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N-ethylmaleimide activates a Cl⁻-independent component of K⁺ flux in mouse erythrocytes

Boris E. Shmukler^a, Ann Hsu^a, Jessica Alves^b, Marie Trudel^e, Marco B. Rust^f, Christian A. Hubner^g, Alicia Rivera^{b,c,*}, and Seth L. Alper^{a,d,*}

^aDivisions of Nephrology and Molecular and Vascular Medicine, Beth Israel Deaconess Medical Center, Boston, MA

^bDepartment of Laboratory Medicine, Children's Hospital, Boston, MA

^cDepartment of Pathology, Harvard Medical School, Boston, MA

^dDepartment of Medicine, Harvard Medical School, Boston, MA

^eInstitut de Recherches Cliniques de Montréal, Molecular Genetics and Development, Faculté de Médecine, University of Montreal, Montreal

^fNeurobiology/Neurophysiology Group, University of Kaiserslautern, Kaiserslautern, Germany

^gInstitute of Human Genetics, University Hosp. Jena, Jena, Germany

Abstract

The K-Cl cotransporters (KCCs) of mouse erythrocytes exhibit higher basal activity than those of human erythrocytes, but are similarly activated by cell swelling, by hypertonic urea, and by staurosporine. However, the dramatic stimulation of human erythroid KCCs by N-ethylmaleimide (NEM) is obscured in mouse erythrocytes by a prominent NEM-stimulated K⁺ efflux that lacks Cl⁻-dependence. The NEM-sensitivity of Cl⁻-independent K⁺ efflux of mouse erythrocytes is lower than that of KCC. The genetically engineered absence of the K-Cl cotransporters KCC3 and KCC1 from mouse erythrocytes does not modify Cl⁻-independent K⁺ efflux. Mouse erythrocytes genetically devoid of the Gardos channel KCNN4 show increased NEM-sensitivity of both Cl⁻-independent K⁺ efflux and K-Cl cotransport. The increased NEM-sensitivity and stimulation magnitude of Cl⁻-independent K⁺ efflux in mouse erythrocytes expressing transgenic hypersickling human hemoglobin SAD (HbSAD) is independent of the presence of KCC3 and KCC1, but absence of KCNN4 reduces the stimulatory effect of HbSAD. NEM-stimulated Cl⁻-independent K⁺ efflux of mouse red cells is insensitive to ouabain and bumetanide, but partially inhibited by chloroquine, barium, and amiloride. The NEM-stimulated activity is modestly

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Correspondence: Seth L. Alper, MD-PhD, Beth Israel Deaconess Med. Ctr. 99 Brookline Ave RN380F, Boston, MA 02215, salper@bidmc.harvard.edu. Alicia Rivera, PhD, Childrens Hospital, Bader 7, 300 Longwood Ave, Boston, MA 02115, arivera@childrens.harvard.edu.

*Equal contributions

Contributions

SLA, AR and BES designed the study. MT, MBR, and CAH provided genetically modified mice. BES, AH, and JA performed experiments and collected data. BES, AR and SLA analyzed data. The manuscript was drafted by SLA and reviewed by the authors.

Conflict of Interest

The authors declare no competing financial interests.

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reduced at pH 6.0, but not significantly altered at pH 8.0, and abolished at 0°C. Although the molecular identity of this little-studied K⁺ efflux pathway of mouse erythrocytes remains unknown, it's potential role in the pathophysiology of sickle red cell dehydration will be important for extrapolation of studies in mouse models of sickle cell disease to our understanding of humans with sickle cell anemia.

Keywords

Red blood cells; K-Cl cotransport; Gardos channel; chloroquine; amiloride

Introduction

Erythrocytes of patients with sickle cell disease (SCD) exhibit increased activity of K⁺ leak pathways. The resulting cell shrinkage produces a population of dense cells with elevated intracellular concentrations of HbS. When these dense, dehydrated erythrocytes are deoxygenated, the lagtime for onset of deoxyHbS polymerization is accelerated, increasing risk of endothelial adhesion and vaso-occlusion [1]. Increased proportions of dense, dehydrated erythrocytes are associated with increased incidence of severe SCD manifestations such as skin ulcer, priapism, and renal dysfunction, and with indices of hemolysis such as elevated serum bilirubin and lactate dehydrogenase [2]. Major K⁺ leak pathways promoting increased dehydration of human sickle cells include the Gardos channel Kcnn4 and the Na⁺-independent K-Cl cotransporters. Pharmacological blockade of these leak pathways remains a therapeutic goal for the adjunct treatment of SCD [3; 4; 5], envisioned as combination therapy with inducers of HbF [6; 7; 8] and likely also with blockers of sickle cell adhesion to activated endothelial cells [9]. The Kcnn4 blocker senicapoc [10], a congener of the antifungal Kcnn4 blocker clotrimazole [11], has been shown to reduce the proportion of densest erythrocytes, increase mean corpuscular volume, decrease intracellular HbS concentration, improve anemia, and decrease indices of hemolysis [12; 13]. Blockers of K-Cl cotransport suitable for clinical use are actively being sought [14], but are not currently available.

