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Topical Review

$K^+ : Cl^-$ Cotransport: Sulfhydryls, Divalent Cations, and the Mechanism of Volume Activation in a Red Cell

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Introduction: The Phenomenon of Regulatory Volume Decrease (RVD)

Upon exposure to moderately dilute, hyposmotic Na^+ media many animal cells acutely swell due to rapid water entry (osmotic phase) and subsequently regain their original volume, a process called regulatory volume decrease (RVD) [48, 49]. The phenomenon of RVD was observed first in certain bird red cells [48], and then in other cells, most notably in Ehrlich ascites tumor cells [42], fish and *Amphiuma* red cells [15–17, 53], lymphocytes [37, 84] and epithelial cells of the cortical collecting tubules of rabbits [20, 36] and of the gallbladder of *Necturus* [52, 83]. With some minor exception, the prime osmolytes extruded across the plasma membrane during RVD in hyposmotic Na^+ media are K (K^+) and Cl (Cl^-) ions. The swiftness with which any of the above cells readjust their hyposmotically expanded volume back to the original volume depends largely on the transport modes utilized to lower intracellular KCl.

For convenience, Fig. 1 depicts three thermodynamically possible and experimentally proven transport modes effecting RVD in a model cell suspended in hyposmotic Na^+ media. The comparatively slowest RVD (i.e., within 1 hr) is mediated by electroneutral $K^+ : Cl^-$ cotransport (Fig. 1*A*), as for example in fish red cells [11, 15, 53] and in *Necturus* gallbladder [52]. A much faster RVD (i.e., within 5–10 min) is seen in *Amphiuma* red cells which possess a K^+/H^+ antiport (Fig. 1*B*) in parallel with a Cl^-/HCO_3^- exchanger [16, 17]. By far the fastest RVD (i.e., less than 5 min) takes place in lymphocytes [37] where K^+ and Cl^- apparently exit via different membrane potential-sensitive pathways (channels, *see* Fig. 1*C*).

Two out of three steps of RVD, common for all cells and studied thus far (i.e., in Fig. 1, step 1, the bulk water entry of the osmotic phase and cell swelling (broken line), and step 3 the shrinkage of the cell to its original volume as indicated by the solid line) will not further be considered here. Instead, emphasis will be placed on function 2, the activation and inactivation of K^+ fluxes effecting RVD. Of particular interest are data which shed light on the regulation of electroneutral $K^+ : Cl^-$ cotransport (Fig. 1*A*). Paradoxically, these data have been gathered from experiments with enucleated human [6, 23, 44, 62, 65, 94] and sheep [22, 25, 26, 55–60] erythrocytes whose total ouabain-resistant (OR) K^+ fluxes are minute ($t_{1/2}$ of OR cation equilibration is in the order of >24 hr for red cells of both species) and thus preclude exact measurements of RVD within reasonable times.

The absence of both Ca^{2+}-activated K^+ channels (Gardos effect [34]) and electroneutral $Na^+ : K^+ : 2Cl^-$ coupled cotransport has made the sheep red cell a most suitable model for extrapolations to regulation of similar transport activities in other cells. Again, as in much earlier work on the kinetic properties of the Na^+K^+ pump [54], it is the genetically low K^+ (LK) but not high K^+ (HK) red cell which emerges as the experimental system to obtain a glimpse at the possible mechanism regulating $K^+ : Cl^-$ cotransport in RVD. It will become clear that chemical modification of sulfhydryl (SH, thiol-) groups leads to stimulation of $K^+ : Cl^-$ cotransport, similar to that seen during cell swelling, and thus becomes an important tool to unravel the mystery of the mechanism regulating the activity of $K^+ : Cl^-$ fluxes during RVD. The model gradually

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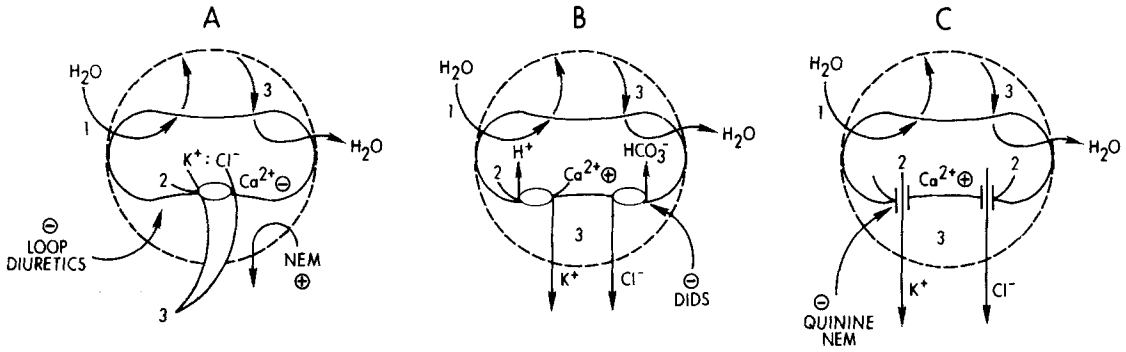

Fig. 1. K^+ flux modes regulating volume decrease by (*A*) $K^+ : Cl^-$ cotransport, (*B*) K^+/H^+ countertransport, and (*C*) electrogenic K^+ and Cl^- channels. Common events preceding activation of all three modes are: water entry and cell swelling (1) in hyposmotic media, the unknown trigger for flux activation (2) of each mode (3), and the accompanying regulatory volume return to the original volume. Activators = +, and inhibitors = −. NEM = N-ethylmaleimide; DIDS = 4,4′ disothiocyano-2,2′-stilbene-disulfonic acid. For further details *see text*

derived throughout this discourse has the character of a personal working hypothesis, as no report has dealt yet with the molecular mechanism of regulation of $K^+ : Cl^-$ cotransport. Recent advances in the general area of Cl^- mediated cation transport systems have just been reviewed [61].

