Serine/threonine protein phosphatases and regulation of K-Cl cotransport in human erythrocytes

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Bize, Isabel, Birol Güvenç, Aеisha Robb, Guido Buchbinder, and Carlo Brugnara. Serine/threonine protein phosphatases and regulation of K-Cl cotransport in human erythrocytes. Am. J. Physiol. 277 (Cell Physiol. 46): C926–C936, 1999.—Activation of K-Cl cotransport is associated with activation of membrane-bound serine/threonine protein phosphatases (S/T-PPases). We characterize red blood cell S/T-PPases and K-Cl cotransport activity regarding protein phosphatase inhibitors and response to changes in ionic strength and cell size. Protein phosphatase type 1 (PP1) activity is highly sensitive to calyculin A (CalA) but not to okadaic acid (OA). PP2A activity is highly sensitive to CalA and OA. CalA completely inhibits K-Cl cotransport activity, whereas OA partially inhibits K-Cl cotransport. Membrane PP1 and membrane PP2A activities are elevated in cells suspended in hypotonic solutions, where K-Cl cotransport is elevated. Increases in membrane PP1 activity (62 ± 10% per 100 meq/l) result from decreases in intracellular ionic strength and correlate with increases in K-Cl cotransport activity (54 ± 10% per 100 meq/l). Increases in membrane PP2A activity (270 ± 77% per 100 mosM) result from volume increases and also correlate with increases in K-Cl cotransport activity (420 ± 47% per 100 mosM). The characteristics of membrane-associated PP1 and PP2A are consistent with a role for both phosphatases in K-Cl cotransport activation in human erythrocytes.

SWOLLEN ERYTHROCYTES of several species return to their original volume by activating mechanisms that achieve loss of KCl by distinct electrogenic K ion and Cl ion channels or by electroneutral K-Cl cotransport (29). Regulation of erythroid K-Cl cotransport activity appears to involve phosphorylation/dephosphorylation reactions in serine/threonine, and tyrosine residues in substrate proteins (2, 12, 13, 21). Direct phosphorylation of the ubiquitously expressed K-Cl transporter, KCC1, has not been demonstrated, but the KCC1 polypeptide contains consensus sequences for phosphorylation by serine/threonine kinases, consistent with the possibility of direct phosphorylation in the regulation of the activity (15). K-Cl cotransport is inhibited by the inhibitors of protein phosphatase type 1 (PP1) and protein phosphatase type 2A (PP2A), okadaic acid (OA), and calyculin A (CalA), suggesting that dephosphorylation is associated with activation of the transporter. At least two other ion transporters involved in red blood cell volume regulation are sensitive to OA and/or CalA: Na/H exchange and Na-K-2Cl cotransport (18).

The signaling pathways regulating the activity of the K-Cl cotransporter protein are incompletely characterized. K-Cl cotransport activity is very low in mature human red blood cells at pH 7.4 in isosmotic conditions, but it can be activated by swelling, acidification, and many other agents, including sulfhydryl reagents, the kinase inhibitor staurosporine, and Mg ion depletion (see reviews, Refs. 18 and 27). The activation of K-Cl cotransport by most of these agents is inhibited by OA and/or CalA (1, 3, 11, 14, 21, 22, 32, 33, 38), indicating that the molecular target of the phosphatase(s) is either the transporter itself or a close upstream regulator. Experimental evidence suggests that the phosphatase(s) that activates K-Cl cotransport is associated with the cell membrane (4, 36). There is also substantial evidence that the phosphatase is PP1 (4, 24). It remains unclear whether PP2A is also involved in K-Cl cotransport regulation (30). We have reported the presence of PP1 activity in the membranes and PP2A activity in the cytosol of human and sheep erythrocytes (4).

Most PP1 and PP2A catalytic subunits are associated with one or more of many different, tissue-specific, regulatory subunits. Regulatory subunits play a role in regulating substrate specificity and targeting of the catalytic subunit to subcellular organelles or to particular substrates. The catalytic subunit isotypes are also tissue specific (37). Mature red blood cells contain PP1 and PP2A catalytic subunits and several of their regulatory subunits (23, 39). The regulatory subunits of red blood cell PP1 have not been identified. In general, the native structure of PP1 is a 1:1 complex between the catalytic subunit and different regulatory subunits (41). Human red blood cells contain the catalytic subunit of PP2A (α) and the regulatory subunits β and δ (30).

