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IS THE RED CELL CALCIUM PUMP ELECTROGENIC?

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SUMMARY

1. In inside-out vesicles of high potassium permeability, prepared from human red cell membranes, volume changes accompanying the action of the Ca^{2+} pump were measured by recording the intensity of light scattered by a suspension of these vesicles. Replacing Cl^- by the impermeant gluconate anion changed swelling into shrinking.

2. Assuming that in Cl^- media two Cl^- ions accompany one Ca^{2+} ion moved by the pump and in gluconate media two K^+ ions are exchanged for one Ca^{2+} ion resulted in a good agreement between relative Ca^{2+} transport rate obtained from the volume change and from direct measurement of ^{45}Ca uptake in the two media.

3. The fact that it is possible to change co-transport of Ca^+ with Cl^- into counter-transport of Ca^{2+} for K^+ rules out that within the pump there is an obligatory coupling of Ca^{2+} movement with movement of another ion species (including the proton). The conclusion, therefore, is that the Ca^{2+} pump must be electrogenic.

4. The combination of measurement of volume change with direct measurement of ^{45}Ca movement yielded 5–6 μl./mg protein for the volume of the vesicles.

INTRODUCTION

It seems a pertinent question whether the red cell calcium pump moves free Ca^{2+} ions across the plasma membrane without providing compensation of charge (electrogenic performance) or whether it exchanges Ca^{2+} for two monovalent cations such as Na^+ or K^+ or transports Ca^{2+} paired with two monovalent anions such as Cl^-. An exchange for Mg^{2+} has been ruled out (Schatzmann, 1975), but Na^+ and K^+ are potential exchange partners in view of the rate of Ca^{2+} transport and ATP hydrolysis being dependent on the presence of Na^+ or K^+ (Sarkadi, Szasz, Macintyre & Gardos, 1978; Sarkadi, Szasz & Gardos, 1980; Romero, 1980; Schatzmann, 1970; Schatzmann & Rossi, 1971; Bond & Green, 1971).

For red cells in a physiological medium the question cannot readily be answered because the rapid passage of Cl^- and HCO_3^- across the non-exchange-limited anion channel short circuits any possible potential set up by the pump. Waisman, Gimble, Goodman & Rasmussen (1981*b*) have recently shown that replacing Cl^- by an impermeant anion (gluconate) drastically slows the rate of Ca^{2+} pumping. This seems to rule out an obligatory, coupled exchange for cations, provided that removal of Cl^- is not as such detrimental to the pump or the Ca^{2+} barrier in the membrane. By adding

phosphate to inside-out red cell vesicles in gluconate medium they could accelerate Ca^{2+} transport (Waisman, Gimble, Goodman & Rasmussen, 1981*a*). Observing a reduction in Ca^{2+} transport rate by poisons renowned to be specific inhibitors of the band III anion transport system, they concluded that phosphate was driven through the anion channel by a potential created by the Ca^{2+} pump.

The following presents an attempt to answer the question by assessing the number of osmotically active particles changing side during Ca^{2+} pumping in inside-out vesicles (i.o.v.s.) made from human red cell membranes. This can conveniently be done by measurement of volume changes via recording changes in intensity of scattered light emitted by a suspension of vesicles. The method of Kometani & Kasai (1978, 1980), used for the same purpose in vesicles from sarcoplasmic reticulum, was adapted to i.o.v.s. When i.o.v.s pump Ca^{2+} into their interior they will swell and light scattering will decrease, provided that Ca^{2+} is accompanied by two Cl-ions. If one Ca^{2+} were exchanged for two alkali cations the vesicles would shrink and light scattering would increase. If swelling in a Cl^- medium turns into shrinking in a medium with an impermeant anion (gluconate), the argument is as follows. Swelling rules out an exchange with other cations and ascertains that it is $CaCl_2$ which enters the vesicles. Shrinking demonstrates that a Ca^{2+}–cation exchange can be artificially induced which excludes the possibility that the Cl^- entry in the first instance was due to a coupling of Ca^{2+} and Cl^- in the pump mechanism. The only possibility remaining then is that Cl^- normally moves, driven by the potential difference set up by the Ca^{2+} pump, through a distinct passive channel.

In a first step it is shown that change in light scattering is consistently related to volume change (brought about by altering the osmolarity ratio across the membrane) and it is demonstrated that entry of different salts can be followed by recording intensity of scattered light and that from such records a rough estimate of their relative permeation times may be obtained from which relative permeabilities of anions and cations involved may be derived. These are required for the interpretation of the calcium experiment. In a second step the Ca^{2+} pumping experiment is presented and its outcome compared to the result obtained by measuring Ca^{2+} movements directly by aid of ^{45}Ca under the same conditions.

METHODS

Inside-out vesicles (i.o.v.s) were prepared from human blood cells obtained from the blood donor service of the Swiss Red Cross one day after collection into acid citrate-glucose solution. Red cells were washed four times in a 154 mM-NaCl solution and the white cells thereby discarded.

Cell membranes were prepared as described previously (Schatzmann, 1973, second method, without freezing). From these *i.o.v.s* were prepared essentially according to the procedure of Steck & Kant (1974) in 0·5 mM-Tris-Cl (pH 8·5 at 22 °C) with 0·05 mM-dithiothreitol and separated from larger cell debris by centrifugation on a dextran cushion (density 1·03 g/cm^3; 8·01 g dextran/100 ml., 0·5 mM-Tris-HCl, 0·05 mM-dithiothreitol, pH 8·5 (22 °C)). The *percentage of inside-out vesicles* was estimated by measuring the acetylcholine esterase activity liberated by Triton X-100. According to this criterium the percentage was on average $52·7 \pm 2·3\%$ ($n = 10$). The method consisted of measuring the rate of acid production in a pH-stat apparatus in the presence of 100 mM-acetylcholine before and after adding 0·165% Triton X-100.

At the end of the preparation the vesicles were suspended in (mM): Tris-Cl, 0·5 (pH 8·5 at 22 °C); dithiothreitol, 0·05; plus some dextran. They were equilibrated first with a chloride or gluconate medium of approximately 114 m-osmole and then with one of about 23 m-osmole as follows.

