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SHAPE CONTROL IN THE HUMAN RED CELL

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SUMMARY

When the human red cell consumes its ATP, the cell loses its discoid character in favour of a spiculated and eventually a spherical form. This discocyte–echinocyte transformation parallels both degradation of phosphatidylinositol 4,5-bisphosphate and phosphatidic acid but not dephosphorylation of cytoskeletal proteins. Dephosphorylation of both spectrin and band 3 lags behind metabolic crenation.

Exogenous vanadate accelerates both shape changes and lipid dephosphorylation in a parallel manner during metabolic depletion. In contrast to its effect on lipids, vanadate reduces the rate of protein dephosphorylation. These observations strongly support a shape control mechanism in the red cell, based on phosphoinositide metabolism and compatible with a bilayer-couple model.

INTRODUCTION

Nearly 25 years ago Nakao and his colleagues (Nakao, Nakao & Yamazoe, 1960; Nakao, Nakao, Yamazoe & Yoshikawa, 1961) discovered that ATP was needed to maintain the normal biconcave shape of human red cells. If the ATP content decreased below a certain level, the discocytes were transformed into crenated forms. Later studies confirmed these observations and showed that a number of stages in the shape transformation can be discerned (Weed, La Celle & Merrill, 1969; Mohandas & Shohet, 1978). These comprise the appearance of spicules on the surface of the membrane (echinocytosis), loss of discoid character in favour of a spherically symmetrical shape, and the elongation and, in due course, separation of the spicules from the cell in the form of microvesicles, with formation of a smooth spherocyte. Up to the point preceding major membrane loss, the sequence is reversible and the discoid shape can be recovered if the cell is allowed to resynthesize ATP. These ATP-dependent shape transformations can be reproduced in sealed red cell ghosts (Palek, Stewart & Lionetti, 1974; Birchmeier & Singer, 1977; Sheetz & Singer, 1977; Fairbanks, Patel & Dino, 1981).

Shape changes similar to those observed during metabolic depletion can also be induced by certain anionic amphiphilic agents, whereas cationic amphipaths transform the cells into stomatocytes (cup-forms) (Deuticke, 1968). To explain the transformations brought about amphiphilic molecules, Sheetz & Singer (1974, 1976) formulated the bilayer-couple hypothesis, which states that cell shape is a function of the relative areas of the inner and outer leaflets of the membrane. Anionic amphipaths, which intercalate mainly in the outer leaflet, increase the area of the

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outer relative to the inner leaflet and thereby produce the typical protrusions that characterize the echinocyte; cationic amphipaths accumulate in the inner leaflet and the increase in area reveals itself as invagination and stomatocytosis.

Persuasive evidence in favour of this model comes from the effects on cell shape of exogenous lysophosphatidyl choline (Mohandas, Greenquist & Shohet, 1978; Lange & Slayton, 1982). When this molecule first enters the membrane it lodges in the outer leaflet, and echinocytosis results; it is then redistributed between the leaflets and the cell reverts to a discocyte. If now the fraction in the outer leaflet is extracted with albumin, stomatocytosis ensues.

The membrane cytoskeleton was long thought to be the agent by which the shape of the cell was controlled, and indeed it is established that such features as the viscosity of the membrane and the lateral mobility of membrane particles are regulated by this complex (reviewed by Gratzer, 1983; and Sheetz, 1983). Singer and co-workers (Birchmeier & Singer, 1977; Sheetz & Singer, 1977) first offered evidence that metabolic shape changes were linked to the phosphorylation of the major structural protein, spectrin, by way of a cyclic AMP-independent kinase, acting in opposition to a phosphatase. According to this model, dephosphorylation of spectrin would lead to rearrangement of the cytoskeleton and contraction of the inner leaflet of the membrane with consequent formation of echinocytes. More recent observations have undermined this scheme: Anderson & Tyler (1980) have observed that echinocytosis largely precedes dephosphorylation of spectrin in intact cells and Patel & Fairbanks (1981) found that Mg-ATP levels can determine the morphological state of ghosts when nearly all the kinase has been extracted from the membrane.

The resemblance in shape and properties of ghosts and even isolated cytoskeletons (obtained by extraction of membranes with non-ionic detergent) to the red cells from which they derive, has been interpreted as support for a shape-controlling function of the cytoskeletal network (Johnson & Robinson, 1976; Hainfeld & Steck, 1977; Lux, John & Karnovsky, 1977; Johnson, Taylor & Meyer, 1980; Tomaselli, John & Lux, 1981). However, more recently, Lange, Gough & Steck (1982*a*) and Lange, Hadesman & Steck (1982*b*) adduced evidence for a passive accommodation of the cytoskeleton to the shape of the membrane. They view the cytoskeleton as a flexible and elastic network that can adopt and stabilize the shape of the membrane but cannot initiate morphological changes.

The current prevailing opinion is that the bilayer-couple hypothesis is the best explanation for shape control of the red cell. One possible mechanism for ATP-dependent shape changes originates from work of Allan & Michell (1975), who found that calcium loading and ATP-depletion, which both cause echinocytosis, lead to increased levels of diacylglycerol in the membrane. It was later found that Ca^{2+} activates a membrane-bound phosphodiesterase that degrades polyphosphoinositides (phosphatidylinositol 4-phosphate (PI-P) and phosphatidylinositol 4,5-bisphosphate (PI-PP)) to diacylglycerol (Allan & Michell, 1978; Downes & Michell, 1981; Allan & Cockcroft, 1983). In the absence of ATP (when the reverse reaction is precluded) the activity of this enzyme would thus lead to a loss of phosphate groups and redistribution of the product, with consequent shrinkage of the inner leaflet of the

membrane. Recently, Ferrell & Huestis (1984) showed that shape changes, induced by ATP-depletion, parallel conversion of PI-PP and phosphatidic acid to phosphatidylinositol (PI) and diacylglycerol, respectively. Their results suggest that metabolic crenation may be caused by a decrease in area of the inner leaflet in accordance with the bilayer-couple hypothesis.

