Inhibition of Deoxygenation-Induced Membrane Protein Dephosphorylation and Cell Dehydration by Phorbol Esters and Okadaic Acid in Sickle Cells

By Hassana Fathallah, Ericque Coezy, René-Serge de Neef, Marie-Dominique Hardy-Dessources, and Françoise Giraud

Deoxygenation (DO) of sickle cell anemia red blood cells (SS cells) induces membrane permeabiliration to Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\) and cell dehydration mostly through the activation of the Ca\(^{2+}\)-dependent K\(^{+}\) channels. We show that DO of both SS cells and normal red blood cells was accompanied by a non-specific dephosphorylation of membrane proteins. After treatment with a protein kinase C activator (phorbol myristate acetate) or a phosphoprotein phosphatase inhibitor (okadaic acid), the level of membrane protein phosphorylation in deoxygenated cells was maintained higher or equal, respectively, to that of the oxygenated controls. We found that these drugs in SS cells (1) inhibited by 40% the DO-induced Na\(^{+}\) and cell dehydration mostly through the activation of the Ca pump and of the Na/H antipporter. Inhibition of SS cell dehydration might be due to an activation of the Ca pump preventing [Ca\(^{2+}\)\(_{i}\)] elevation responsible for the stimulation of the K\(^{+}\) channels and/or to an activation of the Na/H exchange resulting in cell water gain.

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MATERIALS AND METHODS

Preparation of cells. After informed consent was obtained, blood was collected into heparinized tubes either from normal donors or from sickle cell anemia patients homozygous for HbS and kept at 4°C for no more than 3 hours before processing. Part of the plasma was removed after centrifugation of SS blood and the resulting suspension (about 50% hematocrit) was layered onto a discontinuous gradient of Percoll (Pharmacia, St Quentin-Yvelines, France; \(d = 1.084\) and 1.106). The SS cells with 1.084 < \(d < 1.106\) were collected. The fraction used in these experiments was enriched in reticulocytes (10% to 26%) relative to the unfractronated population (6% to 15%). The fraction was washed three times in solution A (140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl\(_2\), 1 mmol/L Na\(_2\)PO\(_4\), 10 mmol/L glucose, 10 mmol/L HEPES-Tris \(pH 7.4\) at \(37°C\), 300 mosm/kg) and kept at 4°C for 12 to 36 hours before the experiments were performed. AA cells, recovered after centrifugation of the blood and elimination of the white blood cells, were washed in solution A and kept under the same conditions. All washing steps were performed at 4°C.

General protocol to all experiments. Just before the experiments were performed, the cells were washed once in solution A and incubated at 15% hematocrit for 45 minutes at \(37°C\) in the same solution containing 2 mmol/L adenine and 10 mmol/L inosine to replete the ATP pool. Cells were washed twice in solution B (140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl\(_2\), 1 mmol/L CaCl\(_2\), 10 mmol/L glucose, 10 mmol/L HEPES-Tris \(pH 7.4\) at 37°C, 300 mosm/kg).

From the Laboratoire de Biomembranes et Messagers Cellulaires, Université Paris XI, Orsay, France; and Unité de Recherche sur la Défibrino-lysine, Pointe-à-Pitre, Guadeloupe.

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Address reprint requests to Françoise Giraud, MD, Biomembranes et Messagers Cellulaires, URA 1116, Bat 447, Université Paris XI, 91405 Orsay Cedex, France.

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mosmol/kg]) and preincubated at 10% hematocrit for 60 minutes at 37°C. Unless otherwise mentioned, PMA (400 nmol/L, P8139; Sigma-Aldrich-Chimie, St Quentin Fallavier, France) was present only during the drug treatment (either 400 nmol/L PMA for the last 60 minutes or 400 nmol/L OK for the last 10 minutes). Cell suspensions were further incubated for 30 minutes under deoxygenated or oxygenated conditions. At selected times, aliquots were centrifuged and the packed cells were lysed at 4°C with 20 vol of 10 mmol/L Tris-Cl (pH 7.8), 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; P7626; Sigma Chimie). Membranes were washed twice with the lysing solution and resuspended in the same solution containing 0.2% sodium dodecyl sulfate (SDS). Aliquots were taken for determination of the protein concentration and for protein resolution by electrophoresis on SDS 8% polyacrylamide gels. Gels were stained in Coomassie brilliant blue, dried, and submitted to autoradiography (Hyperfilms MP; Amersham, Les Ulis, France). Patterns of radioactivity in gels were analyzed by transsection and counting in water. Each section contains at least spectrin and ankyrin (area 1); protein band 3, adducin, and glycophorin A (area 2); protein 4.1 and protein 4.2 (area 3); and protein 4.9 and actin (area 4) (Fig 1). Data were expressed as counts per minute per microgram of total membrane proteins.