Murine models of sickle disease have been valuable tools in the study of human SCD pathogenesis [15; 16], and will continue to be central to development of novel approaches to disease treatment. The SAD mouse model of sickle disease is particularly remarkable for its erythrocyte dehydration phenotype in concert with elevated K-Cl cotransport [17] and Gardos channel pathways [18]. Thus, characterization of mouse erythrocyte K⁺ leak pathways and their regulation is an important prerequisite for understanding the range of conditions in which erythrocytes from mouse models of sickle disease faithfully model the dehydration pathology of human sickle erythrocytes.

The mouse erythroid Ca²⁺-activated K⁺ channel of intermediate conductance, KCNN4 [19], mediates erythroid Gardos channel activity [20]. Mouse erythrocyte K-Cl cotransport activity is mediated largely by KCC3/SLC12A6, with a small contribution from KCC1/SLC12A4 [21]. Mouse erythroid K-Cl cotransport activity is activated by hypotonic swelling, by staurosporine [22], and by acid pH (Shmukler, Casula, LeClair, and Alper, unpublished), as is the corresponding human erythroid activity [23; 24]. However, whereas human erythroid K-Cl cotransport activity is strongly activated by N-ethylmaleimide (NEM) [25], mouse erythroid K⁺ efflux stimulated by 0.5 mM NEM was reduced at most 35% by extracellular Cl⁻ substitution [22]. Only the Cl⁻-dependent component of NEM-stimulated K⁺ efflux was inhibited by phosphatase inhibitor okadaic acid or by K-Cl cotransport inhibitor DIOA ([2-n-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-

yl)oxy]acetic acid). These data suggested the possible presence in mouse erythrocytes of an independent, alkylolation-sensitive K^+ leak pathway not evident in human erythrocytes.

In the current work, we characterize the NEM-stimulated Cl^- -independent K^+ efflux pathway of mouse erythrocytes. We show that its sensitivity to NEM is lower than that of K-Cl cotransport, but is enhanced in the presence of transgenic HbSAD. Neither KCNN4, KCC3, nor KCC1 were essential for NEM stimulation of erythroid Cl^- -independent K^+ efflux. NEM-stimulated Cl^- -independent K^+ efflux was insensitive to activation by low temperature, and was partially blocked by chloroquine, barium, and amiloride, but not by DIDS (4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid) or by other tested cation transport inhibitors.

Materials and Methods

Drugs

All salts were from Sigma-Aldrich (St. Louis, MO), and were of reagent grade. Staurosporine, calyculin A, and 2-aminophenylborate (2-APB) were from Calbiochem (San Diego, CA). XE-991 diHCl was from Tocris (Ellisville, MO). and HC-030031 was from Ascent Scientific (Bristol, UK). All other drugs were from Sigma or Aldrich.

Mouse strains

Mice were housed in humidity- and temperature-controlled rooms in the Animal Research Facility of Beth Israel Deaconess Medical Center, and had free access to water and food. HbSAD heterozygous transgenic sickle mice [26] were bred, genotyped, and maintained as previously described [21; 27], with modifications. *Kcnn4*^{-/-} mice were genotyped as previously described [20]. *Kcc1*^{-/-}; *Kcc3*^{-/-} double knockout mice were bred, genotyped, and maintained as previously described [21], with modifications. *Kcnn4*^{-/-} HbSAD transgenic mice and triple knockout *Kcnn4*^{-/-}; *Kcc1*^{-/-}; *Kcc3*^{-/-} mice were bred, maintained, and genotyped as described by Shmukler et al (manuscript in preparation). Each mutant strain has been bred onto the C57BL6 background for many years. Wildtype mice for comparison with SAD mice were progeny of SAD x WT crosses, and their erythrocytes were indistinguishable from those of JAX C57/BL6/J mice with respect to K-Cl cotransport activity and red cell indices (not shown). Wildtype mice used for comparison with *Kcnn4*^{-/-} mice were the wildtype progeny of *Kcnn4*^{+/-} breeder pairs.