A different mechanism for shape control, though still based on the bilayer-couple hypothesis, has been proposed by Seigneuret & Devaux (1984). Using spin-labelled phospholipids they observed that phosphatidylserine and phosphatidylethanolamine, but not phosphatidylcholine, upon incorporation into the membrane were rapidly transported to the inner leaflet. Since this process was ATP-dependent, they inferred that an active enzyme-regulated mechanism operates in the red cell to maintain the balance of areas of the leaflets demanded by the discoid shape.

A rather different model has been proposed by Jinbu and co-workers. Using resealed red cell ghost membranes they observed two ATP-dependent steps in the shape change, one slow and one rapid (Jinbu, Nakao, Otsuka & Sato, 1983; Jinbu, Sato & Nakao, 1984*a*; Jinbu, Sato, Nakao & Tsukita, 1984*b*). Completion of the slow process conditions the cell, so that it can respond rapidly to addition of ATP, even at 6°C. From this they suggested that membrane protein phosphorylation as well as a non-covalent effect of ATP are necessary for shape change. In addition, they have shown that proteolytic degradation of ankyrin (with no visible damage to any other protein) leads to an immutable shape, unaffected by metabolic depletion or crenating drugs, thus indicating that ankyrin plays an important role in shape control (Jinbu, Sato, Nakao & Nakao, 1982; Jinbu *et al.* 1984*c*). This is supported by the observation that the cytoplasmic fragment of band 3 inhibited ATP-dependent but not ATP-independent smoothing of crenated ghosts (Carter & Fairbanks, 1984). In this case it was inferred that the fragment competed with intact band 3 for binding to ankyrin and thereby affected the anchorage of the cytoskeleton to the membrane.

The complexity of the evidence relating to the mechanism of shape control is further compounded by the recent demonstration that red cell membranes contain myosin and tropomyosin (Fowler & Bennett, 1984; Fowler, Davis & Bennett, 1985; Wang, Kiehart & Pollard, 1985), which reopens the possibility that an actomyosin contractile mechanism might be involved in shape control.

Since ATP is needed to sustain the normal properties of the cell, one must suppose that at least part of the ATP-pool is used for phosphorylation reactions. To assess the role of phosphorylation of proteins and lipids, vanadium compounds have been used to perturb the phosphorylation balance in intact human red cells. Vanadate is known to be a potent inhibitor of a number of enzymes, such as ATPases, kinases and phosphatases (Simons, 1979; Macara, 1980; Ramasarma & Crane, 1981).

Vanadate (at physiological pH the oxyanion of pentavalent vanadium) is accumulated in the red cell, probably via the anion channel; it is reduced in the cell to vanadyl ions (vanadium IV) by a process requiring glutathione (Cantley & Aisen, 1979; Macara, Kustin & Cantley, 1980; Heinz, Rubinson & Grantham, 1982). In this paper it is shown that vanadate is a potent echinocytogenic agent and that this effect can be linked to its intervention in the metabolism of phosphoinositides. This

supports the view that discocyte–echinocyte transformation is related to changes in the phosphoinositide components of the lipid bilayer.

MATERIALS AND METHODS

Fresh human blood was generously provided by the blood bank at the University Hospital, Umeå. Essentially fatty acid-free bovine serum albumin (BSA), glutaraldehyde and lipids were from Sigma Chemical Co, St Louis, MO, U.S.A. Penicillin G and streptomycin mixture was from Gibco and ATP monitoring kit was purchased from LKB, Bromma, Sweden, carrier-free $H_3{}^{32}PO_4$ from Amersham Sweden AB, Solna, Sweden, Silica Gel 60 thin-layer chromatography plates (20 cm × 20 cm) from Merck, Darmstadt, BDR, and GelBond PAG film from FMC Marine Colloids Division, Rockland, ME, U.S.A. All other chemicals were of at least reagent grade.

General methods

Washing of human red cells was carried out at 4°C with ice-cold solutions. All incubations described below were done at 37°C and at a haematocrit of 10%.

Prelabelling with ^{32}P

Human red cells were washed five times with buffer H (130 mM-NaCl, 3·7 mM-KCl, 2 mM-$MgCl_2$, 1 mM-EGTA, 25 mM-HEPES, pH 7·5) and twice with complete buffer H (buffer H containing 100 units ml^{-1} penicillin G, 100 units ml^{-1} streptomycin and 1 mg ml^{-1} BSA). Plasma and buffy layers were removed by aspiration after each centrifugation.

Red cells were labelled with ^{32}P by incubation for 23–39 h with complete buffer H, supplemented with 10 mM-glucose and 1 mM-adenosine. The suspension medium contained about 0·74 MBq ml^{-1} (20 μCi ml^{-1}) $H_3{}^{32}PO_4$. After incubation the cells were washed four times with buffer H and twice with complete buffer H. Some experiments were done in the presence of calcium by washing and incubating cells in buffer H/Ca and complete buffer H/Ca (same as buffer H and complete buffer H, respectively, except that EGTA was replaced by 10 μM-$CaCl_2$).

Metabolic depletion and recovery

Metabolic depletion was initiated by resuspending ^{32}P-labelled cells in complete buffer H or H/Ca, containing the indicated amounts of sodium metavanadate. In the absence of vanadate, this treatment induced ATP depletion and crenation with half-times of 3 and 9 h, respectively, independent of Ca^{2+}. To initiate ATP repletion, 0·1 vol. of 100 mM-glucose, 10 mM-adenosine and $H_3{}^{32}PO_4$ in buffer H or H/Ca was added.

In some experiments a high level of ATP was maintained by resuspending ^{32}P-labelled erythrocytes in complete buffer H, supplemented with 10 mM-glucose and 1 mM-adenosine. These cells retained their biconcave shape and normal glutathione (GSH) level for more than 40 h, whereas the ATP level started to decrease after about 20 h.