'*Chloride vesicles*' were prepared by washing the vesicles twice with 100 volumes of a solution containing (mM): KCl, 50; $MgCl_2$, 4; $CaCl_2$, 0·2; Tris-Cl, 1 (pH 7·4 at 37 °C). They were stored for 2 days at 4 °C in this medium at a concentration of 2·0–2·8 mg of total protein per ml. Before the experiment they were incubated for 30 min at 37 °C at a concentration of 50–70 μg protein/ml. in a medium containing (mM): KCl, 2; $MgCl_2$, 4; $CaCl_2$, 0·2; Tris-Cl, 1 (pH 7·4 at 37 °C).

'*Gluconate vesicles*' (*chloride-free*) were prepared from purified vesicles by washing three times with 100 volumes of MOPS-Tris 1 mM (pH 7·4 at 37 °C) and twice with 100 volumes of a solution containing (mM): K-gluconate, 50; Mg-gluconate, 4; Ca-gluconate, 0·5; MOPS-Tris, 1 (pH 7·4 at 37 °C). They were stored in the same medium for two days at 4 °C. Before the experiment the vesicles were incubated for 30 min at 37 °C at a concentration of 50–70 μg protein/ml. in a medium containing (mM): K-gluconate, 2; Mg-gluconate, 4; Ca-gluconate, 0·5; MOPS-Tris, 1 (pH 7·4 at 37 °C).

The osmolarity of the final external solution was approximately 23 m-osmole. In this medium the vesicles were either transiently strongly distended or ruptured and resealed. At any rate after 30 min incubation they were at equilibrium with the solution because there was no drift in volume after 30 min.

Uptake of ^{45}Ca into i.o.v.s. Vesicles were incubated in different media for 30 min at 37 °C. Then ^{45}Ca was added (specific activity 4–17 × 10^5 counts/min. μmole) and 5 min later the reaction was started by addition of Na-ATP (0·9 mM). Samples were taken in duplicate and filtered immediately through millipore filters with a pore diameter of 0·45 μm, which retained all protein. The filters were washed twice with 4 ml. of a cold solution of 50 mM-MOPS-Tris, pH 7·4 at 37 °C. The filters were transferred to counting vials, disolved in Cellosolve (2-ethoxyethanol) and radioactivity counted in 10 ml. xylene-Triton X-100 (1 :1) containing 0·4 % Omnifluor (NEN). Composition of media is given in the legends to the Figures.

Measurement of volume change by light scattering. The volume change of vesicles was investigated by measuring the intensity of scattered light (436 nm) at 120 deg to the incident beam using the fluorescence attachment of an Eppendorf photometer. The vesicle suspension (50–70 μg protein/ml.) was exposed to the light beam in a 1 cm glass cuvette kept at 37 °C. Light intensity was measured in an arbitrary scale with zero at the intensity coming from the cuvette without vesicles.

In order to establish the relation between the optical response and a known change in volume the osmotic pressure difference across the membrane was changed by three different methods which gave identical results (see Fig. 1). (1) Vesicles were pre-incubated for 24 hr at 4 °C in solutions of various tonicities containing K-gluconate, MOPS-Tris 1 mM (pH 7·4 at 37 °C) with or without $CaCl_2$ 0·5 mM at 60 μg protein per ml. 1 ml. of the suspension was transferred to the cuvette and, after reading the light intensity (I_0), was mixed with 30 μl. of a concentrated K-gluconate solution such that the final tonicity of the medium was brought to either 22·4, 153·2 or 154·7 m-osmole. Mixing took about 5 sec; the new light intensity was immediately read and the observed ΔI corrected for the change of light intensity obtained by the same dilution with an isotonic medium. (2) I.o.v.s were pre-incubated for 30 min in a solution containing (mM): KCl, 2; $MgCl_2$, 4; $CaCl_2$, 0·5; Tris-Cl, 1 (pH 7·4 at 37 °C); 120 μg protein/ml. 0·5 ml. of the suspension was mixed with different amounts of water and the light intensity noted. Then the experiment was repeated with the same volume of isotonic solution (the pre-incubation medium). The difference in light intensity between the two experiments was taken as the change in light intensity due to the hypotonicity. (3) I.o.v.s were pre-incubated for 30 min in a solution containing (mM): KCl, 2; $MgCl_2$, 4; $CaCl_2$, 0·5; Tris-Cl, 1 (pH 7·4 at 37 °C); 60 μg protein/ml. Then 1 ml. was mixed with 5 μl. of KCl (mM): 100, 200, 300, 500, 1000, 2000, respectively, and the immediate change of light intensity due to shrinking was noted. The ensuing slow entry of KCl was followed by recording the light intensity until the curve approached an asymptotic value (see insert, Fig. 1). The difference between the immediate intensity and that corresponding to the asymptote was taken to reflect the volume change. For swelling vesicles were pre-incubated for 30 min in the same medium but at 120 μg protein/ml. 0·5 ml. of the suspension was mixed with an equal volume (which brought the protein concentration to 60 μg/ml.) of different dilutions of the above medium (eventually pure water) giving a series of different hypotonicities. The slow escape of the KCl was recorded and the difference in light intensity between the immediate value and the asymptotic value used as indicator of the volume change (see insert, Fig. 1).

Optical measurement of accumulation of calcium by action of the Ca pump was performed essentially in the same way (for medium see legends to Figs. 2 and 4). However, here the volume

change took place only in the i.o.v.s whereas in the osmotic shrinking and swelling all tight vesicles took part. Thus in order to derive absolute values for the pump-induced volume changes from the osmotic calibration the percentage of i.o.v.s in the vesicle population had to be taken into account. Electronmicrographs showed that nearly all the material was vesiculated. The assumption is made that inside-out and outside-out vesicles attained the same permeability to ions such that their osmotic response was the same. The experiments were initially carried out in the presence of 0·125 mM-digitoxin. When it turned out that there is no interference from the Na–K-pump, digitoxin was omitted from the media.