Preparation of erythrocytes for flux studies

Blood was collected in heparinized syringes by cardiac puncture of mice anesthetized with Avertin according to protocols approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center. Blood was centrifuged at 2,500 rpm in 50 ml Falcon tubes for 5 min at 4°C. After careful removal of the buffy coat by aspiration, packed cells were washed 5 times at 4°C in ~20 volumes of wash solution (in mM: 172 choline Cl, 1 MgCl₂, 10 Tris MOPS), pH 7.40 at 4°C. Cells were resuspended to 30–50% cytocrit in wash solution and kept at 4°C for same-day use in flux studies. Red blood cells counts on 12.5×-diluted specimens were performed with the ADVIA 120 hematology analyzer, with mouse software (Siemens Diagnostic Solutions, Tarrytown, NY) as previously described [28].

Measurement of Cl^- -dependent and Cl^- -independent components of K^+ efflux

For assay of Cl^- -dependent K^+ efflux (K-Cl cotransport), erythrocytes at ~1% cytocrit were incubated at 37°C in isotonic NaCl medium containing (in mM) 160 NaCl, 1 MgCl₂, 10 glucose, 10 Tris-MOPS pH 7.4. For assay of Cl^- -independent K^+ efflux, incubation medium contained (in mM) 160 Na sulfamate, 1 Mg(NO₃)₂, 10 glucose, 10 Tris-MOPS pH 7.4. For

assay at low ionic strength, incubation medium contained (in mM) 320 sucrose, 1 $\text{Mg}(\text{NO}_3)_2$, 10 glucose, 10 Tris-MOPS pH 7.4. All flux solutions contained 1 mM ouabain to inhibit the (relatively ouabain-resistant murine erythrocyte Na^+, K^+ -ATPase) and 10 μM bumetanide (to inhibit Na-K-2Cl cotransport). Some efflux experiments were carried out in the presence of additional candidate inhibitors added at the indicated concentrations. Additional experiments were carried out in media of pH 6.0 or 8.0 (Fig. 6A).

Samples were incubated in the absence or presence of N-ethylmaleimide (NEM) at the indicated concentrations. Aliquots were removed after 5 and 25 min incubation at 37°C (or at 0°C in Fig. 6B), immediately transferred to pre-cooled 4 ml plastic tubes, centrifuged, and supernatants were collected for analysis of K^+ by atomic absorption spectrometry. K^+ efflux was calculated from the slope of the linear regression of extracellular K^+ content vs time. NEM-stimulated K-Cl cotransport activity was estimated as the difference between NEM-stimulated K^+ efflux in the presence and absence of extracellular Cl^- . In these conditions, K^+ efflux was linear at all tested [NEM] up to 0.5 mM. [NEM] > 1 mM was shown previously to predispose mouse erythrocytes to hemolysis [29]. However, 25 min exposure of mouse erythrocytes to 0.5 mM NEM at 37°C was confirmed not to promote hemolysis based on measurements of A_{540} in post-sedimentation supernatants of mock efflux studies (data not shown). Absence of hemolysis in the presence of 50–500 μM [NEM] was also confirmed by visual inspection in all flux experiments.

Statistical analysis—All values are presented as mean $\pm$ standard error of the mean (s.e.m.). Student's *t*-test was used to compare two groups. Comparison of multiple groups was by Kruskal-Wallis test with Dunnett's post-test, or by ANOVA with Tukey's post-test. Results were considered significant with $p < 0.05$.

