Deoxygenation of sickle red blood cells stimulates KCl cotransport without affecting Na\(^+\)/H\(^+\) exchange

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J. Joiner, C. H., M. Jiang, H. Fathallah, F. Giraud, and R. S. Franco. Deoxygenation of sickle red blood cells stimulates KCl cotransport without affecting Na\(^+\)/H\(^+\) exchange. Am. J. Physiol. 274 (Cell Physiol. 43): C1466–C1475, 1998.—KCl cotransport activated by swelling of sickle red blood cells (SS RBC) is inhibited by deoxygenation. Yet recent studies found a Cl\(^-\)-dependent increase in sickle reticulocyte density with cyclic deoxygenation. This study sought to demonstrate cotransporter stimulation by deoxygenation of SS RBC in isotonic media with normal pH. Low-density SS RBC exhibited a Cl\(^-\)-dependent component of the deoxygenation-induced net K\(^+\) efflux, which was blocked by two inhibitors of KCl cotransport, [[dihydroindenyl]oxy]alkanoic acid and okadaic acid. Cl\(^-\)-dependent K\(^+\) efflux stimulated by deoxygenation was enhanced 2.5-fold by clamping of cellular Mg\(^2+\) at the level in oxygenated cells using ionophore A-23187. Incubating cells in high external K\(^+\) or Rb\(^+\) minimized inhibition of KCl cotransport by internal Mg\(^2+\), and under these conditions deoxygenation markedly stimulated KCl cotransport in the absence of ionophore. Activation of KCl cotransport by deoxygenation of SS RBC in isotonic media at normal pH is consistent with the generalized dephosphorylation of membrane proteins induced by deoxygenation and activation of the cotransporter by a dephosphorylation mechanism. Na\(^+\)/H\(^+\) exchange activity, known to be modulated by cytosolic Ca\(^2+\) elevation and cell shrinkage, remained silent under deoxygenation conditions.

Potassium; sodium; erythrocyte; reticulocyte; volume regulation.

DEHYDRATION OF RED BLOOD CELLS containing sickle hemoglobin (Hb S) (SS RBC) contributes to the pathophysiology of sickling because of the high dependence of polymerization on the concentration of Hb S (19). Although the mechanism of dehydration in vivo is not entirely clear, cation loss from sickle cells in vitro appears to involve both sickling-dependent and sickling-independent mechanisms (35).

Sickling-dependent cation loss results from changes in membrane permeability to Na\(^+\), K\(^-\), and Ca\(^2+\) associated with formation of membrane spicules induced by ordered Hb S polymer. In the presence of physiological external Ca\(^2+\) concentrations, sickling-induced K\(^+\) loss exceeds Na\(^+\) gain, as a result of three different mechanisms: 1) imbalance between Na\(^+\) influx and K\(^+\) efflux via the sickling-induced pathway per se (4, 37), 2) unbalanced compensation of the Na\(^+\)/K\(^-\) pump (40), and 3) activation of Ca\(^2+\)-dependent K\(^+\) channels by Ca\(^2+\) influx via the sickling-induced pathway (4, 6). The relative contribution of these pathways to SS RBC dehydration in vivo is not known and may, in fact, vary among different patients and among SS RBC subpopulations in the same patient.

Sickling-independent cation loss from SS RBC may be mediated by the KCl cotransporter, a volume regulatory pathway exhibiting high activity in reticulocytes and capable of rapid K\(^+\) efflux (5, 10). The cotransporter probably contributes to the volume reduction that accompanies normal reticulocyte maturation. KCl cotransport activity is markedly increased in SS RBC (5), perhaps as a consequence of the young age of these cells (10) or possibly secondary to specific interactions of Hb S with the transporter or its regulators (47). The importance of KCl cotransport in dehydration of SS reticulocytes was suggested by our recent finding that reticulocytes that had been dehydrated in vivo had higher apparent KCl cotransport activity than reticulocytes that had remained normally hydrated in vivo (28).

Under isotonic conditions at pH 7.3–7.4, no Cl\(^-\)-dependent component of K\(^+\) loss from oxygenated SS RBC can be demonstrated (7, 10, 36, 39). KCl cotransport is known to be activated in swollen or acidified cells by mechanisms involving dephosphorylation of the transporter or its regulator(s) (3, 25, 32, 33). KCl cotransport activation in SS RBC has generally been considered independent of sickling because deoxygenation inhibits swelling-activated KCl cotransport (11). Binding to deoxygenated Hb of the major cellular Mg\(^2+\) buffers, 2,3-diphospho-D-glycerate and ATP, results in increased intracellular Mg\(^2+\) (24, 48), which inhibits the activated cotransporter (8, 18, 43, 49). In this context, it was not surprising that previous studies found no Cl\(^-\)-dependent K\(^+\) efflux in SS RBC subjected to prolonged, continuous deoxygenation (2, 39). These results were difficult to reconcile, however, with later studies showing that the K\(^+\) efflux and/or density changes of SS RBC subjected to cyclic deoxygenation under isotonic conditions at normal pH were inhibited by the KCl cotransport inhibitor [[dihydroindenyl]-oxy]alkanoic acid (DIOA) (1, 46, 52). Recently we also reported that SS RBC reticulocytes exhibited a significant Cl\(^-\)-dependent change in density when subjected to cyclic deoxygenation in vitro, but not under continuous deoxygenation or oxygenation (29).

