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Membrane protein phosphorylation during stomatocyte-echinocyte transformation of human erythrocytes

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The normal, discoid shape of red blood cells represents an equilibrium between two opposing factors, i.e., stomatocytic and echinocytic transformations. Most stomatocytic agents were found to be inhibitors of calmodulin, a regulator of the phosphorylation of membrane proteins. We determined whether red cell shape transformations could be caused by changes in phosphorylation of membrane proteins, specifically the cAMP-dependent phosphorylation of ankyrin and band 4.1. Red blood cells were incubated with ^{32}P and 100 μM chlorpromazine (stomatocytic transformation) or 30 mM sodium salicylate (echinocytic transformation) for various time intervals. Ghost membrane proteins were examined by polyacrylamide gel electrophoresis and autoradiography. Spectrin (β-chain), ankyrin, band 3, band 4.1 and 4.9 were phosphorylated. No change was found in the degree and pattern of phosphorylation after stomatocytic transformation. Salicylate caused a reversible inhibition of transmembranous phosphate transport in both directions. The results indicate that the stomatocytic transformation induced by chlorpromazine and the echinocytic transformation induced by salicylate do not involve a change in phosphorylation, but that the echinocytic transformation induced by salicylate is associated with an inhibition of transmembranous transport of phosphate. Studies with salicylate suggest that the phosphorylation sites of band 3 are found mainly on the endofacial side of the membrane.

Introduction

The normal shape of a red blood cell at rest is that of a biconcave discocyte. Discocytes can easily undergo either a discocyte-stomatocyte or a discocyte-echinocyte transformation in vitro. Several amphipathic, cationic agents can transform discocytes into stomatocytes, whereas other drugs, mainly anionic agents, can transform discocytes into echinocytes, in dose-dependent manners. These transformations are reversible when the causative agent is removed, or an antagonist drug added [1].

Sheetz and Singer [2] explained these transformations with the 'bilayer couple' theory. The hypothesis is that cationic, stomatocytic agents would accumulate in the inner hemileaflet, whereas anionic, echinocytic agents would concentrate in the outer hemileaflet. Several observations, however, indicate that the bilayer couple theory alone is not sufficient to explain the echinocyte-stomatocyte transformation of red blood cells. Conrad and Singer [3] found only a low degree of binding of stomatocytic and echinocytic agents to red blood cell membranes. Seeman [4] reported that the membrane expansion induced by shape-changing drugs is about 10-times greater than the

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occupational volume of the compound within the membrane.

Many drugs that are known to induce a stomatocytic transformation of red blood cells are also inhibitors of calmodulin [1,5–9]. Calmodulin has been found in red blood cells [10,11], where it is associated with the cytoplasmic surface of the membrane [12]. Recently, Nelson et al. [6] have confirmed a remarkable correlation between stomatocytic activity on red blood cells and inhibition of calmodulin for many stomatocytic agents. Calmodulin is involved in the phosphorylation of membrane proteins; a casein-like kinase, which phosphorylates the β-chain of spectrin and band 3, is modulated by calmodulin [10,13]. A second protein kinase is cAMP-dependent and phosphorylates ankyrin (band 2.1), band 4.1 and band 4.9 [14,15]. The present experiments were performed to investigate the possibility that red blood cell shape transformations are related to changes in phosphorylation of membrane proteins.

Materials and Methods

Reagents

^{32}P as disodium phosphate (NEX-011) was purchased from New England Nuclear (No. Billerica, MA). Materials for polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA). Inosine and adenine were purchased from Sigma (St. Louis, MO), chlorpromazine hydrochloride from Wyeth Lab. (Philadelphia, PA), and sodium salicylate from Fisher Scientific (Pittsburgh, PA). Deionized water was used throughout the experiments.

