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Functional correlation between the Ser/Thr-phosphorylation of band-3 and band-3-mediated transmembrane anion transport in human erythrocytes

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In human erythrocytes, okadaic acid, a potent inhibitor of certain protein phosphatases, promotes a marked increase of Ser/Thr-phosphorylation of membrane proteins, including band-3 protein. Moreover, okadaic acid also increases the band-3-mediated oxalate transport across the membranes, thus suggesting that this process is regulated by Ser/Thr-phosphorylation of transporter band-3 protein.

Introduction

Band-3 protein, the major transmembrane protein of human erythrocytes, is known to display multiple functions, including the ability to transport anions across the membrane. Like other membrane proteins, band-3 protein is phosphorylated, but the physiological role of this modification remains to be clarified. In particular, some observations [1–4] suggested that its Ser/Thr-phosphorylation state is involved in the anion transport.

The results reported here show that human erythrocytes, when treated with okadaic acid, a well-known inhibitor of certain P-Ser/Thr-protein phosphatases [5,6], display an increased level of Ser/Thr-phosphorylation of membrane proteins, including band-3 protein, which is paralleled by an increased transmembrane band-3-mediated oxalate transport. We used oxalate as the anion because its transport has been found [7,8] to be increased in the erythrocytes of idiopathic calcium-oxalate renal stone formers.

Materials and Methods

Human erythrocytes were prepared by centrifugation (at $750 \times g$) for 3 min) of fresh blood collected from healthy donors. To minimize contamination by leucocytes and platelets, the packed red cells were washed three times by centrifugation in buffer A (20 mM Tris (pH 7.5), 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 0.1 mg/ml streptomycin, 25 μg/ml chloramphenicol) and discarding the buffy coat and the upper third of packed red cells layer.

Membrane protein phosphorylation. Packed red cells (1 ml) were preincubated in 9 ml buffer A for 4 h at 35°C to deplete endogenous ATP stores, then incubated with $[^{32}P]P_i$ (0.3 mCi) for 18 h at 35°C in the same buffer A containing glucose (24 mM) and adenosine (1 mM) with gentle stirring.

After 18 h equilibration, okadaic acid (1 μM final concentration) dissolved in dimethylformamide (DMF) was added to half of the sample of ^{32}P-labeled erythrocytes, while the same amount of DMF was added to the remaining half (control). After 90 min incubation, both samples were washed once in the same buffer A containing glucose (24 mM) and adenosine (1 mM) and then hemolysed in 14 ml of hypotonic 5 mM phosphate buffer (pH 8), containing 0.02% NaN_3 and 0.03 mM phenylmethylsulphonyl fluoride (PMSF). The membranes were recovered by centrifugation (at $20\,000 \times g$ for 20 min) and washed twice in hypotonic lysis buffer containing 30 μM vanadate, and twice in 25 mM Tris buffer (pH 8) containing 0.03 mM PMSF, 0.02% NaN_3 and 30 μM vanadate, and then solubilized by addition of 2% sodium dodecylsulfate (SDS) and 1% β-mer-

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captoethanol (final concentration) followed by a 5-min treatment at 100°C.

Solubilized ^{32}P-labeled membranes (60 μg) were submitted to electrophoresis on 0.1% SDS-10% polyacrylamide slab gels, essentially according to Laemmli [9].

After electrophoresis, the slab gels were stained with Coomassie brilliant blue (CB) according to Laemmli [9]. Some gels were treated with a 2 M NaOH solution at 55°C for 1 h and fixed again. Dried gels were autoradiographed as in Ref. 4.

Oxalate transmembrane flux. Oxalate exchange was evaluated in aliquots of human erythrocytes used for membrane protein phosphorylation assay. Erythrocytes were treated as described above, except that [^{32}P]P_i during incubations was omitted. After 90 min incubation in the presence and absence of okadaic acid, the two erythrocyte samples were submitted to the transmembrane oxalate flux assay as previously described [8].