Assay procedure

Morphology. Erythrocytes were fixed by mixing 30 μl of the cell suspension with 0·2 ml 1% glutaraldehyde in 0·1 M-potassium phosphate buffer (pH 7·5) for 60 min at room temperature. They were then examined by phase-contrast microscopy. The extent of crenation was determined by counting at least 400 cells of each sample and classifying the cells as either crenated or uncrenated.

ATP. A sample of the cell suspension (30 μl) was mixed with 0·5 ml ice-cold water and stored frozen until measurement. ATP was determined by the luceferin–luciferase assay (Lundin, Rickardsson & Thore, 1976) using an LKB Luminometer 1250 and standard reagent kit.

GSH. A 0·5 ml sample of the cell suspension was mixed with 1 ml of cold water; 1 ml of this was used immediately to determine the concentration of GSH according to Beutler (1984).

Haemoglobin. The concentration of haemoglobin in lysates was determined from measurements of absorbance at 539 nm using a molar absorptivity of $13{\cdot}4\times10^3\ M^{-1}\,cm^{-1}$ per haem (Noren, Bertoli, Ho & Casassa, 1974).

Lipids. Frozen samples of cell suspension (200 μl) were lysed in 1 ml of ice-cold 10 mM-Tris, 2 mM-EDTA (pH 7·5) and centrifuged for 6 min at 10000 ***g***. This step was repeated twice. The entire ghost membrane pellet was extracted with 100:50:1 (by vol.) methanol/chloroform/concentrated HCl (1·8 ml) followed by addition of water (0·6 ml) and chloroform (0·6 ml). The organic phase was removed and the aqueous phase re-extracted with chloroform (1·2 ml). The organic phases were combined, dried under a stream of nitrogen and dissolved in chloroform (Ferrell & Huestis, 1984). Lipids were separated by thin-layer chromatography on 20 cm × 20 cm Silica Gel 60 plates (not activated) developed in 48:40:10:5 (by vol.) methanol/chloroform/water/concentrated ammonia. This system separated the inositides from the origin and resolved the radioactive lipids. Radiolabelled phospholipids were detected by autoradiography and quantified by liquid scintillation counting of scraped spots. Lipid phosphorus was determined by the spectroscopic method of Bartlett (1959).

Proteins. Samples of cell suspension (4 ml) were lysed in 35 ml ice-cold 5 mM-potassium phosphate buffer (pH 7·5) and centrifuged for 10 min at 46000 ***g***. After one additional wash the ghost membranes were dissolved in 0·5 vol. of 10% sodium dodecyl sulphate (SDS) and 0·3 vol. of sample stock buffer (60 mM-Tris-bicine, 4% 2-mercaptoethanol, 4% SDS, 50% glycerol containing Bromphenol Blue) was added. Immediately before gel electrophoresis the dissolved ghost membranes were immersed for 5 min in boiling water. Electrophoresis was performed in the continuous buffer system of Fairbanks, Steck & Wallach (1971), except that the SDS concentration was 0·1%, using 6% acrylamide gels cast on GelBond PAG film (Nochumson & Gibson, 1983). After staining with Coomassie Brilliant Blue and drying, the gels were autoradiographed. Radiolabelled polypeptides were cut out and quantified by liquid scintillation counting and measurement of absorbance at 630 nm after extraction with 60% (v/v) formic acid.

RESULTS

Effect of vanadate on red cell shape

Under ATP-depleting conditions at 37°C red cells lost their discoid form and became echinocytic, with a half-time of ~9 h. Inclusion of vanadate in the incubation medium increased the rate of echinocytosis considerably; at 100 μM-vanadate the half-time fell to about 1·5 h (Fig. 1A). In contrast, the intracellular ATP and GSH concentrations were not significantly affected by vanadate (Fig. 1B). When EGTA in the incubation medium was replaced by 10 μM-$CaCl_2$, neither shape change nor decline in ATP or GSH were significantly affected; 100 μM-vanadate still reduced the half-time for crenation from 9 to 1·5 h, while the ATP and GSH levels remained very similar to those observed in the presence of EGTA and thus largely independent of vanadate.

Red cells incubated in a medium containing glucose and adenosine, to permit continuing ATP synthesis, retained their discoid form as well as the initial GSH level for more than 40 h. Again vanadate caused a rapid and concentration-dependent shape transformation. In the Ca^{2+}-containing medium 100 μM-vanadate caused complete echinocytosis with a half-time of about 2 h, whereas in the presence of EGTA the shape transformation did not go to completion. As before, the ATP level was largely independent of whether calcium and, or, vanadate were present in the incubation medium. The initial GSH level was unaffected by vanadate during incubation in the presence of EGTA, whereas in the Ca^{2+}-containing medium vanadate caused a rapid decrease in GSH to about 50% of its initial value.

In another experiment, cells were first partially depleted in the presence of external Ca^{2+}, to reduce the ATP concentration to about 10% of its original level,