We examined the effect of treatment with CalA or OA on K-Cl cotransport activity in normal volume and swollen erythrocytes, and we investigated the changes in PP1 and PP2A activity with different degrees of swelling, at constant and variable intracellular osmolarity and ionic strength. The results indicate that both PP1 and PP2A may be involved in K-Cl cotransport regulation, that the activities of membrane-associated phosphatases are increased in red blood cells subjected to an hypotonic challenge, and that two different signal transduction pathways are activated under these conditions. The activation of either or both signal transduction pathways can lead to K-Cl cotransport stimulation and cell volume decrease. The results are relevant to...
the mechanisms of dehydration of sickle erythrocytes, in which K-Cl cotransport is upregulated.

METHODS

Cells

Human red blood cells were obtained from healthy adult volunteers after informed consent. Cells were washed by centrifugation in isotonic choline chloride ("choline washing solution"). This solution contains (in mM) 152 choline chloride, 1 MgCl₂, 5 Tris-MOPS, pH 7.4 at 4°C. For treatment of intact cells, cells were incubated for 20 min at 37°C, 10% hematocrit with 50 nM CalA or 200 nM OA, in media containing (in mM) 145 NaCl, 5 KCl, 5 glucose, 10 Tris-MOPS, pH 7.4 at 37°C, and 1 MgCl₂. Control cells were incubated in parallel without the drugs. CalA and OA were removed by two washes in choline washing solution before the transport assays are beyond the doses required to achieve half-maximal inhibition.

K-Cl Cotransport Activity

K-Cl cotransport activity was determined using⁸⁶Rb influx measurements as previously described (3), except that Cl⁻ was substituted by sulfamate instead of nitrate (35). Control and drug-treated, washed red blood cells were incubated at 10% hematocrit, at 37°C, in flux media containing (in mM) 145 NaCl, 2 KCl, 5 glucose, 1 MgCl₂, 10 Tris-MOPS, pH 7.4 at 37°C, as well as 0.1 mM ouabain and 10 µM bumetanide. Cl⁻-free media contained (in mM) 145 sodium sulfamate, 2 potassium sulfamate, and 1 MgNO₃ instead of the respective CI salts. In some experiments (see Fig. 8A) the ionic strength of the flux media was varied (95–145 mM NaCl, 2 mM KCl) while osmolarity was kept constant (isotonic, 300 mosM) by varying sucrose (100–0 mM). In other experiments (see Fig. 8B), the ionic strength of the flux media was varied and sucrose (0–200 mM) was added to maintain a constant osmolarity of 300 mosM. Fluxes are expressed in millimoles per liter of cells per hour, where liter corresponds to the original cell volume.

Modification of Intracellular Cations: Nystatin Loading

Cells were suspended in the presence of nystatin (40 µg/ml) at 10% hematocrit in media containing (in mM) 10 Tris-MOPS buffer, pH 7.4 at 4°C, 5 glucose, 1 MgCl₂, 10 NaCl, 30 sucrose, and KCl concentrations ranging from 95 to 140 mM. This cell suspension was maintained at 4°C for 20 min and then at 37°C for 10 min. Each lot of cells was pelleted and resuspended in the corresponding loading solutions without nystatin. Density distribution and median density (d₅₀) were measured with phthalate esters (5) at 25°C. The d₅₀ value for cells of normal volume is 1.095 ± 0.001. At this cell density, normal volume cells partition 50% in the upper portion and 50% in the lower portion. Cell lysis was carried out in the corresponding nystatin loading solutions but nystatin was omitted. Cells suspended in hypotonic solutions (in mM: 95 KCl, 10 NaCl, 30 sucrose) in the presence of nystatin have a d₅₀ of 1.088 ± 0.001. In contrast, cells suspended in the same hypotonic solutions (without sucrose or nystatin) have a d₅₀ of 1.066 ± 0.001.

Subcellular Preparation

Isotonic lysis. Red blood cells were lysed in 10 vol of isotonic solutions (300 mosM). Lysis buffer contained 10 mM Tris-MOPS, pH 7.4, 0.1% β-mercaptoethanol, 10 µM phenylmethylsulfonyl fluoride, 25 µg/ml each of leupeptin and aprotinin, and NaCl and KCl such that total NaCl plus KCl was kept at 150 mM. Lysis was accomplished either by freezing/thawing (dry ice with ethanol, 5 min; 30°C, 8 min) or by sonication (Sonic Dismembrator model 60 from Fisher Scientific). Sonic cell disruption was performed by one to three bursts of 5 s each at 0.05 W of power output. Nonruptured cells were removed by pelleting at low speed (500 g for 3 min) before the centrifugation at 30,000 g to separate the membranes from the cytosol.

