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Stimulation of Human Erythrocyte K-Cl Cotransport and Protein Phosphatase Type 2A by n-Ethylmaleimide: Role of Intracellular Mg^{++}

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Abstract. An increase in the activity of membrane-associated protein phosphatase type 1 (mb-PP1) is associated with stimulation of erythrocyte K-Cl cotransport (KCC). We have recently proposed that membrane-associated protein phosphatase type 2A (mb-PP2A) is also involved in KCC regulation by cell swelling (Bize et al., 1999. *Am. J. Physiol.* **277:**C899–C912). We used two protein phosphatase inhibitors, okadaic acid (OA) and calyculin A (CalA), and two KCC activating treatments, n-ethylmaleimide (NEM) and Mg_i^{++}-depletion, and determined KCC transport activity and mb-PP1 and mb-PP2A activities. OA, an inhibitor of erythrocyte mb-PP2A, partially prevents stimulation of KCC activity by NEM but not by Mg_i^{++}-depletion. CalA, an inhibitor of both mb-PP1 and mb-PP2A prevents stimulation of KCC activity by both treatments. NEM and Mg_i^{++}-depletion inhibit mb-PP1 activity, suggesting that activation of KCC can take place in the absence of mb-PP1 activation. Mb-PP2A activity is stimulated in NEM-treated cells but not in Mg_i^{++}-depleted cells. In NEM-treated cells, Mg_i^{++}-depletion inhibits both KCC and mb-PP2A. In Mg_i^{++}-depleted cells, NEM does not stimulate KCC or mb-PP2A. The strong correlation between KCC stimulation and mb-PP2A stimulation provides further support to the idea that mb-PP2A plays an important role in KCC regulation. Our results are consistent with the hypothesis that KCC regulation involves at least two distinguishable phosphorylation sites.

Key words: Okadaic acid — Calyculin A — Magnesium — N-ethylmaleimide — Red blood cells — Protein phosphatase 1 (PP1) — Protein phosphatase 2A (PP2A)

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Introduction

Regulation of cell volume after swelling is accomplished by mechanisms that decrease the amount of cell solutes. In erythrocytes, swelling activates K efflux through K-Cl cotransport (KCC). In addition to cell swelling, KCC can be activated by N-ethylmaleimide treatment (NEM) [40], intracellular acidification [9], protein kinase inhibitors [5, 16, 2], oxidations [51, 6, 1], urea [49, 19, 35], high hydrostatic pressure [24, 26] and increases in temperature [56]. Activation of KCC in isotonic conditions leads to cell dehydration [21, 23].

KCC1 appears to be the prevalent isoform of KCC in erythrocytes [50, 54]. Activation of KCC by several stimuli (including swelling) takes place via a net dephosphorylation of either KCC1 and/or an associated protein [for reviews *see* 33, 48, 28]. KCC1 has consensus sites for phosphorylation by the ser/thr protein kinases PKC and CK2 [25]. There is substantial evidence for a role of membrane-associated protein phosphatase type 1 (mb-PP1) in KCC regulation [52, 34, 7]. Recent findings suggest that KCC activity is also regulated by membrane-associated PP2A (mb-PP2A): when human red cells are swollen in hypotonic media, activation of KCC is associated with an increase in the activity of mb-PP1 and mb-PP2A. Furthermore, stimulation of either mb-PP1 or mb-PP2A alone is associated with KCC activation [8].

In mature human erythrocytes at normal volume and physiological pH, KCC activity is very low [27, 14] but it can be stimulated 10–20-fold by NEM treatment [40, 44, 41, 14, 4, 11, 31, 34]. The effect of NEM is mediated through dephosphorylation of the transporter (or a regulator) [31, 34, 16, 6, 2, 22, 54]. Recently, Jennings has provided indirect evidence for inhibition by NEM of a volume-sensitive kinase involved in KCC regulation in rabbit red cells [32].

Depletion of intracellular Mg^{++} (with A23187 and EDTA) also stimulates KCC. This has been observed in

sheep red cells [40, 3, 18, 43], human sickle red cells [10, 13], in both mature and immature normal human red cells [20, 14, 10] in rabbit red cells [32], and mouse red cells [2]. The effect of Mg^{++}-depletion, like that of NEM, is also mediated through phosphorylation reactions as indicated by its inhibition by OA and CalA [46, 31, 34, 42, 52, 6, 22]. It has been proposed that Mg^{++}-depletion, like NEM, inhibits a volume-sensitive kinase that phosphorylates and inactivates the transporter [30, 46, 52, 47, 26, 22, 32]. In rabbit red cells, the effects of Mg^{++}-depletion and NEM are mediated through inhibition of a single volume-sensitive reaction [32]. In human and sheep red blood cells, however, maximal KCC stimulation by Mg^{++}-depletion is smaller than maximal KCC stimulation by NEM, suggesting that the targets of these two activators do not always coincide [41, 13, 14]. No data have been published on the effects of Mg^{++}-depletion on mb-PP1 or mb-PP2A.

In LK sheep red blood cells, swelling induces changes in transporter V_{max} and the affinity of the transporter for K^+ [18] through two separable processes [3, 37], consistent with the presence of 2 distinct signals for activation of KCC by cell swelling [19]. KCC stimulation by cell swelling is associated with an increase in mb-PP1 and mb-PP2A activity, indicating that a volume-sensitive kinase may not be the only mediator of stimulation of KCC by increases in volume [8]. Furthermore, transport measurements suggest the presence of at least two functionally distinct ser/thr phosphorylation sites in the transporter (or a regulator) [6].

In this work, we determined KCC activity and mb-PP1 and mb-PP2A activities in cells of normal volume (and Mg_i^{++} content), and in cells with KCC activated by NEM or Mg^{++}-depletion, in an attempt to understand the role of membrane-associated phosphatases in KCC regulation. We used okadaic acid (OA) for specific inhibition of mb-PP2A, and calyculin A (CalA) to inhibit both mb-PP1 and mb-PP2A [8].