\[ Ca^{2+} \text{ fluxes in chelator-loaded cells.} \]

\[ Ca^{2+} \text{ fluxes were measured after the incorporation of a Ca}^{2+} \text{ chelator, quin 2, to increase the exchangeable intracellular Ca}^{2+} \text{ pool. Chelator loading can be performed either in the presence or in the absence of Ca}^{2+} \text{ in the medium. In the first case, the endogenous exchangeable Ca}^{2+} \text{ pool was increased up to an equilibrium during the chelator loading and, after } {\text{Ca}^{2+}} \text{ addition, bidirectional } {\text{Ca}^{2+}} \text{ fluxes took place. In the second case, during the chelator loading, Ca}^{2+} \text{ was reduced below levels that activate the Ca pump, so that unidirectional } {\text{Ca}^{2+}} \text{ influx could be measured with minimal contribution of the Ca}^{2+} \text{ pump-mediated efflux. Either one or the other procedure was used, as indicated in the text.} \]

Cells were loaded (37°C, 30% hematocrit) with the Ca}^{2+} \text{ chelator, quin 2-AM (Molecular Probes Inc, Leiden, The Netherlands; 200 μmol/L of cells), for either 45 minutes in solution B or for 75 minutes in solution C (solution B in which CaCl}2 was replaced by 0.1 mmol/L EGTA). At the end of this incubation, the intracellular concentration of free quin 2 was 100 to 140 μmol/L of cells and the ATP concentration was at least 0.5 mmol/L of cells, as previously reported. Chelator-loaded cells were washed three times in solution B or in solution C, resuspended at 10% hematocrit in their respective solution, and incubated with DMSO, PMA, or OK as indicated above.

\[ Ca^{2+} \text{ influx under oxygenated conditions.} \]

\[ Ca^{2+} \text{ influx under oxygenated conditions was measured in cells loaded with the chelator in solution B. Cells were first preincubated for 30 minutes under air in the same solution containing } {\text{Ca}^{2+}} \text{, to equilibrate cell } {\text{Ca}^{2+}} \text{ with that of the medium, before DO. Net } {\text{Ca}^{2+}} \text{ uptake under deoxygenated conditions was measured in cells loaded with the chelator in solution B. Cells were first preincubated for 30 minutes under air in the same solution containing } {\text{Ca}^{2+}} \text{, to equilibrate cell } {\text{Ca}^{2+}} \text{ with that of the medium, before DO. Net } {\text{Ca}^{2+}} \text{ uptake was calculated from the initial rate of } {\text{Ca}^{2+}} \text{ uptake in deoxygenated conditions.} \]

\[ Ca^{2+} \text{ influx under deoxygenated conditions.} \]

\[ Ca^{2+} \text{ influx under deoxygenated conditions was measured in cells loaded with the chelator in solution C. Cells were first preincubated for 10 minutes in the same solution under deoxygenated conditions before the addition of } {\text{CaCl}2} \text{ (final free concentration, 1 mmol/L). It was calculated from the initial rate of } {\text{Ca}^{2+}} \text{ uptake.} \]

In all conditions, incubations were performed at 37°C and 10% hematocrit in a medium containing 1 mmol/L } {\text{CaCl}2} (370 kBq/μmol; Amersham). Samples were withdrawn at different times, washed three times in solution B, and lysed in 50 vol of H2O. Aliquots of the lysate were taken to measure the hemoglobin (Hb) concentration by spectrophotometry at 540 nm and the } {\text{Ca}^{2+}} \text{ content, after precipitation in 6% trichloroacetic acid (TCA), by liquid scintillation counting. } {\text{CaCl}2} \text{ was calculated from the } {\text{Ca}^{2+}} \text{ content and the specific radioactivity of } {\text{Ca}^{2+}} \text{ in the incubation medium and expressed as micromoles per 340 g of Hb.} \]

\[ Na^+ \text{ and } K^+ \text{ contents.} \]

