J. Membrane Biol. 101, 237–246 (1988)

The Journal of
Membrane Biology

Electrogenic Behavior of the Human Red Cell Ca^{2+} Pump Revealed by Disulfonic Stilbenes

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Summary. A systematic study was made of the action of 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) on active Ca^{2+} transport of human erythrocytes. Pumping activity was estimated in inside-out vesicles (IOV's) by means of Ca^{2+}-selective electrodes or use of tracer $^{45}Ca^{2+}$. The stilbenes exhibited an approximately equal inhibitory potency and their action could be overcome by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at low but not at high stilbene concentrations. In the absence of DIDS, Ca^{2+} transport was not affected upon addition of valinomycin, but it was appreciably reduced when vesicles were preincubated with low DIDS concentrations. Such an effect was strictly dependent on the external K^+ concentration and it was abolished when valinomycin was added together with FCCP. Similar results were obtained using IOV's prepared from intact cells which had been previously exposed to the stilbene. The findings clearly demonstrate the presence in human red cells of a partially electrogenic Ca^{2+} pump, exchanging one Ca^{2-} ion for one proton.

Key Words electrogenic Ca^{2+} pump · red cell Ca^{2+} transport · stilbene action on Ca^{2+} pump · IOV's Ca^{2+} uptake · membrane potential and Ca^{2+} pump

Introduction

Evidence is accumulating in support of an obligatory Ca^{2+}/H^+ exchange during operation of the human red cell Ca^{2+} pump (Niggli, Sigel & Carafoli, 1982*a*; Smallwood et al., 1983). However, the stoichiometry of exchange is uncertain, some authors favoring a 1 : 1 or a 1 : 2 Ca^{2+}-to-H^+ coupling ratio (Schatzmann, 1985).

In the above context the possibility of an electrogenic activity arises. This proposal was originally suggested to account for inhibition of Ca^{2+} uptake by inside-out vesicles from human red cells (IOV's), caused by both the replacement of external anions by more impermeant species and the action of Band III inhibitors (Gimble, Goodman & Rasmussen, 1981; Waisman et al., 1981).

The latter view of a Band III-mediated effect is in conflict with early work, showing inhibition by DIDS of the Ca^{2+}-ATPase of human red cells in fragmented membranes (Waisman et al., 1982) or after being reconstituted into asolectin liposomes (Niggli et al., 1982*b*). The possibility exists then, that the action of Band III inhibitors on IOV's Ca^{2+} uptake originates from a direct pump effect. Such a possibility was investigated in the present work by studying the effect of SITS[1] and DIDS on Ca^{2+} transport by IOV's from human red cells.

On the other hand, Ca^{2+} pump activity is markedly stimulated by raising external Na^+ or K^+, as demonstrated in dextran-resealed ghosts after restoring a low permeability to alkali cations (Romero, 1981; Romero & Romero, 1982, 1984).

Furthermore, a concomitant influx of either of these cations appears correlated with the Na^+- or K^+-activated Ca^{2+} efflux. (Romero & Romero, 1982). Since such movements may result from an electrogenic pumping activity, we have also studied the effect of valinomycin and FCCP on active transport both in the presence and absence of stilbenes.

It was found that SITS and DIDS have a direct action on the Ca^{2+} pump. The findings also demonstrate that in the presence of low-stilbene concentrations, the pumping activity decreases when the membrane potential goes more positive internally, as would be expected from an electrogenic Ca^{2+} transport.

[1] *Abbreviations:* SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; FCCP, carbonylcyanide *p*-(trifluoromethoxy)-phenylhydrazone; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; DMSO, dimethylsulfoxide.

Materials and Methods

Analytical quality reagents were used whenever possible. Vanadium-free ATP (disodium salt), EGTA, DIDS, valinomycin, ouabain and FCCP were purchased from Sigma. All other reagents were obtained from the British Drug Houses, Ltd. Human blood (mainly O+ group) was collected in citrate-dextrose solution and used within 1 to 3 days after collection. The pH of all solutions was adjusted at room temperature within a range of ±0.01 units.

Preparation of Red Cells

Between 40 and 80 ml blood were contrifuged at 3,000 × *g* for 20 min at room temperature. After removing white cells, the suspension was adjusted to pH 7.6 and the erythrocytes washed 3 to 4 times with a 150-mM NaCl + 10-mM Tris-HCl (pH 7.6) medium. Cells were finally packed by centrifuging for 15 to 20 min under the above conditions.

Pretreatment of Cells with Stilbenes

In some experiments intact cells were previously exposed to DIDS, essentially as described by Grinstein, McCulloch and Rothstein (1979). Accordingly, cells packed as above were further washed twice in isotonic saline, buffered with 5 mM phosphate (pH 8.0) and then incubated for 30 min at 37°C in a fresh washing medium, containing 5 μM DIDS, after which the cells were washed thrice and packed as outlined above.

Preparation of IOV's

This was performed as referred to by Sarkadi, Szász and Gárdos (1980), but by homogenizing ten times through a 27-gauge needle. IOV's were finally resuspended in a small volume of a 160-mM choline chloride + 1-mM $MgCl_2$ + 10-mM Tris-HCl medium (pH 7.6) to obtain about 25 to 30 mg protein/ml, divided into aliquots of 200 to 400 μl and immediately stored at −70°C until use within not more than three weeks. Confirmatory experiments done on freshly prepared IOV's gave essentially the same results as those stored for three weeks, thus indicating that little if any deterioration occurred during the storage period.

