Coordinate modulation of Na-K-2Cl cotransport and K-Cl cotransport by cell volume and chloride

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Lytle, Christian, and Thomas McManus. Coordinate modulation of Na-K-2Cl cotransport and K-Cl cotransport by cell volume and chloride. Am J Physiol Cell Physiol 283: C1422–C1431, 2002; 10.1152/ajpcell.00130.2002.—Na-K-2Cl cotransporter (NKCC) and K-Cl cotransporter (KCC) play key roles in cell volume regulation and epithelial Cl transport. Reductions in either cell volume or cytosolic Cl– concentration ([Cl–]i) stimulate a corrective uptake of KCl and water via NKCC, whereas cell swelling triggers KCl loss via KCC. The dependence of these transporters on volume and [Cl–]i was evaluated in model duck red blood cells. Replacement of [Cl–]i with methanesulfonate elevated the volume set point at which NKCC activates and KCC inactivates. The set point was sensitive to cytosolic ionic strength. Reducing [Cl–]i at a constant driving force for inward NKCC and outward KCC caused the cells to adopt the new set point volume. Phosphopeptide maps of NKCC indicated that activation by cell shrinkage or low [Cl–]i is associated with phosphorylation of a similar constellation of Ser/Thr sites. Like shrinkage, reduction of [Cl–]i accelerated NKCC phosphorylation after abrupt inhibition of the deactivating phosphatase with calyculin A in vivo, whereas [Cl–]i had no specific effect on dephosphorylation in vitro. Our results indicate that NKCC and KCC are reciprocally regulated by a negative feedback system dually modulated by cell volume and [Cl–]i. The major effect of Cl– on NKCC is exerted through the volume-sensitive kinase that phosphorylates the transport protein.

sodium-potassium-chloride cotransport; intracellular chloride; cell volume regulation; ionic strength; cell water content; sodium-potassium-chloride cotransporter phosphorylation

HOW ANIMAL CELLS DETECT osmotic perturbations and then activate ion transport processes that restore their fluid volume remains incompletely understood. Abrupt swelling typically triggers a corrective release of Cl– via cotransport or separate channels, whereas shrinkage evokes ion accumulation via Na-K-2Cl cotransport, Na/H exchange, or Na+ channels (22, 51).

Studies of red blood cells suggested that swelling-activated and shrinkage-activated ion transporters are coordinately controlled by a common mechanism (40, 43). When duck red blood cells are equilibrated in an osmotic artificial medium, they adopt a relatively stable fluid volume, termed the “volume set point,” at which both swelling-activated K-Cl cotransporters (KCC) and shrinkage-activated Na-K-2Cl cotransporters (NKCC) are nearly inactive (16, 52). Osmotic shrinkage and swelling stimulate one cotransport process and further suppress the other. It has been demonstrated that this set point is modulated by numerous factors. β-Adrenergic stimulation via intracellular cAMP (37, 52), Mg2+ loading (53), deoxygenation (52), calyculin A, and fluoride (37) all reprogram the set point to larger volumes and thereby stimulate NKCC in normal cells and suppress KCC in swollen cells. In contrast, depletion of cytoplasmic Mg2+ (53) or treatment with N-ethylmaleimide or staurosporine (C. Lytle, unpublished results) reduces the set point.

Information on cell volume appears to be transmitted to volume-regulatory ion transporters, at least in part, through protein phosphorylation. In duck red blood cells, osmotic perturbations that displace cell volume from the prevailing set point, or interventions that raise the set point above the prevailing cell volume, promote phosphorylation of NKCC at a similar constellation of serine and threonine sites (27). The degree to which these sites are phosphorylated appears to reflect a simple competition between an unknown volume-responsive protein kinase (28) and a type 1 protein phosphatase (PP1) that assembles with the transporter’s NH2-terminal domain (10, 45).

Another factor that has emerged as a key negative feedback regulator of NKCC is cytosolic Cl– concentration ([Cl–]i) (3, 4, 12, 14, 15, 24, 29, 36, 46, 48, 49, 56, 58), yet the characteristics and mechanism of this regulation remain poorly understood. The present experiments assess whether Cl– ions act allosterically on the transporter itself or on the upstream biochemical reactions that determine its volume-dependent phosphorylation. The results indicate that cytosolic Cl– shifts the set point for activation of NKCC and KCC to smaller volumes. We used this characteristic of the set point to confirm that it determines the steady-state volume of the cell, and we discuss the possible physiological implications of its modulation by Cl–. The results also suggest that the major effect of Cl– on NKCC is exerted through the volume-sensitive kinase that...
phosphorylates the transport protein. Finally, we present evidence that the volume set point is not influenced by the shifts in cytosolic ionic strength (\(\Gamma_u\)) that accompany cell shrinkage and swelling.