After incubation under oxygenated or deoxygenated conditions with or without drug pretreatments, cells were washed three times with a solution containing 108 mmol/L MgCl}2, and 10 mmol/L Tris-Cl (pH 7.8 at 4°C), packed, and lysed with 20 vol of CsCl (2 mmol/L). The lysates were used to measure...
SICKLE CELL DEHYDRATION AND PHOSPHORYLATION

Fig 2. Effect of DO on the level of $^{32}$P incorporation in membrane proteins of SS or AA cells. Cells were preincubated for 2.5 hours with $[^{32}P]$ P. At time 0, they were either kept oxygenated (C) or submitted to DO (○). The values shown are means ± SE of 6 (SS) or 5 (AA) experiments and are expressed as a percentage of the oxygenated controls at time 0 for each gel area (1 to 4, see Fig 1). The absolute values of $^{32}$P incorporation (counts per minute per microgram of protein, n = 4) at time 0 were 26 ± 5 and 27 ± 7 (area 1), 35 ± 9 and 26 ± 5 (area 2), 11 ± 3 and 5 ± 1 (area 3), and 7 ± 1 and 5 ± 1 (area 4), respectively, in SS and AA cells.

the Hb concentration by spectrophotometry and the Na$^+$ and the K$^+$ concentrations, after precipitation with 6% TCA, by flame photometry using suitable standards prepared in CsCl. The cell Na$^+$ and K$^+$ contents were expressed as millimoles per 340 g of Hb.

Density distribution curves. Density profiles were determined after centrifugation of the cell suspensions, at room temperature (and 10 minutes of reoxygenation for the previously deoxygenated samples), through mixtures of dibutyl and diethyl phthalates (BDH, Poole, UK).

RESULTS

Effects of DO, PMA, and OK on the phosphorylation of membrane proteins. Initial experiments were designed to determine whether DO of RBCs altered the phosphorylation state of membrane proteins. SS and AA cells were incubated for 2.5 hours in a medium containing $[^{32}P]$ P, to label the phosphoproteins up to an equilibrium value. Subsequently, the cell suspensions were either maintained oxygenated or deoxygenated for 30 minutes. After cell lysis and electrophoresis separation of membrane proteins, the radioactivity of 4 areas of the gels was measured (Fig 1). The sum of the radioactivity of these areas, which contained well-identified proteins, amounted to about 70% of the total radioactivity of the membrane proteins. The remaining 30% corresponded to a number of weakly labeled bands, which were hardly detectable on the autoradiograms (Fig 1, lane B). The levels of $^{32}$P incorporation in SS cell membrane proteins of areas 2, 3, and 4 were higher (by 40% to 100%) than those of the corresponding areas in AA cells (see Fig 2 legend). These findings are in agreement with those of our previous report, indicating a greater turnover of the phosphate monooester groups of membrane proteins in SS cells. DO of SS and AA cells resulted in a decrease in the $^{32}$P radioactivity of membrane proteins that amounted to about 15% of the value of the oxygenated controls after 30 minutes of incubation. The kinetics of dephosphorylation of the proteins in each of the 4 areas is shown in Fig 2. The reduction in phosphorylation was nonspecific, was already significant after 5 minutes of DO, and was maintained up to 30 minutes (Figs 2 and 3A).

To increase the phosphorylation level of membrane proteins during DO, the cells were pretreated with PMA or OK during the $[^{32}P]$ loading period. The drugs were added under conditions leading to maximal inhibition of DO-induced Ca$^{2+}$ uptake (see below; 400 nmol/L PMA for 60 minutes or 400 nmol/L OK for 10 minutes). As already reported in AA$^{9,10}$ or SS cells,$^{11,12}$ treatment with PMA under oxygenated conditions increased the phosphorylation of the three known PKC substrates adducin (area 2), protein 4.1 (area 3), and protein 4.9 (area 4) (Fig 1, lane C, and Fig 3B). DO of PMA-treated cells induced a dephosphorylation of membrane proteins, but the effect was not statistically significant. Treatment with OK under oxygenated conditions had almost no effect on the phosphorylation of membrane proteins in SS cells. It caused a significant increase in area 1 (spectrin + ankyrin) and area 3 (protein 4.1) in AA cells, in agreement with a previous report ($^{13}$ Fig 1, lane D, and Fig 3C).
Fig 3. Effect of DO and drug treatment on the level of $^{32}$P incorporation in membrane proteins of SS and AA cells. (A) Control; (B) PMA; (C) OK. The experimental protocol is the same as that described in the Fig 2 legend, except that PMA (400 nmol/L) was added for 60 minutes and OK (400 nmol/L) was added for 10 minutes before time 0. The values shown are expressed as a percentage of the oxygenated controls at time 0 for each gel area (1 to 4, see Fig 1), represent $^{32}$P incorporation after 30 minutes of oxygenation (○) or DO (■) and are the means + SE of 4 (SS) or 3 experiments (AA). (†) Significantly different from oxygenated controls; (▲) significantly different from oxygenated untreated controls.

