Ca^{2+} promotes erythrocyte band 3 tyrosine phosphorylation via dissociation of phosphotyrosine phosphatase from band 3

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The anion-exchange band 3 protein is the main erythrocyte protein that is phosphorylated by protein tyrosine kinase (PTK). We have previously identified a band 3-associated phosphotyrosine phosphatase (PTP) that is normally highly active and prevents the accumulation of band 3 phosphotyrosine. Band 3 tyrosine phosphorylation can be induced by inhibition of PTP (vanadate, thiol oxidation), activation of PTK (hypertonic NaCl) or intracellular increased Ca^{2+} (mechanism unknown). We now show that there is inhibition of dephosphorylation of band 3 in Ca^{2+}/ionophore-treated erythrocytes and in membranes isolated from the treated cells. These membranes exhibit phosphatase activity upon the addition of exogenous substrate. Dephosphorylation of the endogenous substrate (band 3) can be activated in these membranes by the addition of Mg^{2+}. Thus the inability of PTP to dephosphorylate the band 3 phosphotyrosine is not due to inhibition of the enzyme itself. Ca^{2+} rise in the erythrocyte causes dissociation of PTP from band 3, thus leaving the kinase unopposed. This is shown by a significant diminution in band 3/PTP co-precipitation. Addition of Mg^{2+} to these membranes leads to reassociation of band 3 with PTP. The Ca^{2+}-induced inhibition of band 3 dephosphorylation may be due to Ca^{2+}-dependent alterations in membrane components and structure, affecting the interaction of band 3 with PTP. The Ca^{2+}-induced tyrosine phosphorylation, involving an apparent PTP inhibition via dissociation from the substrate, may play a role in signal transduction pathways and in certain pathological disorders associated with increased cell Ca^{2+}.

Key words: hypertonic, Mg^{2+}, phosphotyrosine phosphatase 1B (PTP1B), protein tyrosine kinase (PTK), red blood cell.

INTRODUCTION

Phosphorylation of protein tyrosine residues plays a central role in the regulation of various cell processes, such as cell proliferation, differentiation and metabolism. The level of protein phosphotyrosine is regulated by a balance between the activity of protein tyrosine kinases (PTKs) and the opposing activity of protein phosphotyrosine phosphatases (PTPs) [1–4]. Usually, little phosphotyrosine is detected in normal cells. A significant increase in tyrosine phosphorylation can be achieved by various triggering events, and by the use of compounds known to inhibit PTP [1–3]. It has been proposed that the PTPs normally act to maintain a very low level of phosphorylated tyrosine [1].

Erythrocytes contain PTK activity, with band 3 protein being the major substrate for the PTKs [5–7]. Several PTPs have been detected in erythrocytes [8,9]. We have previously identified a PTP associated with band 3 in the human erythrocyte membrane, which is normally highly active and prevents the accumulation of band 3 phosphotyrosine. The PTP appears to be PTP1B [10]. It is reversibly inhibited by vanadate. It is also inhibited by erythrocyte thiol oxidation, which leads to the formation of PTP/band 3 mixed disulphides and abolition of dephosphorylation, allowing the accumulation of band 3 phosphotyrosine [11]. Inhibition of PTP has been shown to be responsible in other cases of oxidative-stress-induced tyrosine phosphorylation [12, 13].

Protein tyrosine phosphorylation has been shown to be induced by increasing Ca^{2+} in several types of cells [14–16]. In the normal erythrocyte, band 3 tyrosine phosphorylation occurs upon an increase in erythrocyte Ca^{2+} [17], and is impaired in Ca^{2+}-treated erythrocytes in Scott syndrome [18]. Tyrosine phosphorylation of band 3 protein is also induced when human erythrocytes are treated with hypertonic NaCl [17] or on an increase in Mg^{2+} [19]. Low levels of band 3 phosphotyrosine are also detected in deoxygenated normal erythrocytes, and high levels are observed in sickle cells [19,20]. The Ca^{2+}- and NaCl-induced tyrosine phosphorylation has been suggested to result from erythrocyte shrinkage [17,21]. However, in skate erythrocytes, band 3 tyrosine phosphorylation is induced by hypotonic volume expansion [22]. In the case of hypertonic NaCl-induced tyrosine phosphorylation, activation of PTK appears to be responsible for the band 3 tyrosine phosphorylation [21]. It has not yet been clarified whether Ca^{2+}-induced tyrosine phosphorylation is due to PTK activation or to PTP inhibition.

Here we show that the Ca^{2+}-induced tyrosine phosphorylation is different from that induced by hypertonic NaCl. An increase in erythrocyte Ca^{2+} leads to band 3 tyrosine phosphorylation which is not reversed by kinase inhibitors, whereas the NaCl-induced tyrosine phosphorylation is reversible ([21] and the present work). Ca^{2+}-induced tyrosine phosphorylation involves dissociation of PTP from band 3, leading to an apparent inhibition of PTP. No such PTP inhibition occurs in NaCl-induced tyrosine phosphorylation. The inability of PTP to dephosphorylate band 3 phosphotyrosine in the Ca^{2+}-treated cells allows band 3 tyrosine phosphorylation by unopposed kinase activity. The overall results are consistent with the idea that the NaCl-induced phosphorylation is due to activation of PTK, whereas the Ca^{2+}-induced phosphorylation is due to inhibition of band 3 dephosphorylation by PTP. Ca^{2+}-induced tyrosine phosphorylation involving PTP dissociation from substrates may play a role in signal transduction pathways and in certain pathological disorders associated with increased intracellular Ca^{2+}.

Abbreviations used: GF, GF 109203X; HRP, horseradish peroxidase; PKC, protein kinase C; p-NPP, *p*-nitrophenyl phosphate; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine; PTK, protein tyrosine kinase; PTP, phosphotyrosine phosphatase.

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EXPERIMENTAL

Erythrocytes and erythrocyte membranes

Fresh blood was obtained from healthy humans using EDTA as an anti-coagulant. Blood was centrifuged, plasma and buffy coat were removed and the erythrocytes washed three times with 150 mM NaCl. Erythrocyte membranes were obtained by haemolysing cells in 5 mM sodium phosphate buffer, pH 8.0/1.0 mM EDTA/0.1 mM PMSF (haemolysing solution). Membranes were washed with haemolysing solution, then further washed with 10 mM NaCl/0.1 mM PMSF to obtain haemoglobin-free membranes (white membranes), as described previously [10].