Physiological Identity of $K^+ : Cl^-$ Cotransport

Thermodynamic Aspects

A relatively variable fraction of OR K^+ fluxes can be attributed to the presence of Cl^--dependent K^+ transport, also called $K^+ : Cl^-$ cotransport. The driving forces for such a coupled system are given by

$$\Delta\mu^{net}_{K^+:Cl^-} = n\Delta\mu_{K^+} + p\Delta_{Cl^-} = RT \ln \frac{[K^+]^n_o\,[Cl^-]^p_o}{[K^+]^n_i\,[Cl^-]^p_i}, \quad (1)$$

where R and T have the usual meanings, and the letters n and p are stoichiometric factors/exponents. It is apparent that, for example, K^+ may be driven by the Cl^- (driver ion) outwardly or inwardly and uphill against its own chemical gradient when the ionic product is greater on the inside, i, or outside, o, of the membrane. The experimental fact, i.e., that Cl^- drives K^+, is difficult to establish due to the high activities of electroneutral Cl^- exchange operating in most membranes studied. (Naturally, in the case of coupled $K^+ : Cl^-$ cotransport as defined by Eq. (1), the membrane potential term is omitted.) Net $K^+ : Cl^-$ outward cotransport can be measured under zero-*trans* conditions, i.e., in the absence of extracellular K^+ in isosmotic media. When the product terms of Eq. (1) are equal in the nominator and denominator, net $K^+ : Cl^-$ cotransport is zero. Experimentally, this point has been determined only in a few cases. In LK sheep red cells net K^+ flux was zero near 15 mM $[K^+]_o$, i.e., at $[K^+]_i/[K^+]_o$ and $[Cl^-]_o/[Cl^-]_i$ ratios of ≈1.4 K^+ was close to chemical equilibrium [55]. In red cells of the Muscovy duck, $[K^+]_o$ was estimated to be 75 mM at close to zero K^+ net flux [48].

Kinetic Properties

For a kinetically clean evaluation of $K^+ : Cl^-$ cotransport it is necessary to correct for OR K^+ fluxes which occur via other mechanisms such as electrodiffusion or countertransport. Of extraordinary convenience is the fact that $K^+ : Cl^-$ cotransport essentially requires the presence of Cl^- and that only Br^- is able to replace Cl^-, while other anions of the Hofmeister series (NO_3^-, I^-, SCN^-) inhibit K^+ movements through the system [22, 23, 25, 26, 50, 55, 62]. Therefore, in cells equilibrated with, for example, NO_3^-, OR K^+ influxes are much lower and approximately linear functions of $[K^+]_o$ [55] and thus thought to represent ion movements in terms of electrodiffusional "ground permeability" [67]. In the presence of Cl^-, the relationship of K^+ transfluxes to $[K^+]_o$ on the *cis* side of the membrane is nonlinear because it derives from the linear component of the K^+ ground fluxes just described and the hyperbolic Cl^--dependent K^+ flux ($K^+ : Cl^-$ cotransport). Equation (2) shows that *cis*, *c*, to *trans*, *t*, K^+ transport specifically through the $K^+ : Cl^-$ cotransporter, $(ctJ^{OR}_K)_{\Delta Cl}$, may be defined as follows:

$$(ctJ^{OR}_K)_{\Delta Cl} = (ctJ^{OR}_K)_{Cl} - [(ctk^{OR}_K)_{NO_3}\,[K^+]_c] \quad (2)$$
$$\text{(A)} \qquad \text{(B)} \qquad \text{(C)}$$

where (A) = $\frac{(ctJ_K^{OR})^{max}_{\Delta Cl}\,[K^+]_c}{K^{K_c^+}_{0.5} + [K^+]_c}$, (B) = OR *cis-trans* (*ct*) K^+ flux in Cl^-, and (C) = the product of the pseudo-first order rate constant, ct_{kK}^{OR}, for *ct* K^+ flux in NO_3^- media and the K^+ concentration on the *cis* side, $[K^+]_c$. The half-maximum saturation constant for $[K^+]_c$, $K^{K_c^+}_{0.5}$ has been found to be in the range of 17–30 mM for LK sheep [55] and human [44] red cells, hence reflecting a low affinity for K^+ compared to other K^+ transporting systems such as the Na^+K^+ pump.

The half maximum saturation concentration for Cl^- has not been determined unequivocally. This fact is due to the experimental difficulty of separating true effects due to Cl^- replacement at the transport site from allosteric modulations or effects of the chaotropic replacement anions on the function of the $K^+ : Cl^-$ cotransporter. This dilemma is born out by relationships of $(ctJ_K^{OR})_{\Delta Cl}$ being sigmoidal [23], hyperbolic-convex or -concave, or linear with respect to $[Cl^-]_o$ for thiol-activated $K^+ : Cl^-$ flux [59]. Nevertheless, based on preliminary measurements for various anions to inhibit $K^+ : Cl^-$ cotransport, the following anion ranking of decreasing transport activity has been established for human and LK sheep red cells alike: $Br^- \geq Cl^- \gg NO_3^- = I^- = SCN^-$ [23, 26, 59]. The physicochemical basis of the specific Cl^- requirements of $K^+ : Cl^-$ cotransport are unknown.