Lysis in solutions of different osmolarities and ionic strength. Lysis was carried out by freezing/thawing or by sonication in hypotonic solutions (210–300 mosM). In experiments where salt and sucrose were used, the ionic strength (mEq/l) was estimated from the salt concentration and the activity coefficient. Either Na or K was kept constant at 10 mM, and the other cation (K or Na) was modified (95–140 mM) to achieve the desired osmolarity and ionic strength. In some experiments (see Fig. 7A) the ionic strength of the lysis solution was increased (95–140 mM KCl, 10 mM NaCl) while osmolarity was kept constant (isotonic, 300 mosM) by the addition of variable amounts of sucrose (100–0 mM) to the suspending medium. In these experiments, d₅₀ at the time of lysis is 1.088 ± 0.001. In other experiments (see Fig. 7B), the ionic strength of the lysis solution was maintained constant (95 mM KCl, 10 mM NaCl), and osmolarity was varied by the addition of sucrose (0–200 mM). In these experiments d₅₀ at the time of lysis ranges from 1.066 ± 0.001 (with no sucrose) to 1.112 ± 0.001 (with 200 mM sucrose).

All preparations were performed at 4°C. Cell lysates were centrifuged (30,000 g for 10 min) and the membrane pellet was washed (3–4 times) by centrifugation and resuspension in "hypotonic lysis buffer" (equal to the isotonic lysis buffer except that NaCl and KCl were omitted). The first supernatant was used as the cytosolic fraction. Preparations were stored frozen at –70°C. The cytosolic fractions were stored in the lysis buffer in which they were prepared at a protein concentration of 15–30 mg/ml. The membrane fractions were stored in hypotonic lysis buffer at a concentration of 2–5 mg protein/ml. Protein concentrations were determined using the Lowry assay (28), with BSA as standard. The amount of protein in the cytosol and membranes isolated by the different lysis procedures did not differ.

Phosphatase Activity

Protein phosphatase activity was determined using ³²P-labeled phosphorylase a (GIBCO BRL). ³²P-labeled substrate was prepared as previously described (4). The phosphatase assay reaction media contained 16.7 mM Tris·HCl, 13.3 mM imidazole (final pH, 7.4), 5 mM caffeine, 0.1 mM EDTA, 0.1% β-mercaptoethanol, 0.1 mg/ml BSA, 0.5% (vol/vol) Triton X-100, and 1 mg/ml ³²P-labeled phosphorylase a. Protein concentration in the phosphatase assay was 50–100 µg/ml for the membrane and 100–250 µg/ml for the cytosol preparations. Phosphatase activity is expressed as U/mg protein, where one unit (U) equals 1 nmol of phosphate released per minute.

The activities of serine/threonine phosphatases PP1 and PP2A do not have an absolute requirement for divalent cations and are routinely assayed in the presence of divalent cation chelators. In preliminary experiments we found that 50 mM NaCl or KCl did not affect PPase activity measure-
Table 1. Distribution of PP1 and PP2A in mature human red blood cells

<table>
<thead>
<tr>
<th></th>
<th>PP1 SA (U/mg protein)</th>
<th>PP1 Per cell</th>
<th>PP2A SA (U/mg protein)</th>
<th>PP2A Per cell</th>
<th>PP1 + PP2A SA (U/mg protein)</th>
</tr>
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<tr>
<td>Cytosol</td>
<td>0.031 ± 0.002</td>
<td>31 ± 2</td>
<td>0.137 ± 0.015</td>
<td>135 ± 15</td>
<td>0.17 ± 0.015</td>
</tr>
<tr>
<td>Membrane</td>
<td>0.384 ± 0.042</td>
<td>5.8 ± 0.6</td>
<td>0.089 ± 0.018</td>
<td>1.3 ± 0.3</td>
<td>0.47 ± 0.045</td>
</tr>
<tr>
<td>Total</td>
<td>0.415 ± 0.042</td>
<td>36.8 ± 3</td>
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Values are means ± SE. Specific activity (SA) is expressed in U/mg cytosolic protein in the cytosol and in U/mg membrane protein in the membrane. Activity per cell is expressed in U/g total cell protein. PP, protein phosphatase.
curves at ~1 nM OA corroborates the finding that, in dilute cell-free extracts, 1–3 nM OA is a valuable tool to distinguish between PP1 and PP2A (10, 41).