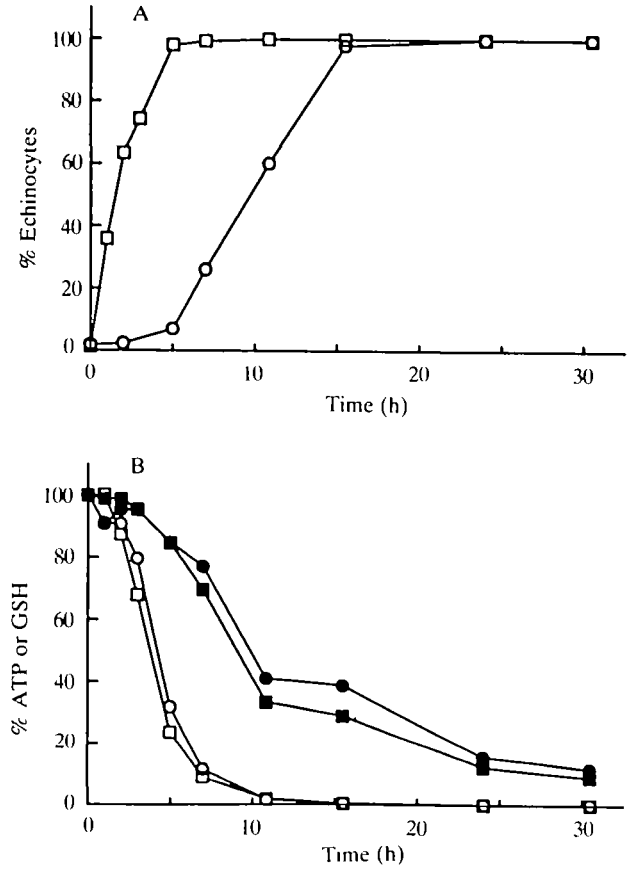

Fig. 1. Metabolic depletion of red cells in EGTA. Washed red cells were suspended at 10% haematocrit in complete buffer H and incubated at 37°C in the absence (circles) or presence (squares) of 100 μM-vanadate. At the indicated times, samples were withdrawn and the morphology, and the ATP and GSH concentrations were determined as described in the text. A. Time course of echinocytosis. B. Time course of changes in ATP (open) and GSH (filled) concentrations; 100% ATP or GSH represent in this and subsequent figures the concentrations (4·5 μmol ATP or 6·2 μmol GSH/g haemoglobin) determined directly after the initial wash.

and then glucose and adenosine were added, both in the absence and presence of vanadate, to replenish the ATP pool. In the absence of vanadate the cells recovered their discoid character immediately, as shown in Fig. 2A, whereas in its presence echinocytosis was increased, rather than reversed; indeed 100 μM-vanadate increased the rate of shape change to the level observed during ATP depletion. At 40 μM-vanadate the rate of echinocytosis was considerably slower, though still faster than during unperturbed ATP depletion. At this concentration the proportion of cells transformed into echinocytes reached a plateau level at 70%. Moreover, the ATP level was, as before, unaffected by vanadate (Fig. 2B).

It was further observed that the pretreatment of cells can influence their response to vanadate. Cells washed in phosphate-buffered saline before suspension in the final incubation medium displayed a considerable lag phase in their response. To investi-

gate the basis of this phenomenon, cells were incubated under ATP-synthesizing conditions in the presence of both phosphate and vanadate. Phosphate was found to reduce both the rate and final extent of echinocytosis. This strongly suggests that phosphate inhibits the transport of vanadate across the membrane and that vanadate exerts its action only within the cell.

Protein phosphorylation

It has been shown that in the presence of nutrients [^{32}P]orthophosphate equilibrates in 25–30 h with the endogenous pool of ATP and causes incorporation of ^{32}P into the γ-position of ATP (Anderson & Tyler, 1980; Harris, Levin & Lux, 1980). The radiolabel enters proteins and lipids under the action of several kinases.

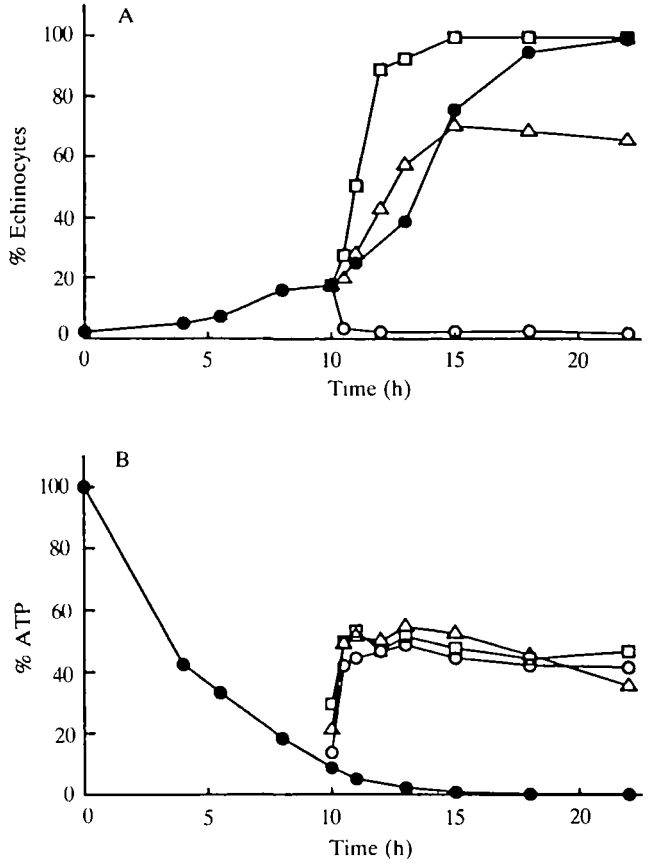

Fig. 2. Recovery of metabolically depleted red cells in the presence of calcium. Washed red cells in complete buffer H/Ca (10% haematocrit) were incubated under ATP-depleted conditions at 37°C. After 10 h of incubation, when the ATP level had decreased to about 10% of its initial value, 0·1 vol. of 100 mM-glucose and 10 mM-adenosine in buffer H/Ca was added to replenish the cells. A. Time course of echinocytosis. The morphology of the cells was determined in the absence (○) or in the presence of 30 μM (△) and 100 μM (□) vanadate as well as in the case of continued depletion (●). B. Time course of change in ATP (symbols as in A).