Results

Mouse erythroid K-Cl cotransport is activated by low concentrations of NEM

Previous studies of K-Cl cotransport in erythrocytes from human, rabbit, and sheep have described specific activation of K-Cl cotransport by 0.5–2 mM NEM [30; 31]. *Xenopus* oocyte studies of mouse Kcc1 [32; 33] and endogenous *Xenopus* Kcc [34] confirmed susceptibility to stimulation by NEM. NEM was shown also to stimulate recombinant human KCC3 activity in HEK-293 cells [35] and in NIH/3T3 cells [36], but NEM-sensitivity of mouse KCC3 activity has not been reported. However, early studies of murine erythrocyte K-Cl cotransport with these concentrations of NEM revealed that most of the activated K^+ efflux was Cl^- -independent [22]. Fig. 1A confirms NEM-stimulation of K^+ efflux from mouse erythrocytes. Fig. 1C shows further that K-Cl cotransport (Cl^- -dependent K^+ efflux) was maximally activated at 50 μM NEM. K-Cl cotransport was appropriately absent from erythrocytes lacking both KCC1 and KCC3 (Fig. 1B). Since KCC3 mediates most if not all K-Cl cotransport in mature mouse erythrocytes [21], NEM at concentrations up to 50 μM is very likely activating KCC3-mediated K-Cl cotransport in these wildtype mouse erythrocytes. NEM-sensitivity of Cl^- -independent K^+ efflux was similar in the absence or presence of KCC1 and KCC3 (Fig. 1D).

Cl^- -independent K^+ efflux from mouse erythrocytes is activated by higher concentrations of NEM than those activating K-Cl cotransport

Cl^- -independent K^+ efflux stimulated by NEM was first evident at an NEM concentration of 50 μM , and continued to increase in magnitude at concentrations of 200 μM and 500 μM (Figs. 1B and 1D). This K^+ efflux occurred in the absence of detectable hemolysis. Cl^- -independent K^+ efflux stimulated by NEM was indistinguishable in the presence and

absence of K-Cl cotransporter polypeptides KCC1 and KCC3 (Fig. 1B, 1D), confirming its mediation by a distinct pathway.

NEM activates Cl⁻-independent K⁺ efflux in *Kcnn4*^{-/-} erythrocytes

The oxidizing agents 1-chloro-2,4-dinitrobenzene (CDNB) and phenazine methosulfate (PMS) have been shown to activate the Gardos channel in normal human erythrocytes [37; 38]. We addressed a possible contribution of the Gardos channel KCNN4 to NEM-stimulated Cl⁻-independent K⁺ efflux in mouse erythrocytes by comparing NEM-stimulated activity in wildtype and *Kcnn4*^{-/-} erythrocytes. Figs. 2A–C show that Cl⁻-dependent K⁺ efflux from *Kcnn4*^{-/-} erythrocytes (K-Cl cotransport) was indistinguishable from that of wildtype erythrocytes stimulated by 200 or 500 μM NEM. However, 50 μM NEM maximally stimulated *Kcnn4*^{-/-} erythrocyte K-Cl cotransport, while stimulation at that concentration was only minimal in wildtype erythrocytes from the same lineage. Thus, absence of KCNN4 in mouse erythrocytes is associated with K-Cl cotransport (likely mediated by KCC3) of increased NEM-sensitivity. In mouse erythrocytes lacking KCNN4 exhibited a proportionately much smaller enhancement of NEM-sensitivity for Cl⁻-independent K⁺ efflux (Figs. 2A, B, D).

The presence of HbSAD enhances NEM-sensitivity of Cl⁻-independent K⁺ efflux from mouse erythrocytes

The presence of HbSAD greatly increases Cl⁻-independent K⁺ efflux activity at low NEM concentrations (Fig 3B, 3D), but the effect is not evident at 500 μM NEM. Interestingly, unlike the enhanced swelling-sensitivity and staurosporine-sensitivity of K-Cl cotransport in SAD erythrocytes, NEM stimulation of K-Cl cotransport in SAD erythrocytes displayed a complex multiphasic concentration-response relationship (Fig. 3B, 3C).

HbSAD-containing erythrocytes lacking KCNN4 exhibit reduced NEM-sensitivity of erythroid Cl⁻-independent K⁺ efflux, but not of K-Cl cotransport

In *Kcnn4*^{-/-} erythrocytes, the presence of HbSAD does not significantly alter the NEM-sensitivity or magnitude of NEM-stimulated K-Cl cotransport (Fig 4A–C). However, NEM-sensitivity and the magnitude of stimulation of Cl⁻-independent K⁺ flux in *Kcnn4*^{-/-} erythrocytes both are increased in the presence of HbSAD (Fig. 4A, B, D). Cl⁻-independent K⁺ efflux in *SAD* erythrocytes was maximally activated at 200 μM NEM, vs. 500 μM in *SAD x Kcnn4*^{-/-} erythrocytes (Fig. 4D). Similarly enhanced NEM-sensitivity and flux magnitude of Cl⁻-independent K⁺ efflux was evident in HbSAD-triple knockout (*SAD;Kcc3*^{-/-};*Kcc1*^{-/-};*Kcnn4*^{-/-}) mouse red cells devoid of NEM-stimulated K-Cl cotransport (Fig. 5C), as compared to triple knockout erythrocytes without HbSAD (Fig 5A, B, D).