We found that stimulation of KCC by NEM and by Mg^{++}-depletion differ in the degree of OA-sensitivity: OA inhibited more than 50% of KCC stimulation by NEM, but inhibited stimulation of KCC induced by Mg^{++}-depletion only slightly. Treatment with NEM indirectly stimulated mb-PP2A activity, in agreement with the OA sensitivity of NEM effect. On the other hand, the slight inhibition by OA of the Mg_i^{++}-depletion effect suggests that the Mg_i^{++}-depletion effect is mostly independent of mb-PP2A activity. Mg_i^{++}-depletion had no effect on mb-PP2A activity (at least in cells not treated with NEM). We conclude that activation of KCC by NEM and by Mg_i^{++}-depletion can occur by different signal transduction pathways of different OA sensitivity.

Stimulation of KCC and of mb-PP2A by NEM requires Mg_i^{++}. Mg_i^{++}-depletion inactivates KCC and mb-PP2A in cells previously activated by NEM. The correlation between KCC activity and mb-PP2A activity is further evidence that mb-PP2A is involved in KCC regulation.

Materials and Methods

Cells

Blood was collected into heparinized Vacutainer tubes from six healthy adult volunteers after obtaining informed consent. Blood was passed through cotton to remove leukocytes and washed four times by centrifugation in a solution containing 152 mM choline chloride, 1 mM $MgCl_2$, 10 mM Tris-MOPS pH 7.4 at 4°C ('choline washing solution'). For treatment of intact red cells, cells were incubated with the desired agents for 20 min at 37°C at 10% hematocrit, in media containing 145 mM NaCl, 5 mM KCl, 5 mM glucose, and 10 mM Tris-MOPS, pH 7.4 at 37°C. For NEM treatments we have chosen to treat with 0.5 mM NEM at 37°C because preliminary experiments showed that this treatment elicits almost maximal activation of KCC, and maximal inhibition of the stimulation by OA (200 nM) and CalA (50 nM). The cells were washed free of drugs in 'choline washing solution' before K-Cl cotransport activity determination and membrane preparation. In most experiments 2 sequential treatments were performed. Cells were washed once between treatments.

Magnesium Depletion and Loading

Washed control or drug-treated cells were incubated at 10% hematocrit with 6 μM ionophore (A23187) and either 1 mM EDTA or 500 μM $MgCl_2$, in a solution containing (in mM): 145 NaCl, 5 KCl, 5 glucose and 10 Tris-MOPS pH 7.4 at 37°C, for 20 min. To insure maximal effect of Mg^{++}, we used 500 μM $MgCl_2$ to load the cells, (intracellular free Mg^{++} of intact cells is about 300 μM [53]). Following incubation, the ionophore was removed by two consecutive washes in the respective loading solutions (either EDTA or $MgCl_2$) containing 1% BSA and no ionophore, and two incubations in the same media for 15 min at 37°C (50% hematocrit). After the ionophore was removed the cells were washed in 'choline washing solution'.

K-Cl Cotransport Activity

KCC activity was determined using K^+ efflux into K^+-free media or $^{86}Rb^+$ influx from a media containing 2 mM KCl. K^+ efflux was determined in washed red cells by measuring the external K concentration after 5 and 35 min of incubation at 37°C, 2–3% hematocrit, in media containing (in mM): 140 NaCl, 10 glucose, 1 $MgCl_2$, 10 Tris-MOPS pH 7.4 at 37°C, 0.1 ouabain and 10 μM bumetanide (290–300 mosM). Cl^--free media contained 140 mM sodium sulfamate (NaSfa) and 1 mM $Mg(NO_3)_2$, instead of the respective chloride salts. When efflux was determined in Mg^{++}-loaded or Mg^{++}-depleted cells, the flux media contained 50 μM EGTA and no Mg^{++}. Efflux was calculated from the slope of the concentration of external K versus time, and the hematocrit of the cell suspension. Fluxes are expressed as millimoles per hour per original liter of cells (i.e., volume of untreated cells in isosmotic conditions). The difference between K^+ efflux into chloride-containing (Cl^-) and chloride-free (Sfa^-) media was taken as the KCC activity. $^{86}Rb^+$ influx was determined as previously described [6], in the same media as above except the media also contained 2 mM K (Cl^- or Sfa^-) and 10–20 μCi ^{86}Rb/ml.

Preparation and Treatment of Membranes

(i) *Hypotonic Lysis:* Red cells were lysed in 15 volumes of 'hypotonic lysis buffer' containing: 10 mM Tris-MOPS pH 7.4, 0.1% β-mercaptoethanol, 1 μM phenylmethyl sulfonyl fluoride and 25 μg/ml each of leupeptin and aprotinin. (ii) *Isotonic Lysis:* Cells were lysed in isotonic solutions by freezing/thawing or by sonication. Cell lysis media contained 'hypotonic lysis buffer' and NaCl and KCl such that NaCl + KCl equaled 150 mM. The red cell suspensions (10–15% hematocrit) were lysed by 1–3 cycles of freezing (dry ice with ethanol, 5 min) and thawing (30°C, 8 min). Alternatively, cells were lysed by sonication with a probe inserted into the tube with the cell suspension. Cell lysates obtained by any of the above procedures were centrifuged (30,000 × *g* for 20 min) and the membrane pellets were washed three times by centrifugation and resuspension in 'hypotonic lysis buffer' (no NaCl or KCl). The membranes were stored frozen (−70°C) in lysis buffer at a concentration of 2–5 mg protein/ml, until use. Protein concentrations were determined using the Lowry assay [45] with bovine serum albumin as standard.

Phosphatase Activity

Phosphatase activity was determined using ^{32}P-labeled glycogen phosphorylase-a as substrate in dilute cell-free extracts, as previously described [7]. Unless indicated the phosphatase assay buffer contained 100 μM EDTA and no added cations. Phosphatase activity is expressed as units/mg protein, where one unit (U) equals 1 nmole of phosphate released per minute. Our PPase measurements were performed with phosphorylase-a, a substrate for both PP1 and PP2A. This substrate however, does not distinguish between different isoforms of PP1.

PP1 and PP2A Activity

The activity of PP1 was determined by assaying phosphatase activity in the presence of 2 nM okadaic acid. The activity of PP2A was determined as the portion of total phosphatase sensitive to 2 nM okadaic acid. This concentration of OA in the phosphatase assay is adequate to distinguish between PP1 and PP2A [29].

Statistics

Data were analyzed using Student's *t* test for paired or unpaired samples depending on the experimental protocol. All error bars are standard error of the mean of several experiments (SEM).