Calculation. Relative change in light intensity is $\Delta I/I_0$ and relative volume change is $\Delta V/V_0$. If the linear relation holds then

$$\frac{\Delta I}{I_0} = k.\frac{\Delta V}{V_0}. \tag{1}$$

Relative volume change is obtained by measuring I_0 and ΔI if k is known. *Calibration*: in vesicles with ideal osmometric behaviour whose solids may be neglected the relative volume change is

$$\frac{V_x - V_0}{V_0} = \frac{\Delta V}{V_0} = \frac{To}{T_x} - 1 \tag{2}$$

where V_0 is the volume at original equilibrium (tonicity of the medium and inside the vesicles T_0) and V_x is the volume at the new distribution of water after T_0 has been changed to T_x. Or with eqn. (1)

$$\frac{\Delta I}{I_0} = k.\left(\frac{T_0}{T_x} - 1\right). \tag{3}$$

From a plot of $\Delta I/I_0$ *vs.* T_0/T_x, k may be found (Fig. 1).

Pump action. If the pump moves osmotically active particles across the membrane an apparent internal change in tonicity (relative to the original volume) is reflected by the volume change observed. The external tonicity remains constant (because the external volume is large). Thus $T_{in,app}/T_{out}$ can be substituted for T_0/T_x in eqn. (3). Inserting the numeric value for k in eqn. (3) then gives $T_{in,app}/T_{out}$ and this is the osmotic equivalent of salt transported by the pump per litre of original vesicle volume as fraction of the external tonicity (which is known). k is the slope of the calibration curve shown in Fig. 1.

($Ca^{2+} + Mg^{2+}$)-ATPase activity was measured as described previously (Schatzmann, 1977), phosphate according to Berenblum & Chain (1938).

The osmotic activity of the different salts used was measured by comparing their freezing point depression to that of known standards of KCl at 60 mM. It is assumed that at low concentrations 1 mM-KCl corresponds to 2 m-osmole. Na_2-ATP was assumed to yield three particles in solution. ATP hydrolysis was less than 3 % of the added concentration (0·9 mM) in 15 min and was neglected.

Protein was measured according to Lowry, Rosebrough, Far & Randall (1951).

Ionophore A 23187 was a gift from Ely Lilly Inc. and was dissolved in dimethylsulphoxyde (DMSO). The final concentration of DMSO was 0·5 %. Digitoxin (Fluka) was dissolved in ethanol. The final ethanol concentration in the samples was 1 %. Valinomycin (Sigma or Fluka) in ethanol was added to samples giving 1 % final ethanol concentration. All other chemicals were Analytical Grade from Merck, Fluka or Sigma. Tap water was passed over an ion exchange column and then distilled in a Scorah all glass still.

RESULTS

Volume calibration of the optical system and ionic permeabilities

Fig. 1 is a plot of the relative change in intensity of scattered light *versus* the ratio of original tonicity (T_0, with which the vesicles are at equilibrium) and final tonicity (T_x) of the medium. The tonicity of the medium was changed from T_0 to T_x by the different techniques described in Methods. Above and below $T_0/T_x = 1$ the result is approximated by two different linear regression lines through the origin ($\Delta I/I_0 = 0$ at $T_0/T_x = 1$), i.e. the optical response is not symmetrical for volume changes above and below resting volume. This is not surprising, since electronmicrographs (not

shown) reveal that at osmotic equilibrium the vesicles are not spheres but doubled-up bags with a cleft-like inner space. The precision of the measurements is not high enough to decide whether the shown regression lines through the origin are adequate or whether a line with a slight upward curvature near $T_0/T_x = 1$ would make a better fit to the experimental points. If the linear regression is accepted as an approximation,

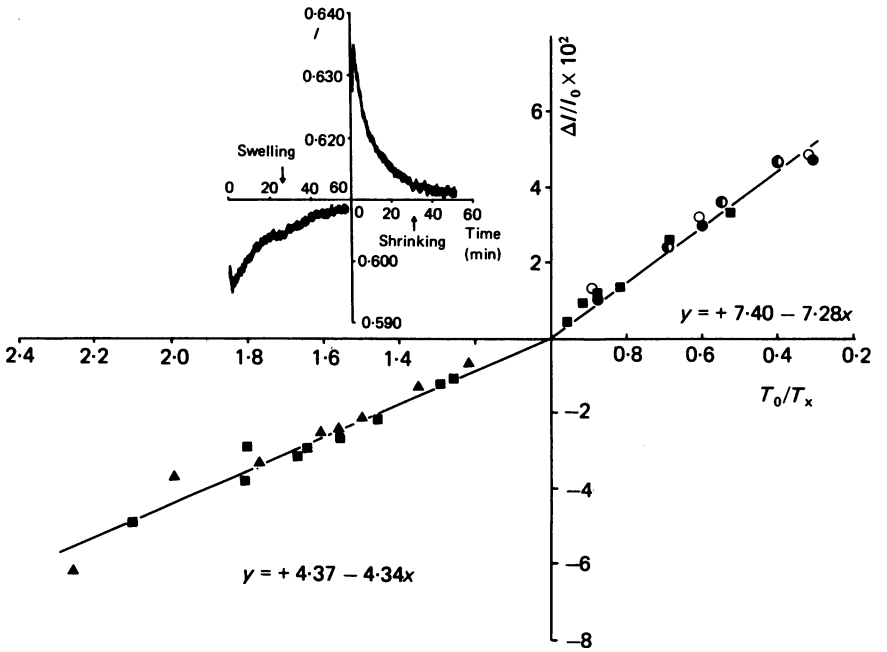

Fig. 1. Dependence of change of intensity of scattered light on osmotically induced volume change in red cell membrane vesicles. Insert: illustration of the experiment (technique 3 in Methods). At zero time the medium is diluted (left) or concentrated (right). Light intensity (I, arbitrary scale) rises abruptly and later falls due to passage of salt. The difference between the asymptotic value and the extrapolated intercept on the I axis is ΔI appearing in the main graph. Main part. Abscissa: ratio of tonicity of medium before (T_0) and after (T_x) the step change. Ordinate: relative change in intensity of scattered light. Circles, technique 1 (○ total tonicity 153·2 m-osmole, no Ca^{2+}; ●, total tonicity 154·7 m-osmole, 0·5 mM-$CaCl_2$; ◐ total tonicity 22·4 m-osmole, 0·5 mM-Ca-gluconate); ▲, technique 2; ■, technique 3 (see Methods). Left side: swelling; linear regression line through $\Delta I/I_0 = 0$, $T_0/T_x = 1$, $r = 0{\cdot}99$, slope $= k = 4{\cdot}34$ (eqn. (3)). Right side: shrinking; linear regression through same origin, $r = 0{\cdot}99$, slope $= k = 7{\cdot}28$ (eqn. (3)).

it may be seen that the proportionality factor (k) of eqn. (3) is 4·34 for swelling and 7·28 for shrinking. The fact that the optical response to a given relative change in tonicity is identical at 22·4 and 154 m-osmole total concentration makes it certain that it reflects volume change rather than some salt effect on the membrane material.