**EXPERIMENTAL PROCEDURES**

**Materials.** \(^{86}\)RbCl was obtained from Dupont NEN, stau-rorosporine and calcylin A were from Biomol or LC Labs, protease inhibitors were from Boehringer-Mannheim, 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS) was from Aldrich Chemical, and reagent grade chemicals, \(\text{NaCl}\), \(\text{KCl}\), \(\text{Na}_{2}\text{PO}_4\), \(\text{KCl}\), and 1-propanesulfonate (CHAPS), ouabain, and nystatin were from Sigma. Salts of methanesulfonic acid (>99%; Fluka) were prepared by neutralization with \(\text{NaOH}\), KOH, or \(\text{N-methyl-d-glucamine}\) (NMDG). Monoclonal antibody T14, directed against the carboxy-terminal 310 amino acids of the human colonic Na-K-Cl cotransporter, was developed and used as described previously (27, 30). Hybridoma cells were grown in the ascites of pristane-primed severe combined immunodeficiency mice (Taconic CB-17 Fox-Chase SCID).

**Preparation of red blood cells.** Blood was drawn from the brachial vein of White Pekin ducks (\(\text{Anas platyrhynchos}\)) into heparinized syringes. After centrifugation, the plasma and ouabain with trace \(^{86}\)Rb and various concentrations of succrose were removed. The cells were resuspended in 30 ml of \(\text{NaCl}\), \(\text{KCl}\), and \(\text{Na}_{2}\text{PO}_4\) with \(\text{MgSO}_4\) and \(0.1\) \(\text{NaHPO}_4\) with 45 \(\mu\)g/ml nystatin in 250 \(\mu\)M DNDS, which does not affect NKCC or KCC activity.

**Alteration of cytosolic Cl\textsuperscript{−}.** Cells were incubated in DFS containing methanesulfonate (MSA\textsuperscript{−}) or sulfamate (SFM\textsuperscript{−}) in place of Cl\textsuperscript{−} for 30 min at 41°C. These monovalent sulfonates equilibrate rapidly (\(<30\) s) across the duck red blood cell membrane via AE1-mediated anion exchange (26) without altering cell volume or transmembrane Na\textsuperscript{+}, K\textsuperscript{+}, or H\textsuperscript{+} distributions (44). In circumstances in which Cl\textsuperscript{−} was replaced by MSA\textsuperscript{−} or SFM\textsuperscript{−} on one side of the membrane only, anion gradients and cell pH were stabilized by inhibition of anion exchange with 250 \(\mu\)M DNDS, which does not affect NKCC or KCC activity.

**Alteration of cytosolic ionic strength.** The electrolyte content of duck red blood cells was altered by equilibration dialysis with nystatin (7). Cells were permeabilized by incubation in an ice-cold medium containing (in mM) 115 KCl, 50 sucrose, 7.5 Na-HEPES (pH 7.65 at 23°C), 5 glucose, 1 \(\text{MgSO}_4\), and 0.1 \(\text{NaHPO}_4\) with 45 \(\mu\)g/ml nystatin. After 20 min, the cells were transferred to in an otherwise identical “leading medium” containing either 45 KCl or 45 ClCl plus 135 K-MSA and incubated for 60 min at 4°C, with viable cells with half-normal or twice-normal ionic strength, respectively, and physiological cell [Cl\textsuperscript{−}] and water content. To restore native membrane permeability, the cells were washed four times at room temperature in the same loading medium containing 0.4% bovine serum albumin instead of nystatin. The dependence of NKCC activity on cell volume was measured by incubating the cells for 10 min at 41°C in flux media containing (in mM) 40 NaCl, 5 KCl, 7.5 Na-TEA (pH 7.4), 5 glucose, 1 \(\text{MgSO}_4\), 0.1 NaHPO\textsubscript{4}, and 0.025 ouabain with trace \(^{86}\)Rb and various concentrations of succrose. For cells loaded with extra electrolyte (135 mM K-MSA), the flux media were supplemented with 135 mM NMDG-MSA to preserve osmotic balance. Bumetanide-sensitive \(^{86}\)Rb influx was plotted as a function of intracellular water content, which was measured on paired cell samples.