All of these findings could be explained by a model in which deoxygenation induces a change in the phosphorylation-dephosphorylation equilibrium of the cell, thereby activating KCl cotransport. Such a deoxygenation-induced change in phosphorylation was recently reported for several red cell membrane proteins and shown to result from activation of okadaic acid-sensitive phosphatase(s) (22). The model predicts that
the “activated” cotransporter would, nevertheless, be inhibited by elevated internal Mg$^{2+}$
 during deoxygenation. On reoxygenation, with lowering of cellular Mg$^{2+}$, the activated KCl cotransporter would be released from inhibition and mediate K$^+$ and Cl$^-$ efflux until the slower process of deactivation of the transporter occurred. The present study was undertaken to test the hypothesis that the KCl cotransporter is activated by deoxygenation, by measuring transport under conditions that controlled cellular Mg$^{2+}$. In addition, we examined whether the Na$^+$/H$^+$ exchanger could contribute to the deoxygenation-induced Na$^+$ influx, since deoxygenation may produce an elevation of cytosolic Ca$^{2+}$ (21) as well as cell shrinkage, both of which could result in the activation of Na$^+$/H$^+$ exchange (12, 20). A preliminary report of these studies has appeared in abstract form (38).

**MATERIALS AND METHODS**

Incubation media. Unless otherwise noted, media were obtained from Sigma (St. Louis, MO) and were reagent grade or better. HEPES-buffered saline (HBS) contained (in mM) 140 NaCl, 20 HEPES (pH 7.4 at 37°C with NaOH), 0.1 EDTA, and 10 glucose. In experiments in which cellular Mg$^{2+}$ was manipulated with A-23187, EGTA replaced EDTA, and total external Mg$^{2+}$ concentration was 0.15 mM, unless indicated otherwise. This concentration of external Mg$^{2+}$ has been shown to maintain constant internal Mg$^{2+}$ and cell volume in ionophore-treated RBC (8, 26). HEPES-buffered K$^+$ medium and Rb$^+$ medium were identical to HBS, except that K$^+$ and Rb$^+$ salts, respectively, replaced Na$^+$ salts. In NO$_3$ media, NO$_3$ salts replaced Cl$^-$ salts. Ouabain was present at 0.1 mM in all solutions. DIOA and okadaic acid were purchased from Research Biochemicals International (Natick, MA).

Blood samples and density separations. After informed consent, blood was obtained by venipuncture into heparinized tubes from volunteers homoygous for Hb S. Unless otherwise stated, all experiments were performed on the least dense 15–25% of the SS RBC population, since these cells have the highest KCl cotransport activity (5, 10). Fresh whole blood was washed in HBS at 4°C without removing the buffy coat and then applied to discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradients. After centrifugation at 3,000 g for 20 min, cells with density ($\rho$) $< 1.085$ were isolated as described previously (28, 30), washed, and filtered through glass wool to remove white blood cells. When stored overnight, cells were suspended in HEPES-buffered solution containing 15 mM NaCl and 125 mM KCl. Spin hematocrit values were measured on oxygenated samples in microhematocrit tubes after centrifugation for 5 min at 13,000 g in a hematocrit centrifuge.

Cellular cation measurements. Cellular cations were measured by methods described in detail previously (37, 39). Briefly, samples containing 5–10 μl of cells were taken into iced tubes containing 110 mM MgCl$_2$ layered over dibutyl phthalate (Fisher, Pittsburgh, PA). Cells exposed to ionophore were not washed before exposure to MgCl$_2$, but there was no indication of cell swelling or hemolysis before centrifugation through phthalate oil. There was also no difference in Na$^+$ or K$^+$ fluxes in NO$_3$ media between cells incubated with or without ionophore, indicating that the short exposure of cells to the combination of isotonic MgCl$_2$ and ionophore at 0°C did not affect the monovalent cation content of the cells. After the tubes had been washed, the oil was removed and the cells were lysed for analysis of cations by flame emission spectroscopy (Perkin-Elmer model 370 atomic absorption spectrophotometer, Norwalk, CT). Hemoglobin was measured at 540 nm using a Beckman DU spectrophotometer (Beckman Instruments). Cation content was calculated as millimoles per kilogram hemoglobin; all cation measurements were made in triplicate, unless otherwise noted.

Net cation fluxes. As in previous studies (37–40), SS RBC were suspended at 2% hematocrit in appropriate media, and ionophore or drugs were added as needed in stock solutions in DMSO. DMSO was present in a final concentration of 0.4–1 vol% and was added to controls. Cells were warmed to 37°C for 10 min, and an initial cation sample was taken. Oxygenated samples were capped, whereas deoxygenated samples were flushed with humidified N$_2$ for 1 h, after which the flasks remained sealed. This deoxygenation protocol did not change the osmolality of media, indicating that N$_2$ humidification was adequate. A second cation sample was taken after 2 h of incubation, and the net flux was calculated from the change in cation content.