Treatments of erythrocytes and of erythrocyte membranes

To study erythrocyte phosphorylation, washed erythrocytes were suspended to 10 % haematocrit in 25 mM Hepes buffer, pH 7.3/150 mM NaCl, containing 10 mM glucose and 1.0 mM adenosine (buffer A). Erythrocyte suspensions were incubated in the presence of 0.01–1.0 mM $CaCl_2$ and 5 μM of the ionophore A23187 (Sigma, St. Louis, MO, U.S.A.; referred to as Ca^{2+}/A23187), or in the presence of 0.1 mM sodium orthovanadate (from hereon called vanadate), or with buffer A containing an extra 250 mM NaCl (final concentration, 400 mM; hypertonic NaCl). To study effects of PMA (Sigma), erythrocyte suspensions were incubated in the presence of Ca^{2+}/A23187 and 1 μM PMA (diluted from stock solution of 1.0 mM in DMSO). To study the effects of inhibitors, erythrocyte suspensions were preincubated at 37 °C for 15 min without or with one of the following reagents (obtained from Calbiochem, La Jolla, CA, U.S.A.), at the final concentrations given: 25 μM calpeptin, 40 μM GF 109203X (GF), 40 μM 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1), 50 μM KN-62 (a Ca^{2+}/calmodulin kinase II inhibitor). Stock solutions were prepared in DMSO, with final concentrations of DMSO below 0.1 %. The suspensions were then incubated further with Ca^{2+}/A23187 or hypertonic NaCl. Other erythrocyte suspensions were first incubated for 30 min with Ca^{2+}/A23187 or with hypertonic buffer, then incubated further with GF or PP1. Aliquots of erythrocytes were removed at intervals, and membranes prepared as described above using 0.1 mM vanadate in the haemolysing and washing solutions.

To study membrane phosphorylation, membranes were suspended at 1.0 mg of protein/ml in 25 mM Hepes/0.1 mM PMSF (buffer B), containing 5 μM ATP and 10 mM Mg^{2+}, and incubated at 30 °C for 15 min, without or with 0.1 mM vanadate, or 0.1 mM $CaCl_2$, in the absence and presence of 5 μM A23187.

Electrophoresis and immunoblotting

Membrane samples were solubilized in Laemmli's SDS buffer (sample buffer), and boiled for 2 min. Proteins of the solubilized membranes were resolved by SDS/PAGE (10 % gels), followed by transfer to Hybond ECL nitrocellulose membranes (Amersham Bioscience). The nitrocellulose membranes were blocked for 1 h at room temperature in a solution of 10 mM Tris, pH 7.4/135 mM NaCl/0.1 % Tween-20 (TNT)/1.0 % BSA. Membranes were then incubated for an additional 1 h at room temperature with one of the appropriate primary antibodies: monoclonal anti-phosphotyrosine PY-20 antibody (Transduction Laboratories, Lexington, KY, U.S.A.); monoclonal anti-PTP antibody FG6-1G (Oncogene Research Products, Cambridge, MA, U.S.A.); monoclonal anti-band 3 antibody (Sigma); polyclonal anti-protein kinase C (PKC)α antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.); monoclonal anti-μ-calpain antibody [23]. After washing with TNT/0.1 % BSA, the membranes were incubated for 1 h with the appropriate secondary antibody [goat anti-mouse or anti-rabbit IgG (H+L), conjugated to horseradish peroxidase (HRP); Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.], washed in TNT and analysed using the ECL detection system (Pierce, Rockford, IL, U.S.A.).

Immunoprecipitation of PTP

Erythrocyte membrane suspension (1 vol. containing about 2.0 mg of protein/ml) was mixed at 4 °C with 1 vol. of extraction buffer containing 50 mM Hepes buffer, pH 7.3, 600 mM NaCl, 2.0 mM EGTA, 0.2 mM vanadate, 2.0 μg/ml aprotonin, 0.2 mM PMSF and 0.6 % Triton X-100 (2 × buffer C). Membrane suspensions were agitated at 4 °C for 45 min, then centrifuged at 40000 ***g*** for 30 min. Aliquots of 200 μl of the supernatants (membrane extract) were mixed with 1.0 μg of monoclonal anti-PTP 1B antibody, FG6-1G. After gentle agitation at 4 °C overnight, 30 μl of Protein A/G–agarose (Santa Cruz Biotechnology) was added and gentle agitation continued for 2 h. The mixtures were then centrifuged at 14000 ***g*** at 4 °C for 5 min, and the pellets washed four times in buffer C. The immunoprecipitates were then solubilized in 40 μl of sample buffer, boiled, electrophoresed and analysed by immunoblotting, as described above. The detection of band 3 was carried out with the primary and secondary antibodies described above. For the detection of PTP on the immunoblot, polyclonal anti-PTP antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.) was used as the primary antibody, followed by Protein A conjugated to HRP (Amersham Bioscience), instead of the secondary antibody HRP-conjugated IgG. The Protein A–HRP was used to prevent the interference by the IgG heavy chain, present in the immunoprecipitates, in the detection of PTP, since both migrate with similar mobilities on SDS/PAGE.

Estimation of PTP activity

PTP activity in the erythrocytes was evaluated by following dephosphorylation of band 3 in the erythrocytes, treated as described above. In addition, PTP activity was evaluated by carrying out dephosphorylation of band 3 in membranes that were prepared from phosphorylated erythrocytes. Membranes were suspended in buffer B containing 1.0 mM dithiothreitol and incubated at 30 °C in the absence and presence of 10 mM Mg^{2+}. Aliquots were removed at intervals, solubilized and boiled in sample buffer, and proteins resolved by SDS/PAGE (10 % gels), followed by anti-phosphotyrosine immunoblotting, as described above. PTP activity in the membranes was also assayed by using *p*-nitrophenyl phosphate (p-NPP) as a substrate, according to published procedures [10].

