Tyrosine phosphorylation of band 3 protein in Ca^{2+} /A23187-treated human erythrocytes

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Human erythrocytes were induced to release membrane vesicles by treatment with Ca^{2+} and ionophore A23187. In addition to the biochemical changes already known to accompany loading of human erythrocytes with Ca^{2+} , the present study reveals that tyrosine phosphorylation of the anion exchanger band 3 protein also occurs. The relationship between tyrosine phosphorylation of band 3 and membrane vesiculation was analysed using quinine (a non-specific inhibitor of the Ca^{2+} -activated K^+ channel, and the only known inhibitor of Ca^{2+} -induced vesiculation) and charybdotoxin, a specific inhibitor of the apamin-insensitive K^+ -channel. Both inhibitors suppressed tyrosine phosphorylation of band 3. In the presence of quinine, membrane vesiculation

was also suppressed. In contrast, at the concentration of charybdotoxin required to suppress tyrosine phosphorylation of band 3, membrane vesiculation was only mildly inhibited (16–23 % inhibition), suggesting that tyrosine phosphorylation of band 3 is not necessary for membrane vesiculation. Phosphorylation of band 3 was in fact observed when erythrocytes were induced to shrink in a Ca^{2+} -independent manner, e.g. by treatment with the K^+ ionophore valinomycin or with hypertonic solutions. These observations suggest that band 3 tyrosine phosphorylation occurs when cell volume regulation is required.

INTRODUCTION

Human erythrocytes, when treated with Ca^{2+} and ionophore A23187, respond by budding to release membrane vesicles [1,2]. This treatment induces several biochemical changes, including K^+ efflux [3], the breakdown of polyphosphoinositides, a rise in 1,2-diacylglycerol and phosphatidate concentrations [2,4–8], cross-linking of membrane proteins catalysed by transamidase [9,10] and a selective loss of glycosyl-phosphatidylinositol (GPI)-anchored membrane proteins [11]. The treatment of erythrocytes with Ca^{2+} and ionophore A23187 can thus be regarded as a model with which to investigate the biochemical mechanisms responsible for membrane vesiculation occurring during erythrocyte life in the circulation.

It has been shown that GPI-linked proteins play a role in membrane vesiculation, since erythrocytes lacking GPI-linked proteins have an impaired ability to vesiculate when treated with Ca^{2+} /A23187 [12]. Data in the literature suggest that, on the cell surface, GPI-linked proteins are complexed to protein-tyrosine kinases [13–17]. Therefore, with the aim of describing the biochemical events related to Ca^{2+} -dependent vesiculation, in the present study we have investigated whether tyrosine phosphorylation of membrane proteins occurs when erythrocytes are induced to release vesicles by treatment with Ca^{2+} /A23187.

Allan and Thomas [7,18] have described several Ca^{2+} -induced biochemical changes in erythrocytes treated with Ca^{2+} /A23187, and have analysed the relationship of these changes to membrane vesiculation. They have shown that, in the presence of increased intracellular concentrations of Ca^{2+} , vesicles are released only under conditions where polyphosphoinositides are broken down and where KCl efflux, leading to cell shrinkage (the Gardos effect [19]), occurs. Since in the present study it was observed that loading of erythrocytes with Ca^{2+} resulted in tyrosine phos-

phorylation of the anion exchanger band 3 protein, the role played by the tyrosine phosphorylation of band 3 in Ca^{2+} -induced membrane vesiculation was investigated. In particular, attention was focused on the Gardos effect, in order to study the role played by Ca^{2+} -induced K^+ efflux and concomitant cell shrinkage in the tyrosine phosphorylation of band 3. For this purpose the extent of tyrosine phosphorylation of membrane proteins was analysed in Ca^{2+} -loaded erythrocytes in the presence of quinine and charybdotoxin (ChTX), which act as inhibitors of Ca^{2+} -induced membrane vesiculation and of Ca^{2+} -induced K^+ efflux and cell shrinkage respectively.