**RESULTS**

The set point for osmotic activation of NKCC depended on [Cl\textsuperscript{−}]. (Fig. 1). When extracellular [Cl\textsuperscript{−}] was maintained at 150 mM and cytosolic Cl\textsuperscript{−} was ex-
changed with MSA\(^-\), the relation between NKCC activity and cell water content shifted to the right and upward (Fig. 1A). Because this maneuver does not significantly alter cytosolic \(\Gamma_c\), osmolarity, [K\(^+\)], [Na\(^+\)], pH, or Donnan charge (26, 44), the causative factor appeared to be the Cl\(^-\) anion itself. Experiments conducted on control cells (in the absence of DIDS) indicated that the effect is rapidly reversible (data not shown). Replacement of Cl\(^-\) on both sides of the membrane produced a similar shift in the relation between cell water content and NKCC protein phosphorylation (Fig. 1B), whereas replacement of extracellular Cl\(^-\) alone had no detectable effect (data not shown). Thus cytosolic Cl\(^-\) influences the reactions that couple NKCC phosphorylation to cell volume.

Certain anions of the lyotropic series are known to perturb cellular processes through their effects on protein charge, conformation, and aggregation (9). In red blood cells, for example, substitution of Cl\(^-\) with NO\(_3\)\(^-\), I\(^-\), or SCN\(^-\), but not with MSA\(^-\) or SFA\(^-\), increases the net negative charge on intracellular protein, causing a loss of permeant anions, a decrease in cell volume, and an acidic shift in cytosolic pH (44). Despite their disruptive nature, NO\(_3\)\(^-\) and SCN\(^-\) have been commonly used as replacements for Cl\(^-\) in studies of volume-responsive ion transport. To determine whether the effects we observed here are caused by removing Cl\(^-\) or by introducing the replacement anion, we compared the effects of MSA\(^-\), NO\(_3\)\(^-\), and SCN\(^-\) on the volume set point. Each of these anions equilibrates within seconds across the red blood cell membrane via AE1, but none is transported by NKCC (17, 26). When 75% of cellular Cl\(^-\) was exchanged with NO\(_3\)\(^-\) or SCN\(^-\), much greater degrees of shrinkage were required to activate NKCC (Fig. 2). The effect of anions on the set point (expressed as 1 cell water/kg cell solid) followed a typical “lyotropic” sequence: MSA\(^-\) (1.6) > Cl\(^-\) (1.5) > NO\(_3\)\(^-\) (1.3) > SCN\(^-\) (1.1). Besides shifting the set point to smaller volumes, NO\(_3\)\(^-\) and SCN\(^-\) also appeared to inhibit ion translocation via NKCC (bumetanide-sensitive \(^{86}\)Rb influx) in maximally shrunken cells.

When duck red blood cells are equilibrated in an artificial isotonic medium, they eventually adopt a water content at which both NKCC and KCC are essentially inactive (16). In this “resting” state, NKCC units remain partially phosphorylated, presumably at sites necessary but not sufficient alone to trigger ion translocation (27, 28). Replacement of Cl\(^-\) with NO\(_3\) reduced this basal phosphorylation and the increment in phosphorylation elicited by norepinephrine 85% and by cell shrinkage 49% (Fig. 3). By contrast, replacement
with MSA\(^-\) (Figs. 1–3) or SFA\(^-\) (Fig. 3) promoted NKCC phosphorylation in resting cells but evoked no further phosphorylation of NKCC units already stimulated by cell shrinkage or norepinephrine. NKCC phosphorylation, whether evoked by cell shrinkage, norepinephrine, or Cl\(^-\) replacement, decreased to undetectable levels within 10 min after inhibition of kinase activity with 1 mM N-ethylmaleimide or 5 μM staurosporine (data not shown). These results indicate that Cl\(^-\), and to a greater degree, NO\(_3\) discourages phosphorylation of NKCC.

The relation between [Cl\(^-\)], and spontaneous NKCC activity is shown in Fig. 4. After altering [Cl\(^-\)],, \(^{86}\)Rb influx via NKCC was assayed in a Cl\(^-\) medium containing DNDS (to minimize AE1-mediated dissipation of the Cl\(^-\)/MSA\(^-\) gradients). NKCC activity increased as [Cl\(^-\)] decreased below ~60 mM. Because Cl\(^-\) participates directly in the cotransport reaction as a transported substrate, part of its effect could reflect \textit{trans}-inhibition, a characteristic of many cotransport mechanisms with ordered substrate binding (54). To distinguish whether the inhibitory effect of [Cl\(^-\)] on inward cotransport is kinetic or regulatory in nature, the experiment was repeated on cells treated with calyculin A, a potent inhibitor of PP1 and PP2A (18). By preventing NKCC dephosphorylation, this agent renders the cotransporter maximally active and refractory to the deactivating influences of cell swelling, N-ethylmaleimide, or staurosporine (27). With NKCC fixed in its phosphorylated form, changes in [Cl\(^-\)] had a much smaller influence on bumetanide-sensitive \(^{86}\)Rb influx (Fig. 4). These results substantiate earlier conclusions, based on ion flux studies with squid axon, that the predominant effect of [Cl\(^-\)] on inward cotransport is regulatory and not related to titration of the internal Cl\(^-\)-transport sites (4, 50).