Inhibitors and Other Determinants

Good inhibitory substances, specific for $K^+ : Cl^-$ cotransport at low concentrations and not interferring with other ion transport systems are not available. Much use has been made of furosemide, which at concentrations of greater than 10^{-4} M may inhibit $K^+ : Cl^-$ cotransport to the extent of Cl^- replacement by NO_3^- [58], but at these drug concentrations interference with Cl^-/HCO_3^- exchange also occurs [13]. Even utilizing the augmenting role of Rb^+ (or K^+) to increase the inhibitory effect [58], the low affinity of $K^+ : Cl^-$ cotransport for furosemide (and bumetanide) excludes its use to establish the molecular identity of the carrier. Nevertheless, the existence of a specific antibody sharply reducing $K^+ : Cl^-$ cotransport in LK sheep red cells has been reported [59, 71]. Interestingly, this antibody, anti-L_L, is present together with anti-L_p, an antibody against the Na^+K^+ pump of the same cells, in alloimmune sera prepared from HK sheep injected with LK sheep red cells (*see* references in [54]). As will be seen below, anti-L_L has been an important tool to establish the identity of $K^+ : Cl^-$ cotransport activated by several mechanisms in LK sheep red cells.

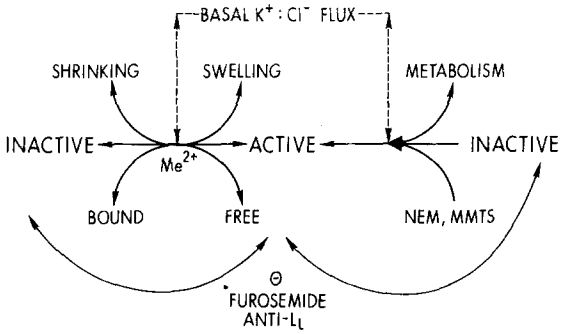

Fig. 2. Operational equilibrium states and modulators of $K^+ : Cl^-$ cotransport in low K^+ sheep red cells. NEM = N-ethylmaleimide; MMTS = methylmethanethiolsulfonate. For details *see text*

Modes of Activation or Inactivation of $K^+ : Cl^-$ Cotransport

General Aspects

Quantitative assessments of K^+ fluxes through the Cl^--dependent and furosemide-sensitive pathways in *isosmotic* media are largely lacking. This is very unfortunate since one would like to correlate "basal" (i.e., not stimulated by osmotic challenge, etc.) with stimulated $K^+ : Cl^-$ cotransport. This is a kinetic and not a thermodynamic question. Figure 2 provides an overview of the operational equilibria of $K^+ : Cl^-$ cotransport in LK sheep red cells to be discussed in this review. It can be seen that various effectors either may inactivate (cell shrinkage or bivalent metal ion (Me^{2+}) binding) or activate (cell swelling, Me^{2+} debinding, N-ethylmaleimide (NEM) or methylmethane thiosulfanote (MMTS)) OR $K^+ : Cl^-$ cotransport to below or above a "basal" activity. The inhibition of all activated $K^+ : Cl^-$ fluxes by loop diuretics or anti-L_L (*see below*) is evidence for molecular identity.

Figures 3 and 4 illustrate the major mechanisms discussed here, known to activate $K^+ : Cl^-$ cotransport in LK red cells and to some extent in other red cells: cell swelling (Fig. 3), thiolalkylation (Fig. 3) and debinding of Me^{2+} (Fig. 4). Figure 3 shows the typical effect of hyposmotic media on $K^+ : Cl^-$ efflux in LK sheep red cells reported earlier by others [22, 25, 26, 71]. After treatment with NEM a 10-fold OR $K^+ : Cl^-$ efflux stimulation is observed in isosmotic media [55, 66], which in hyposmotic media is augmented only to the extent of control cells (ΔV_B) and hence volume-insensitive [66]. Figure 4 shows the powerful activation of $K^+ : Cl^-$ cotransport by A23187 and a Me^{2+} chelator, while A23187 and Ca^{2+} are inhibitory in control cells. In NEM-treated cells A23187 and the chelator were unable to further

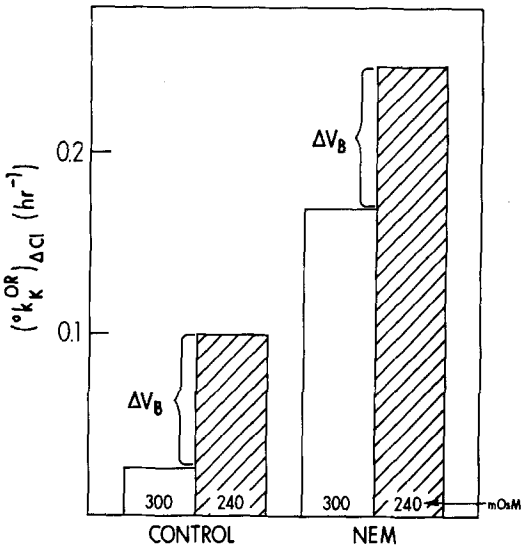

Fig. 3. Stimulation of $K^+ : Cl^-$ cotransport in LK sheep red cells by cell swelling in 240 mOsM Na-media in control and NEM-treated cells. $(^{o}k_K^{OR})_{\Delta Cl}$ = ouabain-resistant, Cl^--dependent K^+ efflux rate constant (hr^{-1}). Numbers = mOsmol/liter. ΔV_B is the swelling-induced $K^+ : Cl^-$ flux increment, which is identical in controls and NEM-treated cells, while the NEM-stimulation, here about sixfold, was volume-independent