Materials

Media components and drugs were obtained from Sigma Chemical (St. Louis, MO). Enzymes for the phosphatase assay were obtained from Gibco BRL (Gaithersburg, MD) or from RBI (Natick, MA). ^{32}P-γ-ATP was obtained from Dupont, New England Nuclear (Boston, MA). Calyculin A and okadaic acid from RBI (Natick, MA). Acationox from Baxter (MgGraw Park, IL), and A23187 from Calbiochem (San Diego, CA).

Results

Effect of OA and CalA on the Stimulation of KCC by NEM and Mg_i^{++}-Depletion

Figure 1*a* shows Cl^--dependent K efflux in cells treated with NEM (after OA), and Fig. 1*b* shows efflux in cells depleted of Mg_i^{++} after OA treatment. Cells were incubated with OA (200 nM, 20 min, 37°C, 10% hematocrit). After removal of OA, cells were incubated ±0.5 mM NEM (20 min, 37°C, 10% hematocrit, Fig. 1*a*), or were loaded with Mg_i^{++} or depleted of Mg_i^{++} (Fig. 1*b*). Figure 1*a* shows that NEM stimulates steady state Cl^--dependent K^+ efflux in control cells (>10-fold). However, the stimulation by NEM is greatly reduced ($P = 0.02$, $n = 4$) in OA-treated cells. A significant OA-sensitive component in NEM stimulation of KCC activity had been shown previously [31, 34]. Similar results were obtained when the order of treatments is reversed (Table 1). The results suggest that full NEM stimulation of KCC may require PP2A activity, which is highly sensitive to OA.

Figure 1*b* shows the effect of lowering intracellular Mg^{++} on KCC activity. There is a small but statistically significant effect of OA pretreatment on the stimulation

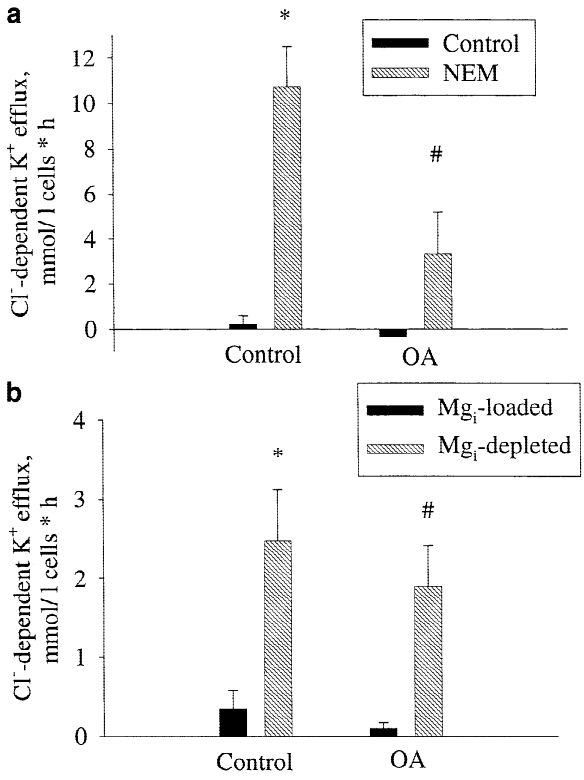

Fig. 1. (*a*) NEM stimulation of KCC is reduced in OA treated cells. Cells were pretreated with OA, washed and then treated ±0.5 mM NEM. Results show Cl^--dependent K^+ efflux (mean ± SEM) of 4 experiments in 4 subjects. Efflux was significantly stimulated by NEM in control cells (*, $P < 0.001$), and in OA treated cells ($^{\#}$, $P = 0.04$). (*b*) EDTA stimulation of KCC is only slightly inhibited in OA treated cells. K-Cl cotransport activity was determined in control cells and in cells pretreated with OA (200 nM). Each group of cells was then loaded or depleted of Mg_i^{++} (500 μM $MgCl_2$ (Mg_i^{++}-loaded) or 1 mM EDTA (Mg_i^{++}-depleted). Results are means ± SEM of 3 experiments in 3 subjects. *, $P = 0.02$, different from control-Mg_i^{++}-loaded cells; $^{\#}$, $P = 0.03$, different from OA-Mg_i^{++}-loaded cells.

Table 1. Effects of OA and CalA on KCC stimulation by NEM and by Mg_i^{++}-depletion (EDTA)

	KCC activity: Cl^--dep K^+ efflux, (mmol/l cells ∗ hr)					
1st treatment	Control A*	NEM	NEM	OA	NEM	CalA
2nd treatment			OA	NEM	CalA	NEM
	0.26 ± 0.14 (9)	9.5 ± 1.1 (7)[1]	4.4 ± 1.2 (3)[2]	3.7 ± 1.5 (4)[3]	3.4 ± 1.1 (3)[4]	0.6 ± 0.4 (4)[5]
1st treatment	Control B*	Mg_i^{++}-dep	Mg_i^{++}-dep	OA	Mg_i^{++}-dep	CalA
2nd treatment			OA	Mg_i^{++}-dep	CalA	Mg_i^{++}-dep
	0.49 ± 0.1 (6)	2.4 ± 0.3 (6)[6]	1.9 ± 0.3 (4)[7]	1.9 ± 0.5 (3)[8]	1.3 ± 0.4 (4)[9]	−0.1 ± 0.1 (3)[10]

Cells were incubated with either 0.5 mM NEM, or with A23187 and 500 μM $MgCl_2$ or 1 mM EDTA. In cells with double treatments, cells were washed once between treatments in control incubation media with 1 mM $MgCl_2$ (except in EDTA experiments). Control A*, no A23187. Control B*, A23187 plus 500 μM $MgCl_2$. [1], different from Control *A*, $P < 0.001$. [2–5], different from NEM: [2], $P = 0.009$; [3], $P = 0.04$; [4], $P = 0.003$; [5], $P = 0.02$. [6], different from Control B, $P = 0.007$. [7–10], different from Mg_i^{++}-depleted [7], $P = 0.05$; [8], $P = 0.04$; [9], $P = 0.007$; and [10], $P = 0.03$. In addition, [9] is different from Mg_i^{++}-depleted + OA, $P = 0.002$.

of KCC by Mg_i^{++}-depletion (<20% inhibition, 2.4 ± 0.2 mmol/l cells ∗ hr, in control *vs.* 1.9 ± 0.3 mmol/l cells ∗ hr in OA, $P = 0.03$). Since there is almost full stimulation by Mg_i^{++}-depletion in OA-treated cells, the results suggest that mb-PP2A (the OA-sensitive PPase) is not playing a major role in KCC activation by Mg_i^{++}-depletion (or that the Mg_i^{++}-inhibited reaction is mostly independent of mb-PP2A).