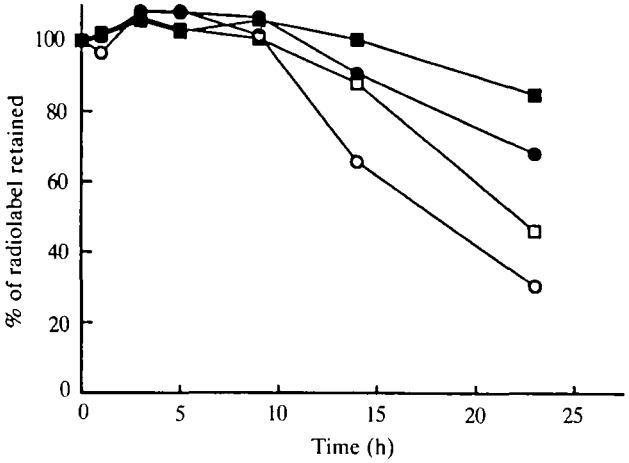

Fig. 3. Protein dephosphorylation during metabolic depletion. Red cells were equilibrated with [^{32}P]orthophosphate before initiating metabolic depletion. At indicated times, samples were withdrawn, and membranes were prepared as described in the text. The fraction of radioactivity retained in spectrin β-chain (○,□) and band 3 (●,■) in the absence (○,●) and presence (□,■) of 100 μM-vanadate was determined by polyacrylamide/SDS gel electrophoresis and Čerenkov counting. (The time course of echinocytosis and those of ATP and GSH concentrations during this experiment matched those depicted in Fig. 1).

The protein predominantly phosphorylated in human red cells is the spectrin β-chain, but bands 3, 4·1 and 4·9 (nomenclature of Fairbanks *et al.* 1971) are also radiolabelled.

To relate phosphorus incorporation to ATP-dependent shape changes, red cells were incubated with nutrients and [^{32}P]orthophosphate. After about 24 h at 37 °C excess radiolabel was removed and the cells were metabolically depleted of ATP in the absence and in the presence of vanadate. SDS/polyacrylamide gel electrophoresis of ghost membrane proteins revealed that shape change largely preceded dephosphorylation of spectrin, in agreement with earlier work (Anderson & Tyler, 1980), as well as that of band 3 (Fig. 3). The dephosphorylation of the other cytoskeletal phosphoproteins, bands 4·1 and 4·9, could not be determined accurately enough because of low incorporation of ^{32}P. Furthermore, vanadate did not affect protein dephosphorylation in the early stages of depletion. After about 10 h of incubation, cells treated with vanadate showed a decreased rate of protein dephosphorylation relative to the controls. Thus dephosphorylation of spectrin or band 3 appears to proceed independently of the process of echinocytosis in the absence as well as in the presence of vanadate.

Lipid phosphorylation

In agreement with earlier observations (Allan & Michell, 1978), red cells labelled with [^{32}P]orthophosphate incorporated the radiolabel most prominently into PI-PP, PI-P and phosphatidic acid. A fourth radioactive spot, so far unidentified (R_F value

0·14), was observed after thin-layer chromatography and autoradiography of the total extracted lipid (not shown). This, however, contributed less than 4% of the total incorporated radiolabel.

When ^{32}P-labelled cells were depleted of ATP in EGTA-containing medium, a distinct redistribution of the radiolabel among the phospholipids occurred (Fig. 4A) in a manner that closely paralleled echinocytosis. The radioactivity associated with PI-PP decreased sharply, while the incorporation into PI-P and phosphatidic acid rose; this is in accord with the observations of Ferrell & Huestis (1984). Addition of vanadate increased the rate of dephosphorylation of PI-PP with a concomitant accretion of PI-P and phosphatidic acid and again the redistribution of ^{32}P paralleled the shape transformation. However, in contrast to the control, the fraction of radiolabel associated with PI-P increased much more rapidly than that associated with phosphatidic acid. In fact, after 30 h depletion about 50% of the label was recovered in PI-P compared to about 38% in the control and phosphatidic acid contained 40% of the radioactivity in the presence of vanadate and 50% in its absence (Table 1). In addition, the specific activity of total phospholipid was significantly higher in vanadate-treated cells, at least after a few hours incubation (Fig. 4B).

Since both metabolic and vanadate-induced shape change and the concomitant decrease in ATP and GSH appeared to be independent of the calcium concentration in the medium, the effect of calcium on the labelling of the phosphoinositides was also examined. Addition of calcium to the incubation medium in the absence of vanadate did not appreciably change the kinetics of ^{32}P-redistribution, even though the initial pattern of labelling was different (Fig. 5). Vanadate again increased the rate of conversion of PI-PP to phosphatidic acid and PI-P but, whereas in EGTA the label entered PI-P more rapidly than phosphatidic acid, the opposite was observed when calcium was present. Thus the mechanism of vanadate-induced shape change is significantly perturbed by calcium.

DISCUSSION

The chemistry of vanadium, as well as its effect on biological systems, is complex. At physiological pH the predominant ion is pentavalent metavanadate (HVO_4^-). Vanadate accumulates in the red cell by a biphasic process. The rapid initial phase ($t_{\frac{1}{2}} \sim 2$ min), corresponding to equilibration across the membrane via the anion channel, is followed by a slower phase ($t_{\frac{1}{2}} \sim 17$ min) (Heinz *et al.* 1982), which has been attributed to a reduction of vanadate (V(V)) to vanadyl (V(IV)) by a non-enzymic process requiring glutathione (Cantley & Aisen, 1979; Macara *et al.* 1980). Vanadate perturbs a number of cellular processes and is a potent inhibitor of many enzymes, including ATPases, kinases and phosphatases (Macara, 1980; Ramasarma & Crane, 1981). Vanadyl, on the other hand, is less inhibitory to most enzymes; exceptions are alkaline phosphatase and ribonuclease. This probably explains the lack of any observable effect on ATP levels during incubation of red cells with

vanadate. If vanadate were the predominant ionic species inside the cell, the rate of ATP production through the glycolytic pathway would be expected to decrease, since vanadate inhibits phosphofructokinase ($k_i \sim 0{\cdot}5$–$3\ \mu M$) and interferes with the substrate phosphorylation catalysed by glyceraldehyde-3-phosphate dehydrogenase (Macara, 1980; Ramasarma & Crane, 1981).