MATERIALS AND METHODS

Materials

Nitrocellulose membranes were obtained from Sartorius (Göttingen, Germany); Protein A-Sepharose 4B and Percoll were bought from Pharmacia Fine Chemicals (Uppsala, Sweden); calcium ionophore A23187, α -chymotrypsin (bovine pancreas), Nonidet P40, anti-(band 3) monoclonal antibody (clone B-III 139), quinine and valinomycin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); ChTX was from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.) and from Peptide Institute (Osaka, Japan); anti-phosphotyrosine monoclonal antibody (clone 4G10) was from UBI (Lake Placid, NY, U.S.A.); rabbit anti-mouse IgG (H+L) was obtained from Calbiochem; goat anti-mouse IgG (H+L) conjugated to horseradish peroxidase was from Bio-Rad Laboratories (Hercules, CA, U.S.A.); Iodo-Gen Iodination Reagent was from Pierce (Rockford, IL, U.S.A.); Na^{125}I and enhanced chemiluminescence (ECL) Western blotting detection reagent were

purchased from Amersham Life Science (Little Chalfont, Bucks., U.K.). Other reagents were of analytical grade.

Collection of blood and filtration of erythrocytes

Fresh blood from normal volunteers was collected using 130 mM citric acid, 152 mM sodium citrate and 112 mM glucose as anticoagulant at a ratio 1:10 (v/v) (anticoagulant/blood). Cells were sedimented at 1000 *g* (r_{av} 11 cm) for 5 min at room temperature and then filtered on cellulose as described by Beutler et al. [20]. Erythrocytes were washed three times with 10 mM Tris/HCl, pH 7.4, buffer containing 150 mM NaCl and 2 mM MgCl₂ (referred to as 'Tris buffer' throughout the text).

Erythrocyte treatment

Treatment with Ca²⁺/A23187

Portions of washed, packed erythrocytes were resuspended at 20% haematocrit in Tris buffer containing CaCl₂ up to 1 mM and ionophore A23187 at a concentration ranging from 4 to 10 μ M. The suspension was incubated at 37 °C, sometimes in the presence of inhibitors or other substances, as described in the Results section. Incubations were terminated (usually after 30 min) by addition of 1/15 vol. of 60 mM EGTA, the cells were sedimented [1000 *g* (r_{av} 11 cm) for 5 min at 20 °C] and the supernatant was carefully collected for the isolation of vesicles (see below).

Treatment with chymotrypsin

An aliquot of cells treated with Ca²⁺/A23187 was washed with Tris buffer and treated with chymotrypsin (1 mg/ml final concentration) in Tris buffer for 15 min at 37 °C. Then 1 vol. of Tris buffer containing 2.0 mM PMSF was added and the cells were sedimented and washed.

Vesicle purification

The vesicle-containing supernatant was centrifuged at 25000 *g* (r_{av} 7 cm) for 15 min at 20 °C. Vesicles were purified from cellular debris and contaminating erythrocytes by centrifugation in a self-forming Percoll gradient, by the procedure described by Iida et al. [21]. The collected vesicles were suspended in Tris buffer and quantified by assaying the total protein content [21] by the Lowry method [22] using BSA as standard. An aliquot of vesicle suspension was immediately mixed with 0.5 vol. of SDS/PAGE sample buffer [50 mM Tris/HCl, pH 6.8, 5% (w/v) SDS, 35% (w/v) sucrose, 5 mM EDTA, 200 mM dithiothreitol, 0.01% Bromophenol Blue] and incubated at 100 °C for 5 min.

Preparation of erythrocyte ghosts

After treatment with Ca²⁺/A23187 or chymotrypsin, erythrocytes were washed twice with Tris buffer. White ghosts were prepared by the method of Marchesi and Palade [23]. Erythrocytes were lysed with 5 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF and 1 mM *p*-nitrophenyl phosphate. Membranes were collected by centrifugation for 30 min at 4 °C and 25000 *g* (r_{av} 7 cm) and further washed until free of haemoglobin. The protein concentration of the membrane suspension was determined by the Lowry method [22] using BSA as standard. An aliquot of ghost suspension was immediately dissolved in 0.5 vol. of SDS/PAGE sample buffer and incubated at 100 °C for 5 min.