Na$^+$/H$^+$ exchange measurements. Blood was centrifuged on Percoll gradients, and a cell density (1.076 < $\rho$ < 1.106) was isolated. Cells were washed in solution A (in mM: 140 NaCl, 5 KCl, 1 MgCl$_2$, 1 Na$_2$HPO$_4$, 2 NaPO$_4$, 20 EDTA, 10 HEPES-Tris (pH 7.4 at 37°C), and 10 glucose) and stored at 4°C for 12–36 h. Just before the experiments, cells were washed once in solution A and incubated at 15% hematocrit at 37°C for 45 min in the same solution containing 2 mM adenine and 10 mM inosine. Cells were then washed four times in solution B (solution A without PO$_4$, and supplemented with 1 mM CaCl$_2$, 0.1 mM ouabain, and 0.01 mM bumetanide) and resuspended in the same solution at 10% hematocrit. Cells were incubated at 37°C for 5 min with 40 μM dimethyl amiloride (DMA) and then for 10 min with 0.4 μM phorbolester 13-acetate (PMA). DMSO from the stock solutions of DMA and PMA was present at a final concentration of 0.4–0.8 vol% and was added to control suspensions. Cells were then either kept oxygenated or flushed with humidified N$_2$, as described previously (22, 23). After 90 min, cells were washed twice in a solution containing 110 mM MgCl$_2$ and 10 mM HEPES-Tris (pH 7.4 at 4°C). Pellets were lysed, and lystate was analyzed for hemoglobin and Na$^+$.

**RESULTS**

Cl$^-$ dependence of K$^+$ efflux in deoxygenated SS RBC. Figure 1 depicts a series of experiments in which ouabain-treated SS RBC were incubated under oxygenated or deoxygenated conditions in either Cl$^-$ or NO$_3$ media and net fluxes of Na$^+$ and K$^+$ were measured. Figure 1A depicts cells under control conditions (without ionophore). In oxygenated cells there was no Cl$^-$ dependent transport of either Na$^+$ or K$^+$; fluxes in NO$_3$ exceeded those in Cl$^-$, as reported by others (7, 39). On deoxygenation, both Na$^+$ and K$^+$ fluxes increased, as expected from previous studies (2, 4, 37, 39), and Na$^+$ influx was independent of Cl$^-$, K$^+$ efflux, however, was 12.5% higher in Cl$^-$ compared with NO$_3$ media (P < 0.04), suggesting KCl cotransport activity stimulated by deoxygenation. This result contrasts with previous findings from our laboratory (36, 39) and by others (2) that there was no Cl$^-$-dependent component to the K$^+$ efflux in deoxygenated SS RBC. This difference may arise from the fact that the current studies utilized a low-density fraction of SS RBC (least dense 25–50% of cells) containing a higher number of young cells with
high KCl cotransport activity (10), whereas previous studies used unfractionated cells. In addition, the deoxygenation-induced K⁺ efflux (difference between fluxes in deoxygenated and oxygenated cells) in this fraction exceeded deoxygenation-induced Na⁺ influx, which is also in contrast to previous results obtained in unfractionated SS RBC showing balanced sickling-induced Na⁺ and K⁺ fluxes (2, 36, 39, 40). The activation of KCl cotransport in this low-density fraction appears to account for this difference.

Figure 1B depicts parallel incubations in the presence of the ionophore A-23187, which causes Mg²⁺ to distribute across the membrane according to the membrane potential, so that free internal Mg²⁺ is approximately twice the external concentration (26). In the experiments in Fig. 1, external Mg²⁺ was 0.15 mM, which in the presence of ionophore “clamps” internal Mg²⁺ at normal levels in oxygenated cells (8, 26) and prevents the increase in cellular free Mg²⁺ associated with deoxygenation (11, 24, 26, 48). There was no effect of ionophore on Na⁺ and K⁺ fluxes in oxygenated cells, as expected, and in deoxygenated cells, no Cl⁻ dependence of Na⁺ influx was noted. However, K⁺ efflux from deoxygenated SS RBC under these conditions was 34% higher in Cl⁻ media compared with NO₃ media (P < 0.0001), whereas K⁺ efflux from deoxygenated SS RBC in NO₃ media was the same without and with ionophore (means ± SD, 33.9 ± 4.9 vs. 34.5 ± 6.5 mmol·kg Hb⁻¹·h⁻¹). Thus the Cl⁻-dependent component of the K⁺ efflux in deoxygenated SS RBC was increased in the presence of ionophore. In this series of experiments, the Cl⁻-dependent K⁺ efflux in deoxygenated SS RBC without ionophore (Fig. 1A) averaged 5.4 ± 3.8 mmol·kg Hb⁻¹·h⁻¹, which is rather small compared with the total K⁺ efflux (38.3 ± 4.9 mmol·kg Hb⁻¹·h⁻¹). However, when internal Mg²⁺ was clamped in the presence of A-23187 (Fig. 1B), Cl⁻-dependent K⁺ efflux was stimulated to 12.4 ± 5.2 mmol·kg Hb⁻¹·h⁻¹ (P < 0.005 vs. ionophore-free control). To establish that this Cl⁻-dependent component of the K⁺ efflux in deoxygenated SS RBC was mediated by KCl cotransport, the effects of pharmacological inhibitors of this transport system were examined.