Although the action of vanadate or vanadyl is not fully understood, the effect of vanadate on red cell shape is large and undoubted. Incubation of cells in the presence of vanadate leads to a rapid discocyte–echinocyte transformation, regardless of the metabolic state of the cells. During ATP-depletion vanadate increases the rate of echinocytosis and when depleted cells are allowed to resynthesize their ATP vanadate inhibits the recovery of the discoid form in a concentration-dependent manner. Even when nutrients are present in the incubation mixture to allow continuous maintenance of the ATP level, vanadate still induces echinocytosis. In contrast to its effect on shape, external vanadate does not significantly affect ATP levels, either during depletion or replenishment. However, resynthesis of ATP after partial depletion appears to be somewhat accelerated in vanadate-treated cells. This implies that ATP is either synthesized more rapidly or hydrolysed more slowly in the presence of vanadyl ions. Since the concentration of ATP under conditions of glycolysis is unaffected by vanadyl ions, although glucose metabolism is stimulated (Ninfali *et al.* 1983), the second explanation appears the more likely. Further, as reduction of intracellular vanadate is slower than its equilibration across the membrane (Heinz *et al.* 1982), there will be, at least initially, a certain concentration of vanadate inside the cell. This could transiently inhibit the major consumers of ATP, the membrane pumps, and increase the initial rate of ATP accretion.

As for ATP, the GSH levels are not significantly influenced by external vanadate during depletion and replenishment in the presence of EGTA. In contrast, a significant amount of cellular GSH is rapidly consumed when vanadate is added to the cells together with calcium under ATP synthesizing conditions. The initial consumption of the reductant may be explained by a higher influx of vanadate (which has been shown to depend on the incubation medium; Heinz *et al.* 1982), which then has to be reduced.

During the first 10 h of depletion, the phosphorylation levels of spectrin and band 3 are hardly affected. Apparently both the spectrin β-chain and band 3 retain their phosphate groups even when the ATP concentration is less than 10% of normal and while 50% of the cells undergo echinocytosis. Thus shape change largely precedes

Fig. 4. A. Kinetics of ^{32}P incorporation during metabolic depletion in EGTA. Red cells were labelled with ^{32}P, washed free of phosphate, and suspended in complete buffer H. After 1·5 h of incubation at 37 °C, vanadate to a final concentration of 120 μM was added. Samples were withdrawn and analysed as described in the text, and the fractions of PI-PP (○, ●), PI-P (△, ▲) and phosphatidic acid (□, ■) in the absence (open) and presence (filled) of vanadate were determined. B. Specific activity of total phospholipid. The content of phosphorus and the radioactivity of the phospholipids were measured before separation by thin-layer chromatography, and the specific activity of the lipid fraction of control (○) and vanadate-treated (●) cells was calculated. (The time course of echinocytosis matched that shown in Fig. 1).

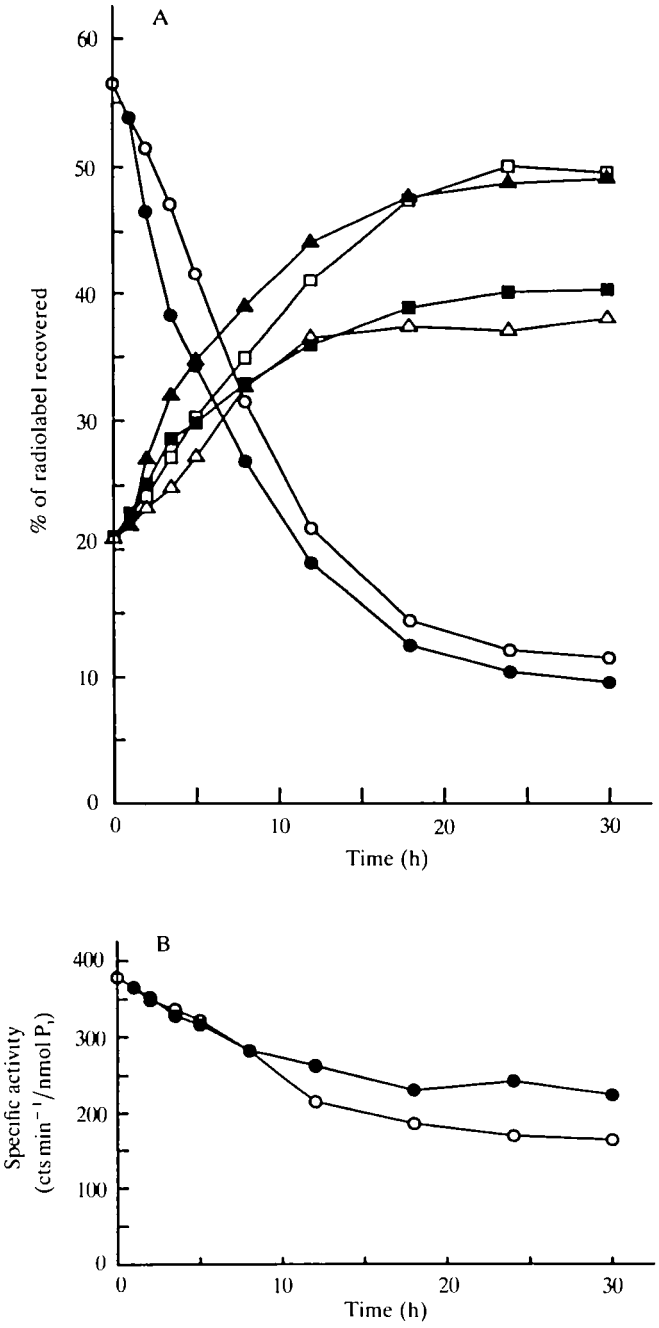
A
% of radiolabel recovered
60
50
40
30
20
10
0
0
10
20
30
Time (h)
B
Specific activity
(cts min^{-1}/nmol P_i)
400
300
200
100
0
0
10
20
30
Time (h)

Fig. 4

dephosphorylation of spectrin, as has been reported before (Anderson & Tyler, 1980), as well as that of band 3. Addition of vanadate does not increase the rate of dephosphorylation, as would be expected if spectrin phosphorylation were responsible for shape control, but rather decreases the rate of phosphorus liberation after long periods of depletion (more than 10 h).

