 2 show that external Cl^- and Br^- increase J_H for proton exit, supporting the notion that MVV proton permeability is regulated allosterically by anions at an external binding site. An additional prediction of this mechanism is that Cl^- gradients would not induce pH gradients. In the absence of a pH gradient, 150 mM external Cl^- did not alter the internal pH of MVV (<0.01 pH unit/min), whereas 150 mM external Na^+ caused MVV

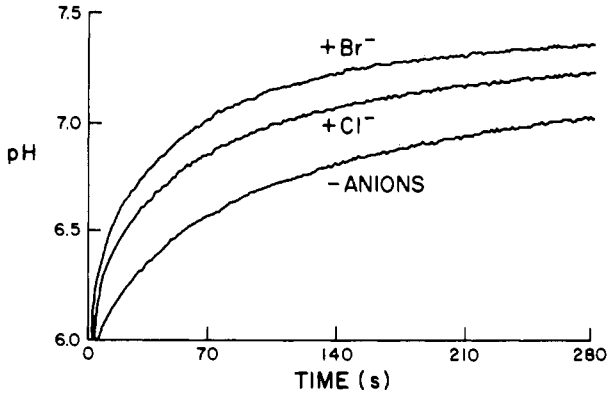

FIGURE 2: Acceleration of outward proton flux by external anions. MVV containing 6CF and valinomycin in 250 mM sucrose, 50 mM HEPES/Tris, and 150 mM potassium gluconate, pH 6.0, were suspended in 250 mM sucrose and 50 mM HEPES/Tris, pH 7.4, 23 °C, and 150 mM potassium gluconate or 150 mM KCl or 150 mM KBr. Time constants for fitted single exponentials were 88 s (−anions), 71 s (+Cl^-), and 61 s (+Br^-).

Table III: Effect of Inhibitors on Proton Flux[a]

inhibitor	J_H [equiv s^{-1} (mg of protein)$^{-1}$]	
	−Cl^-	+Cl^-
none	9.7 ± 0.1	12.5 ± 0.3
H_2DIDS	7.9 ± 0.2[b]	9.4 ± 0.6[b]
DIDS	8.1 ± 0.2[c]	11.7 ± 0.1[c]
DBDS	9.6 ± 0.1	12.7 ± 0.8
furosemide	9.4 ± 0.6	12.6 ± 0.5
amiloride	9.6 ± 0.1	12.4 ± 0.6

[a] J_H for a 1.5 pH unit inwardly directed proton gradient was determined in the absence and presence of inhibitors (100 μM) added just prior to the experiment. Experiments were performed in triplicate; errors are 1 SD. [b] Values differ significantly from J_H measured in the absence of inhibitors with $P < 0.01$ (unapired t test). [c] Same as b but $P < 0.05$.

alkalinization (0.4 pH unit/min) due to Na^+/H^+ countertransport (not shown).

The data in Table III show that H_2DIDS and DIDS partially inhibit proton permeability in the absence and presence of external Cl^-. The K_I for H_2DIDS inhibition of anion-independent proton permeability is 8 μM (Figure 3). The bulkier stilbene inhibitor DBDS did not inhibit proton permeability nor did it block the increase in proton permeability induced by Cl^-. DBDS is a potent inhibitor of erythrocyte anion exchange and undergoes a 100-fold fluorescence enhancement (excitation 360 nm, emission 420 nm) when bound to erythrocyte band 3 (Verkman et al., 1983). To determine whether a DBDS binding site exists on MVV, the fluorescence of DBDS (0–10 μM) and MVV (0.5 mg/mL protein) in 250 mM sucrose and 10 mM HEPES/Tris, pH 7.4, 23 °C, was measured in the absence and presence of 10 μM H_2DIDS. No DBDS binding to MVV was detectable by this method, whereas in parallel experiments using erythrocyte ghost membranes (0.04 mg/mL protein), saturable binding of DBDS was easily detectable. Table III also shows that J_H was not inhibited by amiloride, a Na^+/H^+ countertransport inhibitor, or by furosemide, which has been reported to block MVV anion exchange (Shennan et al., 1986).