Both types of cells, the DO-induced dephosphorylation of membrane proteins was totally prevented by the OK treatment.

Effects of DO, PMA, and OK on Ca$^{2+}$ fluxes. Chelator-loaded SS cells were incubated under oxygenated conditions with $^{45}$Ca. After 30 minutes, $[^{45}\text{Ca}]_i$, calculated from the $^{45}$Ca uptake, was close to a steady-state value (Fig 4A, ○). Further incubation under deoxygenated conditions induced an increase in $[^{45}\text{Ca}]_i$, indicating a net Ca$^{2+}$ uptake by the cells (Fig 4A, ●), in agreement with earlier studies.\textsuperscript{23-26} Preincubation of the cells with PMA (60 minutes at 400 nmol/L) or with OK (10 minutes at 400 nmol/L) inhibited the DO-induced Ca$^{2+}$ uptake from 80 (control) to 32 (PMA) and 55 (OK) pmol/340 g Hb/h in this experiment (Fig 4B). The dose and time dependences of the effect of PMA and OK are shown in Fig 5. Maximal inhibition (40% to 50%) was obtained at 400 nmol/L PMA or 200 nmol/L OK (Fig 5A and B, respectively). PMA (400 nmol/L) had maximal effect on Ca$^{2+}$ uptake after 60 minutes of pretreatment (Fig 5C). OK (200 nmol/L) inhibited Ca$^{2+}$ uptake in less than 10 minutes of pretreatment (Fig 5D). A longer pretreatment time with OK progressively abolished the inhibition of Ca$^{2+}$ uptake, an effect that might be related to intracellular degradation of OK. The structurally related phorbol ester, 4a-phorbol 12,13-didecanoate (400 nmol/L), which does not activate protein kinases C, did not reduce Ca$^{2+}$ uptake (data not shown).

Because the net Ca$^{2+}$ uptake, measured in the experiments reported in Figs 4A and B and 5, results from the balance between the passive influx and the Ca pump-mediated efflux, its inhibition by the drugs could be due either to an activation of the efflux or to an inhibition of the influx. Ca$^{2+}$ influx was estimated from $^{45}$Ca$^{2+}$ uptake measured under conditions of Ca$^{2+}$ pump inhibition in deoxygenated SS cells (Fig 4C). PMA or OK pretreatment had no effect on this influx, suggesting that the drug effect on net Ca$^{2+}$ uptake was mediated by an activation of Ca$^{2+}$ efflux.

Fig 4. Effect of DO and drug treatment on Ca$^{2+}$ uptake in SS cells. Cells were loaded with quin 2-AM either in the presence (A and B) or the absence (C) of CaCl$_2$ in the medium (see Materials and Methods). (A) Control cells under oxygenated (O$_2$) or deoxygenated conditions (N$_2$). (B) Control or drug-treated cells under deoxygenated conditions. (C) Control or drug-treated cells under deoxygenated conditions. PMA (400 nmol/L) was added for 60 minutes and OK (400 nmol/L) was added for 10 minutes before the sampling period. Data shown in (A) and (B) are from the same experiment.
SICKLE CELL DEHYDRATION AND PHOSPHORYLATION

Fig 5. Effects of PMA and OK on the inhibition of the DO-induced Ca2+ uptake in SS cells. (A) PMA concentration dependence (pretreatment for 60 minutes). (B) OK concentration dependence (pretreatment for 10 minutes). (C) PMA pretreatment time dependence (400 nmol/L). (D) OK pretreatment time dependence (200 nmol/L). Values shown are the means ± SE of three to seven experiments or of two experiments.