Immunodetection of membrane tyrosine-phosphoproteins

Cell processing for SDS/PAGE

The phosphotyrosine content of erythrocyte membrane proteins was assayed after SDS/PAGE and Western blotting of either purified ghost membranes or erythrocyte samples. Briefly, aliquots (40 μ l) of the 20% haematocrit erythrocyte suspension were mixed with 360 μ l of SDS/PAGE sample buffer and incubated at 100 °C for 5 min.

SDS/PAGE

SDS/PAGE was performed according to the procedure of Laemmli [24]. SDS/PAGE of dissolved erythrocytes was conducted in large gel slabs (140 mm $\times$ 130 mm $\times$ 1.5 mm), and that of ghost samples on small gels (100 mm $\times$ 70 mm $\times$ 0.5 mm), at 10% acrylamide concentration.

Western blotting

After SDS/PAGE, proteins were transferred to a nitrocellulose membrane for 2 h at 200 mA using the procedure of Towbin et al. [25], with slight modifications as described in [26]. As primary antibodies, either anti-phosphotyrosine monoclonal or anti-(band 3) monoclonal IgGs were used. Horseradish peroxidase-conjugated goat anti-mouse IgG was usually employed as a secondary antibody, in conjunction with ECL detection. In some experiments a radioiodinated rabbit anti-mouse IgG was used. Multiple detection steps with different primary antibodies were performed by stripping and reprobing the same nitrocellulose membrane as described by Kaufmann et al. [27], with slight modifications as reported in the Amersham ECL protocol.

Immunoprecipitation of tyrosine-phosphorylated proteins

Portions of 150 μ l of packed cells were suspended in Nonidet P40 lysis buffer, and phosphoproteins were immunoprecipitated as described by Harrison et al. [28] using anti-phosphotyrosine monoclonal antibody. To displace phosphoproteins, Protein A-Sepharose was treated for 15 min at 4 °C with 50 μ l of 25 mM Hepes/NaOH and 225 mM NaCl containing 2 mM phosphotyrosine. Samples containing immunoprecipitated proteins were collected, immediately mixed with 50 μ l of SDS/PAGE sample buffer and treated at 100 °C for 5 min.

Lactate assay

To 1 vol. of 20% haematocrit erythrocyte suspension in Tris buffer was added 0.25 vol. of ice-cold 1 M perchloric acid. The precipitated proteins were sedimented by centrifugation for 2 min at 4 °C and 15000 *g* (r_{av} 6 cm), and the supernatant was used for lactate determination, as described by Gutmann and Wahlefeld [29] and Beutler [30].

RESULTS AND DISCUSSION

Tyrosine phosphorylation of band 3 protein in Ca²⁺/A23187-treated erythrocytes

After treatment of erythrocytes with Ca²⁺/A23187, a major tyrosine-phosphorylated protein of about 95 kDa was present

Figure 1 Tyrosine phosphorylation of band 3 in Ca^{2+} /A23187-treated erythrocytes

(A) Erythrocytes (20% suspension in Tris buffer) were incubated in the presence of increasing Ca^{2+} concentrations and $2 \mu\text{M}$ calcium ionophore (A23187) at 37°C for 1 h. Lane 1, 3 mM EDTA; lane 2, control; lane 3, $50 \mu\text{M}$ Ca^{2+} ; lane 4, $100 \mu\text{M}$ Ca^{2+} ; lane 5, $200 \mu\text{M}$ Ca^{2+} ; lane 6, $500 \mu\text{M}$ Ca^{2+} ; lane 7, 1 mM Ca^{2+} ; lane 8, membrane vesicles released from erythrocytes treated with 1 mM Ca^{2+} and ionophore A23187. (B) and (C) Erythrocytes (20% suspension in Tris buffer) were incubated in the presence of 0.8 mM CaCl_2 and $2 \mu\text{M}$ calcium ionophore A23187 at 37°C for 1 h. An aliquot of cells was then treated with α -chymotrypsin (1 mg/ml) at 37°C for 15 min. Lane 1, control cells; lane 2, cells treated with Ca^{2+} /A23187; lane 3, cells treated with Ca^{2+} /A23187 and then with chymotrypsin. Ghost membranes were prepared from cells in the presence of tyrosine phosphatase inhibitors. Ghosts and vesicles were diluted with 0.5 vol. of SDS/PAGE sample buffer, treated at 100°C for 5 min and subjected to SDS/PAGE. After transfer to nitrocellulose, band 3 and related peptides were visualized by immunodetection with anti-phosphotyrosine monoclonal antibody in (A) and (B) and with anti-(band 3) monoclonal antibody in (C), followed by ECL. Samples of $30 \mu\text{g}$ of proteins were loaded in each well.