Table 1 shows the results of the above experiments, of analogous experiments with CalA (50 nM), and of experiments where the treatment with PPase inhibitors (OA and CalA) was carried out after the treatment with NEM or Mg_i^{++}-depletion. The table shows results of experiments with treatments with the KCC activators in the absence or presence of PPase inhibitors. In experiments with double treatments, PPase inhibitor exposure either preceded or followed treatments with the activators.

Table 1 shows that control KCC activity (untreated cells in isosmotic conditions) is not different in intact cells and in Mg_i^{++}-loaded (cells treated with A23187 and 500 μM $MgCl_2$, $P = 0.1$). OA and CalA inhibit Cl^--dependent K^+ transport in both types of cells (*not shown*). NEM stimulation is >10-fold, while that by Mg_i^{++}-depletion is ~5-fold, similar to previous data [41, 13, 11]. Treatment with OA or CalA inhibits the stimulation by NEM, when the inhibitors are added either before or after NEM treatment. Pretreatment with CalA prevents stimulation of KCC by NEM and by Mg_i^{++}-depletion. Stimulation of KCC by Mg_i^{++}-depletion is only slightly inhibited by subsequent treatment with OA. Stimulation of KCC by NEM on the other hand has a large OA-sensitive component. The results suggest that stimulation by NEM, but not stimulation by Mg_i^{++}-depletion, is partly mediated by a dephosphorylation event at a mb-PP2A regulated site. The results also suggest that the effect of Mg_i^{++}-depletion is restricted to a site whose phosphorylation state is mostly independent of mb-PP2A activity. The differential effect of OA on NEM stimulation and Mg_i^{++}-depletion stimulation of KCC activity, suggests the presence of at least two phosphorylation sites involved in KCC regulation, and indicates that the sites can be distinguished by their sensitivity to OA.

CalA does not abolish stimulation of KCC in cells pretreated with NEM or Mg_i^{++}-depletion as previously observed [31, 52, 16, 6, 22]. This result suggests that full stimulation of KCC by NEM and by Mg_i^{++}-depletion also involves inhibition of kinases, consistent with results in rabbit red cells [32].

To investigate whether NEM but not Mg_i^{++}-depletion may stimulate mb-PP2A activity, we determined the activity of both phosphatases (PP1 and PP2A) in the membranes and cytosol of control cells and of cells pretreated with NEM or Mg_i^{++}-depleted. We also determined the direct effects of NEM and EDTA on mb-PP1 and mb-PP2A activity.

Phosphatase Activity: Effects of NEM and Mg^{++} in Membrane Preparations

Mb-PP1 and mb-PP2A activities from control cells were determined in the presence of increasing concentrations of NEM (0 to 0.5 mM) in the PPase assay. NEM was added to the membranes, rather than to intact cells. There was no significant direct effect of NEM on either mb-PP1 or mb-PP2A activity. Mb-PP1 activity was 0.32 ± 0.02 (U/mg protein) in control, and 0.35 ± 0.03 (U/mg protein), in the presence of 0.5 mM NEM. Mb-PP2A was 0.1 ± 0.01 (U/mg protein) in control, and 0.08 ± 0.01 (U/mg protein) in NEM. Mb-PP1 and mb-PP2A activities were also determined in the presence of increasing Mg^{++} concentrations. Mg^{++} inhibits mb-PP1 ($IC_{50} \simeq 2$ mM). At 300 μM calculated Mg_i^{++} (close to normal Mg_i^{++}) mb-PP1 activity is inhibited 18 ± 3% ($P = 0.001$, $n = 3$).

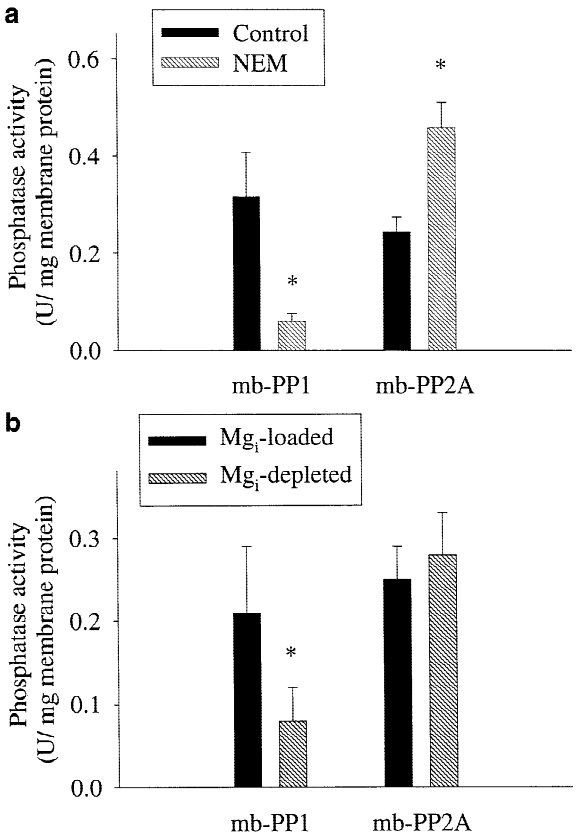

Fig. 2. (*a*) NEM treatment inhibits mb-PP1 and stimulates mb-PP2A activity. PP1 and PP2A activities were determined in the membrane of cells treated ±0.5 mM NEM. Results show mean ± SEM of mb-PP1 and mb-PP2A activity. *, $P < 0.001$, $n = 15$. (*b*) Mg_i^{++} depletion inhibits mb-PP1 activity and does not affect mb-PP2A activity. Cells were loaded with Mg^{++} (500 μM Mg_i^{++}) or depleted of Mg^{++} (1 mM EDTA) as detailed in Materials and Methods. Membrane fractions were isolated from each group of cells and mb-PP1 and mb-PP2A activity were determined. Mb-PP1 activity is decreased in Mg_i^{++} depleted cells (*, $P < 0.001$). Mb-PP2A activity does not change. Results show the mean ± SEM of 18 experiments.