If shrinkage-activated NKCC and swelling-activated KCC respond to the same signal of volume change, alterations in [Cl\(^-\)] might exert tandem effects on their respective set points. Increasing [Cl\(^-\)] from 0 to 70 mM should reduce the set point from ~1.95 to ~1.55 l water/kg cell solid (Fig. 1). To test this prediction, the relation between [Cl\(^-\)] and KCC activity was determined in cells osmotically swollen to 1.95 l water/kg cell solid. As predicted, KCC activity was high in Cl\(^-\)-rich cells (where the set point < cell volume) yet absent in Cl\(^-\)-free cells (where the set point = cell volume) (Fig 5). Comparable results were obtained with SFM\(^-\) rather than MSA\(^-\) in place of Cl\(^-\). Although the [Cl\(^-\)] supporting half-maximal KCC activity (~10 mM) appeared to be somewhat lower than that yielding half-maximal NKCC activity (~30 mM; Fig. 4), the two values cannot be compared directly because they were obtained under different experimental conditions. The fact that KCC activity was measured in swollen cells whereas NKCC activity was measured at normal volume could be especially important, because work on the squid axon (3) demonstrated that cell swelling shifts the relation between NKCC activity and [Cl\(^-\)] to lower concentrations (see Discussion). These results add [Cl\(^-\)] to the list of physiological factors (norepinephrine, deoxygenation, [Mg\(^{2+}\)]) known to produce concerted shifts in the volume dependence of NKCC and KCC in duck red blood cells.

If the coordinate operation of NKCC and KCC actually determines the physiological volume of the cell, increasing the set point by reducing [Cl\(^-\)] should bring about a corresponding increase in steady-state water content. To test this hypothesis, duck red blood cells were incubated in a synthetic, isotonic medium similar to plasma for 1 h—long enough for them to adopt and

![Fig. 3. Effect of different replacement anions on NKCC phosphorylation. Cells were incubated for 12 min in media containing Cl\(^-\), MSA\(^-\), NO\(_3\), or sulfamate (SFM\(^-\)) salts. Where designated, NKCC was activated by 10 μM norepinephrine (+NE) or cell shrinkage (+100 mM sucrose). NKCC phosphorylation was normalized to values in isotonic Cl\(^-\) medium. Data represent means ± SE from no. of experiments indicated above columns.](http://ajpcell.physiology.org/)

![Fig. 4. Concentration dependence of [Cl\(^-\)] effect on NKCC activity. [Cl\(^-\)] was altered by preincubation for 12 min in isotonic media containing various concentrations of MSA\(^-\) in place of Cl\(^-\). After 7 min, calyculin A (0.2 μM) was added to half of the cell suspension to fix NKCC in its phosphorylated state. Both sets were then incubated for 1 min in a volume medium containing 155 mM Cl\(^-\), 250 μM 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS; to inhibit Cl\(^-\)/MSA\(^-\) exchange), and trace \(^{86}\)Rb. Bumetanide-sensitive \(^{86}\)Rb influx rates are expressed as % of maximal values, consistently observed with calyculin-stimulated Cl\(^-\)-free cells. [Cl\(^-\)] was estimated as the product of extracellular [Cl\(^-\)] ([Cl\(^-\)]) and anion equilibrium distribution ratio (0.64). Curves were drawn by eye. Data represent averages ± SD from 3 experiments.](http://ajpcell.physiology.org/)
maintain the so-called “lower steady-state” volume (47) or “volume set point” (40) at which both NKCC and KCC are minimally active (31, 16). To remove potential thermodynamic and kinetic constraints on ion uptake via NKCC, extracellular K⁺ concentration ([K⁺]₀) was raised from 6 mM to 10 mM. This maneuver, by itself, does not affect the volume of resting cells but promotes rapid swelling if NKCC is made active by hormonal (norepinephrine) or osmotic stimulation (31). Thus under these experimental conditions, the extent to which NKCC affects cell volume is determined by regulatory rather than thermodynamic or kinetic factors. Cells were then incubated for 3 h in isotonic media containing different concentrations of Cl⁻, 156, 78, or 31 mM. Because partial substitution of Cl⁻ with MSA⁻ does not alter the distribution ratios ([ion]ᵢ/[ion]ₒ) of Na⁺, K⁺, or Cl⁻ (44), the net chemical potential for inward NKCC should be equivalent in all three batches of cells, as should that for outward KCC. As predicted, reprogramming the set point to larger volumes by adding or depleting twofold in electrolyte by the technique of nystatin dialysis. To avoid ancillary effects of anions, [Cl⁻]ᵢ was held constant at 30 mM and Γᵢ was varied by adding or omitting the inert ion pair K⁺-MSA⁻. Changing Γᵢ over a very broad range had no significant effect on the volume set point for activation of NKCC (Fig. 7).