The insert of Fig. 1 shows that after the initial deviation the light response declines in time, signalling penetration of the salt used to set up the water movement. From such records the half-times for passage of different salts were assessed. These, rather than rate constants, were used to make a rough guess at relative permeabilities (as shown in Table 1) because the decline was not strictly logarithmic in all instances.

The word relative permeability is used loosely, meaning relative rate of penetration at the given driving force (exerted by concentration difference, membrane potential and membrane tension). Thus the figures only indicate the order of magnitude; but the approximation is sufficient for the present purpose. Under the assumption that in the presence of valinomycin in a KCl medium the permeability of Cl^- and in its

TABLE 1. Relative permeability of vesicles to different salts

	−Valinomycin	+Valinomycin
KCl (Ca^{2+} 500 μM)	1·00 (5)	1·36 ± 0·06 (5)
KCl (Ca^{2+} 5 μM)	1·00 (5)	1·36 ± 0·06 (5)
KCl (Ca^{2+} 5 μM + EGTA 1 mM)	0·51 ± 0·02 (5)	1·36 ± 0·06 (5)
K-gluconate	$6{\cdot}14 \pm 0{\cdot}261 \times 10^{-3}$ (2)	$6{\cdot}22 \pm 0{\cdot}155 \times 10^{-3}$ (2)
NaCl	$2{\cdot}45 \pm 0{\cdot}05 \times 10^{-3}$ (2)	$2{\cdot}43 \pm 0{\cdot}04 \times 10^{-3}$ (2)
$CaCl_2$	($< 1 \times 10^{-3}$) (2)	($< 1 \times 10^{-3}$) (2)

From experiments of the type shown in insert Fig. 1, half-times for salt penetration were obtained. (KCl entry into 'chloride vesicles' had a half-time of 5 min ($n = 2$). Half-time of KCl entry into gluconate vesicles was 2·1 ± 0·04 min ($n = 5$). The difference may be due to the different membrane potential). 'Gluconate vesicles' were pre-incubated for 30 min at 37 °C in a solution containing (mM): K-gluconate 2; Mg-gluconate, 4; Ca-gluconate, 0·5 or 5×10^{-3}; MOPS-Tris, 1 (pH 7·4 at 37 °C); with and without 5 μg valinomycin/ml. (60 μg protein/ml.); then 1 ml. of suspension was mixed with 30 μl. of 2 osmole salt solution and the change in light intensity was followed. The reciprocal of the half-time is taken as an estimate of the permeabilities. These are expressed as multiples of that for KCl. Values shown are the mean ± S.E. of the mean, number of experiments in parentheses (three different blood samples). The effect of EGTA shown probably underestimates the contribution of the Ca^{2+}-sensitive K channel to the high K permeability (see text).

absence that of K^+ are rate-limiting, Table 1 allows the following conclusion: K^+ permeability is extremely high (at least 100 times that seen in intact red cells) although definitely less than that of Cl^-.

The high K^+ permeability is to the better part not due to the Ca-sensitive K channel (Gardos, 1958; Blum & Hoffman, 1971; Lew & Ferreira, 1977) since 1 mM-EGTA reduced it only by a factor of 2. The effect of EGTA shown in Table 1 was obtained by measurement of the half-time of penetration of KCl which underestimates the tightening effect of EGTA for the following reason. EGTA acts only on i.o.v.s. Whereas without EGTA the time course of the KCl penetration was roughly logarithmic, adding EGTA changed it into a curve which could be fitted by two logarithmic components (not shown). The size of the apparent slow compartment was very nearly identical with the fraction of inside-out vesicles in the preparation and had a rate constant of about a seventh of that seen without EGTA.

It was observed (experiment not shown) that K^+ permeability decreased as a function of time when the vesicles were exposed to media of high K^+ concentration (50 mM). Permeability to Na^+ is about 40 times less than that to K (and, as expected, not affected by valinomycin), permeability to gluconate is about 160 times less than that to K^+. The value for Ca^{2+} permeability is doubtful, because the optical response to water movement in high Ca^{2+} solutions was anomalous, but it is known from experiments with ^{45}Ca that the Ca^{2+} permeability of the vesicles is low (Weiner & Lee, 1972; Sarkadi *et al.* 1978; Wüthrich & Schatzmann, 1980; present experiments).

Volume changes due to Ca^{2+} pumping

Since the volume change is proportional to the ratio of transported Ca^{2+} per litre of vesicles to the total tonicity, the experiments were invariably done in media of low tonicity (23 m-osmole) in order to obtain large optical responses. Fig. 2 shows active ^{45}Ca accumulation by i.o.v.s in a medium containing 0·2 mM-$CaCl_2$, 4 mM-$MgCl_2$, 0·9 mM-Na_2-ATP and a high and a low KCl concentration. It demonstrates that