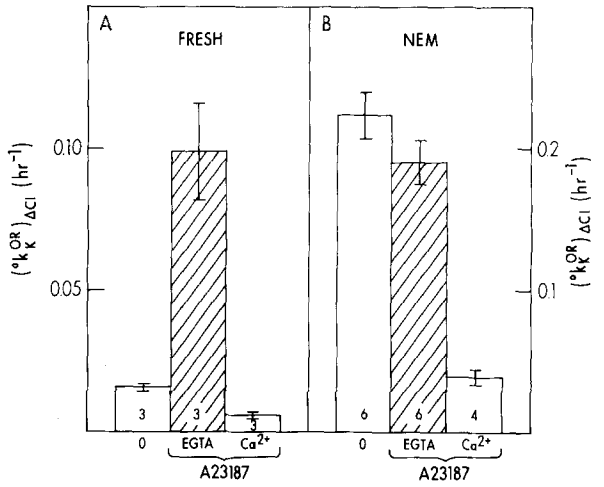

Fig. 4. The effect of divalent ionophore A23187 plus EGTA or plus Ca^{2+} on the rate constant of ouabain-resistant $K^+ : Cl^-$ efflux, $(^{o}k_K^{OR})_{\Delta Cl}$, in fresh (*A*) and NEM-treated (*B*) low K^+ sheep red cells. Numbers indicate experiments, bars ± SD. EGTA = ethylene-glycol-tetraacetic acid. 0 = no treatment-control, which was unaffected by EGTA and Ca^{2+} alone. Note that A23187 and EGTA stimulate $K^+ : Cl^-$ cotransport only in fresh but not in NEM-treated cells, while A23187 plus Ca^{2+} was inhibitory in both cases. There is a scale change on the right-hand abscissa

stimulate OR $K^+ : Cl^-$ transport, but A23187 and Ca^{2+} inhibited most of the NEM-stimulated $K^+ : Cl^-$ flux [60, 64].

There is some evidence that during reticulocyte maturation the number of $K^+ : Cl^-$ transport units decreases [56, 76], and that the basal $K^+ : Cl^-$ fluxes in human red cells vary in their V_{max} but not $K_{0.5}$ values between donors [44, 65]. For a variety of reasons, however, one has to be cautious in interpreting such activity changes only in terms of V_{max} alterations. First, Cl^--dependent Na^+K^+ cotransport of human red cells, earlier shown to have one rather narrow-ranged affinity for each cation [32], was later reported to have significant interindividual difference in these parameters [33] and a metabolic dependence was recently established [1]. Second, the affinity for intra- and extracellular K^+ (as well as for Cl^- as methods become available) should be determined in swollen and shrunken cells (red cells, epithelial cells, wherever $K^+ : Cl^-$ cotransport does exist). Third, why should $K^+ : Cl^-$ cotransport, activated by mechanisms apparently so different from cell swelling such as thiol alkylation [55, 62] or by divalent cation ionophores A23187 or ionomycin [60, 64], exhibit V_{max} changes only? Although in the case of thiol-alkylation by NEM large differences in the $K_{0.5}$ values for $[K^+]_o$ were not observed [60], much more work is required to establish this point. This fact is important since kinetic (save thermodynamic) consequences of the metabolic dependence of $K^+ : Cl^-$ cotransport activated by NEM or divalent ionophores have not been addressed at all. Although it will take some time to come forth with the answers, the questions just raised ought to be kept in mind as each individual activation/inactivation mechanism of $K^+ : Cl^-$ cotransport will be discussed prior to amalgating seemingly independent effects into a coherent model.

Cell Swelling and Shrinkage

That cell swelling activates and shrinkage inactivates OR K^+ fluxes of the type discussed mainly in this review was first discovered in Muscovy duck red cells [48]. It took almost a decade to establish the Cl^--dependence of these K^+ fluxes [23, 50]. A "volume-stat" type mechanism was declared to be responsible for the regulation of $K^+ : Cl^-$ cotransport [48, 49]. The situation in bird red cells, however, is much more complex, since there is a report that $K^+ : Cl^-$ cotransport induced by swelling is overridden by the catecholamine-stimulated $Na^+ : K^+ : 2Cl$ cotransport [40]. Cell swelling has been shown to activate Cl^--dependent K^+ movements in fish red cells [53] and in dog erythrocytes [77].

Of particular interest is the finding that cell swelling by as little as 5% activates Na^+-independent $K^+ : Cl^-$ cotransport in LK (Fig. 3) but not in

Table 1. Some properties of Na^+-independent OR K^+Cl^- fluxes in LK sheep red cells before and after exposure to hyposmotic media or to N-ethylmaleimide

Parameter (units)	Isosmotic controls	Hyposmotically swollen cells	NEM-treated cells
Approx. V_{max} of $Rb^+(K^+)$ influx (mmol/liter cells · hr)	0.6–1.0 [55]	5–14 [22]	47 [55]
Approx. $K_{0.5}$ for Rb_o^+ or K_o^+ (mM)	20–50 [55, 56]	34–47 [22]	27–50 [55, 56]
Approx. $K_{0.5}$ for K_i^+ (mM)	~13 [59]	Unknown	~10 [59]
Anion preference	$Br > Cl \gg SCN = I > NO_3$ [26, 71]	$Br > Cl \gg SCN = I > NO_3$ [22, 71]	$Br > Cl \gg HCO_3 = F \gg I = NO_3 = SCN$ [59]
Metabolic dependence	NO [57, 59]	NO [59]	Yes [57, 59]
Furosemide inhibition (10^{-3} M)	Partially [25, 71]	Partially [71]	Fully with Rb_o^+ [58]
Effect of anti-L_L antibody	Reduction [22, 25, 59, 71]	Reduction [22]	Reduction [59, 71]

Numbers in [ ] indicate references.
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HK sheep red cells without affecting the Na^+/K^+ pump [22, 25, 26]. The alloantibody, anti-L_L, reported earlier to reduce OR K^+ and not Na^+ fluxes in LK cells (*see* in [54]) prevented the swelling induced $K^+ : Cl^-$ flux [59, 71]. Cell shrinkage in hyperosmotic media further reduced $K^+ : Cl^-$ transport below the "basal" level in isosmotic media. In a search to exclude participation of a Ca^{2+}-activated K^+ channel it was found that the divalent ionophore A23187 in the presence of only Ca^{2+} inhibited $K^+ : Cl^-$ cotransport [22, 25]. Aside from the observation that this swelling-induced $K^+ : Cl^-$ flux was reversible, no further enlightment came from this work regarding the important question of the activation mechanism.