Phosphatase Activity: Effects of NEM Treatment and Mg_i^{++}-Depletion of Intact Cells

NEM-Treatment of Erythrocytes Decreases the Activity of mb-PP1 and Increases the Activity of mb-PP2A

Figure 2*a* shows results of the effects of NEM pretreatment on mb-PP1 and mb-PP2A activity. Cells were pretreated ±0.5 mM NEM, washed and membranes were isolated. In untreated cells, mb-PP1 and mb-PP2A have similar activities. However, in NEM-treated cells, mb-PP1 activity is inhibited and mb-PP2A is stimulated.

Mg_i^{++}-Depletion Inhibits mb-PP1 Activity, and Has No Effect on mb-PP2A Activity

Figure 2*b* shows results of the effects of Mg_i^{++}-depletion on mb-PP1 and mb-PP2A activities. Cells were loaded with Mg_i^{++} or depleted of Mg_i^{++}, lysed in isotonic media and membranes were isolated. Mb-PP1 and mb-PP2A activities were assayed in the absence of Mg^{++} (100 μM EDTA). Mb-PP1 and mb-PP2A activities from Mg_i^{++}-loaded cells were similar to PPase activities from cells with unmodified Mg_i^{++} (no A23187 treatment, compare with Fig. 2*a*). In Mg_i^{++}-depleted cells there is a decrease in mb-PP1 ($P < 0.001$, $n = 18$), while there is no significant change in the activity of mb-PP2A.

To investigate whether the changes in mb-PP1 activity and mb-PP2A activity described above are due to redistribution of the phosphatases, we determined the effects of NEM and A23187+EDTA on cytosolic PP1 and PP2A. Table 2 shows a summary of the effects of pretreatment with NEM and A23187+EDTA on the activity of soluble PP1 (cyto-PP1), and soluble PP2A (cyto-PP2A). In NEM-treated cells, cyto-PP1 is significantly decreased, while cyto-PP2A is unaffected. Since mb-PP1 is also inhibited by NEM (Fig. 2*a*), the results indicate that NEM is an indirect inhibitor of the catalytic subunit of PP1, regardless of its subcellular location. NEM treatment does not change cyto-PP2A activity, therefore we conclude that the increase in mb-PP2A elicited by NEM is not due to cellular redistribution of PP2A.

Mg_i^{++}-depletion decreases both cyto-PP1 activity (34 ± 15%) and cyto-PP2A activity (20 ± 4%). Therefore the inhibitory effect of Mg_i^{++}-depletion on mb-PP1 (Fig. 2*b*), like that of NEM-treatment (Fig. 2*a*), is not due to release of PP1 into the cytosol. Since Mg_i^{++}-depletion induces a small but significant decrease in cyto-PP2A activity, and no change on mb-PP2A activity, we conclude (similarly to NEM-treatment) that Mg_i^{++}-depletion-treatment does not affect the cellular distribution of mb-PP2A. In summary, neither inhibition of mb-PP1 by NEM or by Mg_i^{++}-depletion, nor stimulation of mb-PP2A by NEM, appear to be explained by changes in the association of these phosphatases with the red blood cell membrane.

Mg_i^{++}-Dependence of NEM Effects

Mg_i^{++}-Depletion Inhibits KCC Activity in NEM-Treated Cells

Cells were treated ±0.5 mM NEM and then treated with A23187 + 500 μM Mg^{++} or A23187 + 1 mM EDTA. Figure 3*a* shows that Mg_i^{++}-depletion stimulates KCC in control cells ($P = 0.007$, $n = 3$), but it inhibits KCC in NEM-treated cells ($P = 0.007$, $n = 3$). A failure to observe activation by Mg_i^{++}-depletion of Cl^--dependent K^+ fluxes after NEM treatment has been reported [41]. This may be due to a requirement for Mg_i^{++} (or Mg-ATP) on NEM-activated mb-PP2A, among other possibilities.

Table 2. Summary effects of NEM-treatment and Mg_i^{++}-depletion-treatment on soluble PP1 and PP2A

	Phosphatase activity, U/mg protein			
	Control A*	NEM	Control B*	Mg_i^{++}-dep
Cyto-PP1 (U/mg protein)	0.023 ± 0.003 (9)	0.011 × 0.002 (9)*	0.029 ± 0.003 (12)	0.015 ± 0.003 (12)**
Cyto-PP2A (U/mg protein)	0.273 ± 0.05 (9)	0.245 ± 0.03 (9)	0.259 × 0.044 (12)	0.200 × 0.03 (12)***

Values are averages ± SEM. (* and ***, $P = 0.01$; **, $P = 0.001$, different from respective control). Number of experiments in parenthesis. Control A*, no A23187. Control B*, A23187 plus 500 μM $MgCl_2$.

Mg_i^{++}-Depletion Inhibits mb-PP2A Activity in NEM-Treated Cells

Cells were treated as for Fig. 3*a* and membranes were isolated from cells lysed in isotonic media after A23187 removal. Mb-PP1 and mb-PP2A activities were determined. Figure 3*b* shows the results on mb-PP2A (results on mb-PP1 are shown later in Table 3). In cells treated with NEM, mb-PP2A activity is elevated if the cells are not depleted of Mg_i^{++} (*see also* Fig. 2*a*). Figure 3*b* shows that there is no effect of Mg_i^{++}-depletion on mb-PP2A activity in control cells (*see also* Fig. 2*b*) but in NEM-treated cells Mg_i^{++}-depletion reduces the activity of this enzyme to levels similar (or below), to those found in control-Mg_i^{++}-depleted cells, demonstrating that Mg_i^{++}-depletion abolishes the stimulation of mb-PP2A by NEM. This result is consistent with mb-PP2A being required for full NEM stimulation of KCC activity.