An immediate effect of osmotic perturbation is dilution or concentration of cytosolic salt. It has been asserted that changes in Γᵢ initiate or modulate volume regulatory responses (6, 13, 57), and in red blood cells there is evidence that Γᵢ influences the volume set point for swelling-activated K-Cl cotransport (43, 13) and shrinkage-activated Na/H exchange (43). To evaluate this possibility, we compared the relation between NKCC activity and cell volume in cells enriched twofold or depleted twofold in electrolyte by the technique of nystatin dialysis. To avoid ancillary effects of anions, [Cl⁻]ᵢ was held constant at 30 mM and Γᵢ was varied by adding or omitting the inert ion pair K⁺-MSA⁻. Changing Γᵢ over a very broad range had no significant effect on the volume set point for activation of NKCC (Fig. 7).

Two-dimensional phosphopeptide maps of NKCC protein isolated from osmotically shrunken 32P-labeled cells showed a distinctive pattern of tryptic phosphopeptides similar to those obtained previously (27), consistent with heterogeneous phosphorylation at multiple sites. NKCC units phosphorylated by cell shrinkage and by Cl⁻ removal yielded qualitatively indistinguishable maps (Fig. 8). Twelve discrete spots, designated spots 1–12 in Fig. 8, were detected with both stimuli. The relative intensity of the major spots was consistent between experiments. One exception was spot 11, which appeared in some but not all samples of NKCC isolated from shrunken cells. These results suggest that Cl⁻ removal and cell shrinkage promote phosphorylation of a similar constellation of regulatory sites, and by inference, act ultimately through the same protein kinase on NKCC. This view is consistent with our finding that one form of stimulation precludes further NKCC phosphorylation by the other (Fig. 3) and that both stimuli are equipotently blocked (IC₅₀≈ 0.5 μM) by staurosporine (data not shown).

NKCC is deactivated by a PP1 that associates with its NH₂-terminal domain (45, 10). Because the phos-
phosphorylation state of NKCC reflects a competition between concurrent kinase and phosphatase activities (45, 27), net phosphorylation could result from kinase stimulation, phosphatase inhibition, or a combination of both. To determine whether Cl\(^-\) depletion stimulates the kinase, as does cell shrinkage (27, 28), we compared the rate at which NKCC becomes active in cells containing either Cl\(^-\) or MSA\(^-\) after adding calyculin A (Fig. 9). This approach takes advantage of the fact that calyculin A enters the duck red blood cell within seconds and blocks ongoing dephosphorylation of NKCC at all volume-sensitive Ser/Thr residues (27), presumably by inhibiting the transporter’s PP1 subunit. As we found previously (28), calyculin A evoked a progressive increase in both NKCC activity and NKCC protein phosphorylation (Fig. 9), which conformed to a logistic function describing a positive feedback model. In earlier work (28) we showed that the process includes an initial phase (between 0 and 2 min) that depends strongly on cell volume and a secondary phase that does not. Like cell shrinkage, replacement of intracellular Cl\(^-\) with MSA\(^-\) at a normal volume had only a minor influence on the ultimate level of NKCC activity evoked by calyculin A (Fig. 4) whereas it markedly increased the initial rate at which this level was reached. If the initial phase of activation by calyculin A accurately reflects the activity of the volume-sensitive protein kinase that phosphorylates NKCC, the data indicate that this kinase is much more active in cells containing MSA\(^-\) in place of Cl\(^-\) with the same volume, osmolarity, Donnan charge, ionic strength, pH, and protein concentration.