Cl⁻-dependent K⁺ efflux stimulated by deoxygenation is blocked by okadaic acid and DIOA. In a separate series of experiments of similar design, we tested the effects of okadaic acid, a serine/threonine protein phosphatase inhibitor that blocks the swelling-induced activation of KCl cotransport (33), and DIOA, a compound that blocks the cotransport pathway (46, 52). Figure 2 shows net K⁺ efflux in deoxygenated SS RBC. The
small Cl\(^-\)-dependent component of K\(^+\) efflux in deoxygenated SS RBC was apparent in the control (no drug) cells without ionophore; this was not present in oxygenated cells (not shown) and was therefore stimulated by deoxygenation. This Cl\(^-\)-dependent K\(^+\) efflux was augmented by clamping Mg\(^{2+}\), as before, and was completely blocked by both okadaic acid and DIOA, supporting its mediation by the KCl cotransporter.

In Fig. 3, the data have been recalculated, by subtracting the flux in oxygenated cells from that in deoxygenated cells, to yield the deoxygenation-induced K\(^+\) efflux (39). In control samples of these low-density SS RBC fractions, a portion of the deoxygenation-induced K\(^+\) efflux was Cl\(^-\)-dependent without ionophore, and this Cl\(^-\)-dependent component was augmented by clamping cellular Mg\(^{2+}\) at oxygenated levels with ionophore. The Cl\(^-\)-dependent component of these deoxygenation-induced fluxes was abolished by DIOA and okadaic acid both with and without ionophore.

Although not shown in Fig. 3, the deoxygenation-induced K\(^+\) efflux in NO\(_3\) media was equivalent to deoxygenation-induced Na\(^+\) influx under all conditions, as previously described (2, 37), and is presumably mediated by the passive, diffusional pathway activated by sickling and inhibited by stilbenes (2, 34, 39, 40). Although the term "deoxygenation-induced" has been used to describe this pathway, the current data demonstrate that, in low-density SS RBC at least, the deoxygenation-induced K\(^+\) efflux consists of two components: one mediated by KCl cotransport stimulated by deoxygenation and another mediated by the sickling-induced leak pathway.

Stimulation of KCl cotransport by deoxygenation is not a consequence of the Bohr effect. The Bohr effect of hemoglobin (9) produces two changes in RBC on deoxygenation and another mediated by the sickling-induced leak pathway.

Stimulation of KCl cotransport by deoxygenation is a function of Mg\(^{2+}\). We predicted that, if the effect of A-23187 to augment deoxygenation stimulation of KCl cotransport activity in SS RBC is due to clamping of cellular Mg\(^{2+}\) at the normal oxygenated level, raising external Mg\(^{2+}\) in A-23187-treated cells would blunt the deoxygenation stimulation. To test this prediction, SS RBC were deoxygenated as before in the presence of A-23187 at total external Mg\(^{2+}\) concentrations shown in Fig. 5; there was a marked stimulation of Cl\(^-\)-dependent K\(^+\) efflux in deoxygenated cells, as was apparent in previous experiments (Fig. 1). At high Mg\(^{2+}\) (1.5 mM), however, the stimulation of the Cl\(^-\)-dependent component of the K\(^+\) efflux in deoxygenated SS RBC was markedly reduced compared with stimulation at 0.15 mM Mg\(^{2+}\). These data are consistent with the notion that increased cellular Mg\(^{2+}\) in deoxygenated SS RBC (in the absence of ionophore) counteracts the stimulation of KCl cotransport activity by partially inhibiting the transporter under continuous deoxygenation.

The data of Fig. 4 are also consistent with previously reported effects of Mg\(^{2+}\) and other divalent cations on swelling-induced KCl cotransport (8, 11, 18, 43, 49). This effect is illustrated in Fig. 5A. Cells were incubated in hypotonic solutions (220 mosM), and net K\(^+\) efflux was measured over 1 h in Cl\(^-\) or NO\(_3\) media. Cellular Mg\(^{2+}\) was damped at various levels, dictated by the external Mg\(^{2+}\) concentrations shown in Fig. 5; Flatman and Lew (26) have shown that internal Mg\(^{2+}\)}
in human RBC and by Lauf and colleagues (18, 43) in low-K⁺ sheep RBC.