In the membrane fraction (Fig. 1B), the curves for inhibition by OA are markedly different whether the membranes are obtained after freezing/thawing or after sonication. In membranes obtained by sonication, 1 nM OA inhibits only 18 ± 1% of the phosphatase, whereas, in membranes obtained by freezing/thawing, 1 nM OA inhibits 74 ± 7% of the activity. This difference is significant (P = 0.001). OA inhibition in membranes obtained by sonication in isotonic conditions is very similar to OA inhibition from membranes obtained by lysis in hypotonic media from human and sheep red blood cells (4). The shift in IC50 when membranes are prepared by these different lysis methods suggests that either membrane PP2A is activated by freezing/thawing or that membrane PP1 is inhibited (or lost from the membrane) by freezing/thawing, or both.

A comparison of the activities of PP1 in the membrane indicates that freezing/thawing inhibits PP1 in the membrane (60 ± 15%, P < 0.001, n = 4) compared with the SA in membranes isolated by sonication (not shown). The possibility that PP1 is lost from the membrane into the cytosol during freezing/thawing is suggested by a small but significant increase in cytosolic PP1 (25 ± 5%, P = 0.02, n = 4, not shown). On the other hand, there is a tendency for PP2A to be increased by freezing/thawing, although the difference is only significant in the membrane (P = 0.02). This increase is not unexpected since the SA of PP2A is known to be elevated by repeated freezing/thawing in the presence of reducing agents (8). Therefore, the difference in the curves of OA inhibition in membranes isolated by freezing/thawing or by sonication is due to inhibition of PP1 and stimulation of PP2A by freezing/thawing.

CalA inhibition of membrane and cytosolic PPase was also determined, to estimate the activity of the phosphatases in cells prepared for K-Cl cotransport measurements. Untreated cells were lysed by sonication, and PPase activity was determined in the presence of increasing concentrations of CalA. Figure 2 shows that CalA is a potent inhibitor of cytosolic and membrane PPase [IC50 = 0.4 ± 0.1 nM (cytosol) and 1.5 ± 0.5 nM (membrane)]. Because membranes isolated by sonication contain 80% PP1 (Fig. 1B) and cytosols contain 80% PP2A (Fig. 1A), the small difference in the sensitivity to CalA of the membrane and cytosolic preparations supports the view that, in human red blood cells as in other cells (19), CalA is a potent inhibitor of PP1 and PP2A. Moreover, the IC50 for CalA inhibition of cytosolic and membrane phosphatases is nearly the same if CalA inhibition is assayed in the presence of 2 nM OA (PP2A is completely inhibited), indicating that the IC50 for CalA is determined mainly...
by the activity of the phosphatase that is least sensitive to OA (PP1 in both preparations).

A summary of IC50 data for both phosphatases and both inhibitors is shown in Table 2. The IC50 for membrane PP1 inhibition by OA is 40–70 nM and by CalA is 1–2 nM. These results are similar to results obtained with rabbit red blood cells (38), but CalA appears to be more potent in human red blood cells than in dog red blood cells (24). On the other hand, OA inhibits PP2A with similar potency to CalA; the IC50 for cytosolic PP2A inhibition by OA is 0.1 nM, and by CalA is 0.4 nM. These results agree with results with purified PP1 and PP2A preparations (19).

Intracellular concentrations of CalA and OA were estimated by determining the inhibition of cytosolic PP2A in CalA (50 nM)- and OA (50 nM)-treated cells. Free intracellular concentration (nM) of CalA and OA in the cells was estimated by measuring the inhibition of cytosolic PP2A when cytosol from cells pretreated with CalA or OA was added to untreated cytosol. The inhibitory effect of treated cytosol on untreated cytosol was compared with a standard dose-response curve. Our estimation of the free CalA concentration in the cells is 60 nM free CalA, probably a close overestimation, since the cells were incubated with 50 nM. This concentration of CalA is large enough to fully inhibit both PP1 and PP2A. We estimated that free OA concentration, since the cells were incubated with 50 nM. This concentration of OA is 48 nM, in accordance with the incubation conditions and permeability data (31). This concentration of OA is large enough to fully inhibit PP2A and to partially inhibit PP1 (<50% on average).

### Intact Cell Treatment With OA and CalA Inhibits Basal and Volume-Stimulated K-Cl Cotransport

Figure 3 shows the inhibition by CalA (estimated 60 nM) and OA (48 nM) of basal (Fig. 3A) and volume-stimulated (Fig. 3B) K-Cl cotransport. Cells were preincubated with the drugs (20 min at 37°C) and washed. K-Cl cotransport activity was determined in inhibitor-free media using 86Rb influx. Figure 3A shows 86Rb influx in cells suspended in isotonic Cl-containing and Cl-free media (sulfamate). The data show a very low but measurable Cl-dependent 86Rb influx in control cells (P = 0.02), markedly reduced in OA cells and absent in CalA-cells, demonstrating that K-Cl cotransport is not silent in mature normal volume human red blood cells as previously believed (7, 32). It is likely that the difference between the present and previous reports is due to the use of nitrate vs. sulfamate as the anion in Cl-free media. It has been reported that in human red blood cells nitrate induces significant water loss and pH changes and that these changes are avoided using sulfamate as the anion substituting Cl (35).