Phosphorylation experiments

Blood was drawn from healthy volunteers into heparinized vacutainer tubes. The blood was centrifuged for 10 min at $1500 \times g$. Plasma and buffy coat were removed. The red blood cells were washed three times in 5–10 vol. of cold isotonic phosphate-buffered saline (10 mM sodium phosphate, pH 7.2, 150 mM NaCl) according to Shapiro and Marchesi [16].

The washed red blood cells were resuspended in phosphate-buffered saline containing 20 mM sodium phosphate, pH 7.4, 130 mM NaCl, 5 mM glucose, 10 mM inosine, 1 mM adenine [17], and approximately 200 μCi/ml ^{32}P as $Na_2H^{32}PO_4$. Stomatocytic transformation was induced by 100 μM chlorpromazine hydrochloride, echinocytic transformation by 30 mM sodium salicylate. The suspensions were incubated at 37°C in a slow-moving shaking bath. The incubation was terminated by adding 5 volumes of ice-cold phosphate-buffered saline, centrifuging for 5 min at $1500 \times g$ and washing of the packed red blood cells in about 40 vol. of buffer. Ghosts were prepared according to Bennett and Stenbuck [18]. The protein concentration of the ghosts was determined with the method described by Lowry et al. [19]. Ghost membrane preparations of equal protein concentrations were used for SDS-polyacrylamide gel electrophoresis according to Fairbanks et al. [20]. The gels were stained with Coomassie blue, dried and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY) for autoradiography at −70°C for four or more days. Marks on the dried gels and the films allowed their exact superposition for comparison. Densitometric scanning was performed on the dried gels and autoradiographs.

Red cell morphology

The red blood cell morphology was studied by scanning electron microscopy (SEM). Samples were fixed overnight in 1% glutaraldehyde (pH 7.4) at 4°C, post-fixed in OsO_4 for 1 h, and dehydrated in ascending ethanol series. The specimens were air-dried, coated with gold-palladium (Hummer I Sputterer, Technics, Alexandria, VA), and examined by S.E.M. (Model JSM-35, JEOL Corp., Peabody, MA).

Results

The red blood cell morphology is shown in Fig. 1. Discocytes (Fig. 1B) underwent a stomatocytic transformation when incubated with 100 μM chlorpromazine-HCl (Fig. 1A) and an echinocytic transformation when incubated with 30 mM sodium salicylate (Fig. 1C). The transformations were fully developed after 2 min and did not change visibly over 3 h. Washing of these cells in phosphate-buffered saline without chlorpromazine-HCl and sodium salicylate showed that the