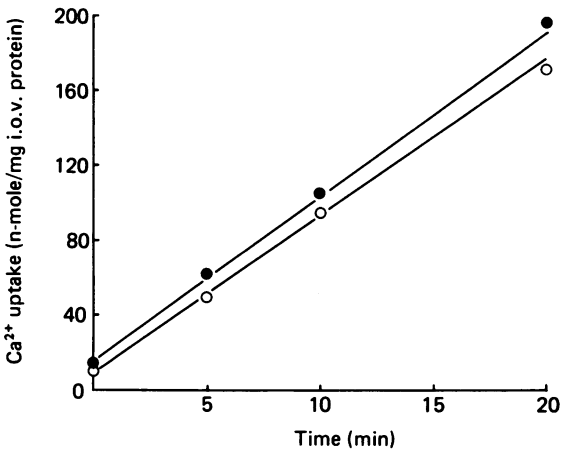

Fig. 2. ^{45}Ca uptake into inside-out vesicles of red blood cells before and after dilution of medium. Vesicles (60 μg protein/ml.) were incubated for 30 min at 37 °C in a solution containing (mM): (○) KCl, 50; $MgCl_2$, 4; $CaCl_2$ (5×10^5 counts/min.μmole ^{45}Ca), 0·2; Tris-Cl, 1 (pH 7·4 at 37 °C); Na_2-ATP, 0·9; digitoxin, 0·125; and (●) KCl, 3; $MgCl_2$, 4; $CaCl_2$ (5×10^5 counts/min.μmole ^{45}Ca), 0·2; Tris-Cl, 1 (pH 7·4 at 37 °C); Na_2-ATP, 0·9; digitoxin, 0·125. All vesicles were first stored in the concentrated medium. Result given in n-mole/mg i.o.v. protein.

transferring the vesicles from the 50 mM-KCl to a 3 mM-KCl solution did not affect the rate of Ca^{2+} transport. Fig. 3 is an example of swelling of vesicles (in the 3 mM-KCl medium) under the influence of Ca^{2+} entry through the pump. The experiment shown was started by adding ATP. In the absence of Ca^{2+}, ATP had no effect but the movement was started immediately upon adding Ca^{2+} (not shown). At the end of the experiment 5 μM-A 23187 was added. It may be seen that the rapid Ca^{2+} release due to the ionophore was faithfully reflected by the optical response. The small displacement in the control curve is probably due to the fluorescence of A 23187.

The next step was to replace Cl^- inside the vesicles and in the medium by the very impermeant gluconate anion. Fig. 4 demonstrates that the active ^{45}Ca accumulation still did occur in a (low tonicity) K-gluconate medium and that valinomycin had no noticeable effect on the rate of the Ca^{2+} uptake. Fig. 5 presents a similar experiment recorded optically. It is obvious that the vesicles shrink and that this shrinking is a consequence of the activity of the Ca pump: the shrinking requires Ca^{2+} and ATP in the medium and can be blocked by vanadate (50 μM). Vanadate is a known inhibitor for the Ca pump (Bond & Hudgins, 1978) which must be present on the internal membrane surface (Rossi, Garrahan & Rega, 1981).

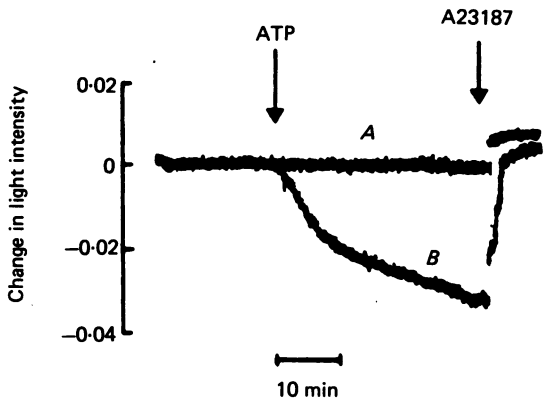

Fig. 3. Transport of calcium causes swelling in a KCl medium. Vesicles (60 μg protein/ml.; i.o.v.s 36%) were incubated for 30 min at 37 °C in a solution containing (mM): KCl, 3; $MgCl_2$, 4; $CaCl_2$, 0·2; Tris-Cl, 1 (pH 7·4 at 37 °C). *A*: control without ATP; *B*: the experiment was started by addition of 0·9 mM-Na_2ATP; 30 min later 5 μM-A 23187 was added in both samples. Ordinate: change in light intensity. The vesicles start swelling upon ATP addition and shrink rapidly when calcium is released by action of ionophore.

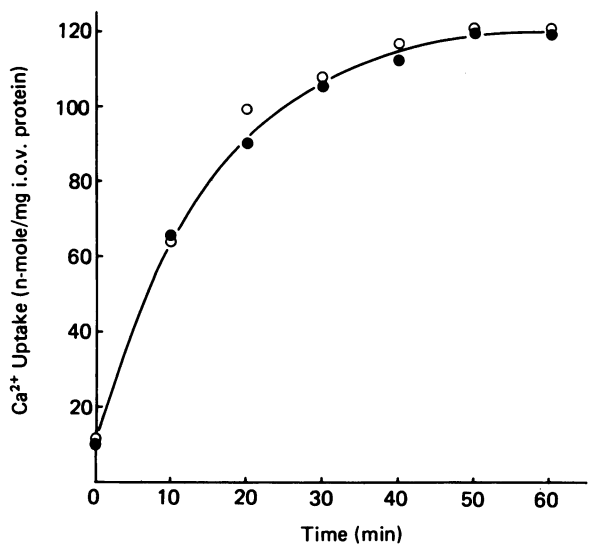

Fig. 4. ^{45}Ca uptake into i.o.v.s in gluconate medium. Effect of valinomycin. An experiment similar to that of Fig. 2 was carried out with vesicles suspended in (mM): K-gluconate, 50; Mg-gluconate, 4; Ca-gluconate, 0·5; MOPS-Tris, 1 (pH 7·4 at 37 °C) and then incubated for 30 min at 37 °C in a solution containing (mM): K-gluconate, 2; Mg-gluconate, 4; Ca-gluconate, 0·5 (4×10^5 counts/min. μmole ^{45}Ca); MOPS-Tris, 1 (pH 7·4 at 37 °C); digitoxin, 0·125 (60 μg protein/ml.); with (●) and without (○) 2×10^{-6} g valinomycin/ml. (ethanol solution; control with the same amount of ethanol). The reaction was started by the addition of 0·9 mM-Na_2-ATP. Results are expressed as n-mole/mg i.o.v. protein. The absence of an effect of valinomycin shows that K movement is not limiting the velocity of Ca pumping.

In the gluconate medium the ionophore A23187 induced a Ca^{2+} escape from Ca^{2+} loaded vesicles which was 5–10 times slower than in a Cl-medium, showing that K^+ permeability was probably farther below that of Cl^- than suggested by the experiment of Table 1 (experiment not presented). The fact that in a gluconate medium valinomycin does not accelerate Ca^{2+} transport (Fig. 4) on the other hand shows that K^+ permeability is high relative to the rate of the Ca pump.