That exogenous vanadate (1) initially induces rapid echinocytosis without causing loss of protein phosphorus and (2) inhibits the phosphatases responsible for dephosphorylation of spectrin and band 3 clearly indicates that neither phosphorylation nor dephosphorylation of these proteins is directly or in any simple way involved in shape control. Taken together with previous observations (Anderson & Tyler, 1980; Patel & Fairbanks, 1981; Lange *et al.* 1982*b*; Ferrell & Huestis, 1984) our results seem greatly to weaken the case for control of cell shape through the cytoskeleton. On the other hand, it must be recognized that arguments to the contrary also exist (Carter & Fairbanks, 1984; Jinbu *et al.* 1984*c*), which will require an explanation if the mechanism of shape regulation is to be fully understood.

Unlike protein phosphorylation, that of lipid is strongly affected by metabolic depletion and recovery in the presence of either EGTA or Ca^{2+}. External vanadate not only increases the rate of echinocytosis but also accelerates the redistribution of ^{32}P between the inositol phospholipids. Regardless of the incubation medium, the interconversion of phosphoinositides and phosphatidic acid parallels shape changes, both in the absence and presence of external vanadate. This is consistent with previous results obtained from both slow metabolic and accelerated (iodoacetamide-induced) depletion (Ferrell & Huestis, 1984), as well as from Ca^{2+}-induced echinocytosis (Allan & Thomas, 1981). Although only the relative amounts of incorporated radiolabel into phosphatidic acid and inositides were determined in this study, it is clear that depletion leads to degradation of PI-PP and that the bisphosphate is degraded differently depending on the conditions during incubation.

In the absence of vanadate the kinetics of ^{32}P incorporation into the phosphoinositides are largely independent of external calcium, and the results agree with those of Ferrell & Huestis (1984). There are, however, differences: during

Table 1. *Incorporation of ^{32}P into the inositol phospholipids*

	EGTA*			Calcium		
	Control		Vanadate	Control		Vanadate
Incubation time	0 h	30 h	30 h	0 h	23 h	23 h
Phosphatidylinositol 4,5-bisphosphate	56·5 ± 1·2	11·6 ± 0·3	9·6 ± 0·6	53·4	7·8	5·0
Phosphatidylinositol 4-phosphate	20·9 ± 0·4	38·2 ± 0·5	49·2 ± 0·8	15·7	33·9	31·6
Phosphatidic acid	21·0 ± 0·9	49·7 ± 0·4	40·4 ± 1·2	26·6	56·8	61·9

^{32}P-labelled cells were incubated under ATP deplete conditions and samples were withdrawn at indicated times. Lipids were extracted and separated as described in the text. The fraction of ^{32}P incorporated was determined by Cěrenkov counting of isolated radioactive spots.

* Values represent average ± S.D. of two experiments as percentage.

incubation in an EGTA-containing medium the ratio of phosphatidic acid to PI-P (calculated from data shown in Fig. 4) increases with time, whereas in the presence of calcium it falls slightly. Given that the concentration of PI-P des not change during depletion (Ferrell & Huestis, 1984), it follows that the concentration of phosphatidic acid increases in the presence of EGTA and decreases slightly with time in the presence of calcium. With vanadate in the medium during depletion the difference becomes much more pronounced. Depletion in the calcium-containing buffer leads to a faster decline in PI-PP and a correspondingly faster accretion in PI-P and phosphatidic acid initially but after 5–10 h of incubation the distribution of ^{32}P approaches the pattern seen in the absence of vanadate (Fig. 5). Replacing calcium by EGTA also leads to a faster decline in PI-PP but the relative rates of accretion of the two hydrolysis products are reversed; the amount of radiolabel recovered in PI-P

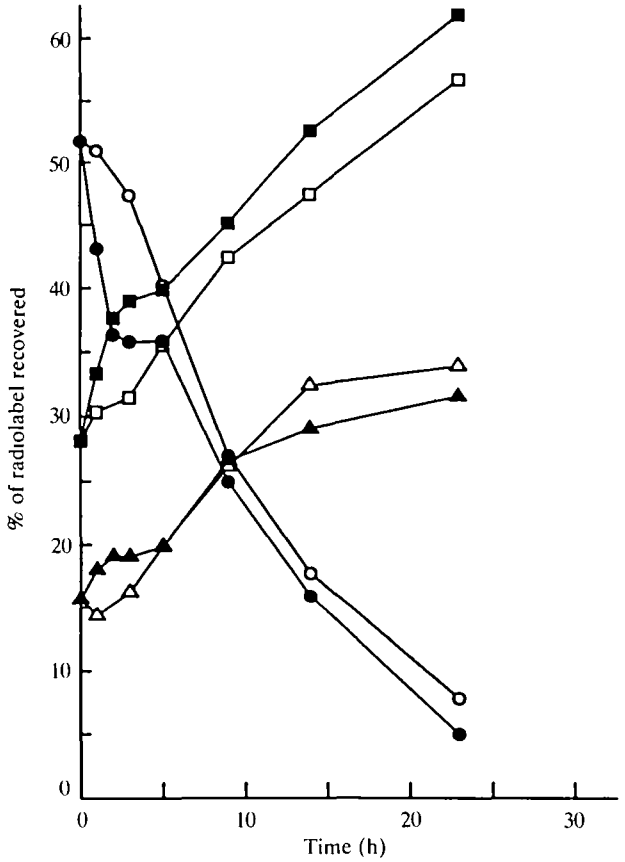

Fig. 5. Kinetics of ^{32}P incorporation during metabolic depletion in the presence of calcium. Red cells were labelled with ^{32}P, washed free of phosphate and incubated in complete buffer H/Ca at 37°C in the absence and presence of 100 μM-vanadate. Samples were withdrawn and analysed, and the fractions of PI-PP (○, ●), PI-P (△, ▲) and phosphatidic acid (□, ■) in control (open) and vanadate-treated (filled) cells were determined.

increases considerably faster than that in phosphatidic acid and never approaches the distribution observed in vanadate-free medium (Fig. 4A). This perturbation of the labelling pattern by vanadate indicates clearly that the degradation and subsequent fate of the phosphoinositides due to metabolic depletion is dependent on whether calcium is present or not.