Figure 3 shows the dependence of J_H on external Cl^- and Br^-. While the Cl^- enhancement was not saturable in the concentration range studied (K_D > 100 mM), the Br^- effect was saturable with a high affinity (K_D = 1.0 mM). To examine whether the increase in protein permeability due to external Cl^- and Br^- is specific to the placental brush border, similar measurements were performed by using brush border

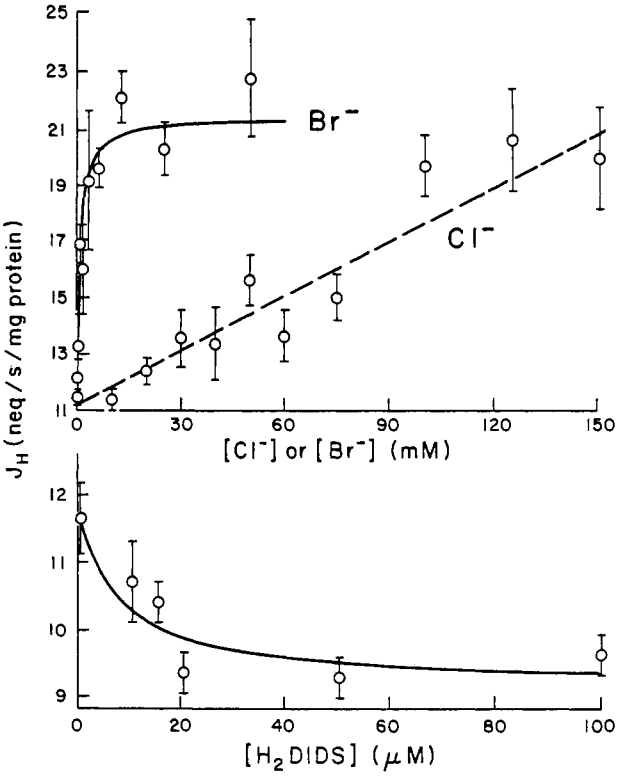

FIGURE 3: Dependence of proton permeability on anion and H_2DIDS concentrations. Top: MVV containing 6CF and valinomycin in 250 mM sucrose, 50 mM HEPES/Tris, and 150 mM potassium gluconate, pH 7.4, were suspended in 250 mM sucrose and 50 mM HEPES/Tris, pH 6.0, and 150 mM potassium gluconate and KCl or KBr. Data were fitted to a single site binding model with K_I = 1.0 ± 0.4 mM (Br^-) and >100 mM (Cl^-, 3 sets of experiments). Bottom: Proton influx in the absence of anions (as in Figure 1, −Cl^-) was measured as a function of [H_2DIDS] added immediately prior to the experiment. The fitted K_I was 11 ● 1 μM.

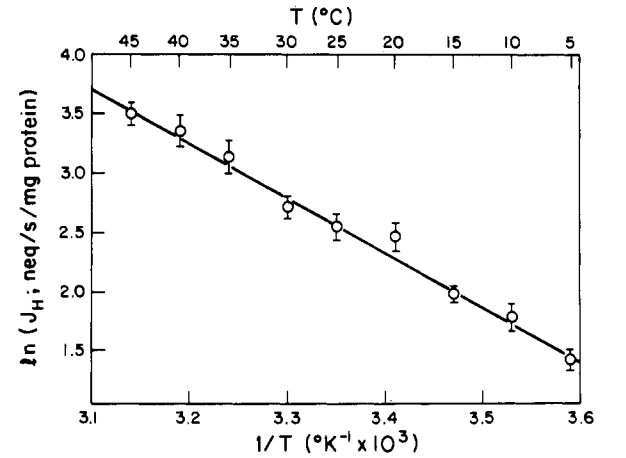

FIGURE 4: Temperature dependence of MVV proton transport. Proton flux for a 1.4 pH unit inwardly directed proton gradient was measured as in Figure 1, −Cl^-. Each point represents the mean of experiments performed in triplicate; error bars are 1 SD. The fitted line of the Arrhenius plot has an activation energy of 9.1 ± 0.3 kcal/mol.

vesicles isolated from rabbit renal cortex. We reported previously that 150 mM Cl^- had no effect on renal vesicle proton permeability (Ives & Verkman, 1986). In experiments identical with those shown in Figure 3 using renal vesicles, 30 mM external Br^- had no effect on the exponential time constant for proton influx (52 ± 5 s, 0 Br^- vs. 51 ± 5 s, 30 mM Br^-). Therefore, enhancement of proton permeability by anions is not a general feature of phospholipid membranes or of brush border membranes containing transmembrane proteins.

Table IV: pH and ΔpH Dependence of Proton Flux[a]

pH_{in}	pH_{out}	ΔpH	J_H [nequiv s^{-1} (mg of protein)$^{-1}$] −Cl^-	J_H [nequiv s^{-1} (mg of protein)$^{-1}$] +Cl^-
7.5	7.0	0.5	4.6 ± 0.1	
7.0	6.5	0.5	2.7 ± 0.2	
6.5	6.0	0.5	1.9 ± 0.2	
6.0	5.5	0.5	1.5 ± 0.1	
7.5	7.0	0.5	4.6 ± 0.1	
7.5	6.5	1.0	13.2 ± 0.5	17.3 ± 0.9[b]
7.5	6.0	1.5	14.7 ± 0.5	16.9 ± 0.2[b]
7.5	5.5	2.0	19.3 ± 0.5	18.4 ± 0.2

[a] In the first four measurements, pH_{in} and pH_{out} were varied in the absence of Cl^-. In the last four measurements, pH_{out} was changed at constant pH_{in} in the absence and presence of 150 mM KCl as described in Table I. Vesicles were voltage-clamped with 50 μg/mg of protein valinomycin. Measurements were performed in triplicate; errors are 1 SD. [b] J_H differs significantly ($P < 0.01$, unpaired t test) for measurements performed in the presence and absence of Cl^-.