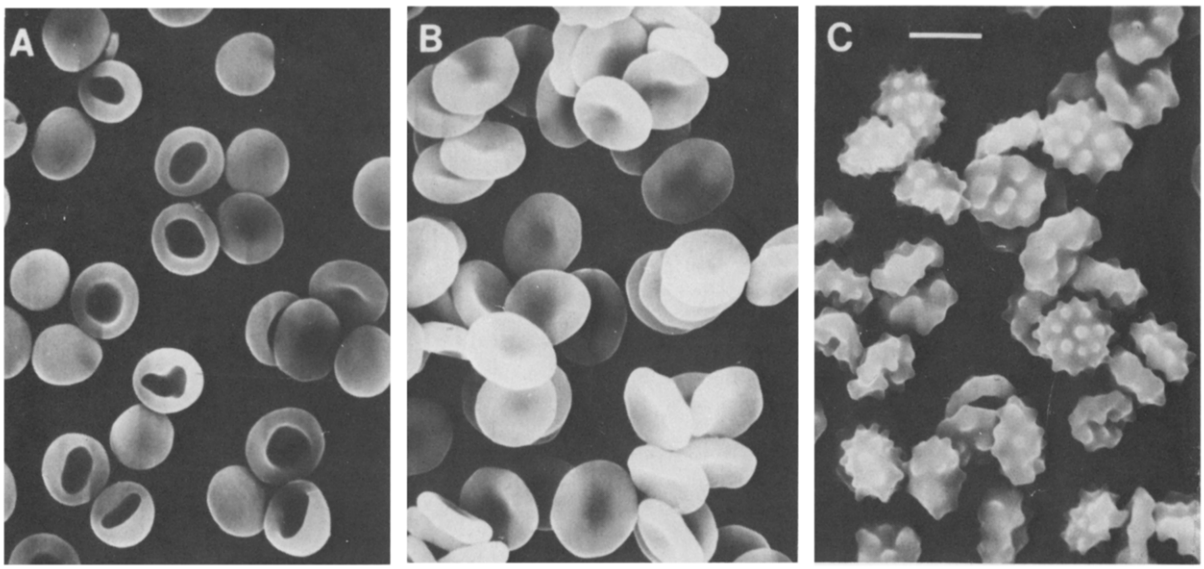

Fig. 1. Scanning electron micrographs of red blood cells showing: A, stomatocytic transformation produced by 100 μM chlorpromazine; B, discocytes under normal conditions; and C, echinocytic transformation produced by 30 mM sodium salicylate. The red blood cells were incubated for 3 h at 37°C. The bar represents 5 μm.

shape changes were reversible.

Polyacrylamide gel electrophoresis of red blood cell ghosts with Coomassie blue staining showed that treatment with either chlorpromazine-HCl or sodium salicylate did not cause any change in the density or relative electrophoretic mobility of the different membrane protein bands (Fig. 2, left side). Autoradiography of the gel from the untreated control sample (Fig. 2, right side) revealed that several bands were phosphorylated, including the β-chain of spectrin (band 2), ankyrin (band 2.1), other minor bands of the 2.1 family (bands 2.2–2.6), and bands 3, 4.1 and 4.9 (molecular weight 48 000). α-Spectrin (band 1) and actin (band 5), both being major membrane skeletal proteins, were not phosphorylated. The pattern of phosphorylation did not change when the red blood cells had been incubated with 100 μM chlorpromazine-HCl, together with ^{32}P, for 3 h (Fig. 2, right side). This was ascertained also on gels where the duration of electrophoresis was 8 h, instead of 4 h, in order to separate the bands 2.1 and 4.1 better from the bands 2 and 3, respectively. Minor differences shown in this example in Fig. 2, e.g. the increase in density of band 2.6, were not found in other experiments. In additional experiments, red blood cells were incubated first with ^{32}P alone for 3 h to allow an equilibration of ^{32}P across the cell membrane, and 100 μM chlorpromazine-HCl was then added for 5 min or 3 h. Again, no change in phosphorylation was observed. Red blood cells incubated with 30 mM sodium salicylate, together with ^{32}P, showed a reduction of the radioactive labeling of all bands (Fig. 2, right side).

The densitometric scanning of the gels in Fig. 2 is shown in Fig. 3. The Coomassie blue stain of the control is given as a reference (bottom panel). By far the strongest phosphorylation is observed on β-spectrin. The band 3 region has two major peaks of phosphorylation. The simultaneous incubation with 30 mM sodium salicylate and ^{32}P for 3 h reduced the radioactive labeling to about 50%. The relative density of phosphorylation with 30 mM sodium salicylate, however, remained the same for all protein bands as in control.

The decrease in radioactive labeling of membrane proteins with salicylate was studied more extensively. The protocol and data are given in Fig. 4. Essentially three types of treatment were performed. In the first group (A) of experiments (lanes 1–4), red blood cell suspensions were preincubated with ^{32}P for 3 h, and then treated for 5