Table 2. IC50 values for inhibition of PP1 and PP2A by okadaic acid and calyculin A

<table>
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<tr>
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<th>PP1</th>
<th>PP2A</th>
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<tr>
<td>Okadaic acid</td>
<td>56 ± 15</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>Calyculin A</td>
<td>1.5 ± 0.5</td>
<td>0.4 ± 0.1</td>
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Values are means ± SE in nM and were derived from the experiments averaged in Figs. 1 (A and B) and 2.
find out whether either or both of these phosphatases were also stimulated when cell volume increases. Membranes were isolated from cells lysed by freezing/thawing or by sonication in solutions of variable tonicities obtained by varying NaCl + KCl from 105 to 150 mM. There was no difference in either PP1 or PP2A activity whether lysis was carried out in 140 mM KCl + 10 mM NaCl, or in 10 mM KCl + 140 mM NaCl (data not shown). Figure 4 shows membrane PP1 activity and membrane PP2A activities (U/mg membrane protein) vs. the osmolarity of the cell suspension during lysis. Eleven experiments were performed and 10 different osmolarities were tested in each experiment. Membrane PP1 activity is elevated in cells lysed in low-osmolar, low-ionic-strength media. The $r^2$ of the linear regression was 0.88. Average stimulation of membrane PP1 activity was $62 \pm 10\%$ per 100 mosM (n = 11). In 210 mosM media $d_{50}$ is $1.066 \pm 0.001$ (n = 3) and K-Cl cotransport is activated. There was no difference in the degree of stimulation in cells lysed by freezing/thawing or by sonication. Cells lysed in media containing nitrate instead of Cl show the same results (n = 2), indicating that the important variable is the ionic strength and/or the cell size, and not the Cl concentration.

Similar results were obtained with membrane PP2A. Membrane PP2A activity is also elevated in cells lysed in 210 mosM media compared with cells lysed in 300 mosM media. Stimulation of membrane PP2A by hypotonic media is more variable and of greater magnitude than stimulation of membrane PP1. The $r^2$ was 0.82. Average stimulation of membrane PP2A was $270 \pm 77\%$ per 100 mosM media (n = 10; 1 experiment was eliminated for this calculation because stimulation was $>1,800\%$). It is interesting to note that the effect of ionic strength and/or cell size persists after cell rupture, suggesting either covalent modification (i.e., phosphorylation) and/or dissociation of regulatory subunits.

Activation of Membrane PP1 Does Not Depend on Cell Size

Experiments were designed to change intracellular ionic strength and osmolarity while minimizing cell volume changes. Cells were suspended and lysed in solutions of different tonicities (210–300 mosM). The tonicity was varied by increasing the concentration of KCl from 95 to 140 mM (NaCl was constant at 10 mM). Cells were suspended in media containing nystatin. Under these circumstances intracellular cations equilibrate with extracellular cations; therefore, the intracellular osmolarity and ionic strength equal extracellular osmolarity and ionic strength. After nystatin removal from the media, cells were lysed by either freeze/thaw or sonication in the corresponding solutions, so that cell volume was normal at the time of lysis ($d_{50}$ at 210 mosM was $1.088 \pm 0.001$, n = 3) (16). The results in Fig. 5 show that the activation of membrane PP1 in 210 mosM media occurs in near-normovolemic cells, indicating that an increase in cell size is not necessary for activation of membrane PP1. There is no difference between the slopes in control cells (no nystatin) and in nystatin-treated cells. In addition, membrane PP1 activity values in control and nystatin-treated cells lysed at 300 mosM were equal (0.29 ± 0.06 and 0.26 ± 0.06, respectively). These findings support the hypothesis that the principal signal for membrane PP1 activation in cells suspended in hypotonic media is the lowering of intracellular ionic strength.
Stimulation of Membrane PP1 in Media With Low Tonicity (and Low Ionic Strength) is Paralleled by a Decrease in Cytosolic PP1 in This Media