To assess whether Cl\(^-\) stimulates the PP1 subunit, we measured the influence of various anions on NKCC dephosphorylation in vitro. Cells were equilibrated with \(^32\)P and osmotically shrunk to label NKCC. When these cells were lysed in 5 volumes of warm permeabilization buffer, the cotransport protein lost \(^32\)P rapidly (Fig. 10A). Dephosphorylation could be prevented by adding either 0.1 \(\mu\)M calyculin A (Fig. 10A) or 7 \(\mu\)g/ml calyculin A (Fig. 10A) or 7 \(\mu\)g/ml.
DISCUSSION

Our results substantiate the concept that NKCC and KCC are reciprocal regulated by a negative feedback system that is dually modulated by cell volume and [Cl\^\textsuperscript{-}]. The data demonstrate that intracellular Cl\^\textsuperscript{-} alters the volume dependence of NKCC by inhibiting the shrinkage-stimulated protein kinase that converts the cotransport protein into an active form. Anions with greater chaotropic character (Cl\^\textsuperscript{-} < NO\textsubscript{3}\textsuperscript{-} < SCN\textsuperscript{-}) cause the cell to behave as if increasingly swollen, apparently by inhibiting the same volume-sensitive kinase.

Since its original description in squid axon (49), modulation by [Cl\^\textsuperscript{-}] has become recognized as a common if not universal property of NKCC in animal cells (2, 4, 12, 14, 15, 24, 29, 36, 46, 48, 56, 58). The present work demonstrates that [Cl\^\textsuperscript{-}] exerts tandem effects on shrinkage-induced NKCC and swelling-induced KCC by shifting their shared volume set point. A growing body of evidence suggests that other volume-responsive ion transport pathways are likewise affected by Cl\^\textsuperscript{-} and foreign anions. For example, like NKCC, activation of Na/H exchange by cell shrinkage is repressed if Cl\^\textsuperscript{-} is replaced with NO\textsubscript{3}\textsuperscript{-} or SCN\textsuperscript{-} (1, 11, 19, 39, 42, 43), whereas substitution with less disruptive or impermeant anions like gluconate has the opposite effect (48). Replacing Cl\^\textsuperscript{-} with NO\textsubscript{3}\textsuperscript{-} or SCN\textsuperscript{-} also reduces the degree of swelling required to activate K-Cl cotransport (23, 42) and Na/Ca exchange (38) in red blood cells. These observations reinforce the concept that volume-activated transporters are coordinately regulated by a common system of sensors and transducers (40).

Our finding that [Cl\^\textsuperscript{-}] modulates the volume dependence of NKCC is fully consistent with observations made by Breitweiser and colleagues (3, 4). Working on the internally dialyzed squid giant axon, they demonstrated that ion influx via NKCC is tonically suppressed by physiological levels of [Cl\^\textsuperscript{-}], and that cell shrinkage relieves this inhibition by shifting the relation between [Cl\^\textsuperscript{-}] and ion influx. Our data, when plotted in the same manner, indicate a similar effect. Thus in both squid axon and duck red blood cell, the regulation of NKCC by cell volume and [Cl\^\textsuperscript{-}] is mutually interdependent, i.e., each signal influences the set point of the other. This linkage is consistent with our evidence that the two signals converge on a protein kinase that phosphorylates NKCC.

The effect of Cl\^\textsuperscript{-} substitution depends on the replacement anion used. Relatively inert anions like MSA\textsuperscript{-} and SFM\textsuperscript{-} (44) cause the duck red blood cell to perceive itself shrunken at normal volume, whereas chaotropic anions like NO\textsubscript{3}\textsuperscript{-} and SCN\textsuperscript{-} have the opposite effect. The sequence of anion effects on the set point regulating NKCC and KCC corresponds to a typical lyotropic series: MSA\textsuperscript{-} < Cl\^\textsuperscript{-} < NO\textsubscript{3}\textsuperscript{-} < SCN\textsuperscript{-}. Besides affecting its biochemical activation, NO\textsubscript{3}\textsuperscript{-} and SCN\textsuperscript{-} seem to interfere with ion translocation by NKCC as their presence reduces the rate of inward cotransport even after changes in phosphorylation are prevented with calyculin A.