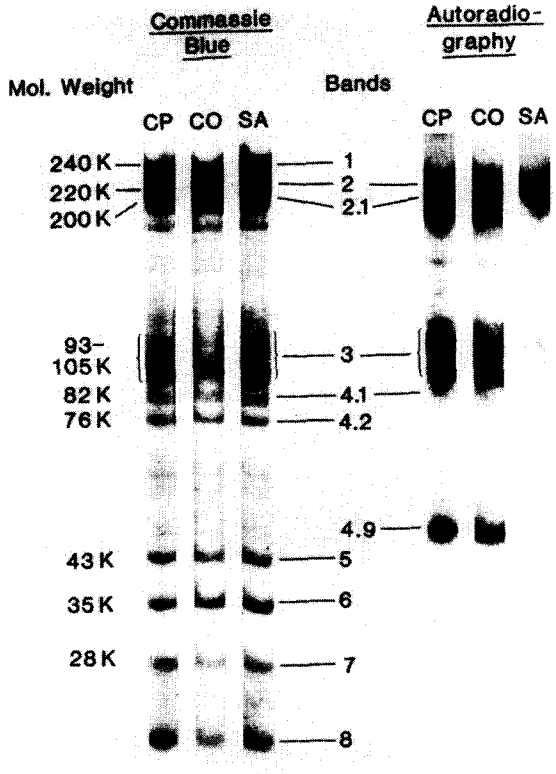

Fig. 2. SDS-polyacrylamide gel electrophoresis of red blood cell ghosts. The intact cells had been incubated at 37°C for 3 h with ^{32}P (about 200 μCi/ml) and either 100 μM chlorpromazine (CP), no drug (CO) or 30 mM salicylate (SA). The left side shows the pattern of the protein bands stained with Coomassie blue; the right side shows the autoradiography of the same gel.

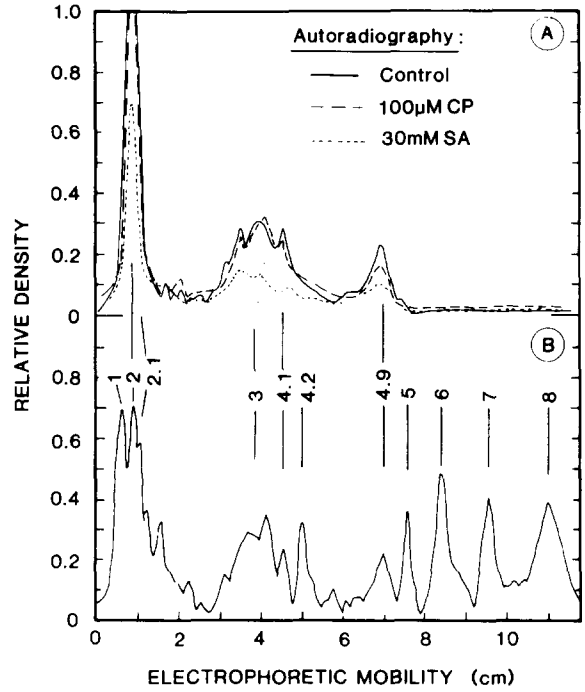

Fig. 3. Densitometry of polyacrylamide gels. A. Autoradiography of red blood cells incubated for 3 h with ^{32}P alone (control), together with 100 μM chlorpromazine or with 30 mM salicylate. The relative density is in arbitrary units. The value of 1.0 was the maximum value at which saturation of the film and/or the densitometer was reached. B. Densitometry of control gel shown in (A) stained with Coomassie blue. The pattern of Coomassie blue stained gels did not differ with treatments.

min with sodium salicylate. In the second group (B, lanes 5–8), red blood cells were subject to the same protocol as in group A, except that the second phase of the experiment studying the effect of sodium salicylate was lengthened from 5 min to 3 h. In the third group (C, lanes 9–12), the protocol was the same as in group B except that sodium salicylate was present in the 3 h pre-incubation period together with ^{32}P for all four lanes.