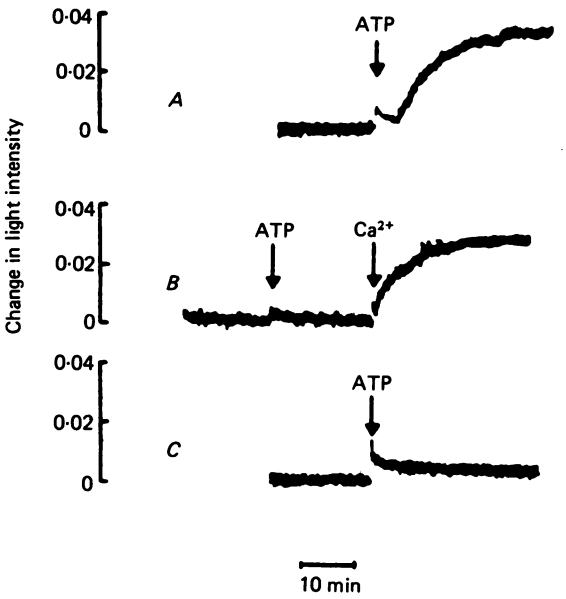

Fig. 5. Transport of calcium causes shrinking in gluconate medium. Vesicles prepared as described in Fig. 4, but without adding ^{45}Ca and valinomycin, were mixed with 0·9 mM-Na_2-ATP and the change of light intensity was followed (*A*). In *B* the vesicles were pre-incubated with 0·5 mM-Tris-EGTA; addition of Na_2-ATP has no effect, addition of 1 mM-Ca-gluconate initiates the reaction. *C*, vesicles pre-incubated as in *A* but with 50 μM-Na-vanadate; addition of Na_2-ATP has no effect. The light intensity changes in opposite direction to Fig. 3 (the vesicles shrink).

Figs. 6 and 7 show what happens when increasing Cl^- concentrations are added to gluconate loaded vesicles in a gluconate medium. Except for the case with 0·5 mM-Cl^- net Ca^{2+} uptake (measured by ^{45}Ca accumulation) increases with increasing Cl^- concentration (Fig. 6). This change is accompanied by a transition from shrinking to swelling (Fig. 7) and it may be seen that the water flow reverses its direction at about 0·5 mM-Cl^-. In these experiments a membrane potential (inside negative) was set up. However it cannot have been large, because Cl^- and K^- permeability were not drastically different. In gluconate media the Ca^{2+} accumulation approaches a considerably lower maximum than in Cl^- media (Fig. 6), but the rate constant is nearly the same ($\sim$ 0·042 min^{-1}). This is probably due to the intravesicular Ca^{2+} concentration increasing more rapidly in the shrinking vesicles (in gluconate).

The simplest interpretation of the qualitative results of these experiments is that in Cl^- media Ca^{2+} moved by the pump is accompanied by two Cl^- ions and in all-gluconate media is exchanged for two K^+ ions. In the first case, there is net inward

movement of three particles, and in the second case there is net outward movement of one particle per pump. An attempt was made to verify whether this simple stoichiometry indeed obtains. Fig. 8 shows the type of experiment used. In experiments under the conditions given in the legend to Fig. 8 the $\Delta I/I_0$ in the interval of 0–10 min was $+1{\cdot}49 \pm 0{\cdot}055 \times 10^{-2}$ (S.E. of the mean $n = 7$) for shrinking in gluconate media

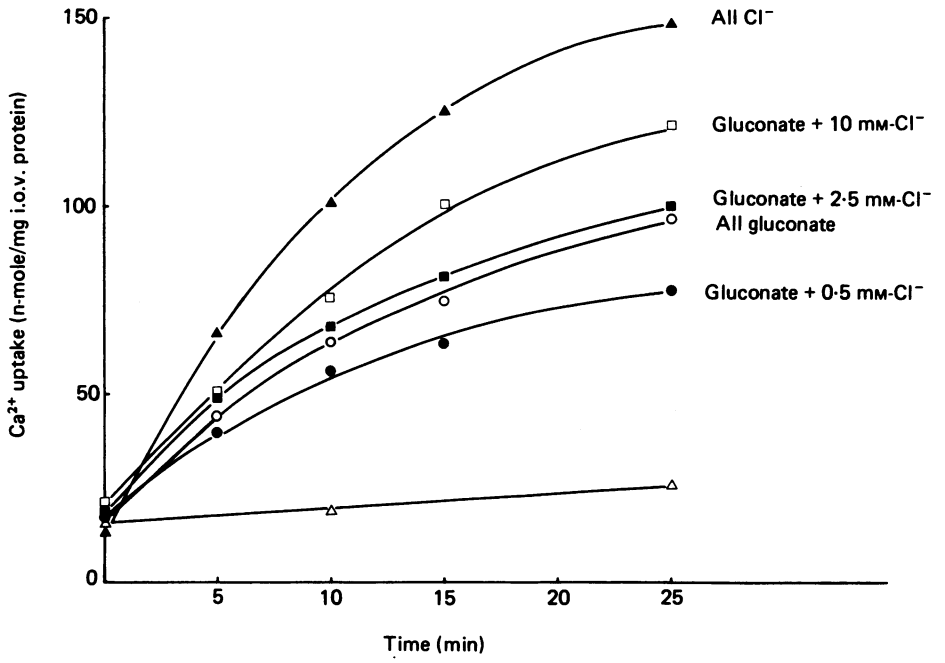

Fig. 6. Effect of chloride on calcium uptake into 'gluconate vesicles' in gluconate medium. 'Gluconate vesicles' (50 μg protein/ml.) prepared as described in Methods were suspended for 30 min in a solution containing (mM): K-gluconate, 2; Mg-gluconate, 4; Ca-gluconate, 0·5 (8×10^5 counts/min . μmole ^{45}Ca), MOPS-Tris, 1 (pH 7·4 at 37 °C) (○); and in the same medium with the addition of 0·5 (●), 2·5 (■) and 10 (□) mM-KCl. The experiment in an all-chloride medium (▲) was carried out in a solution containing (mM): KCl, 2; $MgCl_2$, 4; $CaCl_2$, 0·2 ($1{\cdot}7 \times 10^6$ counts/min . μmole ^{45}Ca); Tris-Cl, 1 (pH 7·4 at 37 °C). The reaction was initiated by addition of 0·9 mM-Na_2-ATP. (△) control without ATP in the all-gluconate medium. Results are expressed as n-mole/mg i.o.v. protein.