Table 1 Membrane vesiculation and cell shrinkage in erythrocytes incubated with Ca^{2+} /A23187 and various substances

Erythrocytes (20% haematocrit) were incubated at 37°C for 30 min in Tris buffer (see the Materials and methods section) with the additions indicated. Treatment of cells (haematocrit 10–20%) with ChTX (10 nM–2 μM) was carried out in Tris buffer for 1 h at 37°C , then the haematocrit was adjusted to 20% and the cells were treated with Ca^{2+} /A23187. nd, not determined. Results are individual values ($n = 1$), ranges ($n = 2$) or means $\pm$ S.D. ($n \geq 3$).

Conditions	Vesiculation (% of max. value)	Shrinkage (%)
Tris buffer (NaCl 150 mM) ($n = 5$)	0	0
Tris buffer (KCl 150 mM) ($n = 1$)	0	nd
Tris buffer (NaCl 150 mM) + 5 μM valinomycin ($n = 1$)	0	nd
Tris buffer (NaCl 150 mM) + 500 mOsm NaCl ($n = 2$)	0	nd
Ca^{2+} /A23187 ($n = 5$)	100	31 ± 3
Ca^{2+} /A23187 + 0.8 mM quinine ($n = 3$)	0	nd
Ca^{2+} /A23187 + 100 nM ChTX ($n = 1$)	90	nd
Ca^{2+} /A23187 + 970 nM ChTX ($n = 2$)	77–84	$15 (n = 1)$
Ca^{2+} /A23187 + 970 nM ChTX + 5 μM valinomycin ($n = 1$)	98	32
Ca^{2+} /A23187 + 970 nM ChTX + 500 mOsm NaCl ($n = 1$)	99	29
Ca^{2+} /A23187 + 2 μM ChTX ($n = 3$)	71 ± 12	0
Ca^{2+} /A23187 + 2 μM ChTX + 5 μM valinomycin ($n = 3$)	78 ± 11	29 ± 4
Ca^{2+} /A23187 + 2 μM ChTX + 500 mOsm NaCl ($n = 2$)	69–75	20–29

both in ghosts prepared from Ca^{2+} -loaded cells and in membrane vesicles (Figure 1A). Another phosphoprotein band of about 60 kDa was consistently detectable in ghost membranes prepared

from Ca^{2+} -loaded and control cells; the amount of this protein progressively decreased as the Ca^{2+} concentration increased (Figure 1A), probably because it was sequestered in high-molecular-mass protein aggregates produced by the activity of Ca^{2+} -dependent transglutaminase [10].

The detection of blotted protein bands with anti-phosphotyrosine antibody and then, after stripping, with anti-(band 3) monoclonal antibody (Figures 1B and 1C) proved that the 95 kDa phosphoprotein is band 3. The 60 kDa phosphoprotein was also recognized by the anti-(band 3) antibody, suggesting that it is derived from band 3 by degradation; this agrees with data reported by Morrison et al. [31] showing the accumulation, in mature erythrocytes, of a 60 kDa fragment of band 3 as a catabolic product of the molecule. The presence of p60 src and fyn in the 60 kDa band was excluded by specific immunodetection.