Presence of Mg^{2+} in the medium used for both resuspension and storage of IOV's was found necessary in order to obtain a consistent response to ionophores.

Membrane sidedness was estimated by measuring latent acetylcholinesterase activity (Steck & Kant, 1974), using acetylcholine iodide as substrate and a double-beam Spectronic 2000 spectrophotometer. The fraction of inverted vesicles was usually between 65 and 75%.

Assessment of Ca Transport

IOV's were preincubated at 37°C for 10 min with and without stilbenes in different media whose detailed composition will be described later, after which ATP (2 mM) was added to initiate active transport. Ionophores when present, were generally added 30 sec before ATP.

Active transport was assessed by measuring the decrease in external free Ca^{2+} concentrations with selective electrodes or by determining the amount of $^{45}Ca^{2+}$ taken up by IOV's, as specified below.

Monitoring Ca^{2+} with Selective Electrodes

Between 1 and 3 mg IOV's protein were incubated at 37°C in 0.5 ml of a medium containing (mM): KCl, 160; $MgCl_2$, 2; ATP-Na_2, 2; $CaCl_2$, 0.1; HEPES (pH 7.6), 10; in a thermostatically controlled Lucite® chamber, provided with a water jacket.

The change in free Ca^{2+} was determined with commercial electrodes (Radiometer F2001 Ca^{2+} Selectrodes) or 500-μm glass minielectrodes containing a neutral Ca^{2+} ligand embedded in polyvinylchloride (Tsien & Rink, 1980). Electrode potentials were measured with an electrometer differential amplifier WPI, FD-223 and continuously registered via a pen chart recorder.

Measurements of $^{45}Ca^{2+}$ Uptake

IOV's were incubated in the presence of 100 μM $CaCl_2$ with tracer Ca^{2+} added (10,000 to 20,000 cpm/nmol) under conditions similar to those outlined above, unless otherwise specified. Samples were withdrawn at regular intervals for up to 10 min and radioactivity was assessed by liquid scintillation counting in a Packard Tricarb instrument, using Liquifluor as scintillant. The uptake was linear with time for up to 10 min under all conditions studied.

Ionophores

FCCP was added as an alcoholic solution whereas valinomycin was dissolved in DMSO.

Ca^{2+} Buffers

Calcium buffers for electrode potential standards were prepared as reported by Tsien and Rink (1980).

Protein

Protein was measured by the Lowry method (Lowry et al., 1951), using bovine serum albumin as standard.

Results

Action of Stilbenes on Ca Transport

Early work has shown that the Ca^{2+},Mg^{2+}-ATPase of human red cells is inhibited by low DIDS concentrations (5 to 10 μM) either in fragmented membranes (Waisman et al., 1982) or after being reconstituted into liposomes (Niggli et al., 1982*b*). In order to determine if disulfonic stilbenes elicit the same effect on Ca^{2+} transport at low and high concentrations as shown for the ATPase, IOV's were preincubated with either SITS or DIDS and trans-

port was immediately assessed by means of both Ca^{2+} electrodes and tracer uptake.

A typical experiment with a Ca^{2+} electrode is shown in Fig. 1. The insert demonstrates a Nernstian response of the electrode down to 1 μM Ca^{2+}. Addition of ATP (curve *a*) immediately produces a fast electrode potential change as free Ca^{2+} becomes chelated by the nucleotide. Thereafter, the potential steadily decreases at a slower rate as Ca^{2+} is taken up by IOV's. Once the uptake has been completed, addition of A23187 (1 μM) fully reverses the electrode potential change, showing that Ca^{2+} was trapped within the vesicles.

When IOV's are preincubated with a low SITS concentration (5 μM), the electrode potential decreases at a very small rate (first part of curve *c*) and it shows no change within the first 10 min after adding ATP to IOV's treated with 50 μM SITS (first part of curve *b*). The Ca^{2+} transport inhibition calculated from the mean slope in the two cases being about 40 and 100%, respectively. Similar results were obtained when SITS was replaced by equimolar DIDS concentrations (*results not shown*). Essentially identical effects of both stilbenes were also obtained by measuring $^{45}Ca^{2+}$ uptake (*results not shown*).

The findings show that SITS inhibits Ca^{2+} transport with a similar potency as DIDS. In addition, they demonstrate that the Ca^{2+} pump is not fully inhibited by a low stilbene concentration.