Results from SS RBC in hypotonic high-Rb⁺ media are depicted in Fig. 5, A and B. Unlike Na⁺, Rb⁺ substitutes for K⁺ as a transported ion on the KCl cotransporter and has been shown to stimulate K⁺ efflux via the transporter in human RBC (41). This trans-stimulation is apparent in hypotonic RbCl media, in which cells show higher K⁺ efflux at all Mg²⁺ concentrations than in hypotonic NaCl (Fig. 5A). A very different response to Mg²⁺ is also noted in high-Rb⁺ media: the swelling-induced K⁺ efflux is less sensitive to increasing Mg²⁺ concentrations, including the range of external Mg²⁺ concentrations (0.1–0.2 mM) that equilibrate with physiological internal Mg²⁺ levels in oxygenated cells (26). On deoxygenation, RBC Mg²⁺ levels increase ~50% (24, 48). Because 0.15 mM external Mg²⁺ reflects the equilibrium concentration in oxygenated cells, deoxygenation would be expected to produce a cellular Mg²⁺ concentration corresponding to an external concentration of 0.2–0.3 mM. As shown in Fig. 5A, this range of concentrations (0.15–0.3 mM) has a profound effect on swelling-activated K⁺ efflux in low-K⁺ medium but a much smaller effect in high-Rb⁺ medium. A similar Mg²⁺ dependence is noted for Rb⁺ influx measured simultaneously in the same cells, as shown in Fig. 5B. Thus swelling-activated K⁺ efflux and Rb⁺ influx mediated by the KCl cotransporter are less susceptible to Mg²⁺ inhibition in high-Rb⁺ (K⁺) buffers. Therefore, if activation of the KCl cotransporter in SS RBC by continuous deoxygenation is partially masked by elevated Mg²⁺ under physiological conditions, then such stimulation should be apparent without Mg²⁺ clamping in cells incubated in high-Rb⁺ (K⁺) buffers. This prediction was borne out in experiments presented below.

KCl cotransport activity in isotonic high-Rb⁺ (K⁺) buffers is stimulated by deoxygenation of SS RBC without manipulating cellular Mg²⁺. Figure 6 depicts

Fig. 5. Effect of Mg²⁺ on swelling-stimulated K⁺ efflux in SS RBC treated with ionophore A-23187. SS RBC were washed in isotonic HEPES-buffered KCl (HBK; or in corresponding KNO₃ buffer) and resuspended at 55% hematocrit. To begin flux, an aliquot of suspension was added to warmed hypotonic medium containing A-23187 (20 μmol/l cells) and the concentrations of external Mg²⁺ shown. Final osmolarity was 220 mosM. After addition of cells, hypotonic HBS media (hypotonic NaCl) contained 94 mM Na⁺, 5.6 mM K⁺, 0.1 mM EGTA, 10 mM glucose, and 0.1 mM ouabain; hypo RbCl was identical except that Rb⁺ replaced Na⁺. In NO₃ media, NO₃ salts replaced Cl− salts of Na⁺ and Rb⁺. K⁺ efflux and Rb⁺ influx were calculated from cellular cation measurements made before addition of cells to hypotonic buffers, and after 1 h of incubation. A: K⁺ efflux in cells in hypotonic Na⁺ and Rb⁺ buffers. B: Rb⁺ influx measured simultaneously in cells in hypotonic Rb⁺ buffers. Fluxes in NO₃ media (hypotonic NaNO₃ or hypotonic RbNO₃) were measured at 0 Mg²⁺. Data are means of triplicate measurements in 1 experiment representative of 3 others; SD, where not shown, were smaller than symbols.

Fig. 6. Rb⁺ uptake in SS RBC incubated in high-Rb⁺ (K⁺) buffer. Cells were preincubated under oxygenated or deoxygenated conditions for 1 h in isotonic HBS or HBN containing 0.1 mM ouabain and 0.1 mM EDTA. Flux was initiated by addition of isotonic HEPES-buffered Rb⁺ medium (Cl⁻ or NO₃⁻) to give 70 mM Rb⁺ and 70 mM Na⁺. Cellular Rb⁺ was measured at indicated times; slopes of these uptake curves represent flux rates. This experiment was representative of 4 others.
an experiment in which Rb\(^+\) influx was measured in SS RBC incubated under oxygenated and deoxygenated conditions (without addition of ionophore) in buffers containing high external Rb\(^+\). Cells were deoxygenated in isotonic HBS or HEPES-buffered NaNO\(_3\) medium containing 0.1 mM ouabain and 0.1 mM EDTA, and at time 0 isotonic Rb\(^+\) buffer (either Cl\(^-\) or NO\(_3\)) was added to give 70 mM Rb\(^+\) (+ 70 mM Na\(^+\)). Cellular Rb\(^+\) uptake was measured at various time points, as depicted in Fig. 6; the slopes of the curves represent flux rates. Under oxygenated conditions, Rb\(^+\) influx was greater in Cl\(^-\) medium than in NO\(_3\) medium, indicating a Cl\(^-\)-dependent component in this experiment; however, this was not a consistent finding, and in the pooled data of Fig. 7 this is not apparent. As illustrated in Fig. 6, deoxygenation increased Rb\(^+\) influx approximately twofold in NO\(_3\) medium, reflecting the sickling-induced increase in passive membrane permeability (39). In Cl\(^-\) media, however, deoxygenation increased Rb\(^+\) influx even more strikingly. Rb\(^+\) influx measured in saline media with external Rb\(^+\) = 5.6 mM showed no significant Cl\(^-\)-dependent component in either oxygenated or deoxygenated cells (not shown), indicating that the deoxygenation stimulation of Cl\(^-\)-dependent Rb\(^+\) influx was unique to the condition of high external Rb\(^+\). These data demonstrate that under conditions of high external Rb\(^+\), a substantial component of the Rb\(^+\) influx stimulated by deoxygenation of SS RBC was Cl\(^-\)-dependent.