NEM-stimulated, Cl⁻-independent K⁺ efflux is partially inhibited by chloroquine and amiloride

The complement of erythrocyte membrane ion transporters remains incompletely defined. Erythrocyte membrane ion transport pathways are characterized by their pharmacological signatures. We therefore screened a series of candidate antagonists for activity against NEM-stimulated Cl⁻-independent K⁺ efflux in wildtype mouse erythrocytes. NEM-stimulated transport was not inhibited (not shown) by Ca²⁺ chelator EGTA (1 mM), H⁺/K⁺-ATPase inhibitor SCH28080 (100 μM), TRPA1 antagonist HC-030031 (10 μM), IP3R/TRP inhibitor 2-aminophenyl-borate (2-APB, 50 μM), and K⁺ channel inhibitor XE-991 (10 and 100 μM). NEM-stimulated Cl⁻-independent K⁺ efflux was inhibited 84% by chloroquine at 2 mM (Fig. 6, p<0.001) and 60% at 500 μM (not shown). Inhibition by 5 mM barium was 57% (p<0.001) and by 1 mM amiloride was 47% (Fig. 6, p<0.05). Additional inhibitory

effects (Fig. 6) falling short of statistical significance (one-way ANOVA or Kruskal-Wallis test) included 1 mM quinacrine (40%), 5 mM TEA (29%), 1 mM quinidine (21%), 1 μ M staurosporine (24%), 50 μ M Gd^{3+} (21%), and 50 μ M ruthenium red (19%). The suggestion of an inhibitory effect of staurosporine on the Cl^{-} -independent component of NEM-activated K^{+} efflux by 25% contrasted to its stimulation of K-Cl cotransport [21]. In a single, triplicate experiment, 100 nM calyculin (adequate to abrogate completely K-Cl cotransport stimulated with 50 μ M NEM) reduced Cl^{-} -independent K^{+} efflux stimulated with 50 or 500 μ M NEM by 40 and 30%, respectively (not shown).

Sensitivity of NEM-stimulated Cl^{-} -independent K^{+} efflux to inhibition by DIDS (0.1–1.0 mM), to clotrimazole (10 μ M), and to the combination of 1 mM EGTA and 10 μ M calmidazolium could not be tested since addition of 500 μ M NEM to mouse erythrocytes in the presence of these inhibitors also led to hemolysis. Clotrimazole and calmidazolium were not hemolytic in the absence of NEM (not shown).

NEM-stimulated, Cl^{-} -independent K^{+} efflux is not further stimulated by low temperature

Bernhardt has described activation in human erythrocytes of a chloroquine-sensitive cation/ H^{+} exchange activity activated by low ionic strength and low temperature [39] [40]. Since NEM-stimulated Cl^{-} -independent K^{+} efflux from mouse erythrocytes was chloroquine-sensitive (Fig. 6) and since Amphiuma erythrocytes exhibit NEM-stimulated K^{+}/H^{+} exchange [41], we tested the dependence of NEM-stimulated K^{+} efflux on extracellular pH. However, the rate of K^{+} efflux was not stimulated when pH was changed from 7.4 to 6.0, but was instead inhibited 26% (Fig. 7A), failing to support a mechanism involving cation/ H^{+} exchange. Moreover, low temperature did not potentiate or sustain NEM-stimulated Cl^{-} -independent K^{+} efflux, but instead completely abolished it (Fig. 7B). The ability of low ionic strength to activate basal or NEM-stimulated K^{+} flux could not be determined, since addition to mouse erythrocytes of 500 μ M NEM in low ionic strength buffer led to hemolysis.

NHA2 is a mammalian cation/ H^{+} exchanger sensitive to 200 μ M phloretin [42]. However, exposure of mouse erythrocytes to phloretin at concentrations of 1 mM or 100 μ M under K^{+} efflux assay conditions led to graded hemolysis, independent of the presence of NEM (data not shown). In contrast, exposure of human erythrocytes to 100 μ M phloretin did not lead to hemolysis. Rarely reported hemolysis promoted by phloretin and other polyphenolics has been attributed to peroxidase-mediated formation of pro-oxidant phenoxyl radicals and generation of additional reactive oxygen species [43].