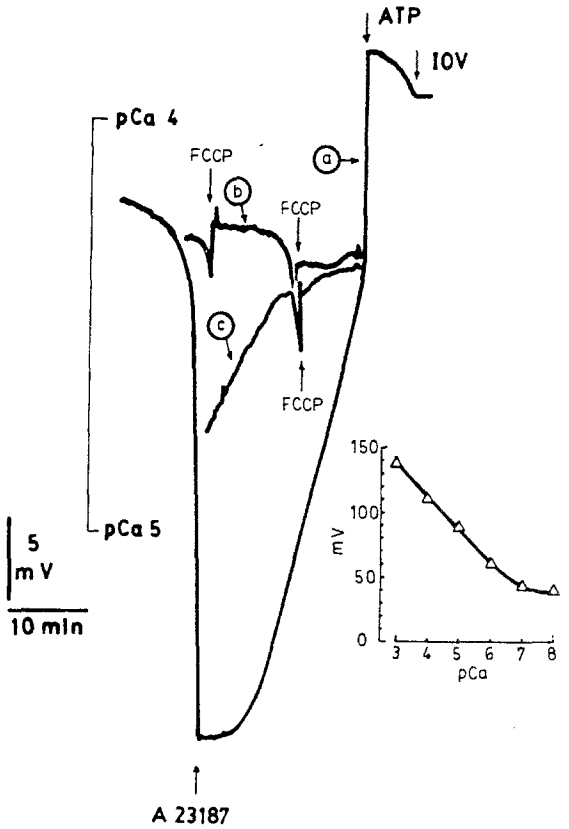

Fig. 1. Inhibition of active transport by SITS. IOV's from human red cells, prepared as described in Materials and Methods, were incubated at 37°C in a high K^+ medium in the presence of 100 μM $CaCl_2$ and different SITS concentrations. Active transport was initiated by adding ATP (2 mM) and assessed by measuring the decrease in free Ca^+ using a Ca^{2+}-selective electrode. The insert represents a typical calibration curve for the electrode. A superimposed recording is shown of the electrode potential changes attained in the absence of stilbene (curve *a*) or in the presence of 5 μM (curve *c*) or 50 μM SITS (curve *b*). For simplicity, the time origin of records (*b*) and (*c*) was eliminated and their inflection point after adding ATP (start of active uptake) was made to coincide with that of record (*a*). The indicated additions of ionophore correspond to 1 μM.

Reversal of Stilbene Inhibition by FCCP

Previous work on Ca^{2+} uptake by IOV's or reconstituted Ca^{2+},Mg^{2+}-ATPase from human erythrocytes, has shown FCCP activation under conditions compatible with an exchange for H^+ ions (Niggli et al., 1982*a*; Smallwood et al., 1983). With a view of determining if FCCP is capable of stimulating Ca^{2+} transport in the presence of stilbenes, IOV's were preincubated at 37°C with 5 and 50 μM SITS. After adding ATP, the incubation was allowed to continue for another 10 min after which FCCP (1 μM) was added.

Ca^{2+} uptake was reactivated by FCCP when IOV's were treated previously with 5 μM SITS (Fig. 1, curve *c*). In the presence of 50 μM SITS, by contrast, addition of FCCP was without effect (curve *b*). Similar findings were obtained by replacing SITS with DIDS, thus suggesting that the protonophore is effective only at low stilbene concentrations. In view of the above results, subsequent experiments were done only with DIDS.

With the interest of investigating the possibility raised above, the effect of a wide range of DIDS concentrations, varying from 0 to 100 μM, was studied. After preincubating with and without FCCP (2 μM), ATP was added and the rate of Ca^{2+} transport was determined.

Results with the different methods of assessing transport agreed well with each other. Thus, in the absence of stilbenes FCCP was stimulatory (Table 1, *also see below*). On the other hand, a marked inhibition of Ca^{2+} transport (nearly 50%) was obtained with 5 μM DIDS and a further rise in concentration to 50 μM caused full inhibition. By contrast, a much lower inhibitory effect of low DIDS concentrations (5 to 10 μM) was attained in the presence of

Table 1. Reactivity of the Ca pump towards DIDS and reversal of inhibition by FCCP.[a] Extent of active transport inhibition

DIDS (μM)	Ca^{2+} electrode (%)		$^{45}Ca^{2+}$ uptake	
	Additions to incubation medium			
	None	FCCP (2 μM)	None	FCCP (2 μM)
0	0	—	0	−22.0
5	43.5	10.0	50.0	15.0
10	58.0	35.0	61.5	31.0
20	93.0	58.0	84.0	53.5
30	99.9	—	89.0	—
50	100	100	99.0	98.0
100	—	—	100	100

[a] IOV's were preincubated for 10 min at 37°C with the various DIDS concentrations indicated above. Thereafter, ATP·Na_2 (2 mM) was added to start active Ca^{2+} transport, which was assessed by means of Ca^{2+}-selective electrodes and by determining $^{45}Ca^{2+}$ uptake. When FCCP was present, it was added 1 min before ATP. Between 0.4 and 6.0 mg IOV's protein/ml were incubated in a medium containing (mM): KCl, 160; $CaCl_2$, 0.1; $MgCl_2$, 2; ouabain, 0.1; HEPES, 10 (pH 7.6). Samples were taken at 0, 1, 2, and 5 min time intervals to measure the amount of $^{45}Ca^{2+}$ taken up, from which initial rates were calculated. The results shown are mean values from two experiments.

FCCP while at higher DIDS levels (50 μM or above) FCCP was without effect.

The present findings clearly show that FCCP only overcomes the inhibition caused by low DIDS concentrations.

pH Dependence of the Reversal of FCCP

In order to investigate if the action of FCCP just described is affected by varying external pH, IOV's were incubated with and without 5 μM DIDS and the initial rate of $^{45}Ca^{2+}$ uptake was determined at different pH values between 7 and 8.

In vesicles which had not been exposed to DIDS, FCCP was without effect at pH 8 while it enhanced Ca^{2+} uptake from about 4 to nearly 6 nmol Ca/mg IOV's protein · min by decreasing pH to 7 (Fig. 2), the stimulatory action being greater the lower the pH. Thus, Ca uptake was stimulated by about 30 and 65% at pH 7.6 and 7.0, respectively.