This Cl\(^-\)-dependence is illustrated in the series of experiments in Fig. 7, in which both Rb\(^+\) influx and K\(^+\) efflux were measured in deoxygenated SS RBC incubated in isotonic high-K\(^+\) (Rb\(^+\)) media, conditions of trans-stimulation of the cotransporter. Final external cation concentrations were 35 mM Na\(^+\), 65 mM K\(^+\), 40 mM Rb\(^+\). These conditions permit measurement of KCl cotransport-mediated K\(^+\) efflux, as well as Rb\(^+\) influx.

In oxygenated SS RBC, there was no Cl\(^-\)-dependent flux and no effect of DIOA or okadaic acid (not shown). However, in deoxygenated SS RBC (Fig. 7), 40% of both K\(^+\) efflux and Rb\(^+\) influx was inhibited in NO\(_3\) medium, without cell swelling, acidification, or manipulation of Mg\(^{2+}\). This deoxygenation-stimulated, Cl\(^-\)-dependent component was substantially inhibited by DIOA and abolished by okadaic acid, confirming its mediation by the KCl cotransporter. Thus, in high-Rb\(^+\)(K\(^+\)) buffers, KCl cotransport is activated by continuous deoxygenation. Under these conditions of trans-stimulation, the increase in cellular Mg\(^{2+}\) produced by deoxygenation has a minimal inhibitory effect on cotransporter activity (Fig. 5).

Na\(^+\)/H\(^+\) exchange is not activated by deoxygenation. To investigate whether a component of the deoxygenation-induced Na\(^+\) influx (Fig. 1) was mediated by the Na\(^+\)/H\(^+\) exchanger, we examined the effect of DMA, an inhibitor of the antiporter (41). These experiments utilized a large density fraction of SS RBC (1.076 < ρ < 1.106); Na\(^+\)/H\(^+\) exchange has been found to be independent of density over this range and decreased only in cells of higher density (12). Net Na\(^+\) influx was measured under oxygenated and deoxygenated conditions in isotonic media at pH 7.4 as described in MATERIALS AND METHODS, with and without DMA. DMA had no effect on Na\(^+\) influx in oxygenated SS RBC or in oxygenated or deoxygenated AA RBC (not shown), indicating that the Na\(^+\)/H\(^+\) exchanger was quiescent under these conditions. The deoxygenation-induced Na\(^+\) influx was calculated as the difference between the values in paired deoxygenated and oxygenated samples, and was normalized to the value in control cells (no added drugs). These normalized values are presented in Fig. 8 as averages of six experiments. Deoxygenation-induced Na\(^+\) influx was not inhibited by DMA, indicating that the Na\(^+\)/H\(^+\) exchanger was not activated by deoxygenation. In contrast, deoxygenation-induced Na\(^+\) influx was increased by 45% after a pretreatment of SS RBC with PMA, as reported previously (22), and this
PMA-stimulated component was completely inhibited by DMA. These data demonstrate that the Na\(^{+}/H^{+}\) exchanger is stimulated by PMA in deoxygenated SS red blood cells, as previously observed in acid-loaded erythrocytes (14) and nucleated cells (45). Interestingly, PMA had no effect on Na\(^{+}\) influx under oxygenated conditions in SS RBC or under oxygenated or deoxygenated conditions in normal RBC (not shown). That activation of Na\(^{+}/H^{+}\) exchange in isotonic media at normal pH occurs only in SS RBC and requires both deoxygenation and PMA implies a complex mechanism of activation. In any case, the data indicate that the Na\(^{+}/H^{+}\) exchanger is present in SS RBC and can be stimulated in deoxygenated SS RBC by PMA but that the exchanger is not activated by deoxygenation alone and thus is not likely to contribute to cation homeostasis under physiological conditions.

**DISCUSSION**

Early studies of cation fluxes in unfractionated SS RBC under continuous deoxygenation in isotonic media at normal pH found no Cl\(^{-}\)-dependent component to the K\(^{+}\) efflux (2, 36, 39). This was consistent with previous findings that the KCl cotransporter was inactive under isotonic conditions at normal pH under oxygenated conditions (7, 11), and, when activated by cell swelling or acid pH, KCl cotransport was inhibited by deoxygenation (11). Canessa et al. (11) showed that this inhibitory effect was a consequence of two well-described phenomena: increased cellular Mg\(^{2+}\) due to binding of organic anions to deoxygenated Hb (24, 26, 48) and inhibition of the cotransporter by internal Mg\(^{2+}\) (8, 11, 18). Deoxygenation also was shown to reduce KCl cotransport in trout and horse RBC incubated in isotonic media at pH 7.0 (15, 31), although the authors of these reports suggested that factors other than cellular Mg\(^{2+}\) were involved.