Briefly, oxalate exchange was evaluated in washed erythrocytes after 2 h incubation in buffer A (pH 7.5), containing 10 mM sodium oxalate. The disappearance of [^{14}C]oxalate from the incubation medium was followed in time. The oxalate flux was expressed as K, an index of oxalate exchange at equilibrium, calculated from the slope of the linear relation:

$$\ln(A_t - A_\infty)/(A_0 - A_\infty) = -K \cdot t$$

where t is time, K the flux rate and A the quantity of [^{14}C]oxalate in the incubation medium at time 0, at time t and at isotope equilibrium (∞).

Miscellaneous. Okadaic acid was purchased from Calbiochem; [^{32}P]P_i from Amersham International. Dimethylformamide was from Prolabo and adenosine from Boehringer-Mannheim.

Protein content was determined according to Lowry et al. [10].

Results and Discussion

When the human erythrocytes were preincubated with [^{32}P]P_i and then treated with 1 mM okadaic acid or with its solvent DMF (control) under the conditions described in Materials and Methods, the ^{32}P-labeling patterns of membrane proteins reported in Fig. 1 were obtained. As shown in Fig. 1 (lanes a,b), the most ^{32}P-labeled proteins are the β-subunit (band 2) of cytoskeletal spectrin and the transmembrane band-3 protein. Their ^{32}P-labeling is due, at least by far predominantly, to the alkali-labile phosphorylation of Ser residues, as indicated by its disappearance in the alkali-treated gels (lanes a',b') [4]. In the presence of okadaic acid (lane b), the ^{32}P-labeling of membrane proteins is markedly increased as compared with control (lane a). Such an increase is due to an enhanced phosphorylation level, taking into account that the okadaic acid does not alter the specific radioactivity of the endogenous [γ-^{32}P]ATP, its content being reported to be unaffected by okadaic acid [5,6].

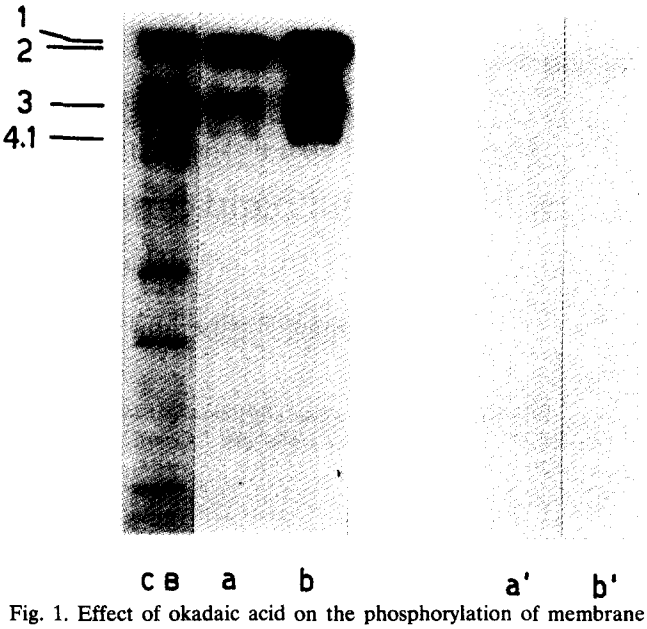

Fig. 1. Effect of okadaic acid on the phosphorylation of membrane proteins in the human erythrocytes. Erythrocytes were preincubated with [^{32}P]ortophosphate and then treated with 1 μM okadaic acid or with its solvent DMF (control). Experimental conditions for the incubation, the membrane preparation and SDS-PAGE analysis of ^{32}P-labeled membrane proteins as described in Materials and Methods. Lane a shows the control, i.e., ^{32}P-labeled membranes from erythrocytes incubated with DMF and in the absence of okadaic acid; lane b, ^{32}P-labeled membranes from erythrocytes incubated in the presence of okadaic acid. Lanes a' and b' show the alkali-treated gels corresponding to the alkali-untreated gels a and b. Lane CB shows the Coomassie-blue-stained gel. Autoradiograms were exposed for 60 h.

It is of interest that the okadaic-acid-treated human erythrocytes exhibit an enhanced band-3-mediated oxalate transport (Fig. 2), thus strongly supporting the view [1–4] that a faster transmembrane anion transport is paralleled by an increased Ser/Thr-phosphorylation of some membrane proteins, including the transporter band-3 protein.