On the other hand, preincubation with DIDS reduced Ca^{2+} uptake by almost half, as was expected. Moreover, FCCP was again stimulatory, as revealed by the apparent lack of effect of DIDS when assayed together with the protonophore (Fig. 2). Such an action of FCCP was obtained at any pH tested and there seemed to be a slight tendency for the effect to increase as the pH was raised from 7 to 8 (Fig. 2).

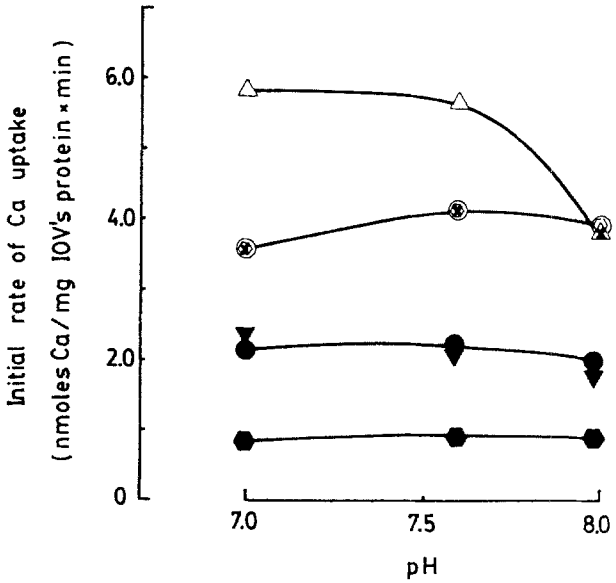

Fig. 2. pH Dependence of stimulation by FCCP and valinomycin action. Initial rates of $^{45}Ca^{2+}$ uptake by IOV's which had been incubated with (× and black symbols) or without (open symbols) 5 μM DIDS, were measured at the different pH values indicated above, as described in the legend to Table 1. The incubation medium contained 10 mM of either of the following pH buffers: imidazole (pH 7.0); HEPES (pH 7.6) and Tris (pH 8.0) and 0.4 to 0.6 mg IOV's protein/ml. Results after incubating with no ionophores (○, ●); 2 μM FCCP (△, ×); 9 μM valinomycin (○, ●) or 2 μM FCCP + 9 μM valinomycin (▼) are shown in the graph as mean values from two experiments performed with different IOV's batches. Ionophores were added 30 sec before adding ATP.

The results demonstrate that FCCP enhances Ca^{2+} transport in a pH-dependent way. The dependence encountered with DIDS-treated vesicles, however, seems the opposite of that found with untreated ones, thus suggesting that FCCP may be acting through different mechanisms.

Modulation of Ca^{2+} Transport by Altering Membrane Potentials

In order to find out if Ca^{2+} transport is affected by conditions altering the electrical membrane potential, IOV's were incubated in a high K medium with and without valinomycin (9 μM) and the rate of uptake was studied at both pH 7.6 and 5 μM DIDS, in the presence and absence of FCCP (2 μM).

In agreement with previous results, FCCP enhanced Ca^{2+} uptake by control IOV's in a statistically significant way. Moreover, valinomycin did not alter the uptake rate but seemed to reduce FCCP stimulation when added together to control IOV's (Table 2). In the presence of DIDS, as expected, Ca^{2+} transport was appreciably inhibited

Table 2. Effects of membrane potentials on Ca^{2+} uptake by IOV's preincubated with DIDS[a]

DIDS concentration (μM)	Initial rate of active Ca^{2+} uptake (nmol Ca^{2+}/mg IOV's protein · min)			
	Additions to incubation medium			
	None	FCCP (2 μM)	Valinomycin (9 μM)	Valinomycin (9 μM) + FCCP (2 μM)
0	3.85 ± 0.33 (7)	5.30 ± 0.38 (6) $P < 0.005$	3.70 ± 0.33 (6) $P > 0.1$	4.45 ± 0.40 (6) $P > 0.1$
5	2.28 ± 0.35 (18)	3.52 ± 0.15 (6) $P < 0.005$	1.00 ± 0.35 (18) $P < 0.005$	2.72 ± 0.41 (5) $P > 0.1$

[a] IOV's (between 0.4 and 0.6 mg protein/ml) were preincubated with the DIDS concentrations shown above, as described in the legend to Table 1 to determine $^{45}Ca^{2+}$ uptake. Ionophores were added 30 sec before ATP. Results are shown as mean value ±1 SD of the number of experiments given within parentheses. P denotes the probability obtained from Student's t-tests.

and restored to control levels when FCCP was added. Contrasting with the above results, valinomycin inhibited Ca^{2+} uptake by DIDS-treated IOV's in a statistically significant way and the effect was fully reversed by FCCP (Table 2). In addition, the inhibitory action of valinomycin was not affected by varying pH between 7 and 8 (Fig. 2). Reversal by FCCP, by contrast, seemed to decrease by raising pH to 8 (Fig. 2).

The findings indicate that Ca^{2+} pump activity is modified by conditions altering the electrical potential across IOV's.

Modification of Membrane Potential in IOV's from Membranes Previously Exposed to DIDS

Early work has reported a preferential action of DIDS on the Band III protein when accessed from the extracellular surface of the human erythrocyte membrane. Thus, in normally oriented vesicles, 5 μM DIDS inhibits sulfate efflux by nearly 95% in comparison with a figure of about 10% attained in IOV's with a similar stilbene concentration (Grinstein et al., 1979).