The identity of the 95 kDa phosphoprotein was confirmed by chymotryptic treatment of intact erythrocytes. After chymotrypsin digestion of Ca^{2+} -loaded erythrocytes, the 95 kDa phosphoprotein disappeared and a 60 kDa phosphopeptide, recognized by the anti-(band 3) antibody, was detected (Figures 1B and 1C). Such a result was expected on the basis of chymotrypsin fragmentation [32] and of the tyrosine-phosphorylation patterns of band 3 reported in the literature [33–35].

Specificity of the detection and immunoprecipitation of phosphoproteins

To avoid possible artifacts resulting from the preparation of ghost membranes from Ca^{2+} -loaded cells, and to better preserve the phosphorylation state of the proteins, the cells, after treatment with Ca^{2+} /A23187, were immediately solubilized with SDS sample buffer and subjected to SDS/PAGE and Western blotting. Under these conditions the anti-phosphotyrosine antibody specifically recognized phosphotyrosine residues on band 3 protein (results not shown) when either ECL detection or radioiodinated secondary antibody was used. This ruled out possible artifactual results due to the ECL method, which is routinely adopted to detect phosphoproteins. Moreover, the identity of the 95 kDa protein as band 3 was confirmed by the presence of this protein in the immunoprecipitate of phosphoproteins from Ca^{2+} -treated erythrocytes, but not from control cells (results not shown).

The transmembrane anion transporter band 3 protein has been recognized as one of the major *in situ* substrates for the human erythrocyte protein tyrosine kinases for several years [33,34,36], and recently the enzyme responsible for the tyrosine phosphorylation of band 3 was identified as the non-receptor protein tyrosine kinase p72^{syk} [28]. The tyrosine phosphorylation site has been identified at the extreme N-terminus of the cytoplasmic tail of band 3 [33–35]. We did not perform a detailed investigation of the tyrosine phosphorylation site; however, our observations agree with data reported in the literature.

Relationship between vesicle release and tyrosine phosphorylation of band 3 in Ca^{2+} /A23187-treated erythrocytes

Ca^{2+} /A23187 treatment in the presence of quinine or in isotonic K^+ buffer

In an attempt to analyse whether the tyrosine phosphorylation of band 3 is necessary for vesiculation to occur and is related to KCl efflux and cell shrinkage resulting from increased intracellular concentrations of Ca^{2+} , we treated erythrocytes with Ca^{2+} /A23187 in the presence of quinine, a non-specific inhibitor

Figure 2 Tyrosine phosphorylation of band 3 in erythrocytes treated with Ca^{2+} /A23187 in the presence of inhibitors of the Gardos effect

Erythrocytes (20% suspension in Tris buffer) were incubated in Tris buffer at 37 °C for 30 min in the presence of 0.8–1.0 mM CaCl_2 and 2–10 μM calcium ionophore A23187 (C), with the addition of 0.8 mM quinine (Q), the replacement of 150 mM NaCl with 150 mM KCl, or the addition of 970 nM ChTX. After incubation, an aliquot of the suspension was collected, diluted with 9 vol. of SDS/PAGE sample buffer and treated at 100 °C for 5 min. Samples of 2 μl of packed cells were loaded into each well. After SDS/PAGE and transfer to nitrocellulose, phosphotyrosine was visualized by immunodetection with an anti-phosphotyrosine monoclonal antibody and ECL. Under all three sets of conditions where the Gardos effect was inhibited (Q and ChTX) or suppressed (KCl), tyrosine phosphorylation of band 3 was suppressed.

of the Gardos effect [37,38] and the only known inhibitor of Ca^{2+} -induced vesiculation [7]. On inhibiting the Gardos effect with 0.8 mM quinine, vesicle release was completely blocked (Table 1). Under these conditions the tyrosine phosphorylation of band 3 was also suppressed (Figure 2), suggesting that band 3 phosphorylation is related either to membrane vesiculation or to the K^+ efflux and cell shrinkage induced by Ca^{2+} treatment.