Later studies, however, suggested that the K\(^{+}\) loss from deoxygenated SS RBC was mediated, at least in part, by the KCl cotransporter. Beuzard and colleagues (46, 52) reported that a portion of the K\(^{+}\) efflux from deoxygenated cells was inhibited by DIOA. Apovos et al. (1) found that cyclic deoxygenation of SS RBC resulted in dense cell formation that was independent of the presence of Ca\(^{2+}\) and inhibited by DIOA. Franco et al. (29) found that Cl\(^{-}\)-dependent dense cell formation in SS reticulocytes occurred with cyclic, but not continuous, deoxygenation under isotonic conditions at normal pH. We proposed an explanation for these findings that takes into account the increase in Mg\(^{2+}\) associated with deoxygenation (24, 48) and its effect on KCl cotransport (11, 18, 49) and the alteration in protein phosphorylation equilibrium induced by deoxygenation (22). Fathallah et al. (22) found that deoxygenation of SS RBC, as well as AA RBC, resulted in non-specific dephosphorylation of RBC membrane proteins. We proposed (29) that the KCl cotransporter or its regulators might be dephosphorylated on deoxygenation of SS RBC but its activity attenuated by the accompanying increase in cellular Mg\(^{2+}\) (24, 26). On reoxygenation, Mg\(^{2+}\) levels fall rapidly, whereas dephosphorylation may persist for some period of time, during which KCl cotransport could take place.

In the present study, we have demonstrated a small Cl\(^{-}\)-dependent K\(^{+}\) efflux in continuously deoxygenated, low-density SS RBC (Fig. 1A). The Cl\(^{-}\)-dependent component of the deoxygenation-induced K\(^{+}\) efflux was augmented more than twofold in cells treated with ionophore A-23187 to minimize the increase in cellular Mg\(^{2+}\) associated with deoxygenation (Fig. 1B). This supports the proposed mechanism whereby the activated KCl cotransporter is partially inhibited by elevated cellular Mg\(^{2+}\) in continuously deoxygenated SS RBC. Inhibition of the Cl\(^{-}\)-dependent component of the deoxygenation-induced flux by DIOA (Fig. 2) identifies it as a manifestation of KCl cotransport activity. Blockade by the phosphatase inhibitor okadaic acid further supports the idea that the stimulation is due to dephosphorylation of the cotransporter (or its regulators) by deoxygenation.

Thus stimulation of KCl cotransport by deoxygenation could also be demonstrated by flux measurements in high-Rb\(^{+}\)(K\(^{+}\)) medium. Under these conditions of trans-stimulation, it was shown that the transporter was more resistant to inhibition by cellular Mg\(^{2+}\), presumably due to the kinetic alterations induced by the trans-stimulation of the transporter in high-K\(^{+}\) medium (41). In terms of a kinetic model for KCl cotransport activity, this phenomenon could be explained if the return of the empty carrier (outside to inside) were rate limiting to KCl cotransport activity at low external K\(^{+}\) and were also inhibited by internal Mg\(^{2+}\). In high external K\(^{+}\)/(Rb\(^{+}\)), if return of the filled carrier were no longer rate limiting (hence, the trans-stimulation), transport inhibition by Mg\(^{2+}\) might be reduced. The data in Fig. 5 are consistent with this hypothesis, but further experiments will be required to provide rigorous kinetic support. Nevertheless, the stimulation of Cl\(^{-}\)-dependent K\(^{+}\) and Rb\(^{+}\) fluxes by deoxygenation of SS RBC is clear in high-Rb\(^{+}\)(K\(^{+}\)) medium (Figs. 6 and 7). As with the K\(^{+}\) flux in Mg\(^{2+}\)-clamped cells in low-K\(^{+}\) medium, these fluxes were inhibited by okadaic acid and DIOA, indicating respectively their activation by a dephosphorylation mechanism and mediation by KCl cotransport.

Stimulation of KCl cotransport in these experiments occurred in the absence of Ca\(^{2+}\), indicating that sickling-induced Ca\(^{2+}\) influx, with K\(^{+}\) channel activation, cellular dehydration, and acidification as proposed by Bookchin et al. (4), was not the trigger for cotransport activation by deoxygenation under these conditions.
The simplest explanation of our data is the direct activation of KCl cotransport by deoxygenation of SS RBC, presumably by a dephosphorylation event. Blockade of this activation by okadaic acid implicates a serine/threonine phosphatase in the process, probably protein phosphatase 1 (PP1), at least in the case of swelling-activated KCl cotransport (33). Involvement of tyrosine kinases and/or phosphatases in the regulation of KCl cotransport has recently been suggested. However, depending on species, tyrosine kinase inhibitors were reported either to inhibit (25, 50, 53) or to stimulate (3, 17, 25) cotransport. In fact, Flatman et al. (25) reported that the stimulation of KCl cotransport by one kinase inhibitor, staurosporine, was blocked by another inhibitor, genistein, suggesting two separate tyrosine phosphorylation sites. Inhibition of PP1 activity and direct activation of the cotransporter have been proposed to account for these effects, although the precise targets of tyrosine phosphorylation are not known and may vary among species. The involvement of both tyrosine kinase and PP1 in the activation of KCl cotransport implies the existence of two additional regulatory proteins: a tyrosine phosphatase and a serine/threonine kinase. Each of these enzymes is a potential target for modulators of KCl cotransport activation such as cell swelling, pH, Mg\(^{2+}\), oxidation, and phosphorylation-dephosphorylation. Furthermore, interactions among these modulators are likely to be quite complex. It is known that cell swelling, pH, and Mg\(^{2+}\) levels alter the effect of each other on KCl cotransport activity (8, 18, 43, 49). In addition, there is evidence for dual effects of Mg\(^{2+}\) on the cotransporter, both altering phosphorylation (18) and inhibiting the transporter directly (49). The interactions of multiple phosphorylation-dephosphorylation events (25, 50) in the activation of KCl cotransport and how they are affected by deoxygenation remain to be determined.