Thiolakylation

At pH 7, N-ethylmaleimide (NEM) preferentially reacts with SH-groups in proteins [35]. At high pH values and high concentrations, reactions also occur with other nucleophiles. As early as 1957 it was found that NEM selectively increased the K^+ permeability of duck red cells [90]. In the Fall of 1978 (working in Munich with Jochen Duhm on the NEM-inhibitable Na^+/Na^+ exchanger and with Günter Valet on maturation of sheep red cells) I observed in a control experiment involving ^{86}Rb that NEM dramatically increased K^+ influx in LK but not in HK sheep red cells [66]. This was a somewhat puzzling finding since an earlier report on human (high K^+) red cells showed that NEM treatment caused initial K^+ loss and cell shrinkage in Na^+ media followed much later by a Na^+ gain and cell swelling [43]. With the simultaneous findings from several laboratories of Cl^--dependent RVD in red cells of birds [50] and fish [53], further experiments revealed that also the NEM-stimulated K^+ flux required the presence of Cl^- and was present also in LK goat red cells [66]. Unlike in the human red cell [62], there was no effect of NEM on OR Na^+ effluxes which in choline media remained at the level of control cells not treated with NEM [55, 56].

Table 1 compares the properties of the basal, volume-dependent $K^+ : Cl^-$ fluxes with those of the NEM-activated $K^+ : Cl^-$ transport. It can be seen that the internal and external affinities for K^+ are relatively low and that the prime effect of cell swelling or NEM treatment seems to be on V_{max} of $K^+ : Cl^-$ cotransport. Did, then, NEM recruit new $K^+ : Cl^-$ transport units since only V_{max} changes were found? Several key findings speak against this possibility. First, in contrast to the basal $K^+ : Cl^-$ flux, the NEM-stimulated flux was volume-insensitive [59]. Second, the fractional reduction of the basal and the NEM-induced $K^+ : Cl^-$ fluxes by anti-L_L were identical, and from anti-L_L absorption studies there was no evidence for an increase (or decrease) in L_L sites/cell [59]. Third, the apparent IC_{50}-value for furosemide to inhibit 50% of the NEM-stimulated $K^+ : Cl^-$ flux was modulated by external Rb^+ (or K^+) [58]. Fourth, as Table 1 shows, only the NEM-stimulated but not the basal $K^+ : Cl^-$ flux was dependent on cellular metabolism since metabolic depletion by starvation or by 2-deoxy-D-glucose feeding of LK sheep red cells reversibly inhibited the NEM-stimulated but not the basal $K^+ : Cl^-$ flux [57]. In human red cells a similar metabolic dependence of $K^+ : Cl^-$ transport stimulated by NEM was found as shown in Fig. 5 [65].

In order to accommodate these various findings complicating the *a priory* assumption of generating new $K^+ : Cl^-$ sites, I proposed [59] that in LK sheep red cells both stimuli, thiolalkylation as well as cell volume, affect one major protein molecule (perhaps the $K^+ : Cl^-$ transport moiety itself) with two func-

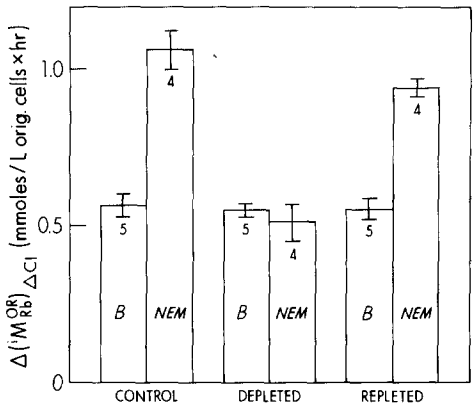
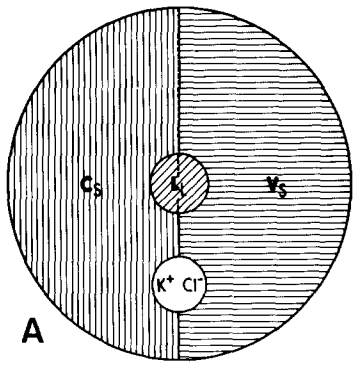


Fig. 5. The NEM-stimulated $K^+ : Cl^-$ cotransport in human red cells is reversibly metabolically dependent. Shown are the total ouabain-resistant Rb^+ influxes in control (*B*) and NEM-treated (NEM) red cells in Cl^- media. Note the disappearance of the NEM-specific fraction in metabolically starved human red cells and its reappearance in repleted cells, fed again with glucose plus adenosine. Numbers indicate experiments; bars = SE. From reference [65] with permission of publisher