The mean values from different experiments are shown in Table 1. As already observed in the experiments of Fig 4, the drugs inhibited net Ca2+ uptake in deoxygenated SS cells, whereas they did not significantly affect Ca2+ influx. Preincubation of SS cells with both PMA (400 nmol/L) and OK (400 nmol/L) did not induce any greater inhibition of Ca2+ uptake than that caused by each drug alone, suggesting that PMA and OK activate Ca2+ efflux through a common mechanism. Table 1 also shows that, under oxygenated conditions, either in SS or in AA cells, PMA or OK had no effect on Ca2+ fluxes. In addition, the drugs had no effect on Ca2+ fluxes in deoxygenated AA cells (data not shown).

**Table 1. Effect of PMA or/and OK on Ca2+ Fluxes in SS or AA Cells Incubated Under Deoxygenated (N2) or Oxygenated (O2) Conditions**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PMA</th>
<th>OK</th>
<th>PMA + OK</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SS cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>Net Ca2+ uptake</td>
<td>82 ± 8</td>
<td>39 ± 9</td>
<td>49 ± 9</td>
<td>54 ± 16</td>
</tr>
<tr>
<td>N2</td>
<td>Ca2+ influx</td>
<td>132 ± 23</td>
<td>137 ± 11</td>
<td>155 ± 18</td>
<td>ND</td>
</tr>
<tr>
<td>O2</td>
<td>Ca2+ influx</td>
<td>30 ± 4</td>
<td>29 ± 4</td>
<td>31 ± 4</td>
<td>ND</td>
</tr>
<tr>
<td><strong>AA cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O2</td>
<td>Ca2+ influx</td>
<td>25</td>
<td>22</td>
<td>26</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are micromoles per 340 g of Hb per hour. The data are means ± SE of n experiments. Experimental protocols are described under Materials and Methods (PMA at 400 nmol/L for preincubation for 60 minutes or/and OK at 400 nmol/L for 10 minutes).

Abbreviation: ND, not determined.

**Effects of DO, PMA, and OK on the Na+ and K+ content.**

SS or AA cells were preincubated without or with PMA (400 nmol/L for 60 minutes) or OK (400 nmol/L for 10 minutes) and incubated under either oxygenated or deoxygenated conditions for 60 minutes. The DO-induced changes in the Na+, K+, and Na+ + K+ contents were calculated as the differences between the values measured in the deoxygenated and in the oxygenated samples after 60 minutes (ΔNa, ΔK, and ΔNa + K, respectively). The individual values from 6 experiments derived from the blood from 5 patients are presented in Table 2. As already reported,1-3,25 DO of SS cells in a medium containing Ca2+
Table 2. Effect of PMA and OK on DO-Induced Changes in the Na and K Contents of SS or AA Cells

<table>
<thead>
<tr>
<th></th>
<th>ΔNa</th>
<th></th>
<th>ΔK</th>
<th></th>
<th>ΔNa + K</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na</td>
<td>Control</td>
<td>PMA</td>
<td>OK</td>
<td>K</td>
<td>Control</td>
</tr>
<tr>
<td>SS cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.A.</td>
<td>10.2</td>
<td>+1.0</td>
<td>+1.9</td>
<td>+2.0</td>
<td>71.1</td>
<td>-14.4</td>
</tr>
<tr>
<td>T.A.</td>
<td>10.9</td>
<td>+1.6</td>
<td>+2.6</td>
<td>+2.9</td>
<td>77.0</td>
<td>-15.6</td>
</tr>
<tr>
<td>B.N.</td>
<td>7.8</td>
<td>+5.4</td>
<td>+6.1</td>
<td>+4.4</td>
<td>99.4</td>
<td>-8.2</td>
</tr>
<tr>
<td>F.C.</td>
<td>22.2</td>
<td>+5.2</td>
<td>+6.6</td>
<td>+4.2</td>
<td>82.6</td>
<td>-10.4</td>
</tr>
<tr>
<td>C.B.</td>
<td>28.3</td>
<td>+1.5</td>
<td>+3.2</td>
<td>+4.1</td>
<td>57.7</td>
<td>-6.1</td>
</tr>
<tr>
<td>B.L.</td>
<td>12.5</td>
<td>+5.1</td>
<td>+7.3</td>
<td>+7.0</td>
<td>81.7</td>
<td>-7.4</td>
</tr>
<tr>
<td>Means</td>
<td>15.3</td>
<td>+3.3*</td>
<td>+4.6*</td>
<td>+4.1*</td>
<td>78</td>
<td>-10.3*</td>
</tr>
<tr>
<td>±SE</td>
<td>±3.3</td>
<td>±0.9</td>
<td>±0.7</td>
<td>±0.7</td>
<td>±6</td>
<td>±1.6</td>
</tr>
</tbody>
</table>