RESULTS

Band 3 tyrosine phosphorylation in intact erythrocytes and in isolated erythrocyte membranes

Erythrocytes were incubated in the presence and absence of Ca^{2+}/A23187, membranes prepared, solubilized and analysed for phosphoprotein by anti-phosphotyrosine immunoblotting (Figure 1, upper panel). No tyrosine phosphorylation was observed in cells incubated with EDTA (Figure 1, upper panel, lane 1). Tyrosine phosphorylation of band 3 (a major band of approx. 95 kDa, a variable minor band of 60 kDa and variable traces of 41/43 kDa, identified as band 3 by antibody to band 3) was observed in Ca^{2+}/A23187-treated erythrocytes, with

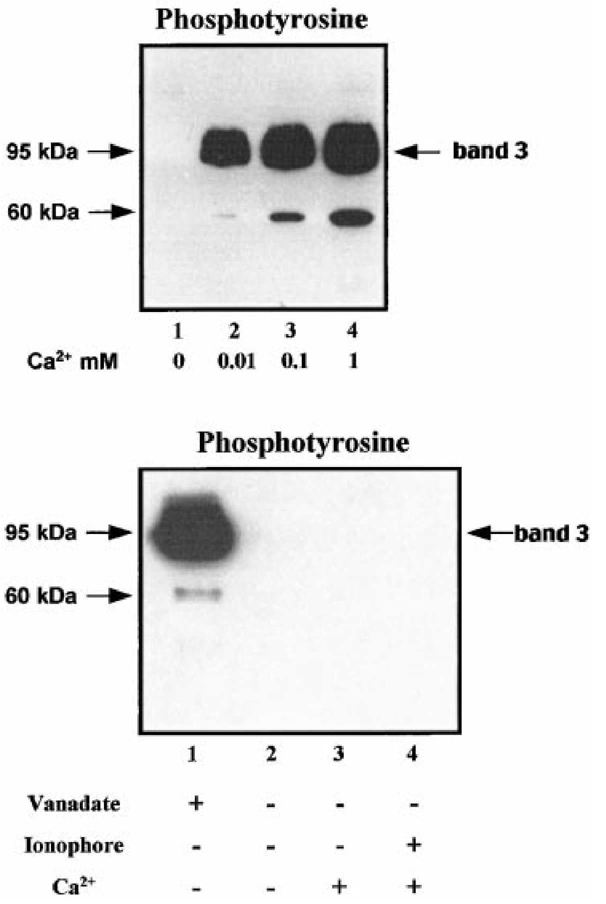

Figure 1 Band 3 tyrosine phosphorylation in erythrocytes and in erythrocyte membranes

Upper panel: band 3 tyrosine phosphorylation in Ca^{2+}/A23187-treated erythrocytes. Erythrocyte suspensions were incubated at 37 °C for 60 min with and without increasing concentrations of Ca^{2+} and 5 μM A23187. Membranes were then prepared, solubilized and analysed by SDS/PAGE and immunoblotting, using anti-phosphotyrosine antibody. Lane 1, no additions; lanes 2–4, A23187 with the indicated concentrations of Ca^{2+}. Lower panel: band 3 tyrosine phosphorylation in erythrocyte membranes. Erythrocyte membranes were incubated at 30 °C for 15 min with 5 μM ATP and 10 mM Mg^{2+}, in the presence or absence of 0.1 mM vanadate, 0.1 mM Ca^{2+} and 5 μM A23187. Membranes were then solubilized, and analysed as described above. Lane 1, vanadate; lane 2, no additions; lane 3, Ca^{2+}; lane 4, Ca^{2+} and A23187. Both panels are representative of three experiments.

increasing levels of phosphorylation observed with increasing concentrations of added Ca^{2+} (Figure 1, upper panel, lanes 2–4). Incubation of erythrocytes in the presence of vanadate or hypertonic NaCl buffer also resulted in band 3 tyrosine phosphorylation (results not shown). These results are consistent with previously published results [17].

Erythrocyte membranes, isolated from untreated cells, were incubated with ATP and Mg^{2+}. Band 3 tyrosine phosphorylation was observed when the membranes were incubated in the presence of vanadate (Figure 1, lower panel, lane 1), as described previously [10]. No tyrosine phosphorylation was observed when the membranes were incubated in the presence of Ca^{2+}, with or without the ionophore (Figure 1, lower panel, lanes 2–4). These results indicate that the cell membrane structure and/or factor(s) present in the intact erythrocyte, but which are either altered in or missing from isolated membranes, are important for the Ca^{2+}-induced band 3 tyrosine phosphorylation.

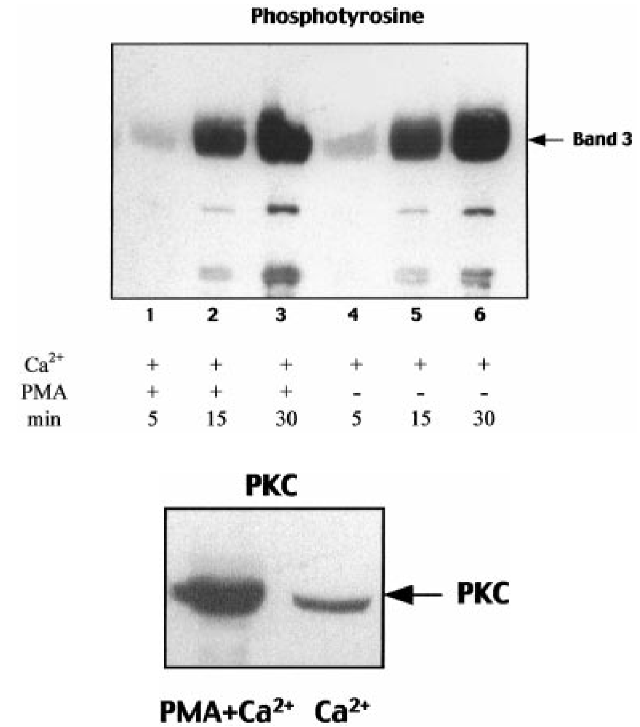

Figure 2 Effects of PMA on band 3 tyrosine phosphorylation and on PKCα translocation to the membrane in Ca^{2+}/A23187-treated erythrocytes

Erythrocytes were incubated with 0.1 mM Ca^{2+}/5 μM A23187, in the presence and absence of 1.0 μM PMA. Aliquots were removed at 5, 15 and 30 min, and analysed as described in the Experimental section. Upper panel: phosphotyrosine. Lanes 1–3, Ca^{2+}/A23187 and PMA; lanes 4–6, Ca^{2+}/A23187. Lower panel: PKCα. Panels are representative of three experiments.