The presence of allosteric regulation of proton permeability, specific inhibition by stilbenes, and anion saturability suggest that MVV proton permeability is, in part, mediated by a membrane protein. Figure 4 shows a linear Arrhenius plot for anion-independent proton transport with an activation energy of 9.1 kcal/mol. In studies of the temperature dependences for proton permeability in lipid bilayers (Rossignol et al., 1982) and in biological membranes in which a lipid pathway for proton transport is postulated (Ives & Verkman, 1985), the activation energies range from 18–25 kcal/mol, much higher than the value for proton transport in the MVV. These data support the existence of a protein-mediated proton-transport mechanism.

The nature of the MVV proton pathway was further characterized from the dependences of J_H on pH in the absence and presence of external Cl^- (Table IV). For a constant 0.5-unit pH gradient in the absence of Cl^-, J_H increased 3-fold as initial pH increased from 5.5 to 7.0. This dependence is not compatible with simple passive diffusion of H^+/OH^- because $[H^+]/[OH^-]$ decreases/increases 32-fold as initial pH varies from 5.5 to 7.0. The measured 3-fold change is consistent with saturable transport of H^+ by a protein carrier or with transport of H^+/OH^- by a lipid pathway (Cafiso & Hubell, 1983; Gutknecht, 1984; Nichols & Deamer, 1980). At constant pH_{in} in the absence of Cl^-, J_H increased almost linearly with pH, again a finding compatible with either saturable protein-mediated transport or lipid transport driven by the electrochemical H^+ potential. Interestingly, the fractional increase in J_H by external Cl^- diminishes as external pH decreases, suggesting that Cl^- binding to the external regulatory site is pH-dependent.

Discussion

The experimental data suggest that HCO_3^--independent passive proton permeability in placental microvillus membranes is in part protein-mediated. Proton transport is voltage-dependent, inhibited partially by low concentrations of disulfonic stilbenes (H_2DIDS, $K_D = 8$ μM) and has a lower activation energy (9.1 kcal/mol) than proton transport in lipid bilayers (18–22 kcal/mol). In addition, the absolute proton permeability coefficient ($P_H = J_H/[\Delta H^+]$), estimated at pH 7 from the data in Table IV, the MVV surface-to-volume ratio of 3×10^5 cm^{-1} (Bissonnette et al., 1981), and the conversion factor of 2 μL MVV volume/mg of protein, is 2×10^{-2} cm/s, much higher than P_H measured in artificial lipid bilayers (10^{-4}–10^{-8} cm/s).

Placental MVV proton transport is regulated by external anions with specificity $I^- > Br^- > Cl^- \gg SO_4^{2-}$ or PO_4^-. The anion effect is blocked by H_2DIDS. Electrogenic coupling between anion gradients and proton transport was ruled out by showing that the increase in proton transport occurred with an adequate K^+ and valinomycin voltage clamp. Electroneutral coupled transport of anions and protons was ruled out by demonstrating that (1) external Cl^- increased both the rates of proton efflux and influx, (2) proton transport was dependent only on the presence of external anions rather than on anion gradients, and (3) there was no effect of inwardly directed anion gradients on proton transport in the absence of a voltage clamp. Findings 1 and 2 also rule out the possibility of coupled electrogenic transport processes such as cotransport of two H^+ with one Cl^-. There have been no previous reports of voltage-dependent proton transporters regulated allosterically by monovalent anions similar to the transport system described in these studies.

Human erythrocyte band 3 is known to transport anions and H^+/OH^- in a 1:1 electroneutral manner (Jennings, 1978; Knauf, 1979; Pitterich & Lawaczeck, 1985). Proton transport is inhibited by disulfonic stilbenes and has activation energies of 27 kcal/mol (<13 °C) and 16 kcal/mol (>13 °C). Recently, Gunn (1986) reported that erythrocyte Cl^- coupled H^+/OH^- transport was relatively insensitive to pH (4-fold change in J_H over pH range 4–7) and had a low affinity for Cl^- ($K_{1/2} = 73$ mM, pH 5), similar to findings for the MVV proton permeability reported here. Similar electroneutral anion/proton transporters have been described in the intestinal brush border (Liedtke & Hopfer, 1982) and in the Necturus microvillus membrane (Seifter & Aronson, 1984). In the rabbit renal brush border (Seifter et al., 1984; Ives & Verkman, 1986), anion/proton coupled transport is absent, and anions do not modify the magnitude of voltage-dependent proton permeability. It is not known whether the anion/proton electroneutral systems described above mediate conductive transport, and if so, whether the conductive transport is modified by anions.