Since long time it is well known that the transmembrane band 3 protein (95 kDa) is phosphorylated [11] primarily, if not exclusively, on Ser/Thr residues [12,13] and on Tyr residues [14–23] of its N-terminal portion (43 kDa), which protrudes as a flexible polyanionic finger into cytoplasmic space.

It may be that the Ser/Thr-phosphorylation of its large cytoplasmic domain induces allosterically a conformational change of its transmembrane segment mediating the anion transport.

A functional correlation between band-3-mediated anion transport and membrane protein Ser/Thr-phos-

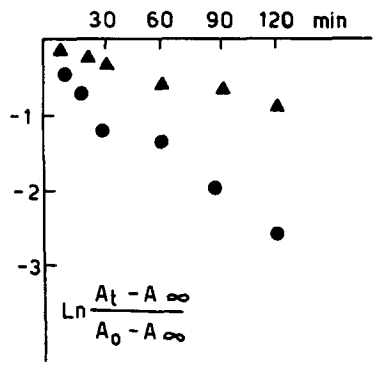

Fig. 2. Effect of okadaic acid on the oxalate transmembrane transport. Oxalate transmembrane self-exchange rate was evaluated in the erythrocytes previously incubated in the presence of 1 μM okadaic acid (●) ($K = -0.70 \cdot 10^{-2}$ min^{-1}) or of its solvent DMF (control, ▲) ($K = -0.21 \cdot 10^{-2}$ min^{-1}). The flux rate was evaluated as described in Materials and Methods.

phorylation was also based on the following lines of evidence: (a) The abnormal higher oxalate-exchange flux rate displayed by the erythrocytes of idiopathic nephrolithiasic patients was associated with an increased phosphorylation of some membrane proteins, including band-3 protein, displayed by their ghosts [2] when incubated in the presence of [γ-^{32}P]ATP. Moreover, a linear relationship between the two processes has been found in the erythrocytes of 13 idiopathic renal stone formers (data not shown); (b) Hydrochlorothiazide, a drug reducing the transmembrane oxalate flux both in vitro and in vivo in erythrocytes of nephrolithiasic patients, has been found to decrease the ghost phosphorylation of some membrane proteins, including band-3, as well [2,8]. (c) Metabolic ATP depletion of normal human erythrocytes, decreasing the transmembrane anion self-exchange [1], has been found to decrease the Ser/Thr-phosphorylation of membrane proteins, including band-3 [4]. The decrease of both processes is reversed by the following metabolic repletion [1,4]. (d) DIDS (4,4′diisothiocyanostilbene-2,2′-disulfonate), a well-known inhibitor of anion transport by binding to the external segment of band-3, has also been found to reduce the Ser/Thr-phosphorylation (but not the Tyr-phosphorylation) of membrane proteins, including band-3 [24].

These results might raise the question whether the band-3-mediated anion transport is regulated by the phosphorylation state of the transporter band-3 protein itself, catalyzed predominantly, if not exclusively, by cAMP- and Ca^{2+}-independent casein kinase (CKI) [25,26], or also by the phosphorylation state of the band-3-linked cytoskeletal proteins (ankirin, spectrin and band 4.1) which are involved in the control of many of the mechanical properties (such as cell shape and deformability) and are phosphorylated by other protein kinases, such as cAMP-dependent protein kinase [27] and Ca^{2+}-phospholipid-dependent protein kinase C [27,28].

However, observations that effectors of protein kinase C and cAMP-dependent protein kinase do not affect oxalate transport [29] supported the view that CKI-catalyzed Ser/Thr-phosphorylation of membrane proteins, including band-3, is necessarily involved in regulating anion transport.

In conclusion, these findings may provide new insights into the physiopathology of cellular anomalies found in idiopathic calcium oxalate nephrolithiasis [2,7,8]. The possibility of an imbalance between Ser/Thr-protein kinase and *P*-Ser/Thr-protein phosphatase activities, or between their effectors, could be taken into account.

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