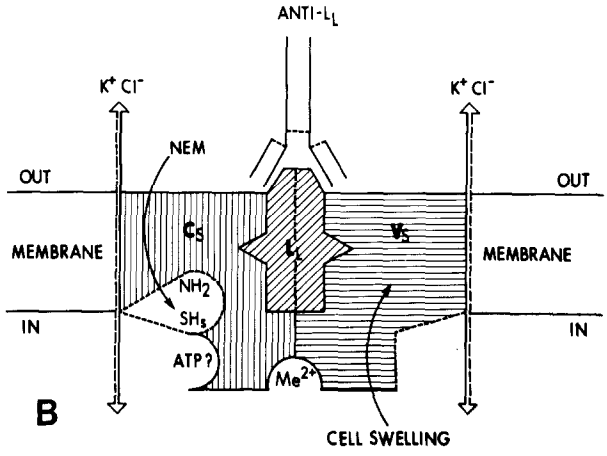


Fig. 6. A two-domain model accounting for the finds of operationally distinct but immunologically identical K^+Cl^- transport in LK sheep red cells stimulated by N-ethylmaleimide (NEM) or cells swelling. *A* = the membrane exofacial aspect of the K^+Cl^- transport system, *B* = membrane cross-section of the transporter. C_s and V_s = chemically and volume-stimulated functional domains or subunits of one component, respectively. In C_s, SH_s is the SH group through which NEM stimulates K^+Cl^- transport via one pathway (*A*) which in (*B*) is doubled to indicate also control by V_s. The apparent low pK_a of the SH_s group may be due to nearby NH_2 groups. ATP is placed into the metabolically controlled site of C_s to indicate the role of metabolism, that has not been shown yet for V_s. The L_L-antigen (L_L) and the NEM reactive SH_s groups of C_s and V_s are independent from each other, while there is functional interdependency between the SH_s groups and the metabolically controlled (ATP) site. The changes in V_{max} induced by cell swelling or NEM are not yet specifically addressed in this model (*see* text). Also, no account is made for the documented [71] presence of inhibitory SH groups. The complex effects of Ca^{++} and its ionophore A23187 are discussed in the text. (Reproduced from reference [59] with permission of the publisher)

tionally separate domains (Fig. 6): A volume-sensitive, functional domain, V_s, operates in terms of Kregenow's [48, 49] "volume-stat," and a chemically stimulated domain, C_s, may be thiolalkylated by NEM or methane-methyl-thiosulfonate (MMTS) at an internal SH group since impermeable maleimides were without effect [59] and, in addition, was found to be metabolically dependent [57]. Interfaced within the two functional domains is the L_L antigen through which anti-L_L reduced both the NEM- and the volume-sensitive $K^+ : Cl^-$ flux activity [59]. In this model I have not given special emphasis to the potential role of inhibitory SH groups. It has been claimed that at low concentrations of NEM there is a minute inhibitory action on $K^+ : Cl^-$ cotransport in LK sheep red cells [71].

Divalent Cations, Ionophores, and Chelators

The divalent ionophore A23187 is known to exchange Me^{2+} and, under certain conditions, Me^{2+} for H^+ when inserted into artificial [9, 78, 80] or biological membranes [68, 92]. The major divalent cation in red cells is Mg^{2+} with a cellular concentration by several orders greater than that of Ca^{2+} [27, 28]. In the presence of A23187 Ca^{2+} may elicit the Gardos effect in human red cells [67]. Hence it was of considerable interest to see whether the NEM-induced $K^+ : Cl^-$ flux could be reduced by removing cellular divalent cations Me^{2+} with A23187 in the presence of an external chelator. We were surprised to find (*see* Fig. 4) that A123187 (or ionomycin, *unpublished*) in the presence of EGTA (ethylene glycol tetracetic acid) or EDTA (ethylene diamine tetracetic acid) dramatically activated OR $K^+ : Cl^-$ cotransport in untreated control but not in NEM-exposed LK sheep red cells which have an already

Table 2. Summary of modulation of ouabain-resistant K^+Cl^- fluxes by A23187 ≠ EGTA or ± Ca^{2+} in fresh and NEM-treated LK sheep red cells

Condition	Ouabain-resistant K^+Cl^- fluxes	
	Untreated cells	NEM-treated cells
Fresh cells		
Control	Unaltered (basal)	Stimulated
A23187 + EGTA	Stimulated	No further stimulation
A23187 + Ca^{2+}	Inhibited	Inhibited
Metabolically depleted cells		
Control	Unaltered (basal)	Abolished
A23187 + EGTA	No stimulation	Not tested (not relevant)
A23187 + Ca^{2+}	Inhibition at $>10^{-5}$ Ca^{2+}	Not tested (not relevant)

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activated $K^+ : Cl^-$ cotransporter [60, 64]. Moreover, the maximal activation of $K^+ : Cl^-$ cotransport was much greater in osmotically swollen than in shrunken cells [60]. Finally in metabolically depleted LK red cells A23187 plus EGTA failed to stimulate $K^+ : Cl^-$ cotransport, an effect which was reversible upon metabolic repletion [60]. A23187 plus Ca^{2+}, however, always inhibited the basal $K^+ : Cl^-$ fluxes [60, 64].