The present experiments define the role of anions in voltage-dependent proton transport but do not address directly whether anions are transported by the proton carrier or whether anion transport occurs in response to induced proton gradients. Recently Shennan et al. (1986) described an anion exchanger in placental microvillus vesicles inhibitable by DIDS ($K_{1/2} \sim 20$ μM) and furosemide (50% decrease in $^{36}Cl^-$ uptake at 100 μM). The capacities of 10 mM Cl^- or Br^- to exchange with $^{36}Cl^-$ were very similar. The proton transporter described here is also stilbene-inhibitable; however, it is not furosemide-inhibitable and has very different potencies for Cl^- and Br^-, suggesting that MVV anion exchange and proton pathways are separate.

Placental transfer is the sole means by which catabolic products are eliminated from the fetal circulation. The placental microvillus membrane and the corresponding basolateral membrane form the interface between maternal and fetal circulation. Transfer of H^+/OH^- across these membranes is important for fetal acid–base homeostasis and for transport of substances such as Na^+ and amino acids which are coupled to H^+/OH^- movement. From our studies, the placental microvillus membrane contains an electrogenic proton pathway that is regulated by external monovalent anions, in addition to an electroneutral Na^+/H^+ countertransporter. Because little is known about other ion and organic acid transport pathways in the placental microvillus and basolateral membranes, it is difficult to define the physiological importance of the microvillus proton pathway. Although maternal Cl^- concentration rarely varies more than 5–10%, the concentrations of organic

monovalent anions may vary and therefore modulate microvillus membrane proton permeability. Another possibility is that the anion-dependent proton permeability may be a developmental refinement of a transport protein which functioned initially as a Cl^-/OH^- countertransporter.

ACKNOWLEDGMENTS

We thank Floyd Rector for his support of this project, Brian Davis for helpful discussions, and James A. Dix for synthesis of DBDS.

Registry No. H_2DIDS, 61481-03-6; DIDS, 53005-05-3; H^+, 12408-02-5; Cl^-, 16887-00-6; Br^-, 24959-67-9; I^-, 20461-54-5; valinomycin, 2001-95-8.

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Properties of Chemically Modified Protein S: Effect of the Conversion of γ-Carboxyglutamic Acid to γ-Methyleneglutamic Acid on Functional Properties†

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Received February 19, 1986; Revised Manuscript Received May 20, 1986

ABSTRACT: Protein S, the protein cofactor for activated protein C in the proteolytic inactivation of factor Va, was chemically modified with a mixture of morpholine and formaldehyde. This treatment resulted in the conversion of the γ-carboxyglutamic acid (Gla) residues of this vitamin K dependent protein to γ-methyleneglutamic acid. With a 10000-fold molar excess of morpholine and formaldehyde over protein S it was found that between 10 and 11 Gla residues could be modified. The degree of modification was proportional to the concentration of the modifying reagents used. The modification of as few as two residues resulted in the 70% loss of activity. Calcium inhibited the modification of several residues. In the presence of 3.2 mM calcium ion, a derivative with 2.5 residues modified was prepared that appeared to have full activity. Modification of protein S resulted in the alteration of a number of its properties. The quenching of intrinsic fluorescence by calcium decreased. The quenching effect of terbium ions was also decreased. However, the modified protein and the native protein were equivalent when protein-dependent terbium fluorescence was measured. When modified, protein S would no longer bind to phospholipid vesicles. Finally, the ability of protein S to self-associate was decreased by modification. These findings suggest that the γ-carboxyglutamic acid residues of protein S may play several roles in the maintenance of structure.

Protein S is a vitamin K dependent protein found in blood plasma (DiScipio & Davie, 1979; Stenflo & Jonsson, 1979). It is believed to function as a cofactor for the expression of the anticoagulant activity of activated protein C (Walker, 1980, 1981b). Protein S forms a complex with activated protein C on the surface of membranes that contain negatively charged phospholipids (Walker, 1981a; Suzuki et al., 1983). This complex is thought to be the main form in which activated protein C is active as an anticoagulant that functions through the proteolytic inactivation of coagulation factors V (Walker, 1981a) and VIII (Lawrence, 1985). This is supported by the observation that plasma depleted of protein S is much less

† This work was supported by a grant-in-aid from the American Heart Association with funds contributed in part by the Indiana Affiliate. This work was also supported by Grant HL 26069-06 from the National Institutes of Health. F.J.W. is a recipient of an Established Investigatorship of the American Heart Association with funds contributed in part by the Indiana Affiliate in support of this work.
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0006-2960/86/0425-6305$01.50/0