and $-4{\cdot}73 \pm 0{\cdot}195 \times 10^{-2}$ ($n = 4$) for swelling in Cl^- media. With the slopes of the calibration curve given in Fig. 1 and the total osmolarity of the medium of 23·29 and 24·04 m-osmole/l. respectively, the assumed stoichiometry gives a shift per 10 min of $9{\cdot}35 \pm 0{\cdot}35$ m-mole Ca^{2+}/l. i.o.v.s from shrinking in the gluconate medium and $16{\cdot}28 \pm 0{\cdot}67$ m-mole Ca/l. i.o.v.s from swelling in the Cl^- medium. Uptake of ^{45}Ca gave $49{\cdot}8 \pm 5{\cdot}4$ n-mole/mg i.o.v. protein ($n = 4$; four experiments with four preparations) in gluconate, and $89{\cdot}0 \pm 1{\cdot}7$ n-mole/mg i.o.v. protein in Cl^- ($n = 3$; three experiments with two preparations) per 10 min. The result is summarized in Table 2. It may be seen that the ratio between the inward Ca^{2+} flux in gluconate and Cl^- media was nearly the same when determined by the ^{45}Ca uptake on one hand and the optical method on the other. Dividing the Ca^{2+} uptake rate (in n-mole/min . mg protein) from the

^{45}Ca uptake experiments by the Ca^{2+} flux calculated from the optical method (in m-mole/min.l. vesicles), the vesicle volume (in μl./mg protein) is obtained. From Table 2 it can be seen that the volume thus calculated from the gluconate and Cl^- experiments is very similar. The good agreement between the ^{45}Ca experiment and the volume change experiment seems to indicate that the optical method is valid.

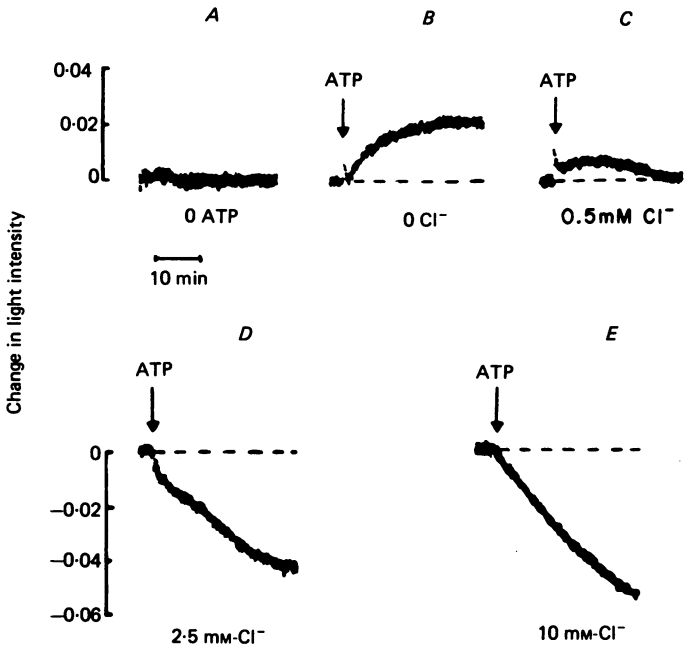

Fig. 7. Effect of chloride on calcium transport into 'gluconate vesicles' in gluconate medium, followed by light scattering. The preparation of vesicles was the same as in the experiment of Fig. 6, but without the addition of radioactive tracer. The experiment was started by adding 0·9 mM-Na_2-ATP. *A*, control (base line) without ATP and without Cl^- (addition of Cl^- did not influence the base line). *B*, gluconate medium (Cl^--free). In *C–E* variable amounts of KCl were added to the sample 30 min before starting the experiment with 0·9 mM-Na_2-ATP.

The nearly identical result for the vesicular volume in gluconate and Cl^- medium is in agreement with the stoichiometry of 1 Ca^{2+}:2 Cl^- when the vesicles swell in a Cl^- medium and 1 Ca^{2+}:2 K^+ when the vesicles shrink in a gluconate medium.

DISCUSSION

The question whether the plasma membrane Ca pump moves Ca^{2+} ions as such, in exchange for other cations, or together with Cl^- is unsettled. As for exchange, Mg^{2+} has conclusively been ruled out as an exchange partner because it does not move when Ca^{2+} is transferred (Schatzmann, 1975) but alkali cations have not. It is true that the pump is not stopped by removal of K^+ or Na^+ (Schatzmann, 1975) but it is slowed (Sarkadi *et al.* 1978, 1980; Romero, 1980). The same holds for ATP hydrolysis by the system (Schatzmann, 1970; Schatzmann & Rossi, 1971; Bond & Green, 1971).

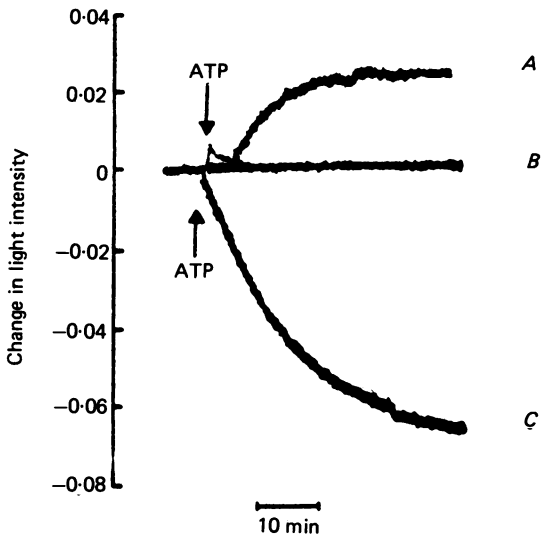

Fig. 8. Type of experiment used in the quantitative comparison of swelling to shrinking of i.o.v.s in different media (Table 2). Calcium transport was followed by light scattering of (*A*) 'gluconate vesicles' and (*C*) 'chloride vesicles' (see Methods). The reaction was initiated by adding 0·9 mM-Na_2-ATP. *B*, control (base line) without addition of ATP in chloride medium. The base line without ATP in gluconate medium was identical.