Cells were treated with nystatin to equilibrate cell Na and cell K with the concentrations in the suspension media and to minimize volume changes as previously described. Na and K concentrations in the cell suspension media were modified to obtain the required intracellular osmolarity. Membrane and cytosolic PP1 were assayed in the same preparations. The results are shown in Fig. 6, where PP1 activity in the cytosol and in the membrane (percent of that measured in fractions prepared from cells lysed at 300 mosM) is plotted against the osmolarity (ionic strength) of the lysis solution. The increase in membrane PP1 and the decrease in cytosolic PP1 in 210 mosM buffer suggests that the localization of PP1 is regulated by the osmolarity and/or ionic strength of the intracellular space and that, in swollen cells, the enzyme is preferentially bound to the membrane. The results suggest that the increase in membrane PP1 activity may be a result of translocation of the enzyme from the cytosol to the membrane, rather than activation of an enzyme already associated with the membrane. However, since only 16% of PP1 is localized in the membrane, the 30% increase in membrane PP1 cannot fully account for the 20% decrease in cytosolic PP1. We have no explanation for the decrease in cytosolic PP1, in excess of the increase in membrane PP1.

Activation of Membrane PP1 Depends on Ionic Strength

Experiments to investigate the role of ionic strength and osmolarity avoiding nystatin were designed. In these experiments we maintained one variable constant while we modified the other variable.

Variable ionic strength, constant osmolarity (constant cell volume). Cells were lysed at constant low extracellular ionic strength (95 mM KCl + 10 mM NaCl, 210 meq/l) in the presence of 105–150 mM total cations and 100–0 mM sucrose to maintain osmolarity at 300 mosM. Cell density (d50) was constant at 1.096. Data in Fig. 7A shows that membrane PP1 activity was elevated in cells lysed in low ionic strength, isotonic buffer (300 mosM). Stimulation was 74 ± 32% per 100 meq/l (n = 3, P = 0.02) and average r² was 0.81 ± 0.08. The results suggest that a decrease in ionic strength in the lysis buffer is sufficient to stimulate membrane PP1 activity, even in isosmotic media, supporting the view that membrane PP1 responds to ionic strength and not cell volume changes. Membrane PP2A activity was also assayed, but the results were more variable and no conclusion could be reached.

Variable osmolarity (variable cell volume). Cells were lysed at constant low extracellular ionic strength (95 mM KCl + 10 mM NaCl, 210 meq/l) in the presence of
increasing sucrose concentrations (0–200 mM) to increase medium osmolarity from 210 to 380 mosM and, accordingly, decrease cell size. The $d_{50}$ at the time of lysis was $1.066 \pm 0.001$ at 210 mosM and $1.112 \pm 0.001$ at 380 mosM ($n = 3$). Figure 7B shows membrane PP1 and PP2A activity vs. the osmolarity of the lysis medium. Changes in cell volume do not result in a clear change in membrane PP1 activity, suggesting that a change in cell size is not sufficient for stimulation of membrane PP1 activity, as previously concluded. In cells incubated without sucrose, the expected intracellular ionic strength would be decreased (since cell size is increased); however, this decrease in intracellular ionic strength does not result in stimulation of PP1 activity, possible because PP1 activity is already stimulated in cells incubated in 95 mM KCl with sucrose. On the other hand, despite the variability in membrane PP2A activity, the results show that PP2A can be stimulated by changes in cell size. Seven experiments were performed in five membrane preparations. The average of values between 210 and 260 mosM (plotted at 235) was $0.137 \pm 0.036$ U/mg membrane protein, the average of values between 270 and 310 mosM (plotted at 290) was $0.077 \pm 0.021$ ($P = 0.02$, ANOVA), and at 320–380 mosM (plotted at 360) the average of values was $0.073 \pm 0.02$ U/mg membrane protein ($P = 0.02$, ANOVA).

**K-Cl Cotransport Activity Is Stimulated by Low Ionic Strength at Constant Osmolarity**

K-Cl cotransport activity was determined in experiments analogous to those in which membrane PP1 activity was measured. Washed cells were preincubated in media containing 95–145 mM NaCl, 2 mM KCl, and sucrose (100–0 mM) to achieve 300 mosM, for 10 min at 37°C, at 10% hematocrit. The media also contained buffer, glucose, ouabain, and bumetanide at concentrations detailed in METHODS. Under these conditions the intracellular ionic strength in cells suspended in (in mM) 2 KCl, 95 NaCl, and 100 sucrose is expected to be lower than the intracellular ionic strength in cells incubated in (in mM) 2 KCl, 145 NaCl. At 95 mM NaCl, the pH inside the cells is expected to rise from 7.2 to 7.34 (6); therefore K-Cl cotransport is expected to be inhibited. After the initial incubation, the cells were pelleted and the flux was started with the addition of the corresponding media (containing $^{86}$Rb). The flux was terminated after 45 min with the addition of cold “Na washing solution.” Figure 8A shows stimulation of $^{86}$Rb influx by low ionic strength at constant osmolarity in media containing Cl. Stimulation at 200 meq/l (compared with 300 meq/l) was $54 \pm 10\%$ ($P = 0.04$, $n = 4$). The stimulation of K-Cl cotransport is present despite the intracellular alkalization. There was no stimulation in Cl-free media ($n = 2$, not shown).