Effects of modulation of Ca^{2+}-activated enzymes on erythrocyte band 3 phosphorylation

Ca^{2+}-activated enzymes may play a role in band 3 tyrosine phosphorylation in the intact erythrocyte, either by activation of PTK and/or inhibition of PTP. To probe the possibility that the phosphorylation is due to effects via Ca^{2+}-activated enzymes, we tested the effects of several reagents. The Ca^{2+}-activated PKC is known to activate PTK [24], and in some cases may inhibit PTP [25,26]. PKCα, known to be present in the human erythrocyte and translocated to the membrane in Ca^{2+}/A23187-treated cells [27,28], was present in membranes isolated from Ca^{2+}/A23187-treated cells. PMA, which promotes the translocation of PKC to the membrane, enhanced significantly the amount of membrane-bound PKCα, but did not increase Ca^{2+}-induced band 3 tyrosine phosphorylation when added to the erythrocytes with Ca^{2+}/A23187 (Figure 2). PMA alone did not lead to tyrosine phosphorylation (results not shown).

The Ca^{2+}-dependent protease calpain [29], which activates membrane-bound PKC [30], was translocated to the membranes in Ca^{2+}/A23187-treated cells (Figure 3, upper panel). Calpeptin, which inhibits calpain activity [31], had little effect on calpain translocation (Figure 3, upper panel) or on band 3 phosphorylation in Ca^{2+}/A23187-treated cells (Figure 3, lower panel). The addition of the Ca^{2+}/calmodulin kinase II inhibitor KN-62 also did not have any effect on the phosphorylation (results not shown).

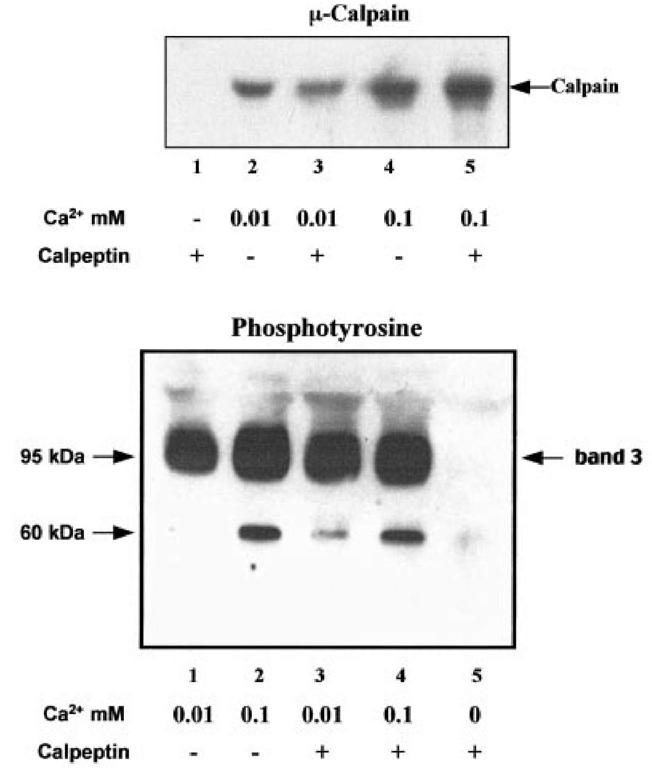

Figure 3 Effects of calpeptin on band 3 tyrosine phosphorylation and on calpain translocation to the membrane in Ca^{2+}/A23187-treated erythrocytes

Erythrocyte suspensions were incubated at 37 °C for 15 min in the presence and absence of 25 μM calpeptin, then further incubated for 30 min without or with Ca^{2+} and 5 μM A23187. Membranes were then prepared and analysed by SDS/PAGE and immunoblotting. Upper panel: immunoblotting with anti-μ-calpain antibody. Lower panel: immunoblotting with anti-phosphotyrosine antibody.

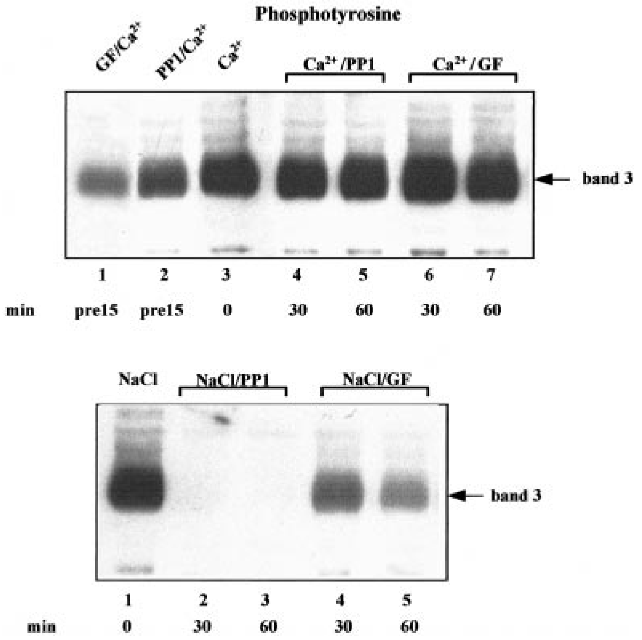

Figure 4 Effects of the kinase inhibitors PP1 and GF on tyrosine phosphorylation in erythrocytes

Upper panel: band 3 tyrosine phosphorylation in Ca^{2+}/A23187-treated erythrocytes. Aliquots of erythrocyte suspensions were preincubated at 37 °C for 15 min (pre15) with 40 μM GF or PP1, then further incubated for 30 min with 0.1 mM Ca^{2+} and 5 μM A23187 (GF/Ca^{2+} and PP1/Ca^{2+}, respectively). Other aliquots were first incubated with Ca^{2+}/A23187, then PP1 or GF were added, and incubation continued for 60 min (Ca^{2+}/PP1 and Ca^{2+}/GF, respectively). Membranes were analysed by immunoblotting with anti-phosphotyrosine antibody, as described in the Experimental section. Lane 1, preincubation with GF; lane 2, preincubation with PP1; lane 3, erythrocytes incubated with Ca^{2+}/A23187; lanes 4 and 5, erythrocytes incubated with PP1 for 30 and 60 min after Ca^{2+}/A23187; lanes 6 and 7, erythrocytes incubated with GF for 30 and 60 min after Ca^{2+}/A23187. Representative of two experiments. Lower panel: band 3 tyrosine phosphorylation in hypertonic-NaCl-treated erythrocytes. Erythrocyte suspensions were incubated at 37 °C for 30 min with hypertonic NaCl, then 40 μM PP1 or GF added and incubation continued for 60 min. Aliquots were removed and analysed as described above. Lane 1, NaCl; lanes 2 and 3, NaCl then PP1; lanes 4 and 5, NaCl then GF. Representative of three experiments.