NEM Does Not Stimulate KCC Activity in Mg_i^{++}-Depleted Cells

Figure 4*a* shows KCC activity in Mg_i^{++}-depleted cells subsequently treated with NEM. KCC activity was determined using Cl^--dependent K^+ efflux. Similar results were obtained with $^{86}Rb^+$ influx (*see* Table 3). Figure 4*a* shows that there is a large stimulation of KCC by NEM in cells loaded with Mg_i^{++} ($P = 0.003$, $n = 3$) similar to (or larger than) that of cells with unmodified Mg_i^{++} (compare to Fig. 1*a*). However, all stimulation by NEM is prevented by Mg_i^{++}-depletion.

NEM Does Not Stimulate mb-PP2A in Mg_i^{++}-Depleted Cells

Mb-PP1 and mb-PP2A activities, assayed in cells treated as above, were determined in the absence of Mg^{++} (100 μM EDTA). Figure 4*b* shows that while NEM stimulates mb-PP2A in control (Mg^{++}-loaded) cells, it does not stimulate mb-PP2A in Mg_i^{++}-depleted cells. This result may partially explain the lack of effect of NEM on KCC activity in Mg_i^{++}-depleted cells (Fig. 4*a*).

Table 3 shows a summary of the changes in KCC activity, mb-PP1 and mb-PP2A activities in NEM-treated and Mg_i^{++}-depleted cells. NEM stimulates KCC, as determined by Cl^--dependent $^{86}Rb^+$ influx from a 2 mM K^+-containing media. NEM treatment also stimulates mb-PP2A activity and inhibits mb-PP1 activity. Mg_i^{++}-depletion stimulates KCC activity (to a lesser extent than NEM-treatment), has no effect on mb-PP2A activity, and inhibits mb-PP1 activity. In NEM-pretreated cells, Mg_i^{++}-depletion completely blocks the stimulation of KCC and mb-PP2A activities by NEM. In these cells, Mg_i^{++}-depletion further inhibits mb-PP1 activity. In Mg_i^{++}-depleted cells, NEM fails to activate further KCC activity and mb-PP2A activity, but it does reduce the activity of mb-PP1.

In summary, our results show that in addition to the inhibitory effects on a volume-sensitive kinase involved in KCC regulation [32], NEM and Mg_i^{++}-depletion may regulate KCC activity in human red cells by additional pathways. Activation of KCC by NEM is associated with inhibition of mb-PP1 and stimulation of mb-PP2A, whereas activation of KCC by Mg_i^{++}-depletion is accompanied by inhibition of mb-PP1 and no effect on mb-PP2A.

Discussion

Several lines of evidence suggest that mb-PP1 and mb-PP2A are positive regulators of KCC activity. Starke and Jennings [52] showed pharmacological evidence that KCC is regulated by protein phosphatase type 1 (PP1). Krarup and Dunham [39] showed that PP1 can reactivate KCC in CalA-treated dog red cell ghosts, demonstrating that this phosphatase can regulate KCC activity. Incomplete inhibition of KCC activity by OA [46, 31, 34, 42, 52, 48, 6, 22], suggests that mb-PP2A (in addition to mb-PP1), may be involved in KCC regulation.

In the present report, we present data on the effects of two activators of KCC activity on mb-PP1 and mb-PP2A activity. The results indicate that activation of KCC by NEM is mediated by mechanism sensitive to OA and CalA, consistent with stimulation of mb-PP2A by NEM, and with mechanism resistant to OA and CalA, consistent with inhibition of kinases. Activation of KCC

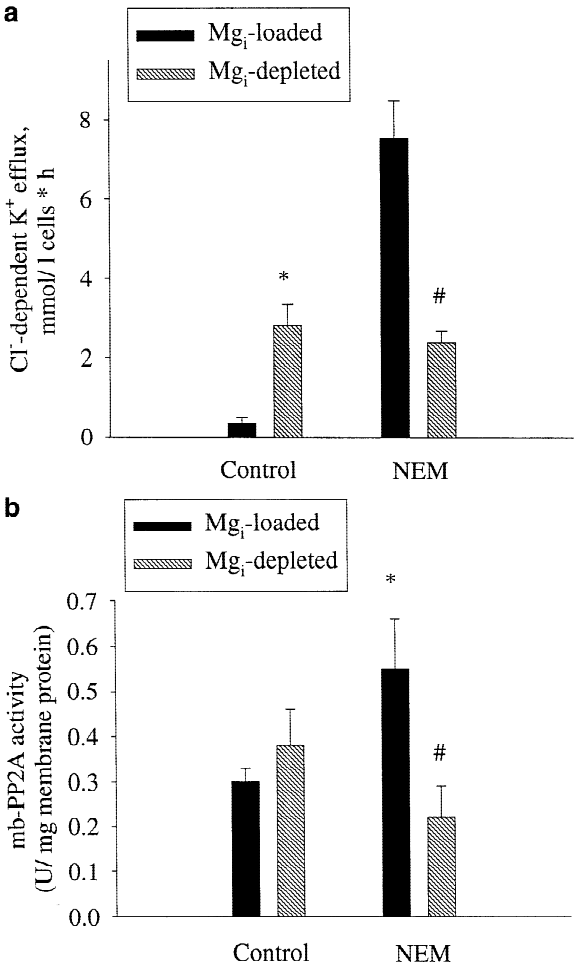

Fig. 3. (*a*) Mg_i^{++} depletion inhibits KCC activity in NEM treated cells. The effect of Mg_i^{++}-loading and Mg_i^{++}-depletion on KCC activity was determined in control cells and in NEM-treated cells (0.5 mM). Control and NEM-treated cells were loaded with Mg_i^{++} (500 μM) or depleted of Mg_i^{++} (1 mM EDTA), and Cl^--dependent K^+ efflux was determined. Results are means ± SEM of 3 experiments in different subjects. (* and $^{\#}$, $P = 0.007$, different from respective controls (Mg_i^{++}-loaded cells). (*b*) Mg_i^{++} depletion inhibits mb-PP2A in NEM treated cells. The effect of Mg_i^{++}-loading and Mg_i^{++}-depletion on mb-PP1 and mb-PP2A activities was determined in membranes from control cells and NEM-treated cells. Phosphatase activity was assayed in the presence of 100 μM EDTA. The increase in mb-PP2A elicited by NEM pretreatment (*, $P = 0.03$, different from control cells) is abolished by Mg_i^{++}-depletion ($^{\#}$, $P = 0.04$, different from NEM-treated, Mg_i^{++}-loaded (control) cells). Results show mean ± SEM of 4 experiments in 2 individuals.