**K-Cl Cotransport Activity Is Stimulated by Low Osmolarity**

Washed cells were incubated in media containing 95 mM NaCl, 2 mM KCl, and sucrose (0–150 mM) to achieve osmolarities ranging from 200 to 370 mosM, for 10 min at 37°C, at 10% hematocrit. The media also contained buffer, glucose, ouabain, and bumetanide as above. $^{86}$Rb influx was determined as in experiments for Fig 7A. Figure 8B shows stimulation of $^{86}$Rb influx by low osmolarity at constant external Cl. Stimulation was 420 ± 47% per 100 mosM (from 200 to 300 mosM, $P = 0.02$, $n = 4$). The stimulation was Cl dependent (not shown, $n = 2$). These results together with those of Fig. 7B show that K-Cl cotransport can be stimulated in the absence of an increase in membrane PP1. Furthermore, the results show that stimulation of K-Cl cotransport in swollen cells is accompanied by stimulation of membrane PP2A (Fig. 7B).

**DISCUSSION**

Regulation of K-Cl cotransport is a complex phenomenon involving a number of tyrosine and serine/threonine kinases and phosphatases (27, 40). Erythroid K-Cl cotransport is believed to be mediated by the KCC1 protein. KCC1 has consensus sequences for phosphorylation by serine/threonine kinases (protein kinase C and creatine kinase 2) (15), and inhibition of
serine/threonine kinases and phosphatases have large inverse effects on KCl cotransport activity, although it remains uncertain whether KCC1 itself is phosphorylated. Our results show that the activities of membrane PP1 and membrane PP2A are increased in cells suspended in hypotonic media, where K-Cl cotransport is elevated, suggesting that both PP1 and PP2A may regulate K-Cl cotransport activity.

OA and CalA, inhibitors of PP1 and PP2A (19), inhibit volume-stimulated K-Cl cotransport in a variety of mammalian red blood cells. The greater effectiveness of CalA than OA in the inhibition of K-Cl cotransport in the red blood cells of many species has been reported earlier (38, 13) and supports the involvement of PP1 in the regulation of K-Cl cotransport (38).

Nonetheless, K-Cl cotransport activity in dog red blood cell ghosts can be reactivated by exogenous PP1 (but not PP2A) (24), supporting a role for PP1 in K-Cl cotransport regulation.

Our phosphatase assay studies indicate that red blood cell PP1 and PP2A are very sensitive to CalA (0.4 < IC50 < 1.5 nM), but only PP2A is very sensitive to OA (IC50 = 0.1 nM). The IC50 of PP1 by OA ranges between 40 and 70 nM (Table 2). In cells pretreated with CalA or OA, our estimate of the intracellular free concentration at the beginning of the flux is 60 nM for CalA and 48 nM for OA. Thus, in CalA-treated cells, both PP1 and PP2A are both inhibited, whereas in OA-treated cells only PP2A is reliably inhibited. Our K-Cl cotransport studies show that pretreatment of cells with CalA inhibits all hypotonic-media-stimulated K-Cl cotransport, whereas pretreatment of cells with OA only partially inhibits volume-sensitive K-Cl cotransport. Our interpretation of the results is that the inhibition of K-Cl cotransport by OA in our experiments is due to inhibition of PP2A, whereas the inhibition by CalA is due to inhibition of PP1 and PP2A, supporting a role for both phosphatases in K-Cl cotransport regulation. The lack of response of K-Cl cotransport to exogenous PP2A in CalA-pretreated resealed red blood cell ghosts may be due to the presence of a regulatory subunit in the preparation used (24).

Both PP1 and PP2A Are Elevated in Cells Suspended in Hypotonic Media

The elevated membrane PP1 and PP2A activities in cells lysed in hypotonic media (Fig. 5) also support a role for both phosphatases in the signal transduction pathways that lead to activation of K-Cl cotransport in swollen cells. Furthermore, our results indicate that suspension of cells in hypotonic media activates two different signal transduction pathways. In one of these pathways, PP1 activity is predominantly modulated by the dilution of intracellular components, rather than by changes in cell size (see Figs. 4 and 7A). In the other pathway, the activity of PP2A is modulated predominantly by the increase in cell size (see Figs. 4 and 7B).