PTP activity in erythrocytes and erythrocyte membranes

Erythrocyte suspensions were incubated with Ca^{2+}/A23187 or hypertonic NaCl. Some aliquots were preincubated in the presence of the Src kinase inhibitor PP1, shown to significantly inhibit pervanadate-induced band 3 tyrosine phosphorylation [32], or with the PKC-selective inhibitor GF, to inhibit PKC activation of PTK [24,33], and then Ca^{2+}/A23187 or hypertonic NaCl were added. Other aliquots were first incubated with Ca^{2+}/A23187 or hypertonic NaCl, and then PP1 or GF was added. Both PP1 and GF significantly diminished tyrosine phosphorylation when added to the cells prior to treatment with Ca^{2+}/A23187 (Figure 4, upper panel, lanes 1 and 2), or with hypertonic NaCl (results not shown). The results indicate that, under the conditions used here, PP1 and GF inhibit significantly PTK activity in the erythrocytes. When PP1 or GF were added to the erythrocytes after Ca^{2+}/A23187-induced tyrosine phosphorylation, no dephosphorylation was observed (Figure 4, upper panel, lanes 3–7). This is in contrast with the rapid dephosphorylation that occurred when PP1 and GF were added to the cells after NaCl-induced tyrosine phosphorylation (Figure 4, lower panel, lanes 1–5). The results show that the Ca^{2+}/A23187-induced tyrosine phosphorylation is different from that induced by hypertonic NaCl. In the case of NaCl-induced tyrosine phosphorylation, the rapid dephosphorylation after the addition of kinase inhibitors indicates that the PTP remains active, and that the tyrosine phosphorylation is due to the NaCl-induced activation of the PTK, as concluded previously [21]. In contrast, the lack of dephosphorylation in the Ca^{2+}/A23187-treated erythrocytes indicates an inability of PTP to dephosphorylate band 3 phosphotyrosine in these cells.

We found previously that phosphorylated membranes, isolated from vanadate-treated erythrocytes, are dephosphorylated when incubated in the absence of vanadate, showing that PTP is active when vanadate is removed [10]. Similarly, tyrosine phosphorylation induced in erythrocytes by thiol oxidation is reversed in membranes prepared from these cells upon reduction of the PTP/band 3 mixed disulphides in the isolated membranes [11].

To find out whether inhibition of PTP is involved in the tyrosine phosphorylation observed here, membranes were prepared from cells prephosphorylated by treatment with Ca^{2+}/A23187, vanadate or hypertonic NaCl. The isolated phosphorylated membranes were incubated in the absence of these reagents. As shown in Figure 5 (upper left panel), very little dephosphorylation, if any, occurred in membranes prepared from Ca^{2+}/A23187-treated cells (lanes 1–3). In contrast, dephosphorylation was observed in membranes prepared from vanadate-treated cells (Figure 5, upper left panel, lanes 4–6), and in those from

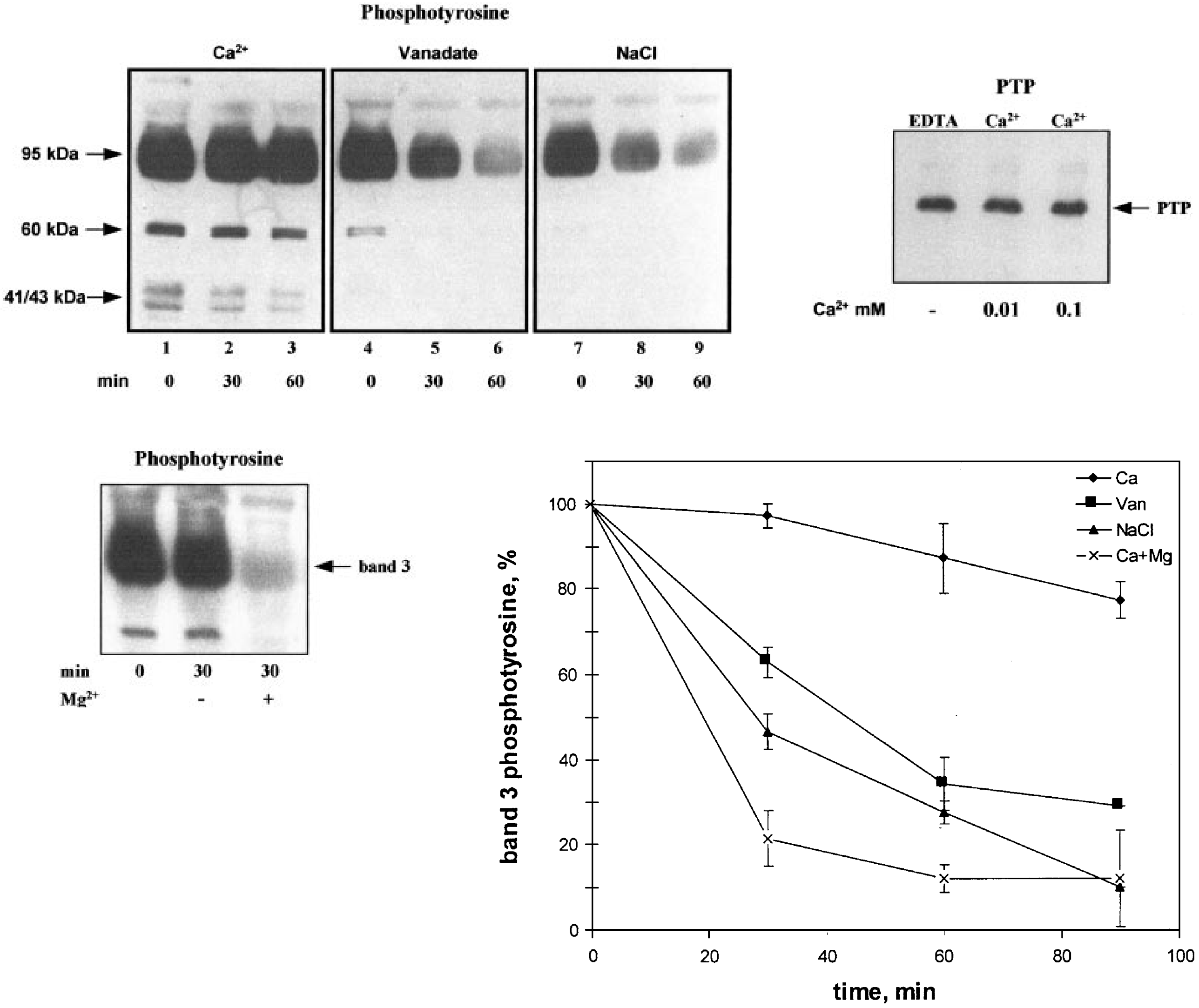

Figure 5 Dephosphorylation of band 3 in membranes prepared from tyrosine-phosphorylated erythrocytes and presence of PTP in the membranes