With the interest of confirming an involvement of Band III both in the inhibition of IOV's Ca^{2+} uptake caused by low DIDS concentrations and subsequent modulation of transport rate by ionophores, intact erythrocytes were previously incubated with and without 5 μM DIDS, as described in Materials and Methods. Thereafter, IOV's were prepared from these cells and the action of ionophores on Ca^{2+} transport rate was then tested. In addition, the effect of exogenous calmodulin was also investigated.

Preincubation with DIDS resulted in a diminution of Ca^{2+} transport by about 40% when compared to control IOV's (Table 3). On the otherhand, valinomycin inhibited Ca^{2+} uptake in a highly statistically significant way only in IOV's obtained from membranes previously treated with DIDS. Thus, the pumping rate of this type of vesicle was diminished by almost 65% in the presence of valinomycin (Table 3). Addition of DMSO at the same final concentration attained in the assay medium produced no significant effect on transport rate.

Confirming preceding experiments with IOV's in the presence of low DIDS concentrations, the inhibitory effect of valinomycin is fully reverted by FCCP (Table 3).

Essentially similar qualitative results were found when valinomycin was added together with calmodulin. Thus, calmodulin caused almost a threefold stimulation of IOV's Ca^{2+} uptake and valinomycin inhibited by nearly 50% the uptake rate only in IOV's derived from membranes which had been previously exposed to DIDS (Table 3).

The results clearly indicate that the action of DIDS on IOV's Ca^{2+} uptake and valinomycin in DIDS-treated vesicles are both associated to Band III inhibition.

Inhibition of Ca^{2+} Uptake by K^+ Diffusion Potentials

The above results suggest that valinomycin exerts its inhibitory action by allowing a positive potential

Table 3. Effects of membrane potentials on Ca^{2+} uptake by IOV's prepared from cells previously treated with DIDS[a]

Pretreatment with:	Calmodulin added	Initial rate of Ca^{2+} uptake (nmol/mg IOV's protein · min) after incubation with:			
		No addition	DMSO (0.5%)	Valinomycin (9 μM)	Valinomycin (9 μM) + FCCP (2 μM)
No DIDS	−	4.35 ± 0.80 (6)	4.01 ± 0.82 (4) $P > 0.1$	3.56 ± 0.74 (5) $P > 0.1$	4.03 ± 0.65 (5) $P > 0.1$
	+	12.57 ± 2.38 (7)	12.46 ± 3.10 (6) $P > 0.1$	9.37 ± 2.69 (5) $P > 0.1$	n.t.
5 μM DIDS	−	2.56 ± 0.59 (6)	2.28 ± 0.49 (5) $P > 0.1$	0.89 ± 0.33 (5) $P < 0.005$	3.45 ± 0.43 (6) $P > 0.1$
	+	8.86 ± 2.03 (9)	8.87 ± 1.81 (7) $P > 0.1$	4.72 ± 1.49 (9) $P < 0.005$	n.t.

[a] Intact cells were preincubated with 5 μM DIDS for 30 min at 37°C, after which IOV's were subsequently prepared. Vesicles (0.4 to 0.45 mg IOV's protein/ml) were incubated with the additions shown above, under conditions described in the legend to Table 1. Calmodulin was added to a concentration of 0.013 to 0.06 μg/μg IOV's protein. Results are shown as mean values ±1 SD of the number of experiments given within parentheses. *P* is the Student's *t*-test probability. n.t. stands for not tested.

to be developed within DIDS-treated IOV's. If this were the case then, there should be a tight correspondence between the magnitude of inhibition and that of the associated membrane potential.

Accordingly, diffusion potentials of variable magnitude were developed by both incubating with valinomycin (9 μM) and increasing external K^+ concentrations. The effect on transport was assessed thereafter by measuring the rate of $^{45}Ca^{2+}$ uptake in the presence of low DIDS concentrations.

The K^+ content of the IOV's used in these experiments was just about 1.2 mM before incubation. This value was obtained by assuming that all the K^+ was inside the vesicles (in a choline medium) and using a value of 9 μl/mg protein for the vesical volume, as reported by Sarkadi et al. (1980). The above concentration is similar to that present in the medium employed for preparing the vesicles (namely 1 mM), thus suggesting that a low K^+ permeability was attained.

It is also of interest to point out that the action of valinomycin was studied at different protein concentrations, originally with the intention of optimizing conditions for experiments. Unexpectedly, it was found that DMSO (used as dissolvent of valinomycin) at concentrations between 0.25 and 0.5%, dramatically decreased the amount of Ca^{2+} taken up by IOV's at low protein concentrations, thus obscuring an effect of valinomycin (Fig. 3*a*). By contrast, DMSO had practically no effect at 0.4 mg IOV's protein/ml. For such a reason, the experiments reported here were done with a final protein concentration at or above that level.

As originally described by Sarkadi, MacIntyre and Gárdos (1978), the rate of Ca^{2+} uptake was enhanced by external K^+, roughly increasing by about 70% by raising K^+ from about 1.6 to 160 mM (*results not shown*). On the other hand, the inhibitory action of valinomycin was strictly dependent on the presence of external K^+. A linear relationship was found between the magnitude of effect and the log of the K^+ concentration up to 160 mM (Fig. 3*b*), as would be expected from a Nernstian response of a K^+ electrode.

These results clearly show that Ca^{2+} transport is markedly inhibited when unfavorable K^+ diffusion potentials are originated inside IOV's.