Discussion

The ability of nucleated cells to maintain a cytosolic composition of high $[K^{+}]$ and low $[Na^{+}]$ while bathed in extracellular fluid of high $[Na^{+}]$ and low $[K^{+}]$ has long focused interest on the identity and structure of transmembrane proteins that regulate membrane cation permeability. This interest has prompted investigation of the molecular components of the “leak” that contribute to the established pump-leak balance of the erythrocyte. Erythroid membrane cation “leak” has been widely defined as cation transport insensitive to both the inhibitor of Na^{+},K^{+} -ATPase, ouabain, and to the inhibitor of Na-K-2Cl cotransport, bumetanide. Inhibition of pathways contributing to this ouabain- and bumetanide-insensitive permeability can control volume and intracellular Hb concentration in circulating erythrocytes, with therapeutic potential in SCD as illustrated by clinical trials of clotrimazole [44] and its more specific congener, senicapoc [13].

In the current work, we identify a novel cation leak pathway in mouse erythrocytes that may contribute to the defense of erythrocyte integrity in pro-hemolytic settings. This leak

pathway may be of physiological importance in mouse models of sickle disease. A potentially similar pathway of NEM-sensitive Cl^- -independent K^+ efflux has been noted in human erythrocytes, although requiring NEM concentrations $> 0.5 \text{ mM}$ [45]. Peroxynitrite treatment of human erythrocytes also stimulated Cl^- -independent K^+ flux, in a manner inhibited 75% by deoxygenation and reduced 40% by 0.2 pH units extracellular acidification [46]. In contrast, NEM inhibited Cl^- -independent K^+ flux in trout erythrocytes [47].

The identity of the K^+ transport pathway activated by NEM remains unclear. The pathway does not require the presence of Gardos channel KCNN4 or K-Cl cotransporters KCC3 and KCC1, but like these pathways is sensitive to the presence of Hemoglobin S or other sickling hemoglobins. Although partially sensitive to chloroquine and amiloride, lack of dependence on temperature or extracellular pH and inability to test for phloretin-sensitivity fail to provide additional support for $\text{K}^+(\text{Na}^+)/\text{H}^+$ exchange as the NEM-stimulated Cl^- -independent K^+ efflux pathway.

The Gardos channel has been reported to be NEM-insensitive [37], but NEM has multiple effects on human erythrocytes (beyond that on K-Cl cotransport) that suggest consideration as possible modifiers or regulators of Cl^- -independent K^+ efflux in mouse erythrocytes. Among the human erythrocyte transport activities inhibited by mM NEM are $^{45}\text{Ca}^{2+}$ uptake induced by plasmalemmal Ca^{2+} -ATPase inhibitor vanadate [48], the nonselective voltage-dependent cation conductance [49], sulfate transport [50], low affinity cationic amino acid transport (system y+L) [51], and equilibrative nucleobase transport [52]. Multiple transport systems may be indirectly modulated by NEM's promotion of the tetramer-to-dimer transition of spectrin, attributed to weakened interaction of ankyrin with AE1/Band 3 [53]; by NEM's activation of phospholipid scramblase, inhibition of aminophospholipid translocase (flippase), and the NEM-associated reduced Ca^{2+} requirement for phospholipid scrambling in both mouse and human erythrocytes [54]; by NEM's inhibition of erythroid pertussis toxin-sensitive G proteins [55; 56; 57]; by NEM's inhibition of serine phosphatases PP1 and PP2A [58]; by NEM's enhanced plasma membrane translocation of calpain-1 [59] and of tyrosine phosphatase SHP1 (independent of Band 3 tyrosine phosphorylation) [60]; and by NEM's inhibition of calpain at multi-mM concentrations [61]. The Cl^- -free conditions required for detection of NEM-stimulated Cl^- -independent K^+ flux may also activate WNK1 kinase by depletion of intracellular Cl^- [62], with consequences to multiple transport systems in addition to the K-Cl cotransporters.

Sulfhydryl group redox state also regulates several pertinent transport activities not yet known to be present in circulating erythrocytes. Among these, NEM activates the isothiocyanate-activated cation channel TRPA1 [63] and the voltage-gated K^+ channels (M channels) Kv7.2, Kv7.4, and Kv7.5 [64] (also activated by H_2O_2 in a dithiothreitol-reversible manner [65]).