How anions affect the generation or the transmission of the volume signal to NKCC and KCC remains unclear. Both Cl\^\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} appear to inhibit NKCC by impeding its phosphorylation without affecting its de-
phosphatase-1 in vitro. Cells were prelabeled with $^{32}$P by incubation for 3 h with $[32P]$orthophosphate and then rendered shrunken by NKCC phosphorylation. Cells were then incubated in 5 vols of “lysis medium” containing 25 mM Na-TES (pH 7.3), 2 mM MgSO$_4$, 1 mM EGTA, 0.1% Triton X-100, and 50 µM ouabain at 30°C. Aliquots of lysed cells were removed at the indicated times. The $^{32}$P content of NKCC protein in each aliquot was determined by immunoprecipitation and autoradiography. A: time course of NKCC dephosphorylation after cell lysis; curve defines fit to a single exponential function. B: rate of dephosphorylation in lysis medium supplemented with 150 mM Na$^+$ salt of Cl$^-$, MSA$^-$, or NO$_3^-$ or with 10 mM NaF.

Fig. 10. Cl$^-$ does not affect dephosphorylation of NKCC by protein phosphatase-1 in vitro. Cells were prelabeled with $^{32}$P by incubation for 3 h with $[32P]$orthophosphate and then rendered shrunken by incubation in a hypertonic (400 mosmol/kg H$_2$O) medium to promote NKCC phosphorylation. Cells were then incubated in 5 vols of “lysis medium” containing 25 mM Na-TES (pH 7.3), 2 mM MgSO$_4$, 1 mM EGTA, 0.1% Triton X-100, and 50 µM ouabain at 30°C. Aliquots of lysed cells were removed at the indicated times. The $^{32}$P content of NKCC protein in each aliquot was determined by immunoprecipitation and autoradiography. A: time course of NKCC dephosphorylation after cell lysis; curve defines fit to a single exponential function. B: rate of dephosphorylation in lysis medium supplemented with 150 mM Na$^+$ salt of Cl$^-$, MSA$^-$, or NO$_3^-$ or with 10 mM NaF.

phosphorylation. This view is compatible with evidence from the squid axon that phosphatase inhibition with okadaic acid stimulates inward cotransport only if [Cl$^-_i$] is reduced (2). Our evidence that low [Cl$^-_i$] and cell shrinkage promote phosphorylation of NKCC in a nonadditive manner at common sites implicates a single protein kinase modulated jointly by [Cl$^-_i$] and cell volume. If osmotic activation of this kinase involves macromolecular crowding or molecular confinement, anions like Cl$^-$ and NO$_3^-$ could alter the structure or hydration of proteins and hence their ability to crowd or be crowded (41, 5). An alternative explanation is that Cl$^-$ ions discourage phosphorylation of NKCC by interacting with the cotransporter itself. For example, titration of one or both internal Cl$^-$ transport sites could induce a conformation that is less accessible to the shrinkage-stimulated kinase.

A curious feature of NKCC in most cells is that it responds much more strongly to an episode of shrinkage caused by a loss of KCl and water (isosmotic shrinkage) than to one caused by a loss of water alone (hypertonic shrinkage). As noted by O’Neill (35), this can be explained by differences in [Cl$^-_i$]; the decrease in [Cl$^-_i$] that attends isosmotic shrinkage would act to elevate the volume set point and thereby amplify the perceived degree of shrinkage, whereas the increase in [Cl$^-_i$] that attends hypertonic shrinkage would blunt the volume signal.

Cl$^-$ may affect cotransport activity beyond its influence on thermodynamic driving force (36, 35) and NKCC phosphorylation (20). In endothelial cells, shrinkage stimulates $K^+$ influx via NKCC (36, 33) along with NKCC phosphorylation (21, 34). Curiously, net salt uptake is not evident, even though energetically favorable, unless [Cl$^-_i$] is decreased (36, 20). A similar phenomenon has been noted in Ehrlich ascites tumor cells (25). O’Neill (35) proposed that physiological [Cl$^-_i$] impedes reorientation of the unloaded transporter, compelling the transporter to engage in an unproductive exchange of extracellular for intracellular ions instead of net salt uptake. Reducing [Cl$^-_i$] would eliminate this kinetic barrier and permit net salt movement. The proposed action of [Cl$^-_i$] is analogous to that of aerobic metabolism on the antiport/uniport switch mechanism of glucose transporters in avian red blood cells (8). If this form of regulation by [Cl$^-_i$] exists, it is not apparent in duck red blood cells, where the net uptake and the Na/Na exchange modes of cotransport change in fixed proportion as [Cl$^-_i$] is varied from 6 to 90 mM (26).