Table 2 summarizes the results of these experiments from which the following conclusions may be drawn.

a) In the presence of EGTA or EDTA, A23187 removes from the LK sheep red cell Me^{2+}, which in controls and under isosmotic conditions (i.e., in the absence of A23187 and chelator) apparently are natural inhibitors of volume-sensitive $K^+ : Cl^-$ cotransport. This finding reminds one of the "Mg^{2+} carrier break hypothesis" in mitochondria [21], which proposes that A23187 removes matrix Mg^{2+} controlling the activity of an electroneutral K^+/H^+ exchanger [21, 24]. Indeed, there is extensive literature on the removal of Mg^{2+} from human and chicken red cells by A23187 [27, 28, 38, 39, 68]. Although it is inviting to envision Mg^{2+} as crucial to maintain normal $K^+ : Cl^-$ cotransport activity, at this point it is not clear which Me^{2+} is involved (*see below*). Interestingly, passive Na^+ fluxes have been reported to be higher in human red cell ghosts with low rather than with high intracellular Ca^{2+} [81]. It is not known whether the Ca^{2+} debinding from the proposed cell membrane [81] activates Na : K : 2Cl cotransport.

b) In NEM-treated cells, A23187 plus EGTA do not further stimulate $K^+ : Cl^-$ cotransport because either thiol-alkylation causes a conformational change in the transport protein (effect equivalent to Me^{2+} removal by A23187 plus EGTA) or Me^{2+} debinding from a site in the proximity of or controlled by the SH-group reacting with NEM. Such a proposition is not unreasonable, since release of ghost membrane-bound Ca^{2+} by radiation or SH-reagents such as NEM has been reported [89]. However, the fact that Ca^{2+} (in the presence of A23187) still inhibited the NEM-stimulated as well as the control $K^+ : Cl^-$ fluxes implies that Ca^{2+} acts at a site less dependent on or not involving SH-groups [60]. An optional explanation is the presence of a common rate-limiting step preventing no further stimulation by A23187 plus EGTA after maximal activation by NEM.

c) There is a striking dependence on metabolism of $K^+ : Cl^-$ flux stimulated by both A23187 plus EGTA [60] or by NEM [57], suggesting that ATP or other intermediate metabolites (2.3 DPG?) either directly (by binding?) or indirectly by Me^{2+} chelation [28] participate in the activation step of $K^+ : Cl^-$ cotransport. However, the inhibitory action of Ca^{2+} on the basal $K^+ : Cl^-$ flux was not affected by metabolic manipulation indicating a second, clearly inhibitory site. Alternatively, metabolic depletion may, through an altered redox state, affect the oxidation state of SH groups crucial for reactivity with NEM.

Figure 7, a magnification of partial aspects of Fig. 6, accommodates the findings discussed above. For simplicity, *A* is the metabolically dependent site from which Me^{2+} may be dislodged by A23187 and EGTA in swollen or shrunken controls, or by thiolalkylation with NEM, both processes leading to $K^+ : Cl^-$ contransport activation. Inhibition of transport occurs by Me^{2+} interaction with site *I*, which is independent of SH-groups and metabolism. The tentative distinction of at least two sites is based on work with several Me^{2+} in control and NEM-treated cells and on the important assumption that the work was done close to equilibrium.

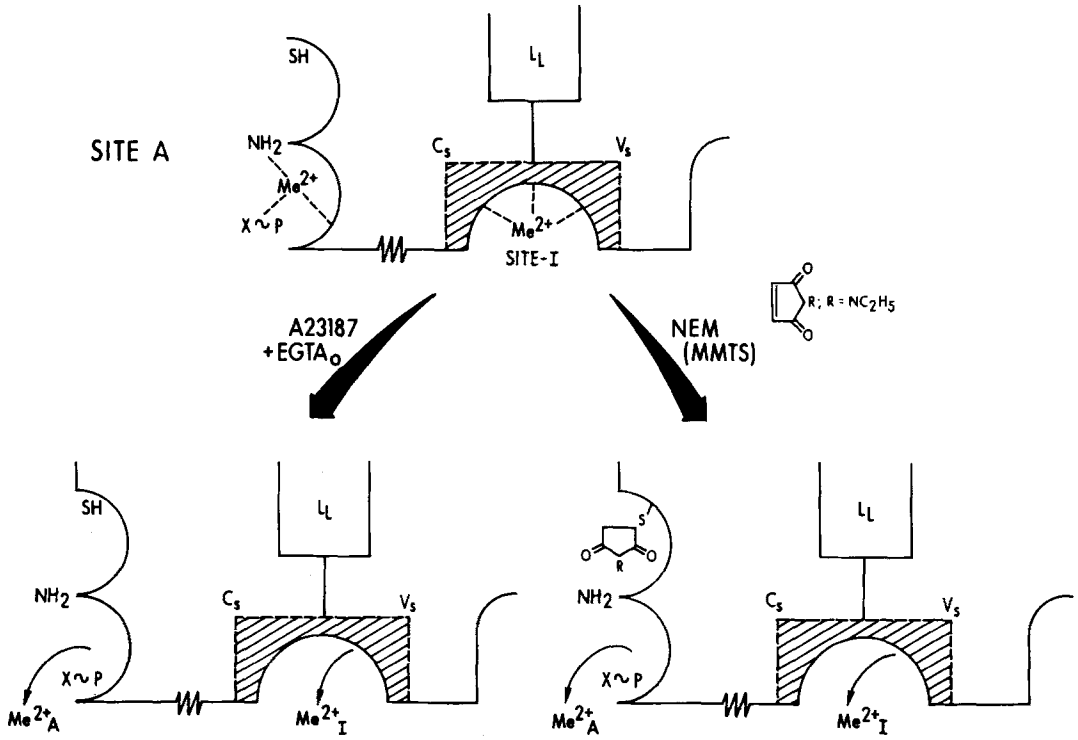

Fig. 7. How A23187 plus $EGTA_o$ and thioalkylation by NEM or MMTS may activate $K^+ : Cl^-$ cotransport in low K^+ sheep red cells. Shown are expansions of part of the model presented in Fig. 6. Site *A* is conjectured as the site of interaction with Mn^{2+} and Mg^{2+}, which may be metabolically dependent on some form of intermediate ($X \sim P$). Site *I* is the site which in both controls and NEM-treated cells reacts with Me^{2+}, which on basis of their radii also could interact with calmodulin (*see* text). Both A23187 or NEM (MMTS) treatment (*see* text) lead to dislodging of Me^{2+} from sites *A* and *I*. It is not excluded that all events occur via one large Me^{2+} binding site that is modulated by metabolism, calmodulin, and the L_L-antigen