Upper left panel: immunoblotting with anti-phosphotyrosine antibody. Erythrocyte suspensions were incubated at 37 °C for 60 min in isotonic buffer containing 0.1 mM Ca^{2+} and 5 μM A23187 (lanes 1–3), or 0.1 mM vanadate (lanes 4–6) or with hypertonic buffer (lanes 7–9). Membranes were prepared and incubated without additions at 30 °C, aliquots removed at 0, 30 and 60 min, solubilized and analysed as described above. Upper right panel: PTP in membranes prepared from Ca^{2+}/A23187-treated erythrocytes. Erythrocyte suspensions were incubated at 37 °C for 60 min in the presence of EDTA, without Ca^{2+} (lane 1), with 0.01 mM Ca^{2+} and 5 μM A23187 (lane 2) and with 0.1 mM Ca^{2+} and 5 μM A23187 (lane 3). Membranes were prepared and analysed by SDS/PAGE and immunoblotting with anti-PTP1B antibody. Representative of two experiments. Lower left panel: effects of Mg^{2+} on tyrosine dephosphorylation in membranes from Ca^{2+}/A23187-treated erythrocytes. Erythrocyte suspensions were incubated at 37 °C for 60 min in isotonic buffer containing 0.1 mM Ca^{2+} and 5 μM A23187. Membranes were prepared and incubated at 30 °C for 30 min without or with the addition of 10 mM Mg^{2+}, then solubilized and analysed by immunoblotting, using anti-phosphotyrosine antibody. Lane 1, no incubation; lane 2, incubation without Mg^{2+}; lane 3, incubation with Mg^{2+}. Lower right panel: densitometry of results presented in the left-hand panels. Means ± S.E.M. from 3–5 experiments are shown; Van, vanadate.

hypertonic NaCl-treated cells (Figure 5, upper left panel, lanes 7–9). These results are consistent with the notion that PTP is active in the case of NaCl-induced phosphorylation, whereas phosphorylation of band 3 tyrosine residues observed in the presence of high intracellular Ca^{2+} involves inhibition of band 3 dephosphorylation by PTP, and that such an inhibition is maintained in the membranes isolated from these cells.

Under the conditions used here, PTP protein (as observed by immunoblotting) was present to a similar level in the membranes prepared from the control and Ca^{2+}/A23187-treated cells (Figure 5, upper right panel). These results indicate that the PTP is not lost from the membranes of Ca^{2+}/A23187-treated erythrocytes.

We previously found that the band 3-associated PTP activity in the human erythrocyte membrane is enhanced by Mg^{2+} [10]. To find out whether the lack of band 3 dephosphorylation results from irreversible alteration in the PTP or if the inhibition can be modulated, we tested the effects of Mg^{2+} added to the membranes. As shown in Figure 5 (lower left panel), Mg^{2+} significantly enhanced the dephosphorylation in the membranes prepared from Ca^{2+}/A23187-treated erythrocytes. Under the conditions used here, about 10–20 % of dephosphorylation was achieved in membranes from Ca^{2+}/A23187-treated erythrocytes upon incubation for 60–90 min, whereas 70–90 % dephosphorylation was observed in similarly incubated membranes that were prepared from vanadate- or hypertonic NaCl-treated cells. In the case of Mg^{2+}-treated membranes isolated from Ca^{2+}/A23187-treated erythrocytes, 80 % dephosphorylation occurred within 30 min of incubation (Figure 5, lower right panel). These results

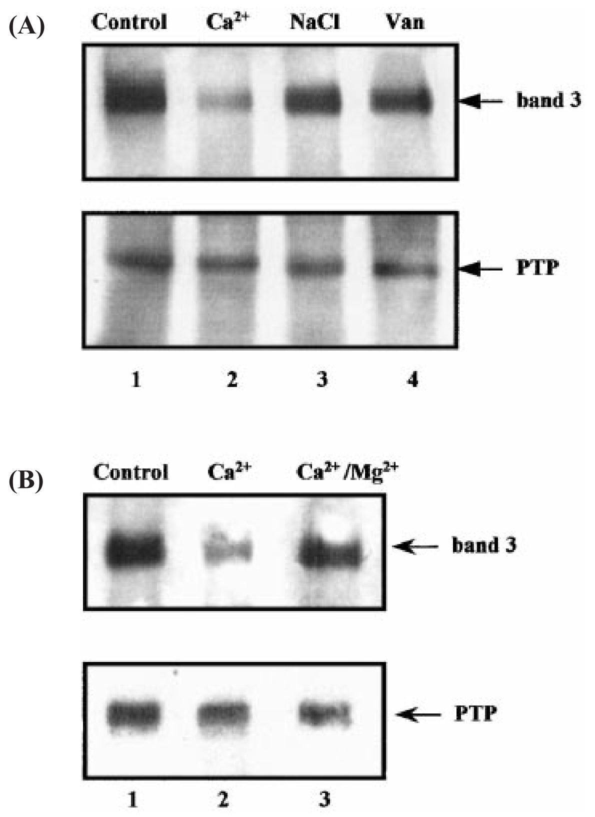

Figure 6 Immunoprecipitation by anti-PTP1B antibody and identification of band 3 and PTP in the immunoprecipitates

(**A**) Erythrocyte suspensions were incubated at 37 °C for 60 min in isotonic buffer in the absence and presence of 0.1 mM Ca^{2+} and 5 μM A23187, hypertonic NaCl or 0.1 mM vanadate (Van). Membranes were prepared, extracted with buffer containing Triton X-100 and immunoprecipitation was carried out using anti-PTP1B antibody. Immunoprecipitates were solubilized, analysed by SDS/PAGE and immunoblotting. Upper panel: anti-band 3 antibody. Lower panel: anti-PTP1B antibody. Lane 1, control; lane 2, Ca^{2+}/A23187; lane 3, hypertonic NaCl; lane 4, vanadate. (**B**) Erythrocyte suspensions were incubated at 37 °C for 60 min in isotonic buffer in the absence and presence of 0.1 mM Ca^{2+} and 5 μM A23187. Membranes were prepared and incubated at 30 °C for 30 min without or with the addition of 10 mM Mg^{2+}, then further processed, as described above. Upper panel: anti-band 3 antibody. Lower panel: anti-PTP1B antibody. Lane 1, control; lane 2, Ca^{2+}/A23187 (Ca^{2+}); lane 3, membranes of Ca^{2+}/A23187 incubated with Mg^{2+} (Ca^{2+}/Mg^{2+}).

indicate that the inhibition of PTP is not due to irreversible effects of Ca^{2+}, but can be overcome under some conditions.