For AA cells (n = 3)

|          | Mean | +0.8 | -0.4 | -0.6 | 94 | +0.5 | -1.1 | +0.1 | +1.3 | -1.5 | -0.5 |
|          | ±SE  | ±0.2 | ±0.2 | ±0.5 | ±9 | ±0.9 | ±2.4 | ±0.5 | ±1.1 | ±2.6 | ±0.9 |

Abbreviation: n, number of experiments.

* Significantly different from corresponding values in AA cells (P < .05).
† Significantly different from corresponding values in paired control (see text; PMA, P < .05; OK, P < .01).

SS or AA cells were preincubated either for 60 minutes with PMA (400 mmol/L) or for the last 10 minutes with OK (400 nmol/L) and incubated for 60 minutes either under oxygenated or deoxygenated conditions. Na and K are the initial Na and K intracellular concentrations; ΔNa, ΔK, and ΔNa + K were calculated as the differences in Na, K, and Na + K concentrations (millimoles per 340 g of Hb) between deoxygenated and oxygenated conditions.

causes an unbalanced Na⁺ gain and K⁺ loss, resulting in a net loss of cations. In these cells, both PMA and OK induced a small increase in ΔNa and a small decrease in ΔK. As a result, ΔNa + K was decreased by the drug treatment. To eliminate the large variations of the data between the different patients, the percent decrease in ΔNa + K induced by the drug treatment was calculated in each experiment, providing mean values of -46% ± 18% (P < .05) and -57% ± 14% (P < .01), respectively, for PMA and OK (Table 2). The drugs had no effect on Na⁺ or K⁺ concentrations in SS cells under oxygenated conditions (data not shown). In AA cells, there was no effect of DO on the Na⁺ and K⁺ contents, either in the absence or in the presence of the drugs.

Effects of DO, PMA, and OK on the SS cell density distribution. Figure 6A shows that, after 60 minutes of DO of SS cells, a small increase in cell density was seen reflecting cell dehydration. This finding is in agreement with those of previous studies, when DO was performed in the presence of external Ca²⁺.25,27,28 Treatment with PMA (400 nmol/L for 60 minutes; Fig 6B) or with OK (400 nmol/L for 10 minutes; Fig 6C) did not modify the density distribution curve in oxygenated conditions when compared with DMSO control samples. However, the den-
sity shift induced by DO was canceled by pretreatment of the cells with PMA or OK.

DISCUSSION

The present study shows that DO induces a nonspecific dephosphorylation of membrane proteins not only in SS cells but also in AA cells. The steady-state level of protein phosphorylation is dependent on the balance between the activities of protein kinases and phosphoprotein phosphatases localized in the cytosol or associated with the membrane. The kinase activities are dependent on ATP and Mg²⁺, whereas the phosphatase activities are generally stimulated by Mg²⁺.

Some kinases are inhibited by 2,3-diphosphoglycerate (2,3-DPG) and some phosphatases by both 2,3-DPG and ATP. Because of the increase in deoxygenated hemoglobin, cell pH increases, the free concentrations of ATP and 2,3-DPG decrease, and the level of free Mg²⁺ increases.

In addition, the glycolytic activity of the cells is accelerated in deoxygenated conditions, increasing the formation of lactate and of 2,3-DPG. It is thus difficult to predict the changes in the cellular concentrations of these metabolites and how they will affect the activities of the kinases or phosphatases. In fact, we found that the phosphatase inhibitor OK (400 nmol/L) prevented the DO-induced dephosphorylation of membrane proteins. In human RBCs, phosphoprotein phosphatases are mostly cytosolic and classified as PP2A, with a minor amount of PP2C. In vitro assays, PP2A are completely inhibited by 1 nmol/L OK, whereas in isolated cells, higher concentrations of the inhibitor may be needed in the medium because of the actual intracellular concentration of the phosphatases and the limited permeability of the cell membrane to OK.