Band 3 phosphorylation and vesicle release were also inhibited when NaCl in the medium was replaced by KCl during Ca^{2+} /A23187 treatment (Figure 2 and Table 1). Under these conditions the passive K^+ efflux through the Gardos channel and resultant cell shrinkage are prevented by the high extracellular concentration of K^+ .

Band 3 tyrosine phosphorylation and glycolytic rate

Since it has been reported that tyrosine phosphorylation of band 3 occurs under conditions where glycolysis is stimulated [39], experiments with quinine were also useful for investigating whether the observed tyrosine phosphorylation of band 3 was related to the increased rate of glycolysis produced by Ca^{2+} loading of erythrocytes. We found that during Ca^{2+} /A23187 treatment the stimulation of glycolysis (evaluated as lactate production) occurred at different levels in erythrocytes from different donors; however, the presence of quinine completely abolished both vesiculation and band 3 phosphorylation (Figure 2 and Table 1) without affecting the rate of glycolysis (Table 2). We cannot exclude the possibility that tyrosine phosphorylation of band 3 could modulate glycolysis under physiological conditions. However, when we artificially increased the intracellular Ca^{2+} to a non-physiological concentration by treatment with ionophore A23187 in the presence of quinine, the overall increase in the glycolytic rate was attained without the occurrence of band 3 phosphorylation. Presumably the increased rate of glycolysis is required to fuel the Ca^{2+} -ATPase involved in removing the extra intracellular Ca^{2+} .

Ca^{2+} /A23187 treatment in the presence of ChTX

A further approach to investigating the correlation between tyrosine phosphorylation of band 3 and vesiculation was carried out using ChTX, a 4.3 kDa polypeptide acting as a specific inhibitor of apamin-insensitive Ca^{2+} -activated K^+ channels [40]. We performed Ca^{2+} /A23187 treatment of erythrocytes in the presence of ChTX at concentrations ranging from 10 nM to

Table 2 Treatment of erythrocytes with Ca^{2+} /A23187 in the presence of quinine: enhancement of rate of glycolysis

Erythrocytes (20% suspension in Tris buffer) were incubated in the presence of 1 mM CaCl_2 , 2 μM A23187 and 5 mM glucose with or without 0.8 mM quinine at 37 °C for 30 min. The stimulation of glycolysis (expressed as lactate production) was evaluated (lactate concentration is referred to cell water). The presence of quinine did not affect the Ca^{2+} /A23187-dependent enhancement of the glycolysis rate.

Expt.	Time (min)	Lactate production			
		– Quinine		+ Quinine	
		(μM)	(% of control)	(μM)	(% of control)
1	0	766	100	766	100
	30	1627	212	1467	191
2	0	175	100	175	100
	30	837	478	829	474
3	0	112	100	112	100
	30	845	754	638	570

2 μM , and observed significant inhibition of band 3 phosphorylation starting at 100 nM ChTX (results not shown). At 970 nM ChTX the tyrosine phosphorylation of band 3 was abolished (Figure 2), while vesicles were released in significant amounts (Table 1). The mild inhibition of vesiculation produced by ChTX treatment questions the possible role played by tyrosine phosphorylation in membrane vesiculation, and suggests that the two events are unrelated to each other.

In the presence of ChTX, the suppression of band 3 tyrosine phosphorylation was observed under conditions where the Gardos effect was only partially inhibited, so that K^+ efflux and cell shrinkage allowed vesiculation to occur at high levels [7]. In these conditions, it was possible, as observed by Allan and Thomas [7], to relieve the slight ChTX inhibition of Ca^{2+} -induced vesiculation by addition of valinomycin, which causes KCl efflux and cell shrinkage in a Ca^{2+} -independent manner [41], or by treatment with hypertonic NaCl buffer, which induces cell shrinkage (Table 1).

On raising the ChTX concentration to 2 μM the Gardos effect was completely inhibited but, unexpectedly, vesiculation was not suppressed (Table 1). This suggests that K^+ efflux and cell shrinkage are not necessary for vesiculation to occur, although these events increase the extent of vesiculation. In the samples containing 2 μM ChTX, treatment with valinomycin or hypertonic NaCl buffer slightly relieved the inhibition of Ca^{2+} -induced vesiculation (Table 1).