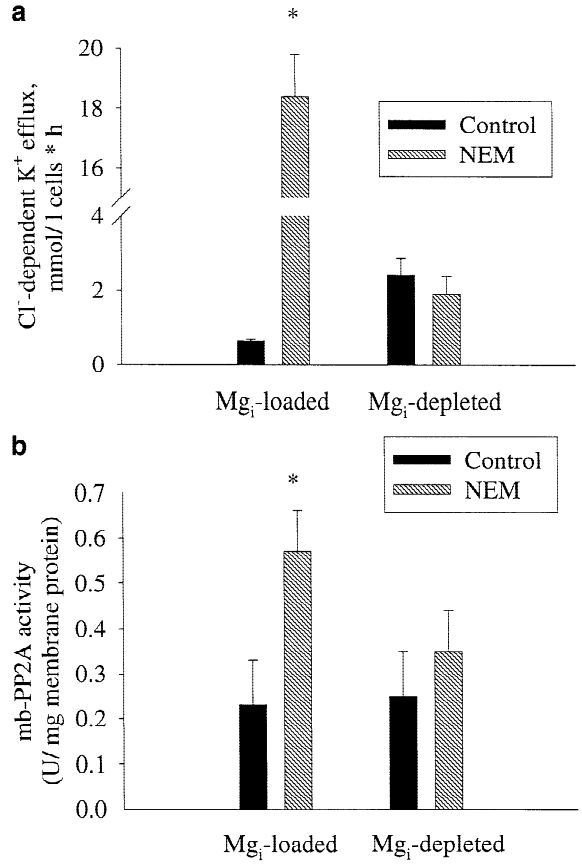

Fig. 4. (*a*) NEM does not stimulate KCC activity in Mg_i^{++}-depleted cells. The effect of NEM on K-Cl cotransport activity was determined in cells loaded with Mg_i^{++} (500 μM) and depleted of Mg_i^{++} (1 mM EDTA). After removal of the ionophore, Mg_i^{++}-loaded cells and Mg_i^{++}-depleted cells were treated ± 0.5 mM NEM. K^+ efflux was measured in isotonic media. Results are means ± SEM of 3 experiments in different subjects. (*, $P = 0.003$). (*b*) NEM does not stimulate Mb-PP2A in Mg_i^{++} depleted cells. Cells were treated as in Fig. 4*a*, and mb-PP2A activities was determined. Results are mean ± SEM of 9 experiments. (*, $P = 0.01$, different from Mg_i^{++}-loaded (control) cells).

by Mg_i^{++}-depletion, on the other hand, is inhibited by CalA but it is only slightly sensitive to OA, consistent with inhibition of a kinase that phosphorylates a site dephosphorylated by mb-PP1.

Two lines of evidence suggest that NEM and Mg_i^{++}-depletion stimulated KCC activity through inhibition of a kinase that inactivates KCC. First, the effects of both treatments require ATP [43], and second, both treatments activate KCC with a delay [32]. Our results show that after activation of KCC by NEM or Mg_i^{++}-depletion, there is only incomplete inhibition by CalA, consistent with the effects of NEM and Mg_i^{++}-depletion being mediated by an inhibitory kinase.

Our present results also show that stimulation of KCC and mb-PP2A by NEM is prevented in cells depleted of Mg_i^{++}, indicating a strong correlation between mb-PP2A and KCC activity, supporting the hypothesis that mb-PP2A regulates KCC activity. In addition, the large inhibition by OA of NEM-stimulated KCC also suggests that NEM may stimulate mb-PP2A. Determination of mb-PP2A activity demonstrates that this is indeed the case: mb-PP2A activity is indirectly stimulated by NEM (Fig. 2*a*).

Our results demonstrate indirect inhibition of mb-PP1 by NEM and by Mg_i^{++}-depletion. Thus, stimulation of KCC by NEM and by Mg_i^{++}-depletion occurs in spite

Table 3. Summary effects of NEM-treatment and Mg_i^{++}-depletion-treatment on KCC, mb-PP2A, and mb-PP1

1st treatment 2nd treatment	Control*	NEM	Mg_i^{++}-dep	NEM Mg_i^{++}-dep	Mg_i^{++}-dep NEM
Cl^--dep $^{86}Rb^+$ influx (mmol/l cells * hr)	0.03 ± 0.003 (3)	0.38 ± 0.09 (3)[1]	0.15 ± 0.05 (3)[2]	0.14 ± 0.05 (3)[3]	0.18 ± 0.05 (3)[4]
mb-PP2A (U/mg protein)	0.23 ± 0.04 (12)	0.49 ± 0.07 (12)[5]	0.27 ± 0.06 (12)[6]	0.26 ± 0.06 (5)[7]	0.35 ± 0.09 (9)[8]
mb-PP1 (U/mg protein)	0.31 ± 0.05 (15)	0.10 ± 0.02 (15)9	0.09 ± 0.02 (12)[10]	0.06 ± 0.02 (5)[11]	0.04 ± 0.02 (7)[12]

Control*, A23187 plus 500 μM $MgCl_2$. KCC activity (Cl^--dependent $^{86}Rb^+$ influx), mb-PP2A and mb-PP1 activities were determined as detailed in Materials and Methods. [1,2,5,9], and [10], different from respective controls: [1], $P = 0.01$; [2], $P < 0.001$; [5], $P = 0.002$; [9] and [10], $P < 0.001$. [3,4,7,8,11] and [12], different from NEM: [3], $P = 0.006$; [4], $P = 0.006$; [7], $P = 0.002$; [8], $P = 0.002$; [11], $P = 0.003$; [12], $P = 0.03$. [11] and [12], different from Mg_i^{++}-depleted: [11], $P = 0.001$; [12], $P = 0.006$.

of inhibition of mb-PP1. This finding argues against dephosphorylation by mb-PP1 being an absolute requirement for KCC activation. However, it is possible that a particular isoform of mb-PP1 [55] (insensitive to NEM and/or Mg_i^{++}-depletion) may be involved in KCC regulation. Therefore, a firm conclusion about this requirement can only be reached when mb-PP1 activity is assayed with the relevant substrate, not yet identified.