The finding that dephosphorylation was inhibited by OK suggests that DO results in an activation of phosphatase(s) rather than in an inhibition of kinase(s). If this interpretation is correct, PMA should not suppress the dephosphorylation. However, because this PKC activator enhanced greatly the protein phosphorylation, it is conceivable that a small dephosphorylation escaped detection at a statistically significant level. In conclusion, the dephosphorylation of membrane proteins in deoxygenated AA and SS cells would result from a shift in the kinase/phosphatase equilibrium in favor of the latter. In drug-treated cells, the level of membrane protein phosphorylation during DO remained higher (PMA) or equal (OK) to that observed in oxygenated controls.

Both PMA and OK were shown to reduce by about 40% the DO-induced net Ca²⁺ uptake in SS cells, whereas they were unable to inhibit Ca²⁺ influx under the same conditions. Although further experiments are required to show that the drugs activate the Ca pump by increasing its phosphorylation state in deoxygenated SS cells, a number of data in the literature argue in favor of this interpretation. Indeed, it has been shown that PKC phosphorylates the erythrocyte Ca pump at both serine and threonine residues and stimulates Ca²⁺ uptake into inside-out vesicles from erythrocytes by a direct effect on the pump protein. In addition, PMA or OK stimulate Ca²⁺ efflux from Ca²⁺-loaded erythrocytes with the ionophore A23187 and increase the phosphorylation state of the Ca pump. The lack of additive effect on the net Ca²⁺ uptake when both drugs were added together in the present study suggests that the putative activation of the Ca pump would be mediated by the phosphorylation state of the same (phospho) serine/threonine residues rather than by PKC or dephosphorylated by PP2A. However, PMA or OK had no apparent effect on the Ca²⁺ fluxes under oxygenated conditions in AA and SS cells or under deoxygenated conditions in AA cells. The increase in [Ca²⁺], occurring in deoxygenated SS cells, but not in AA cells, could permit the drug-induced activation of the Ca pump, in agreement with previous studies.

It has been recently shown that DO of AA or SS cells causes a slight inhibition of the Ca pump. One possibility is that this inhibition results from the pump dephosphorylation at regulatory sites. However, although DO led to a 10% to 15% dephosphorylation of the membrane proteins contained in area 2 (which included the 140-KD Ca pump), more specific methods would be required to determine the phosphorylation state of the pump.

Because PMA or OK reduced the Ca²⁺ uptake in deoxygenated SS cells, they could, at least partially, prevent the increase in [Ca²⁺], that can activate the Gardos channels and induce cellular dehydration. In conclusion, the density shift induced by DO in SS cells was totally abolished after a pretreatment with PMA or OK. However, the DO-induced K⁺ efflux was only slightly decreased by these drugs. OK has been reported to inhibit partially the K/Cl cotransport, after its activation by acid pH or cell swelling under oxygenated conditions, either in AA or in SS cells.

It is unlikely that the small decrease in K⁺ efflux brought about by OK in this study was due to an inhibition of this transporter, because it is not activated in SS cells under continuous DO and, when stimulated by cell swelling, it is inhibited by DO.

Both PMA and OK consistently increased to a small extent DO-induced Na⁺ gain in SS cells. Activation of the Na/H antiport might be involved in this effect. In SS cells, this antiport is activated by cell shrinkage. In several cells, including erythrocytes, the antiport can be directly stimulated by PMA or OK and this stimulation correlates with an increase in phosphorylation of the exchanger protein on serine residues. We have observed that the small effect of PMA and OK on Na⁺ gain was restricted to deoxygenated SS cells. No drug-induced change in the Na⁺ concentration was detected in oxygenated SS cells or in AA cells (deoxygenated or deoxygenated).

An increase in [Ca²⁺], could be necessary to trigger the drug-induced activation of the antiport, in agreement with previous reports. However, other experiments are needed to demonstrate directly that PMA and OK enhance the DO-induced Na⁺ influx by activating the Na/H antiport in SS cells. In conclusion, PMA or OK prevented both membrane protein dephosphorylation and SS cell dehydration under deoxygenated conditions. The drugs could exert their protecting effect on the reduction of the cell volume by activating the Ca pump and the Na/H antiport. AA and SS cells are expected to regulate their [Ca²⁺], by active extrusion through the high-capacity Ca pump and SS cells to respond to cell shrinkage by activating the Na/H antiport. This lack
of regulation may result from the dephosphorylation of these transporters occurring as soon as the cells are deoxygenated.

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H Fathallah, E Coezy, RS de Neef, MD Hardy-Dessources and F Giraud