The different amounts of vesicles released by Ca^{2+} -loaded erythrocytes in the presence of quinine or ChTX may be due to the different specificities of these inhibitors for the K^+ channel, or to their interaction with different sites on the channel, or to the effects of quinine on other unknown events relevant to membrane vesiculation. In this respect it is notable that quinine has been observed to inhibit Na^+ fluxes [42] and phospholipase A and C activity [43,44].

Importance of cell shrinkage for tyrosine phosphorylation of band 3: treatment of erythrocytes with valinomycin and hypertonic NaCl

In order to induce K^+ efflux and cell shrinkage independently of Ca^{2+} loading, erythrocytes were treated with valinomycin, a specific K^+ ionophore causing K^+ loss and cell dehydration [41]. Under these experimental conditions band 3 tyrosine

Figure 3 Erythrocyte shrinkage and tyrosine phosphorylation of band 3

Erythrocytes (20% suspension in Tris buffer) were incubated at 37 °C for 30 min in the presence of 1 mM CaCl_2 and 5 μM valinomycin (A), 0.8 mM CaCl_2 and 10 μM A23187 (B), Tris buffer containing 800 mOsm NaCl (final concentration) (C), or 0.8 mM CaCl_2 , 10 μM A23187 and 800 mOsm NaCl (D). At the indicated times, an aliquot of suspension was collected, diluted with 9 vol. of SDS/PAGE sample buffer and treated at 100 °C for 5 min. In each well, 2 μl equivalent packed cells were loaded. After SDS/PAGE and transfer to nitrocellulose, phosphotyrosine was visualized by immunodetection with an anti-phosphotyrosine monoclonal antibody and ECL.

phosphorylation occurred, whereas vesiculation was absent (Figure 3 and Table 1). That cell dehydration and shrinkage are sufficient for band 3 phosphorylation to occur was also confirmed by the behaviour of erythrocytes treated with hypertonic NaCl (Figure 3).

Conclusions

In this paper we have shown that, when erythrocytes are induced to vesiculate by treatment with Ca^{2+} /A23187, tyrosine phosphorylation of band 3 occurs. It was possible to exclude a relationship between band 3 phosphorylation and membrane vesiculation, since in experiments where loading of erythrocytes with Ca^{2+} was carried out in the presence of non-saturating concentrations of ChTX, band 3 phosphorylation was suppressed while membrane vesiculation occurred at high levels (Table 1). Experiments with ChTX also provided new insight into the role played by the Gardos effect in Ca^{2+} -induced membrane vesiculation. Having observed the release of significant amounts of membrane vesicles when the Gardos effect was completely inhibited by ChTX, it was possible to argue that the Gardos effect is not necessary for Ca^{2+} -ionophore-induced membrane vesiculation (although it can increase the extent of vesiculation).

Band 3 phosphorylation in Ca^{2+} -loaded cells is thus not related to membrane vesiculation, but is the consequence of the cell shrinkage produced by Ca^{2+} /A23187 treatment, since it is observed regardless of the conditions that are adopted to induce cell shrinkage, such as treatment of erythrocytes with valinomycin or with hypertonic buffer (in the absence of membrane vesiculation).

Several protein tyrosine kinases have been reported to show increased activity following a decrease in the cellular hydration state [45]. This could also be the case for p72^{syk}, the protein tyrosine kinase responsible for band 3 phosphorylation in intact cells [28], although the activity of p72^{syk} was not observed to increase when assayed at high ionic strength, using the cytoplasmic domain of band 3 as substrate, in *in vitro* studies (P. S. Low, personal communication).

Tyrosine phosphorylation has been described as a means of controlling the activity of ion channels [46] via mechanisms as yet unknown. In the human erythrocyte, tyrosine phosphorylation of band 3 seems to be an event related to the cell volume regulatory mechanisms or to volume-associated cytoskeletal alterations that lead to changes in cell shape.

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