Despite the intracellular conversion of vanadate to vanadyl, residual vanadate may effect cation transport (Fuhrmann, Huttermann & Knauf, 1984). For instance, it is known that micromolar concentrations of vanadate inhibits the calcium pump in human red cells (Bond & Hudgins, 1980), as well as other ATPases (Simons, 1979; Macara, 1980; Ramasarma & Crane, 1981). If the calcium pump is inhibited by the presence of external vanadate, passive Ca^{2+} influx will elevate the intracellular Ca^{2+} level with a number of consequences. Recently it was shown that even traces of external Ca^{2+} are sufficient to activate passive K^+ efflux (Gardos effect) when red cells are depleted in the presence of vanadate (Fuhrmann *et al.* 1984). It is not clear how early this would ensue under the experimental conditions used, but loss of K^+ and dehydration could lead to consequences unrelated to metabolic crenation *per se*. It was also shown that stimulation of the Gardos system is completely eliminated when a calcium chelator, like EDTA or EGTA, is added to the medium. Ca^{2+} influx may also activate the polyphosphoinositide phosphodiesterase, though under physiological conditions this enzyme is not believed to be active at Ca^{2+} concentrations below 10 μM (Allan, 1982).

Depletion in the presence of external vanadate and calcium may therefore lead to degradation of PI-PP and PI-P to phosphatidyl inositol (PI) by way of phosphomonesterases, but also to diacylglycerol by way of the diesterase. Metabolic replenishment of cells previously depleted under these conditions (not shown) implies a vanadate-dependent conversion of phosphoinositides to phosphatidic acid and would thus support this view. Inhibition of passive Ca^{2+} influx by a calcium chelator should, on the other hand, lead to accumulation of PI. Since the concentration of PI during depletion was not determined in these experiments it is not possible to confirm this. It may be inferred, however, from the increased recovery of ^{32}P in PI-P and the higher specific activity of phospholipids upon vanadate-induced echinocytosis that, at least in the presence of vanadate, less PI-PP and PI-P are degraded to diacylglycerol and phosphatidic acid. In addition, it was recently shown that metabolic depletion in the absence of external added Ca^{2+} and chelator results in accumulation of PI (Ferrell & Huestis, 1984).

Passive Ca^{2+} influx can probably explain the anomaly seen in Fig. 5: after 1·5 h of depletion in the presence of Ca^{2+} and vanadate, that is when 50% of the cells are echinocytic, the fractions of ^{32}P recovered in PI-PP, PI-P and phosphatidic acid are about 0·40, 0·18 and 0·36, respectively. In the absence of vanadate, less than 10% of the cells are echinocytic at the same distribution of radiolabel (after about 5 h). In the EGTA medium this anomalous behaviour is not observed. Besides, during conditions of ATP synthesis in EGTA medium higher concentrations of external vanadate are needed to produce the same degree of echinocytosis. It therefore appears that external Ca^{2+} enhances the effect of vanadate.

The slower decline in specific activity of total phospholipids (Fig. 4B) caused by exogenous vanadate implies that the action of vanadyl probably resides in its effect on the enzymes responsible for dephosphorylation of the phosphoinositides. Since the radiolabel recovered in PI-P increases to much higher levels in the presence of exogenous vanadate it appears that vanadyl mainly affects the phosphomonoesterase catalysing the conversion of PI-P to PI. In addition, the elevated specific activity also shows that the accelerated decline in the relative amount of label in PI-PP cannot be due to a reduced specific activity of ATP.

As has been described, the effects of vanadate (especially in EGTA-containing medium) and of metabolic starvation on shape change and phosphoinositide conversions, during both depletion and recovery, run in parallel. This suggests that the interconversion of phosphoinositides and phosphatidic acid is a primary event in metabolically controlled shape changes and thus strengthens the argument for a causal link between lipid metabolism and shape control inferred by Ferrell & Huestis (1984).

The observed effects can most rationally be explained in terms of the bilayer-couple hypothesis (Sheetz & Singer, 1974, 1976). Since PI-PP is believed to be localized mainly in the inner leaflet of the membrane and should translocate only very slowly because of its highly charged head-group, degradation to PI would be expected to shrink the surface area of the inner leaflet. Ferrell & Huestis (1984) have calculated that a lower limit for the contraction of the inner leaflet of the bilayer (corresponding to no flip-flop of PI) would amount to 0·33%. If phosphatidic acid is also degraded to diacylglycerol, which redistributes readily between the leaflets (Allan, Thomas & Michell, 1978), a larger contraction would result. The estimates agree well with the theoretical prediction of Beck (1978) that a bilayer imbalance of 0·4% should convert discocytes to type 3 echinocytes.

Upon metabolic recovery, PI is phosphorylated to PI-PP, probably by way of PI-P with a concomitant increase in the surface area. Most probably phosphatidic acid is also generated and this will expand the area of the inner leaflet even more. In any event, since both PI-PP and phosphatidic acid return to their normal levels (Ferrell & Huestis, 1984), the bilayer imbalance would be diminished and the cells revert to discocytes.

It should be noted that although ATP-dependent shape changes in the red cell apparently are closely linked to phosphoinositide metabolism this does not preclude the cytoskeleton or even an actomyosin-based contractile system from playing an active role in shape control.

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