Our data suggest a new stimulus for activation of the KCl cotransporter, deoxygenation, which does not require the abnormal conditions of cell swelling or acidification and may be important both physiologically in normal RBC and pathologically in SS RBC. The magnitude of the K\(^{+}\) efflux via the KCl cotransporter stimulated by cyclic deoxygenation of RBC in vivo would be a complex function of circulatory transit times, dynamics of dephosphorylation-rephosphorylation of target proteins in relation to changing cellular Mg\(^{2+}\) levels, and the number of transporter molecules in individual cells. In vitro studies of the activation and inactivation of KCl cotransport in SS RBC by swelling and shrinking revealed delay times for activation of 1.7 ± 0.3 min and for inactivation of 3.6 ± 0.4 min (13). These delay times are somewhat longer than overall circulatory transit time (total blood volume divided by cardiac output), estimated at ~1 min in the healthy adult (44). However, transit times vary considerably among different vascular beds, bone and bone marrow having transit times 10-fold longer than kidney, brain, and lung (44). In addition, the adherence of SS reticulocytes to the endothelium of postcapillary venules (42) may further prolong circulatory times. Thus it is quite possible that individual cells might experience, at least intermittently, oxygenation-deoxygenation cycles in vivo that could activate the KCl cotransporter, with important effects in these cells on volume regulation.

Recently we reported that KCl cotransport activity was higher in SS reticulocytes that had become moderately dense in vivo relative to cells that retained normal density as reticulocytes (28), suggesting that KCl cotransport plays a role in reticulocyte dehydration in vivo. The fact that moderately dehydrated reticulocytes have the same levels of fetal hemoglobin (Hb F) as normally hydrated reticulocytes (27) implies that KCl cotransport, which is independent of Hb F (30), may be more important in the process of moderate reticulocyte dehydration than sickling-dependent mechanisms, which are inhibited by Hb F (19). Sickling-dependent processes, facilitated by moderate dehydration of reticulocytes, may contribute to more severe dehydration of certain reticulocytes (particularly those without Hb F), and to dehydration of more mature cells.

The abnormalities of volume regulation in SS RBC could theoretically arise from perturbations of Na\(^{+}\) transport pathways. The Na\(^{+}\)/H\(^{+}\) exchanger is an important Na\(^{+}\) uptake pathway in human red blood cells (20), and pharmacological and kinetic data suggest that red blood cells express at least the NHE1 isoform (14, 20). SS RBC exhibit elevated Na\(^{+}\)/H\(^{+}\) exchange activity (12), and we hypothesized that deoxygenation might stimulate this pathway. The Na\(^{+}\)/H\(^{+}\) exchanger in RBC is activated by cell shrinkage (12) or, in the presence of a pH gradient, by incubation with PMA or with Ca\(^{2+}\) and ionophore (20), both treatments resulting in protein kinase C\(\alpha\) (PKC\(\alpha\)) translocation to the membrane (23). Deoxygenation of SS RBC in the presence of external Ca\(^{2+}\) leads to transient elevation of cytosolic Ca\(^{2+}\) and subsequent cell volume reduction (via activation of Ca\(^{2+}\)-dependent K\(^{+}\) channels) (4, 6). The present data, however, show that Na\(^{+}\)/H\(^{+}\) exchange activity was not stimulated by deoxygenation alone. The transient rise in Ca\(^{2+}\) associated with deoxygenation is not sufficient to induce PKC\(\alpha\) translocation (23), which may explain the failure of deoxygenation to activate the transporter. Likewise, the addition of PMA to oxygenated SS RBC was insufficient to activate the transporter, although under these conditions PMA induces translocation of PKC\(\alpha\) to the membrane (23). The combined requirement for deoxygenation of SS RBC and the presence of PMA implies that both elevated Ca\(^{2+}\) and PKC\(\alpha\) activation are required for stimulation of Na\(^{+}\)/H\(^{+}\) exchange, in agreement with the proposed mechanism of NHE1 activation in other cells (45). Nevertheless, it does not appear that the Na\(^{+}\)/H\(^{+}\) exchanger mediates a component of deoxygenation-induced Na\(^{+}\) influx under physiological conditions.

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