Discussion

The electrogenic nature of active Ca^{2+} transport in human red cells is a matter of controversy. Thus, some authors have suggested that the pump catalyzes an electroneutral exchange of Ca^{2+} for protons (Niggli et al., 1982*a*; Smallwood et al., 1983). By contrast, others favor the idea of an electrogenic pump, whereby uncoupled Ca^{2+} extrusion occurs

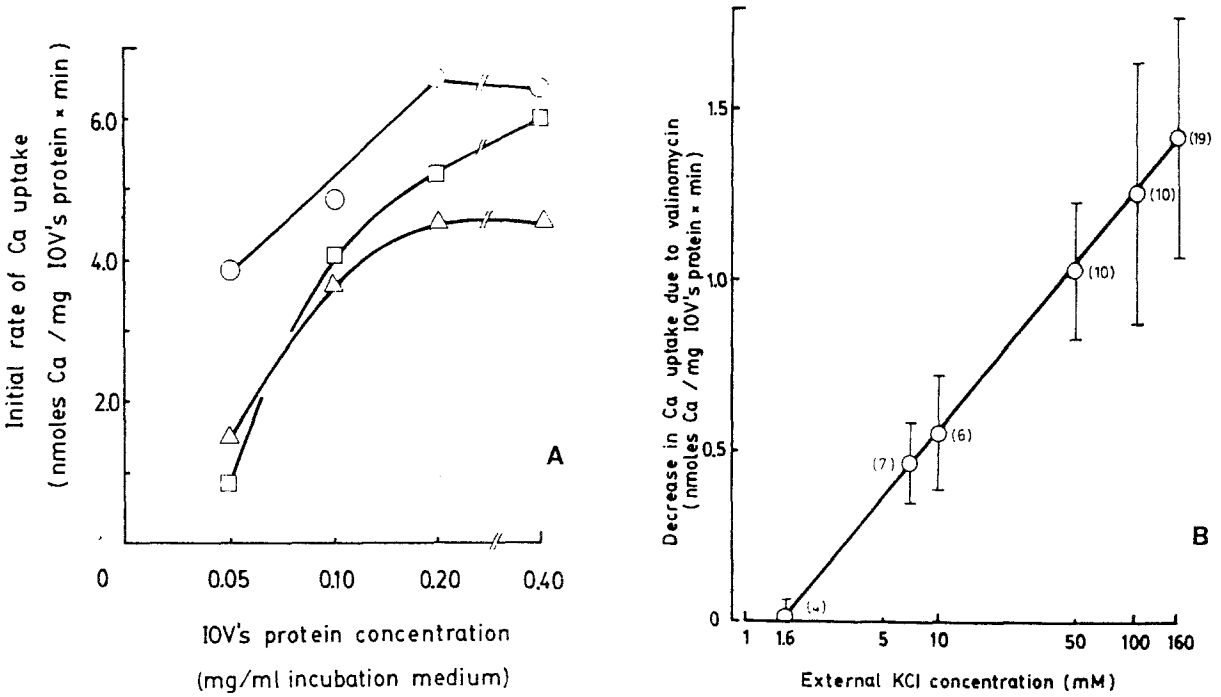

Fig. 3. Valinomycin inhibition of Ca^{2+} uptake by DIDS-treated IOV's. Initial rates of $^{45}Ca^{2+}$ uptake were measured at pH 7.6 in the presence of 5 μM DIDS, as described in the legend to Table 1. Part (*a*) shows results obtained with different protein concentrations after incubating in a high K^+ medium with no additions (○), 9 μM valinomycin (△) or 0.5% (vol/vol) DMSO (□), which was the final DMSO concentration attained in the assay medium after valinomycin addition. Part (*b*) presents results from different experiments (number in parentheses), where the inhibition of Ca^{2+} uptake by valinomycin was assessed in various media containing 0.4 to 0.6 mg IOV's protein/ml and increasing K^+ concentrations. Isotonicity was maintained by replacing osmotically equivalent amounts of choline for K^+. Results are given as mean values ±1 SD of mean (vertical bars)

(Gimble et al., 1981; Waisman et al., 1981; Gimble et al., 1982; Rossi & Schatzmann, 1982; Romero & Romero, 1984).

A crucial argument in the above context is the fact that Ca^{2+} uptake by IOV's is inhibited by reagents for amino groups, such as SITS and DIDS. This effect has been currently ascribed to specific blockage of the Band III anion channel. According to this view, Ca^{2+} transport inhibition originates from the IOV's inability in dissipating either the electric gradient created by an electrogenic uptake or the pH gradient resulting from an obligatory exchange for protons.

The results to be discussed below clearly indicate that although the Ca^{2+} pump is inhibited directly by the above stilbenes, it is still electrogenic and therefore sensitive to blockage of the red cell anion channel.

Inhibitory Action of Disulfonic Stilbenes

The present work has demonstrated that both SITS and DIDS inhibit Ca^{2+} uptake in the same concentration range that affects the Ca^{2+},Mg^{2+}-ATPase (Niggli et al., 1982*b*; Waisman et al., 1982), thus suggesting that their effect is largely due to a direct interaction with the Ca^{2+} pump, as was already pointed out by others (Niggli et al., 1982*b*). This statement applies only if stilbenes have access to the cytoplasmic aspect of the Ca^{2+} pump, where both catalytic and high-affinity Ca^{2+} binding sites are located.