In order to find out whether PTP is inactive towards substrates other than its endogenous substrate, we tested the activity of the isolated membranes on p-NPP. The hydrolysis of p-NPP by samples from Ca^{2+}/A23187-treated cells was similar to that of control samples (results not shown). The results support the conclusion that the PTP is not altered irreversibly, but that its interaction with its endogenous substrate is altered, while retaining activity towards exogenous artificial substrates.

Co-precipitation of band 3 with PTP

We previously found that when PTP was immunoprecipitated by anti-PTP1B antibody from erythrocyte membrane extracts, band 3 was co-precipitated, indicating that the immunoprecipitated PTP was associated with band 3 in the erythrocyte membrane [10]. To find out whether such an association exists under conditions in which band 3 tyrosine phosphorylation is observed, PTP was immunoprecipitated from extracts of membranes prepared from control cells and from cells incubated with either Ca^{2+}/A23187, hypertonic NaCl or vanadate. The immunoprecipitates were analysed by immunoblotting for band 3 and PTP. As shown in Figure 6(A), band 3 was co-precipitated to a similar extent in the samples derived from untreated erythrocytes and from those treated with hypertonic NaCl and vanadate (Figure 6A, upper panel, lanes 1, 3 and 4). In contrast, little band 3 was observed in the immunoprecipitated sample from Ca^{2+}/A23187-treated erythrocytes (Figure 6A, upper panel, lane 2). Electrophoretic mobility of the immunoprecipitated PTP appeared to be similar for all samples (Figure 6A, lower panel, lanes 1–4). To compare the extent of band 3 co-precipitation with PTP among the various samples, densitometric analysis of the band 3 versus PTP was carried out. A significantly diminished amount of band 3 was found in the immunoprecipitates from Ca^{2+}/A23187 samples as compared with the control, whereas the ratios of band 3 to PTP in the samples of hypertonic NaCl and vanadate were similar to that of the control [$29 \pm 7.7\,\%$ ($n = 4$) for Ca^{2+}/A23187, $94 \pm 11.5\,\%$ ($n = 3$) for NaCl and 90–124 % ($n = 2$) for vanadate; means ± S.E.M]. When membranes isolated from Ca^{2+}/A23187-treated erythrocytes were incubated for 30 min in the presence of Mg^{2+} (leading to band 3 dephosphorylation; Figure 5, lower left panel), band 3 was found to be co-precipitated with PTP to an extent similar to that of the control (Figure 6B; 110–125 %, $n = 2$). The results indicate a reassociation of the relevant band 3 sites with PTP.

DISCUSSION

PTPs are integral components of signal transduction pathways, and are involved in the control of a variety of cellular tyrosine kinases, such as receptor kinases [34]. PTP1B is involved in processes such as platelet aggregation and the promotion of cell differentiation, and is implicated in the negative regulation of insulin signalling [35–38]. Information is lacking on PTP1B endogenous substrates, and the factors involved in the regulation of the phosphatase activity remain largely unknown [38]. The erythrocyte anion-exchange band 3 protein and its associated PTP1B is a convenient system to study properties and regulation of PTP activity. Band 3 tyrosine phosphorylation is achieved when the PTP is inhibited by the phosphatase inhibitor vanadate [5,6,10], indicating that the erythrocyte contains higher overall activity of PTP versus PTK. Band 3 tyrosine phosphorylation is also observed upon altered cell volume, deoxygenation, increased Mg^{2+} and increased cell Ca^{2+} ([17–22] and the present work). In the case of volume shrinkage by hypertonicity, the phosphorylation appears to be due to activation of PTK [21]. The fact that dephosphorylation is achieved in the hypertonic-NaCl-treated erythrocytes upon inhibition of PTK, but not in the Ca^{2+}-incubated cells similarly treated (Figure 4), indicates that the mechanism for Ca^{2+}-induced phosphorylation is different from that induced by NaCl. In the present study, we also found that membranes that are isolated from erythrocytes prephosphorylated in the presence of hypertonic NaCl show a rapid dephosphorylation, indicating that PTP is active, and is able to dephosphorylate the band 3 phosphotyrosine once PTK cannot act in the isolated membranes (i.e. in the absence of MgATP). In contrast, little dephosphorylation occurs in membranes isolated from Ca^{2+}/A23187-treated erythrocytes (Figure 5, upper left and lower right panels), indicating that PTP's inability to dephosphorylate band 3 is involved in the accumulation of phosphotyrosine in these erythrocytes. However, the fact that an exogenous small substrate is dephosphorylated by the PTP in these membranes and that band 3 dephosphorylation is achieved when these same membranes are treated with Mg^{2+} indicates that the PTP activity towards band 3 is not irreversibly inhibited. The results suggest that an alteration in the interaction of PTP with band 3 occurs when intracellular Ca^{2+} is increased, resulting in loss of accessibility of the substrate-phosphorylated sites to the phosphatase.

The results are consistent with the idea that the hypertonic-NaCl-induced phosphorylation is due to activation of PTK, whereas the Ca^{2+}/A23187-induced phosphorylation is due to inhibition of PTP. Both hypertonic NaCl and Ca^{2+}/A23187 cause erythrocyte shrinkage, but the hypertonic-induced cell shrinkage is not equivalent to that induced by Ca^{2+}/A23187 [39,40]. The associated membrane biochemical and structural alterations appear to be different. Erythrocytes incubated in the presence of hypertonic NaCl exhibit mainly flattened shapes, with little crenation and no vesiculation [17,21], and the concentration of internal KCl rises with the rise of external osmolarity. Ca^{2+}/A23187 causes K^+ efflux with little change in intracellular tonicity, transformation to echinocytes and vesiculation [39,40]. It has been shown that when NaCl in the medium is replaced by KCl during Ca^{2+}/A23187 treatment, band 3 phosphorylation is inhibited [17]. Under these conditions, K^+ efflux, cell shrinkage and vesiculation are inhibited [40], supporting the notion that the Ca^{2+}-induced band 3 phosphorylation is related to the K^+-efflux-induced changes [17]. It should be noted that Ca^{2+}/A23187 causes various alterations in membrane components (e.g. loss of phospholipid asymmetry [18], polyphosphoinositide breakdown, accumulation of 1,2-diacylglycerol, protein cross-linking and degradation [39,40]). Thus the differences observed between the effects of NaCl and Ca^{2+} on PTK and PTP may be related to differences in NaCl- and Ca^{2+}-induced membrane biochemical alterations, leading to different conformational changes and topology of the substrate versus PTK and PTP. Further studies are necessary to define membrane molecular alterations which may explain the differences between the effects of hypertonic NaCl and Ca^{2+}/A23187.