Increase in Membrane PP1 in Swollen Cells Is Associated With a Decrease in Cytosolic PP1

The inverse correlation between membrane PP1 and cytosolic PP1 activity (Fig. 6) suggests that a dilution of intracellular components induces translocation of PP1 activity from the cytosol to the membrane. It has been suggested that a cytosolic form of PP1 composed of the catalytic subunit and I-2 (a regulatory subunit) serves as a pool of inactive phosphatase from which the catalytic subunit can be recruited (41). It is possible that a similar phenomenon occurs in red blood cells.

K-Cl Cotransport Activity Is Stimulated by Low Ionic Strength in Cells of Normal Volume

The increase in membrane PP1 by low ionic strength suggested that K-Cl cotransport activity would be stimulated in cells suspended in isotonic media of low ionic strength. The results in Fig. 7A show that this is the case: K-Cl cotransport is more active in cells suspended in low-ionic-strength media at constant cell volume. These results are different from results in fish red blood cells where K-Cl cotransport inhibition was associated with a decrease in intracellular ionic strength (17). The observed stimulation of K-Cl cotransport in low-ionic-strength media (Fig. 7A) is associated with stimulation of membrane PP1 activity (Fig. 7A). On the other hand, the results in Figs. 7B and 8B show that an increase in membrane PP1 is not required for K-Cl cotransport stimulation, since activation of K-Cl cotransport can be elicited, without a change in membrane PP1 activity, by increases in cell size (Fig. 8B), where PP2A is elevated (Fig. 7B). Therefore, a comparison of Figs. 7B and 8B indicates that the signal transduction pathways mediated by PP1 and PP2A are independent.

How changes in cell size control the activity of kinases and phosphatases that regulate K-Cl cotransport is unknown. At least part of the signal transduction pathway appears to involve a decrease in the activity of a volume-sensitive kinase (20). Although protein kinase C activity has been shown to be modulated by cell volume changes (26), its role in the regulation of K-Cl cotransport is doubtful (2, 38). Our data indicate that the activation of K-Cl cotransport in swollen cells also involves an ionic-strength-sensitive PP1 and a cell-size-sensitive PP2A. Regulation of volume-sensitive ion transporters by more than one signal transduction pathway appears to be a general phenomenon (18).

A number of parameters have been postulated to act as volume “signals,” including the cytoskeletal architecture, cellular ion concentration, and concentration of cytosolic macromolecules. Our results show that a decrease in intracellular ionic strength results in an increase in membrane PP1 activity and an increase in K-Cl cotransport activity. These results do not support the idea that the swelling signal for the increase in membrane PP1 is a decrease in macromolecular crowding, since a decrease in ionic strength would result in...
an increase in macromolecular crowding (34). Questions regarding how a molecule can "sense" changes in ionic strength, what type of molecule is the "sensor," and whether cell volume changes the phosphorylation state of the phosphatases (and therefore their activity) have not been addressed here and remain unanswered.

Both PP1 and PP2A activities toward a particular substrate are mostly regulated by interactions with regulatory or targeting subunits (8). The activity of their catalytic subunits also appears to be modulated by phosphorylation on serine/threonine and tyrosine residues (9). In some systems phosphorylation results in increased activity and in other systems phosphorylation results in decreased activity (41). In this context, it has been proposed that a src-like kinase is a negative modulator of a phosphatase involved in K-Cl cotransport regulation (12).

The response of cells to osmotic challenge is being studied in several cell models. In human neutrophils, hyperosmotic challenge results in activation of several tyrosine kinases. Activation of tyrosine kinases is paralleled by activation of the Na/H exchanger involved in volume regulation (12). In human neutrophils, tyrosine kinases are involved in volume changes. The increased activity of either PP1 or PP2A in the membrane results in a concomitant increase in cell size. The increased activity of either phosphatase is elevated in cells exposed to hypotonic media. PP1 responds to the decrease in cell size and to the increase in intracellular osmolarity or ionic strength (25).

In summary, our data indicate that both membrane PP1 and PP2A may be involved in the regulation of K-Cl cotransport activity in human red blood cells. The activity of both phosphatases is elevated in cells exposed to hypotonic media. PP1 responds to the decrease in intracellular ionic strength while PP2A responds to the increase in cell size. The increased activity of either PP1 or PP2A in the membrane results in a concomitant increase in K-Cl cotransport activity.

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