When red blood cells had been pre-incubated with ^{32}P for 3 h, the subsequent addition of sodium salicylate, for either 5 min (lane 1 vs. 3) or 3 h (lane 5 vs. 7), had no effect on the pattern of phosphorylation. In contrast, the presence of sodium salicylate from the very beginning reduced the overall ^{32}P incorporation into membrane proteins, as shown by comparisons of group C (lanes 9, 10 and 12) with corresponding experiments in group B (lanes 5, 6 and 8). The effect of sodium salicylate in decreasing ^{32}P incorporation was reversible, as shown in lane 11 in which phosphorylation of membrane proteins resumed after the removal of sodium salicylate. The reduction in ^{32}P incorporation brought about by sodium salicylate can be explained by a decrease in phosphate permeability across the red cell membrane, rather than an inhibition of the phosphorylation reaction itself. This explanation is supported by experiments in which red cell membrane pre-equilibrated with ^{32}P was placed in ^{32}P-free media with and without sodium salicylate (lanes 6 and 8, respectively). The presence of sodium salicylate in the ^{32}P-free medium (lane 6) caused a higher density of labeling than when it was absent in the

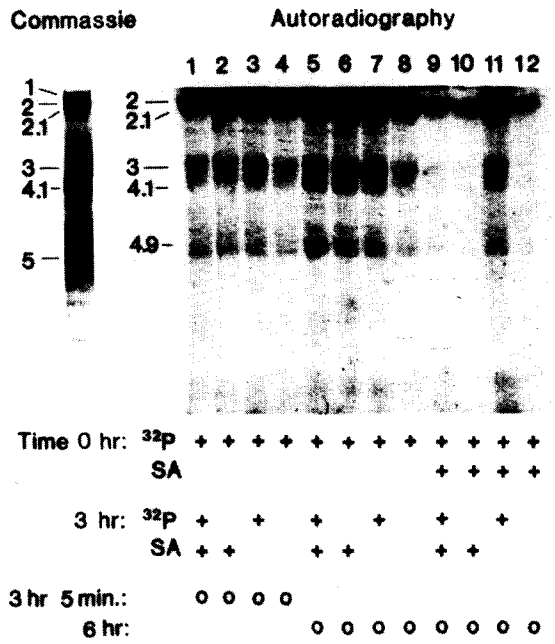

Fig. 4. Autoradiography of SDS-polyacrylamide gel electrophoresis of red blood cell ghosts. The intact cells were incubated with ^{32}P and 30 mM sodium salicylate for different time intervals. The time course of incubation is given on the bottom of the figure: in the first group (A) of experiments (lanes 1–4), red blood cell suspensions were pre-incubated with ^{32}P for 3 h and then treated as follows. For lane 1, sodium salicylate (30 mM) was added for 5 min with continued presence of ^{32}P; for lane 2, sodium salicylate (30 mM) was added with ^{32}P removed; for lane 3, no sodium salicylate was added but ^{32}P continued to be present; for lane 4, no sodium salicylate was added and ^{32}P was removed. In the second group (B, lanes 5–8), red blood cells were subject to the same protocol as in group A (lanes 1–4, respectively), except that the second phase of the experiment was lengthened from 5 min to 3 h. In the third group (C, lanes 9–12), the protocol was the same as in group B (lanes 5–8, respectively) except that sodium salicylate (30 mM) was present in the 3 h pre-incubation period, together with ^{32}P, for all four lanes. O represents the end of incubation. The pattern of the protein bands stained with Coomassie blue showed no differences between the various treatments. One representative pattern (taken from lane 9) is shown on the left side.

^{32}P-free medium (lane 8), suggesting that the added sodium salicylate caused a reduced efflux of ^{32}P into ^{32}P-free medium. The effect of sodium salicylate in reducing the labeling density was also seen when it was present for only 5 min (lanes 2 vs. 4), though to a lesser degree. Thus, sodium salicylate reduced phosphate permeability across the membrane in both directions.

Discussion

Phosphorylation of several ghost membrane proteins was found after ^{32}P labeling of intact red blood cells. The results are in general agreement with earlier reports [21–23]. The smallest phosphorylated band had a molecular weight of 48 000–52 000 and was referred to as band 4.9 [15,24].