In summary, we have characterized an NEM-activated Cl^- -independent K^+ transport pathway previously noted in mouse erythrocytes but not further investigated. This pathway is independent of the Gardos channel KCNN4 and the K-Cl cotransporters KCC3 and KCC1, and exhibits lower NEM-sensitivity than that of mouse erythroid K-Cl cotransport. The presence of HbSAD increases both the NEM-sensitivity and the magnitude of stimulation of the Cl^- -independent K^+ efflux pathway. The pathway is inhibited by mM concentrations of chloroquine and amiloride, but does not share several other properties of $\text{K}^+(\text{Na}^+)/\text{H}^+$ exchange as described in human erythrocytes. This K^+ efflux pathway is also partially inhibited by Ba^{2+} . Additional studies will be needed to establish the functional and molecular identities of the protein(s) that mediate NEM-stimulated Cl^- -independent K^+ efflux of the mouse erythrocyte, and to learn whether the reported corresponding activity of human erythrocytes might be orthologous. Erythrocytes of the *Kcnn4*^{-/-}; *Kcc1*^{-/-}; *Kcc3*^{-/-}

triple knockout mouse will be useful in maximally isolating NEM-stimulated Cl^- -independent K^+ efflux for further study, and in characterizing its regulation by HbSAD or other sickling hemoglobins in oxygenated and deoxygenated states.

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Abbreviations

NEM	N-ethylmaleimide
KCC3	Slc12a6 K-Cl cotransporter
KCC1	Slc12a4 K-Cl cotransporter
IK1	KCNN4 intermediate conductance K^+ channel, or Gardos channel
DIDS	4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid
EGTA	ethylene glycol tetra-acetic acid
DIOA	[(2-n-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid
CDNB	1-chloro-2,4-dinitrobenzene
PMS	phenazine methosulfate

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Figure 1. The Cl⁻-independent component of NEM-stimulated erythroid K⁺ efflux does not require the presence of K-Cl cotransporters.

A. K⁺ efflux from *Kcc1^{+/+}Kcc3^{+/+}* mouse red cells exposed to the indicated [NEM] in the presence (gray bars) or absence of Cl⁻ (black bars, sulfamate replacement). **B.** K⁺ efflux from *Kcc1^{-/-}Kcc3^{-/-}* double knockout mouse red cells exposed to the indicated concentrations of NEM in the presence (gray bars) or absence of Cl⁻ (black bars). Values in panels A and B are means ± s.e.m. from (n) determinations in triplicate. **C.** [NEM] response curves of K-Cl cotransport (Cl⁻-dependent K⁺ efflux) in wildtype red cells (gray circles) and in *Kcc1^{-/-}Kcc3^{-/-}* red cells (black squares).

*, p<0.005; **, p < 10⁻⁶ vs. filled squares at same [NEM]. **D.** [NEM] response curves of Cl⁻-independent K⁺ efflux from wildtype red cells (gray circles) and from *Kcc1^{-/-}Kcc3^{-/-}* red cells (black squares). Values in panels C and D are derived from panels A and B.

Figure 2. The Cl⁻-independent component of NEM-stimulated erythroid K⁺ efflux does not require Gardos channel KCNN4

A. K⁺ efflux from *Kcnn4*^{+/+} (WT) mouse red cells exposed to the indicated [NEM] in the presence (gray bars) or absence of chloride (black bars, sulfamate replacement). **B.** K⁺ efflux from *Kcnn4*^{-/-} mouse red cells exposed to the indicated [NEM] in the presence (gray bars) or absence of chloride (black bars). Values in panels A and B are means ± s.e.m. from (n) determinations in triplicate. **C.** [NEM] response curves of K-Cl cotransport in *Kcnn4*^{+/+} (WT) red cells (gray circles) and in *Kcnn4*^{-/-} red cells (black squares). *, p < 10⁻⁴ vs. WT at same [NEM]. **D.** [NEM] response curves of Cl⁻-independent K⁺ efflux from *Kcnn4*^{+/+} (WT) red cells (gray circles) and *Kcnn4*^{-/-} red cells (black squares). *, p < 0.05 vs WT at same [NEM]. Values in panels C and D are taken from panels A and B.