The same inhibitory action of the stilbenes was also observed on IOV's prepared from cells which had been previously incubated with DIDS, under conditions favoring a covalent reaction with external membrane sites (Grinstein et al., 1979). Since neither SITS nor DIDS affect the Ca^{2+},Mg^{2+}-ATPase when the inner membrane surface is preserved from exposure to stilbenes (Waisman et al., 1982), pump inhibition by externally added DIDS must be associated to an indirect effect arising from interactions with the Band III protein (Knauf et al., 1977; Cabantchik, Knauf & Rothstein, 1978), as will be discussed below.

Effect of FCCP on Ca^{2+} Transport

An interesting finding was that FCCP stimulates the Ca^{2+} pump at concentrations that make membranes

permeable to H^+ ions. This effect was observed both in the absence or presence of low stilbene levels (about 5 μM). By contrast, at higher DIDS concentrations (50 μM or more) FCCP is without any effect. Such an activating effect in the absence of stilbenes was shown previously on the purified red cell Ca^{2+}-ATPase reconstituted into asolectin liposomes (Niggli et al., 1982; Villalobo & Roufogalis, 1986).

The results suggest that DIDS exerts a dual action depending on its concentration. At low levels, the effect may be related to Band III inhibition while at high concentrations it can be ascribed to full pump inhibition.

Nature of Activation by FCCP

The red cell pump operates as an obligatory exchanger of Ca^{2+} for protons (Niggli et al., 1982*a*; Smallwood et al., 1983; Villalobo & Roufogalis, 1986). Accordingly, stimulation by FCCP would occur when the pump becomes rate limited by reduced protons availability.

We are not certain if the IOV's presently used reached equilibrium with the storage medium (160 mM choline chloride + 10 mM Tris-HCl; pH 7.4). The pH dependence of Ca^{2+} uptake suggests an IOV's pH of 8 (*see* Fig. 2), identical to that at which they were prepared. Furthermore, their K^+ concentration is very similar to that of the vesiculation medium, thus suggesting that vesicles did not equilibrate with the storage medium. In such a case, IOV's are likely to be weakly pH buffered.

In the absence of a pH gradient, protons for the exchange during pump operation would be provided by water dissociation. In vesicles weakly H^+-buffered, the interior would become alkaline as Ca^{2+} is pumped in, the effect being more evident in DIDS-treated IOV's, where the anion channel is blocked. Under these conditions, Ca^{2+} uptake would also be diminished due to scarce H^+ ions availability. Addition of FCCP, would therefore reactivate Ca^{2+} transport by both providing protons and dissipating the pH gradient.

Consistent with the above view, a decrease in external pH was found to enhance the effect of FCCP. This is an expected behavior for a Ca^{2+}/H^+ exchanger since more protons are made available to the pump by the ionophore as the H^+ gradient is increased. In the presence of DIDS, by contrast, the pH dependence of FCCP activation is absent.

The latter finding suggests that FCCP is not only dissipating a pH gradient in DIDS-treated IOV's. It seems possible that by allowing protons to move down their electrochemical gradient, FCCP would dissipate an unfavorable membrane potential created by an electrogenic pumping activity, as may arise from a one-for-one Ca^{2+}/H^+ exchange. Such a possibility was suggested recently to account for by the effect of some ionophores on Ca^{2+} uptake by the reconstituted human red cell enzyme (Villalobo & Roufogalis, 1986).

An alternative explanation for the action of FCCP may arise from the following considerations. During Ca^{2+} transport, the translocating site must reduce its affinity in order to release Ca^{2+} within IOV's. It is likely that the affinity of this site increases with a rise in pH, as shown for the sarcoplasmic reticulum Ca^{2+} pump (Verjoski-Almeida & de Meis, 1977). As alkalinization of IOV's would restrain Ca^{2+} release from the translocating site, it is thus expected that by dissipating the pH gradient with FCCP, Ca^{2+} transport would become enhanced.

Conductive Anion Permeability in DIDS-Trested IOV's

The involvement of Band III in a dual action of DIDS on IOV's that were exposed to the stilbene after preparation, might seem questionable on the basis of both its sidedness of effect and the electrogenicity of the catalyzed ion transfer. Nonetheless, there are pieces of evidence in support of such a view. Thus, albeit the anion channel mainly promotes electroneutral exchanges, it is not electrically silent as net fluxes occur through it (Knauf et al., 1977; Cabantchick et al., 1978).

On the other hand, DIDS is known to inhibit the anion exchanger of human red cells mostly from the outside. However, it is slightly inhibitory when added to the cytoplasmic side (Grinstein et al., 1979). Finally, the inhibition of Ca^{2+} uptake found with IOV's prepared from cells which had been reacted previously with DIDS, is strong evidence in favor of an involvement of the anion channel in modulating the pump rate.

Inhibition of the Ca^{2+} Pump by Valinomycin

Perhaps the most important result and contrary to previous work (Niggli et al., 1982*a*) is that valinomycin in the presence of K^+ gradients exerts a highly significant inhibitory effect on Ca^{2+} transport only if IOV's are treated with DIDS (Tables 2 and 3). The effect was strictly dependent on the presence of external K^+ and a linear relationship was found between the extent of inhibition and the log of the K^+ concentration.