Huestis et al. [10] reported that Ca^{2+} stimulated the phosphorylation of spectrin in isolated red blood cell membranes by a calmodulin-dependent protein kinase and that the calmodulin-inhibitor trifluoperazine inhibited the phosphorylation thus stimulated. In our experiments, however, chlorpromazine, which has been shown to be an inhibitor of calmodulin [5], did not affect the phosphorylation of spectrin. Our results support the findings of others [25,26] that the state of spectrin phosphorylation does not determine the red cell shape. Patients with hereditary sphero(stomato)cytosis were found to have a decreased phosphorylation of spectrin, which was normalized after splenectomy [27] despite the persistence of abnormal red blood cell shapes.

The phosphorylation of bands 2.1 and 4.1 is regulated by a cAMP-dependent kinase [14,15]. If calmodulin can stimulate cAMP phosphodiesterase activity in red blood cells, as has been shown in other tissues such as the brain [28,29], this would decrease cAMP, inhibit the cAMP-dependent protein kinase, and reduce the phosphorylation of bands 2.1 and 4.1. Accordingly, a stomatocytic agent such as chlorpromazine, by inhibiting calmodulin, would then be expected to lead to an increase in phosphorylation of bands 2.1 and 4.1. Chlorpromazine, however, did not affect the phosphorylation of bands 2.1 and 4.1. Chlorpromazine, however, did not affect the phosphorylation of bands 2.1 and 4.1 in our study. This result and those of others [30] seem to suggest that the red blood cell has no significant phosphodiesterase activity. The finding that agents inducing stomatocytic transformation also cause an inhibition of calmodulin [6] may be explained by postulating either that these two effects are

parallel, independent events, or that the inhibition of calmodulin may cause stomatocytic transformation of red blood cells by mechanisms other than membrane protein phosphorylation. The role of the cAMP-dependent protein kinase in erythrocytes remains unresolved [31,32]. Mature red blood cells have no significant adenylate cyclase activity [33].

Echinocytic transformation induced by sodium salicylate did not correlate with changes in phosphorylation of any specific membrane protein. An interesting finding was that sodium salicylate reduced the permeability of the red blood cell membrane for phosphate in both directions. An inhibition of the efflux of chloride [34] and phosphate [35] from red blood cells incubated with salicylate has been observed. Besides salicylate, several drugs have been shown to be reversible anion-transport inhibitors [35,36]; most of them are echinocytic agents, but some are stomatocytic agents, e.g. tetracaine [36] and reserpine [35]. The mechanism by which salicylate inhibits anion transport is not known; it is possible that interaction with band 3, the anion channel of the red blood cell membrane, is involved.

In our experiments, when ^{32}P was available in the external medium but its influx was inhibited by sodium salicylate, there was a marked reduction in the phosphorylation of the integral protein band 3 (lane 12 in Fig. 4). This indicates that there are few phosphorylation sites on band 3 accessible from the exofacial surface of the RBC membrane. When the efflux of ^{32}P was inhibited by sodium salicylate after ^{32}P loading of the red blood cell interior (lane 6 in Fig. 4), however, band 3 showed a high degree of phosphorylation; therefore, the phosphorylation sites of band 3 accessible under the experimental conditions are found mainly on the endofacial side of the membrane. It is interesting to note that a 201-residue fragment from the cytoplasmic end of band 3 has revealed the presence of 10 serine residues, the potential phosphorylation sites for cAMP-dependent protein kinase [37].

In summary, the stomatocytic shape transformation of human red blood cells induced by chlorpromazine-HCl is not mediated by changes in phosphorylation of membrane proteins. The echinocytic transformation induced by sodium salicylate also does not involve any change in phosphorylation pattern of various membrane protein components, but it is associated with an inhibition of transmembrane transport of phosphate in both directions.

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