Animal cells seem capable of detecting not only the severity of a volume perturbation but also its cause (13, 32, 35, 55). For example, trout red blood cells engage different volume-regulatory mechanisms depending on whether swelling is caused by a gain of KCl plus water or of water alone (13, 32). Hypotonic swelling, which lowers [Cl$^-_i$], evokes a pathway for efflux of organic osmolytes that resembles the volume-sensitive organic anion channel VSOAC (6, 57). In contrast, isosmotic swelling, which raises [Cl$^-_i$], triggers only K-Cl cotransport. Although the differential response has been attributed to changes in $\Gamma_i$, the experimental findings do not exclude the possibility that the discriminating parameter is instead [Cl$^-_i$]. Our finding that the volume set point varies inversely with [Cl$^-_i$] could explain why KCC is more responsive to isotonic vs. hypotonic swelling.

Parker et al. (43) reported that the set point common to swelling-activated KCC and shrinkage-activated Na/H exchange in dog red blood cells varies inversely with $\Gamma_i$. Analogous effects of $\Gamma_i$ were not apparent in
duck red blood cells: raising or lowering $\Gamma_i$ twofold produced no significant change in the volume set point. The discrepancy might be explained by their use of NO$_3^-$ vs. our use of MSA$^-$, to elevate $\Gamma_i$. In duck red blood cells, NO$_3^-$ reduces the volume set point to an even greater degree than Cl$^-$ itself through a mechanism that is entirely independent of $\Gamma_i$ (Fig. 2).

The relevance of volume-activated transport processes in vitro to volume homeostasis in vivo remains uncertain (35, 40). Two observations suggest that duck red blood cells seek their volume set point through the controlled operation of NKCC and KCC. First, when cells from freshly drawn blood are incubated in a synthetic, isotonic medium similar to plasma, they gradually shrink $\sim 5\%$ to the so-called lowered steady-state volume (47, 16). The loss of salt and water is mediated by KCC (16) and is associated with a corresponding shift in the set point to the lower steady-state volume that occurs when the cells are removed from the influence of endogenous plasma catecholamines (52). Second, when the set point is experimentally altered by manipulating [Cl$^-$], the cells adopt and maintain the new set point volume (Fig. 6).

The effect of Cl$^-$ on the volume set point for NKCC and KCC in duck red blood cells is especially powerful in the range of [Cl$^-$] normally found in other vertebrate animal cells (10–50 mM). If other cells employ a cognate system of volume sensors and effectors, physiological fluctuations of [Cl$^-$] could have an important influence on their volume set point and hence water content. Cells would perceive their volume as lower in circumstances in which [Cl$^-$] is reduced. This modulation could enable the cell to gauge not only the severity of the volume perturbation but also its underlying cause so as to implement a corrective strategy that restores both volume and [Cl$^-$]. For example, if the cell loses both KCl and water (isosmotic shrinkage), the attendant decrease in [Cl$^-$], would potentiate phosphorylation of NKCC and corrective salt uptake. On the other hand, if the cell loses only water (hypertonic shrinkage), the increase in [Cl$^-$], would desensitize NKCC to the shrunken state and avert further enrichment of cytolsolic Cl$^-$. In a similar manner, modulation of the response to cell swelling by [Cl$^-$], would help restore ionic balance, e.g., swelling due to Cl$^-$ uptake would potentiate activation of KCC. This modulation could function to stabilize [Cl$^-$], and defend against potentially deleterious changes in cytolsolic $\Gamma_i$.

Cl$^-$ might play a special role in red blood cells, which are much richer in Cl$^-$ (~74 mM) and protein (~8 mM hemoglobin) than most other animal cells. At this high concentration, the effect of Cl$^-$ on the volume set point is near maximal and flat. Although its effect is clearly not modulatory in the red blood cell, high [Cl$^-$] could act in a continuous manner to maintain the set point at a smaller volume. This could create the permissive environment necessary for red blood cells to package exceptionally high concentrations of soluble protein (hemoglobin). Thus, following the macromolecular crowding theory of cell volume perception (40), high [Cl$^-$] might serve to offset the effect of inordinate macromolecular crowding on the volume set point, which if unopposed, might make the red blood cell seek a swollen state with adverse rheological consequences. This teleological rationalization assumes that volume-sensing mechanisms based on macromolecular crowding are shared by red blood cells and other cells alike, which may not be so.

In summary, these results suggest that in duck red blood cells water content and [Cl$^-$], are maintained at interdependent set points by the coordinate control of separate KCl-loading (NKCC) and KCl-extruding (KCC) transporters. The cell's perception of its fluid volume is influenced by cytosolic [Cl$^-$], and vice versa, but not ionic strength. Information on cell volume and [Cl$^-$], appears to be transduced to NKCC by the same protein kinase. This regulation could enable the cell to gauge not only the severity of the volume perturbation but also its underlying cause so as to implement a corrective strategy that restores both volume and [Cl$^-$].

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