TABLE 2. Ca transport during 10 min, estimated by ^{45}Ca movement and volume change (optical method)

		Gluconate medium (a)	Cl^- medium (b)	Ratio (a/b)
A.	^{45}Ca uptake (n-mole/mg protein . 10 min)	49·8 ± 5·4	89·0 ± 1·7	0·560
B.	Optical method (m-mole/l. 10 min)	9·34 ± 0·35	16·28 ± 0·67	0·574
C.	Calculated vesicular volume (μl./mg protein)	5·33	5·47	

Calculation in B: $\Delta I/I_0 . 1/k . T_M . 1/A . 1/F = R$ (m-mole Ca^{2+}/l. 10 min). With R = rate, A = osmotic activity of species moving, T_M = tonicity of medium, k = slope in Fig. 1, $\Delta I/I_0$ = observed optical effect in 10 min, F = fraction of i.o.v.s.

For gluconate: 1·49 × 0·1374 × 24·04 × 1 × 1·898 = 9·34 m-mole/l. 10 min.

For chloride: 4·73 × 0·2304 × 23·29 × 0·338 × 1·898 = 16·28 m-mole/l. 10 min.

C is A divided by B.

The observation by Waisman *et al.* (1981*b*) that replacing Cl^- by gluconate markedly slows Ca^{2+} transport in red cell i.o.v.s militates against a coupled exchange of cations. Waisman *et al.* (1981*a*) further found that phosphate reverses this inhibition by moving with Ca^{2+} and that the phosphate-dependent Ca^{2+} movement was slightly more sensitive to *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulphonate (NAP-taurine) than the rest and was also inhibited by 4-isothiocyano-4′-acetamido stilbene 2-2′-sulphonate (SITS), assumed to interfere with band III protein specifi-

cally. This was justly considered to be in accord with the notion that Ca^{2+} movement creates a potential difference, driving phosphate through the anion channel.

In the present experiments inside-out vesicles with a high K^+ permeability were used. When Ca^{2+} pumping was started by adding ATP to the medium, the vesicles swelled in a Cl^- medium and shrank in a gluconate medium. Quantitatively the volume changes were consistent with the assumption that in Cl^- media one Ca^{2+} ion was accompanied by two Cl^- ions and in gluconate media one Ca^{2+} ion exchanged for two K^+ ions. This rules out the possibility that there is either an obligatory coupling of Ca^{2+} with Cl^- or an obligatory exchange of Ca^{2+} for K^+ (see Introduction). The good agreement between the relative rate of Ca^{2+} transport in Cl^- and gluconate medium found by direct measurement of ^{45}Ca movement and measurement of shrinking and of swelling (Table 2) allows no other conclusion than the following. According to the conditions chosen either Cl^- or K^+ movements through passive channels parallel to the pump provide electroneutrality of the over-all ionic shift. The Ca pump itself is electrogenic.

High K^+ permeability of the vesicles after hypotonic treatment was favourable to the experiment in K-gluconate. It is partly due to the Ca^{2+}-sensitive K^+ channel (Gardos, 1958; Blum & Hoffman, 1971; Lew & Ferreira, 1977) because removing Ca^{2+} by EGTA reduced it by a factor of from 2 to 7. However, even a factor of 7 is not sufficient to bring the K^+ permeability anywhere near the normal. Thus the high K^+ permeability is partly due to another cause which must be in connexion with the hypotonic treatment of the vesicles. In fact, incubating such vesicles in media with 50 mM-KCl reduced the K^+ permeability in a time-dependent way (not shown).

On the other hand, Cl^- permeability had to be larger than K^+ permeability, if swelling in Cl^- media was to be obtained. This certainly was the case. Different estimates indicate that Cl^- permeability was anywhere between 1·4 and 10 times that of K^+. This may be seen from Table 1 which shows a small difference. It may also be inferred from the fact that in Fig. 7, 0·5 mM-Cl^- was able to offset the effect of 3·5 mM-K^+ with respect to the direction of water movement, and from the fact that Ca^{2+} escape from ^{45}Ca loaded vesicles under the action of A 23187 was 5–10 times slower in gluconate i.o.v.s than in Cl^- i.o.v.s (not shown). In the experiments of Fig. 7 there was a gradient of K^+ and Cl^- from outside to inside. The fact that K^+ and Cl^- permeability were much nearer each other than in intact cells prevented the formation of a large membrane potential. Whatever the potential difference may have been after 30 min, it did not affect Ca^{2+} movement, because without ATP there was no volume change at any Cl^- concentration in the medium.

As a by-product a figure for the vesicular volume was obtained from the experiments (Table 2, last row). The volume of 5–6 μl./mg protein is the resting volume without any strain on the membrane. Electronmicrographs showed that in this state the vesicles are not hollow spheres, but have, in analogy to red cells, extreme 'stomatocyte' shape. This might be the reason why the optical response below and above the resting volume is not symmetrical (Fig. 1). Sarkadi *et al.* (1980) arrived at a volume of 9–10 μl./mg protein in measuring inulin-accessible space. The agreement between the two measurements is reasonably good. The difference is easily explained by a different amount of spectrin removed during preparation of i.o.v.s in the experiments of Sarkadi *et al.* and the present study.

The possibility that the pump might exchange Ca^{2+} for protons needs some comment. In a system with a pH buffer (Tris-MOPS, Tris-Cl) on both sides of the membranes, the change in osmotic activity by a Ca^{2+}:2 H^+ exchange system, and hence the change in volume, would certainly be less than that corresponding to one moving particle (as measured in the gluconate medium) and would not invert its sign and reach the equivalent of three particles upon replacing gluconate by Cl^- in the system. The hypothesis of a Ca^{2+}–proton exchange, for which no compelling evidence has been brought forward so far, can therefore be dismissed.

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