PTP is associated with band 3 in the normal human erythrocyte, as shown by co-precipitation of band 3 when PTP is immunoprecipitated [10]. We show here that when PTP is immunoprecipitated from Ca^{2+}/A23187-treated cells, significantly less band 3 is co-precipitated than in the control samples, whereas when PTP is immunoprecipitated from the hypertonic NaCl-treated erythrocytes band 3 co-precipitation is similar to that of the control. Thus the PTP appears to be dissociated from its substrate in erythrocytes treated with Ca^{2+}/A23187. The dissociation of PTP from band 3 may thus be responsible for the apparent inhibition of PTP, and be due to Ca^{2+}-induced alterations in membrane components, and/or the substrate. It has been shown that Ca^{2+} binds to band 3, resulting in conformational changes of the protein [41]. We have recently found that significantly more band 3 oligomers are present in the membranes of Ca^{2+}/A23187-treated erythrocytes than in control cells (Y. Zipser, A. Barbul, N. S. Kosower and R. Korenstein, unpublished work). Thus altered band 3 subunit association and conformation may contribute to weakening of PTP interaction with band 3. PTP, which is known to have hydrophobic interactions [38], remains bound to the cell membrane (as shown in Figure 5, upper right panel, and Figure 6). That the Ca^{2+}-induced alterations may be modulated is attested to by the effect of Mg^{2+}, which leads to reactivation of PTP and band 3 dephosphorylation in membranes isolated from Ca^{2+}/A23187-treated cells. It is also of interest to note that phosphatidic acid enhances PTP–epidermal growth factor receptor association and leads to epidermal growth factor receptor dephosphorylation [42]. In view of the Ca^{2+}/A23187-induced biochemical alterations in the erythrocyte membrane [39,40], the participation of some erythrocyte factors in the Ca^{2+}-induced apparent inhibition of PTP and in the modulation of such an effect is not excluded, and further work is needed to clarify this point.

Band 3 tyrosine phosphorylation can be achieved in isolated membranes when ATP, Mg^{2+} and vanadate are added [5,6,10], indicating that white membranes have both PTK and PTP activities, and are able to phosphorylate band 3, provided PTP is inhibited by vanadate. In the present work, we show that Ca^{2+} induces band 3 protein tyrosine phosphorylation in the intact erythrocyte (using Ca^{2+}/A23187), but not when added to erythrocyte membranes that have been isolated from control cells. These results suggest that altered membrane structure and/or factor(s) present in the intact cell, but absent from control white membranes, participate in the Ca^{2+}-induced phosphorylation. The erythrocyte contains several cytoplasmic Ca^{2+}-dependent enzymes, including PKC, calmodulin-dependent kinase and calpain, that are translocated to the membrane and activated when cell Ca^{2+} is increased [43]. PKC, which phosphorylates protein serine/threonine residues, is known to phosphorylate both PTK and PTP [24–26,44]. PKC has been shown to phosphorylate and activate the kinase $p72^{syk}$ [24]. Phosphorylation of PTP by PKC may result in PTP inhibition [25,26]. Thus PKC may have been involved in the Ca^{2+}-induced band 3 tyrosine phosphorylation observed here. However, the results reported here do not support an effect of PKC on PTP in Ca^{2+}-induced tyrosine phosphorylation. PKCα was associated with the membranes in Ca^{2+}/A23187-treated cells, in which band 3 tyrosine phosphorylation occurred, but PMA, which enhances PKC translocation to the membrane, did not have an effect on the phosphorylation. In addition, the PKC inhibitor GF did not lead to dephosphorylation when added to erythrocytes after Ca^{2+}-induced phosphorylation. If PTP were to be inhibited via PKC activity, such inhibition would be expected to be reversed by inhibiting PKC, resulting in active PTP, unless dephosphorylation of phosphorylated PTP is quite slow. Further work is necessary to clarify this point. As shown here, calpain is translocated to the cell membranes under the conditions used. Calpain is known to cause the transformation of the membrane-bound, Ca^{2+}-dependent PKC to soluble, Ca^{2+}-independent PKC, followed by its down-regulation [30,45]. The fact that calpain inhibition does not alter the level of band 3 phosphotyrosine suggests that calpain is not involved in this phosphorylation process, either directly or indirectly via effects on PKC.

It is of interest to note that increased band 3 tyrosine phosphorylation occurs in some haemoglobinopathies [20,46], disorders known to have increased erythrocyte Ca^{2+}. In the case of sickle cells, recent data indicate that the phosphorylation in deoxygenated cells is due to PTP inhibition via thiol oxidation [20]. It would be of interest to study the behaviour of PTP in erythrocytes from thalassaemias. In addition, it should be noted that the deficiency in Ca^{2+}-induced band 3 phosphotyrosine formation observed in erythrocytes from Scott syndrome has been ascribed to a defect in Ca^{2+}-induced phospholipid scrambling [18]. It would be of interest to study PTP in these cells, i.e. to find out whether PTP inactivation and/or dissociation from band 3 do not occur in the Ca^{2+}-treated Scott syndrome cells.

The physiological role of band 3 tyrosine phosphorylation and the significance of dephosphorylation are not known. Band 3 is the anion-exchange protein and also binds various cytoskeletal proteins as well as haemoglobin and cytoplasmic glycolytic enzymes [5,6,17,21]. Phosphorylation of band 3 has been proposed to regulate glycolysis [5,6,22]. Modulation of band 3-associated PTP may thus be important for band 3 function in erythrocytes and in other cells which have proteins analogous to band 3 protein.

In conclusion, the Ca^{2+}-induced band 3 tyrosine phosphorylation appears to involve PTP dissociation from band 3. Since Ca^{2+} is involved in many physiological and pathological processes, such PTP inhibition may play a role in tyrosine phosphorylation observed in various cells under conditions of increased Ca^{2+} [14–16].

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Received 4 March 2002/15 July 2002; accepted 13 August 2002
Published as BJ Immediate Publication 13 August 2002, DOI 10.1042/BJ20020359