The findings undoubtedly demonstrate that the action of valinomycin arises from its ionophoric

properties, thereby setting up internally positive K^+ diffusion potentials, capable of counteracting active Ca^{2+} transport (*see* Fig. 4). These potentials would be effectively developed in DIDS-treated IOV's, where conductive anion permeability can be blocked by the stilbene. If calculations are made with the above-cited values, it turns out that the pumping rate is reduced by about 55% by setting up a +100 mV diffusion potential inside IOV's.

It might be argued that the combined action of valinomycin and K^+-gradients described above, may arise from an indirect effect of the electrical potential on an electroneutral H^+/Ca^{2+} exchanger. Accordingly, as a positive potential is being set up within IOV's, protons would start to move along its electrochemical gradient. Such a situation would inhibit Ca^{2+} uptake by both reducing H^+ availability and alkalizing the IOV's interior. It is therefore expected that there be a marked dependence on pH of the valinomycin effect. Obviously, this was not the case when pH was decreased from 8 to 7 (Fig. 2). Furthermore, if protons were approaching equilbrium under the above conditions, addition of FCCP would inhibit Ca^{2+} uptake even more instead of reactivating it.

It is clear that the effect of valinomycin described above is consistent with the operation of an electrogenic but not electroneural H^+/Ca^{2+} exchanger.

The present results with valinomycin are in conflict with those reported recently by Villalobo and Roufogalis (1986) for the reconstituted red cell Ca^{2+},Mg^{2+}-ATPase. The authors found 80 to 90% increase in the rate of ATP hydrolysis after adding valinomycin in the presence of K^+. The discrepancies may arise from the fact that they did not measure initial rates of hydrolysis but rather activity after a prolonged incubation period, where K^+ diffusion potentials are likely to become dissipated.

Partial Electrogenicity of the Ca^{2+} Pump

The above results taken together, strongly suggest that the Ca^{2+} pump of human red cells operates under a partially electrogenic mode, presumably arising from an equimolar Ca^{2+}/H^+ exchange. In this way, the pump would be directly stimulated by any permeable cation moving downhill, as seems to occur by raising external Na^+ or K^+ in dextran-resealed ghosts (Romero, 1981; Romero & Romero, 1982, 1984).

The electrogenic behavior of the Ca^{2+} pump clearly accounts for the different pH dependences of FCCP stimulation found in the presence or absence of DIDS. Accordingly, in addition to providing protons for the exchange, the ionophore may originate a H^+ diffusion potential in DIDS-treated IOV's which would counteract Ca^{2+} uptake as external pH is decreased (*see* Fig. 2). Ca^{2+} pump stimulation would result from a compromise between an unfavorable electrical potential and the necessity of H^+ for an obligatory exchange.

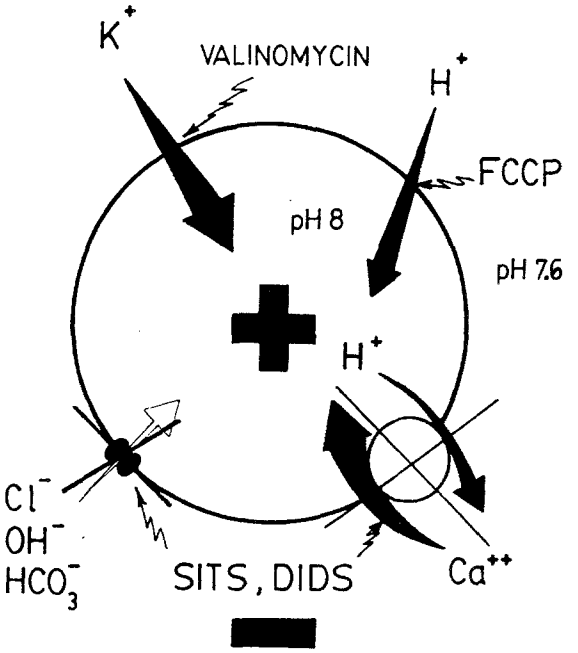

Fig. 4. Diagrammatic representation of the action of various modifiers on Ca^{2+} uptake by IOV's. The pump operates in a partially electrogenic way, exchanging one Ca^{2+} ion for one proton. The electric potential gradient thus produced is normally dissipated by rapid anion influx or counterflow of a permeable cation (Na^+ or K^+). Stilbenes block Band III anion channel, thereby reducing anion permeability at low concentrations and inhibit directly Ca^{2+} pump at higher concentrations. Valinomycin would reduce active transport by setting up K^+ diffusion potentials in stilbene-treated IOV's. FCCP would stimulate Ca^{2+} transport by providing protons for the exchange in the absence of stilbenes, whereas in their presence, transport enhancement would occur by dissipating unfavorable membrane potentials produced by electrogenic pumping activity

This view is compatible with the findings of Smallwood et al. (1983), who showed that FCCP markedly stimulates Ca^{2+} uptake by DIDS-treated IOV's only when incubated in the presence of lipophilic anions such as thiocyanate or nitrate, but has no effect in the presence of anions that penetrate the membrane or neutral acids, as acetate or maleate.

The authors wish to acknowledge financial support from the CDCH of UCV (Grants NO. C-03.2/82; C-04.6/86). They are also grateful to Drs. M. Alfonzo and E. Leal for their generous gift of $^{45}Ca^{2+}$ and Millipore filters, and the Blood Bank Unit of Maternidad Concepcion Palacios for kindly providing the blood. The skillful technical assistance of Mrs. E. Romero in some parts of the work is also acknowledged.

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Received 6 August 1987; revised 12 November 1987