Figure 3. NEM-stimulated Cl⁻-independent K⁺ efflux is enhanced in red cells of SAD mice

A. K⁺ efflux from WT mouse red cells exposed to the indicated [NEM] in the presence (gray bars) or absence of chloride (black bars, sulfamate replacement). **B.** K⁺ efflux from SAD mouse red cells exposed to the indicated [NEM] in the presence (gray bars) or absence of chloride (black bars). Values in panels A and B are means ± s.e.m. from (n) determinations in triplicate. **C.** [NEM] response curves of K-Cl cotransport in WT red cells (gray circles) and SAD red cells (black squares). **, p<10⁻⁶ vs. SAD cells at same [NEM]. **D.** [NEM] response curves of NEM-stimulated Cl⁻-independent K⁺ efflux from WT red cells (gray circles) and SAD red cells (black squares). *, p<0.05; **, p<10⁻⁶ vs. WT red cells at same [NEM]. Values in panels C and D are taken from panels A and B.

Figure 4. Expression of HbSAD increases stimulation of K⁺ efflux by submaximal [NEM] in *Kcnn4*^{-/-} red cells

A. K⁺ efflux from HbSAD transgenic mouse red cells exposed to the indicated [NEM] in the presence (gray bars) or absence of chloride (black bars, sulfamate replacement). **B.** K⁺ efflux from HbSAD transgenic *Kcnn4*^{-/-} mouse red cells exposed to the indicated [NEM] in the presence (gray bars) or absence of chloride (black bars). Values in panels A and B are means ± s.e.m. for (n) determinations in triplicate. **C.** [NEM] response curves of K-Cl cotransport in red cells from HbSAD transgenic mice (gray circles) and from HbSAD transgenic *Kcnn4*^{-/-} red cells (black squares). **D.** [NEM] response curves of Cl⁻-independent K⁺ efflux from red cells of HbSAD transgenic mice (gray circles) and from red cells of HbSAD transgenic *Kcnn4*^{-/-} mice (black squares). *, p<0.05; ** p<10⁻⁶ vs. SAD;*Kcnn4*^{-/-} red cells at same [NEM]. Values in panels C and D are taken from panels A and B.

Figure 5. Expression of HbSAD enhances NEM-sensitivity of Cl⁻-independent K⁺ flux in red cells from *Kcnn4*^{-/-};*Kcc1*^{-/-};*Kcc3*^{-/-} triple knockout mice

A. K⁺ efflux from red cells of *Kcnn4*^{-/-};*Kcc1*^{-/-};*Kcc3*^{-/-} mice exposed to the indicated [NEM] in the presence (gray bars) or absence of chloride (black bars, sulfamate replacement).

B. K⁺ efflux from red cells of *Kcnn4*^{-/-};*Kcc1*^{-/-};*Kcc3*^{-/-} HbSAD transgenic mice exposed to the indicated [NEM] in the presence (gray bars) or absence of chloride (black bars).

Values in panels A and B are means ± s.e.m. for (n) determinations in triplicate. **C.** [NEM] response curves of K-Cl cotransport in red cells from *Kcnn4*^{-/-};*Kcc1*^{-/-};*Kcc3*^{-/-} mice in the absence (gray circles) and presence of HbSAD (black squares). **D.** [NEM] concentration response curves of Cl⁻-independent K⁺ efflux from red cells of *Kcnn4*^{-/-};*Kcc1*^{-/-};*Kcc3*^{-/-} mice in the absence (gray circles) and presence of HbSAD (black squares).

*, p<0.05 vs value in cells lacking HbSAD at same [NEM]. Values in panels C and D are taken from panels A and B.

Figure 6. Pharmacological inhibition profile of NEM-stimulated Cl⁻-independent K⁺ efflux in mouse red cells

Cl⁻-independent K⁺ efflux stimulated by 500 μM NEM in the absence of inhibitors is normalized to 100%. Values represent % residual efflux in the presence of the indicated inhibitors at the listed concentrations (means ± s.e.m. from (n) trials, each in triplicate). **, p<0.001; *, p<0.01 vs. no drug (Kruskal-Wallis test with Dunnett's post-test).

Figure 7. Sensitivity to pH and to temperature of NEM-stimulated Cl⁻-independent K⁺ efflux from mouse red cells

A. Cl⁻-independent K⁺ efflux in the absence and presence of NEM (500 μM) at the indicated extracellular pH values (pH_o), with values normalized to those at pH 7.4. *, p<0.01 vs. pH 7.4. **B.** Cl⁻-independent K⁺ efflux in the absence and presence of NEM (500 μM) at assay temperatures of 37°C and 0°C. **, p<0.001 vs 0 NEM; *, p<0.001 vs 500 μM NEM at 37°C. (ANOVA with Tukey's post-test).