The mechanisms by which these treatments affect mb-PP1 and mb-PP2A activities are unknown; our data show that enzyme relocalization is an unlikely possibility. Kamibayashi et al. (1991), have shown that NEM can activate PP2A by inducing the release of an inhibitory subunit [36]. It is possible that in erythrocytes NEM indirectly activates PP2A by a similar mechanism. The mechanisms by which Mg_i^{++}-depletion decreases the activity of mb-PP1 is also unknown. It is likely that mb-PP1 activity in erythrocytes is controlled by kinases, as in other cells [55], and therefore Mg^{++} and Mg-ATP are indirectly required for PP1 activity.

Jennings has recently demonstrated that during activation of KCC by cell swelling, the rate constant for the rate-limiting inactivation event (supposedly a ser/thr kinase), is strongly dependent on cell volume, suggesting that the main 'signal event' in swelling-activation of KCC of rabbit erythrocytes involves inactivation of a kinase [30, 18, 32]. In sheep red cells, swelling appears to induce changes in more than one rate constant, indicating that there might be more than one 'signal' for activation of KCC by cell swelling [18].

It has been proposed that there are two independent signal transduction pathways for activation of KCC by cell swelling [19]. Consistent with that proposal, we have observed that stimulation of KCC in hypotonic media is also the result of activation of two different signal transduction pathways [8]. In one of these pathways, the decrease in intracellular ionic strength stimulates mb-PP1 (or increases its binding to the membrane). In the other pathway, the increase in cell size activates mb-PP2A [8].

The simplest model of regulation in a system with two phosphorylation sites involves independent regulation of each site. We name s-1 a site dephosphorylated by mb-PP1 and phosphorylated by PK1 (an unidentified kinase), and we name s-2 a site dephosphorylated by mb-PP2A and phosphorylated by PK2 (an unidentified kinase, which could be the same as PK1), *see* Fig. 5. The model assumes that a decrease in phosphorylation at either site will result in an increase in transporter activity. This is not unlikely to occur [31].

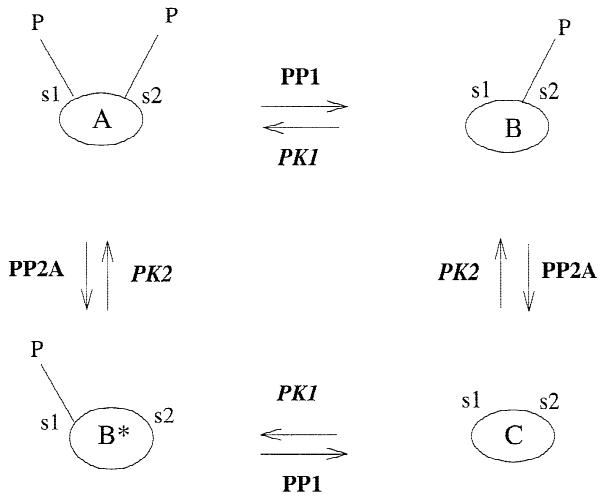

Fig. 5. Diagram of proposed 'activity states' in a transporter with 2 phosphorylation sites.

The finding that full KCC activation (i.e., by NEM) is possible in spite of partial mb-PP1 inhibition (by NEM), suggests that dephosphorylation at s-1 (by mb-PP1) is not required for dephosphorylation at s-2. On the other hand, there is almost full stimulation of KCC by Mg_i^{++}-depletion in cells pretreated with OA, therefore it appears that dephosphorylation at s-2 (by mb-PP2A) is not required for dephosphorylation at s-1. Since the accumulated evidence suggests that mb-PP1 and mb-PP2A are involved in KCC activation, and neither is an absolute requirement of KCC activation, the results suggest that activation of KCC, most likely is not 'ordered' with respect to dephosphorylation of individual sites.

We propose that KCC can exist in 4 different activity states ('A', 'B', 'B*', and 'C') according to the phosphorylation status of each of two distinct ser/thr phosphorylation sites. Data with OA and Cal A (Table 1) indicate that to a large extent only mb-PP1 is involved in the Mg_i^{++}-depletion-stimulated event, therefore, we have assigned Mg_i^{++}-depletion sensitivity to the reactions that regulate phosphorylation of s-1 (mb-PP1 and PK1). The inhibitory effect of Mg_i^{++}-depletion on mb-PP1 cannot explain the increase in flux elicited by this agent. Therefore we assume that Mg_i^{++}-depletion inhibits PK1 in agreement with data in rabbit red cells suggesting that Mg_i^{++}-depletion inhibits a kinase [32]. The kinase that phosphorylates KCC has not been identified. Recently, Klein et al. (1999), have demonstrated that a volume-sensitive kinase (JNK) phosphorylates the Na-K-Cl_2 transporter [38]. Whether this same kinase phosphorylates KCC is unknown.

As in any model of this type, the measured flux depends on the intrinsic flux rate of each state, and the relative amounts of transporters in each state. This last component is determined by the relative rates of phosphatases and kinases that regulate the transporter. Clearly, a change in one rate constant can be balanced by a change in another rate constants, and the flux can remain unchanged. In our view, the large stimulation of KCC by NEM is due to a large decrease in PK1 activity, and a large increase in mb-PP2A activity. The increase in flux in Mg_i^{++}-depleted cells is mostly due to a large decrease in PK1 activity.

The model represents an oversimplified approximation of KCC regulation since transporter activity is also regulated by phosphorylation/dephosphorylation of tyrosyl residues either of the transporter itself or of a regulator(s). The activity of protein tyrosine kinases (TPKs) and protein tyrosine phosphatases (PTPs) proposed to be involved in KCC regulation [5, 16, 22, 17], have not been taken into account. Since TPK inhibitors inhibit KCC activity [5, 22], effects of NEM in addition to those envisioned here are also likely, and may include tyrosine phosphorylation of the transporter or regulatory proteins [57]. Furthermore, some effectors (e.g., pH) may have direct effects on the transporter [43, 47].

Since KCC is elevated in SS red cells, where it contributes to the cell dehydration that marks the onset of a crisis [12, 15, 21, 23], the model presented here may be useful for understanding the events leading to KCC activation and sickle cell dehydration.

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