Figure 8 contains very recent data presenting evidence for the presence of more than one Me^{2+} site. In control cells treated with A23187 (Fig. 8*A*), the following Me^{2+} series with decreasing potency to inhibit $K^+ : Cl^-$ cotransport was determined on the basis of extracellular Me^{2+} concentrations [60]: $Mn^{2+} \gg Ca^{2+} > Mg^{2+} \gg Sr^{2+}$ with Ba^{2+} being ineffective. In NEM-treated cells (Fig. 8*B*) the sequence was $Ca^{2+} \gg Sr^{2+}$, with Mn^{2+} and Mg^{2+} now ineffective at concentrations found to be effective in controls [63]. Consistent with the two-site model proposed above, this finding suggests that NEM treatment conformationally altered site *A* through which Mn^{2+} and Mg^{2+} may modulate $K^+ : Cl^-$ cotransport, while site *I*, independent of SH-groups, binds Ca^{2+} and Sr^{2+}.

The most interesting bivalent metal cations shown to affect $K^+ : Cl^-$ cotransport were Mn^{2+}, which in fresh cells may occur as Mn (II) in very small quantities (certainly below 10^{-6} mol/liter cells [46]), and Mg^{2+}, which we found to be in the range of <2 mmol/liter cells. When in water, Mn (II) forms various hydroxylated complexes [19] with varying net positive charges, which may be recognized by a binding site that accommodates the octahedral Mn (II) via some of its six coordinates to SH and NH_2-groups. In contrast, Mg^{2+} is oxygen-liganded, i.e., to carboxyl groups, suggesting that Mg^{2+} might even bind to another site. However, work on the lactase-synthase complex has shown that Me^{2+} as different as Mn^{2+} and Ca^{2+} (another oxygen-liganded Me^{2+}) and others may bind to the same site [12, 82]. The NEM-effect of abolishing the inhibitory action of Mn^{2+} and Mg^{2+} then may be understood by assuming a conformational change in the complex binding site(s) *A* from which in controls also A23187 and external EGTA remove these Me^{2+}.

It has been shown that SH-groups may be oxidized to sulfonic acid residues (i.e., cystein to cysteic acid) in the presence of MnO_4^-, i.e., Mn (VII), which in turn becomes reduced to MnO_2 [2]. Hence another, although less likely, event due to near neutral pH and the presence of superoxide dismutase [31], is oxidation of Mn (II) to Mn (IV), with subsequent SH oxidation and reduction of Mn (IV) to (II), the only form that inhibits $K^+ : Cl^-$ flux via site *A* as depicted by the scheme in Fig. 7. Clearly,

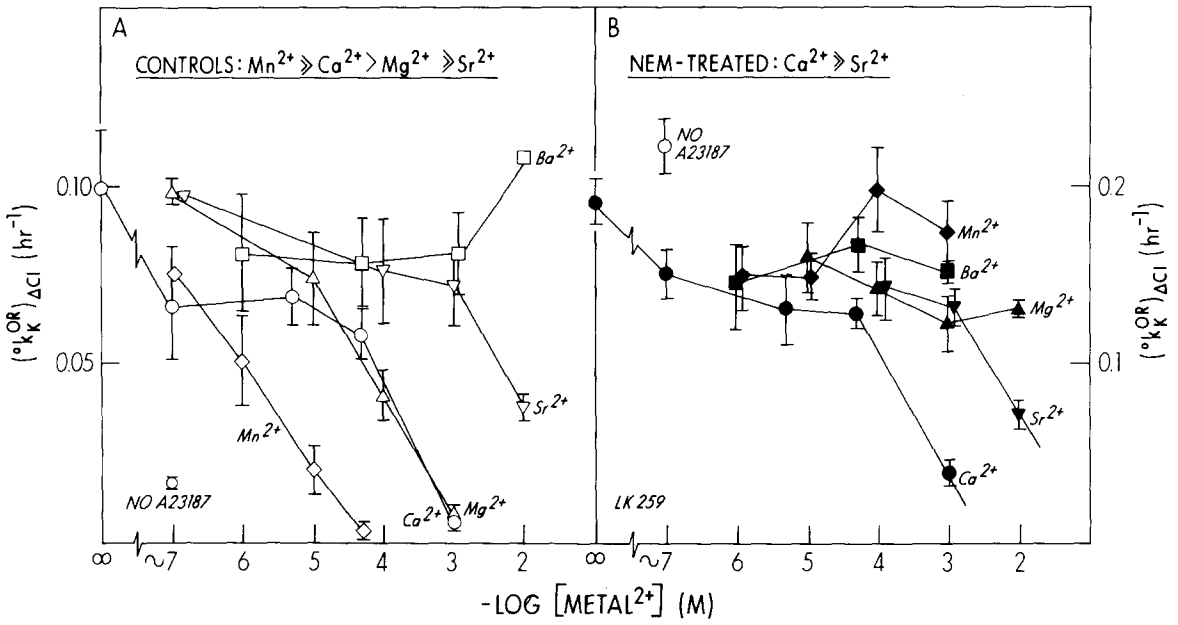

Fig. 8. Evidence for two distinct Me^{2+} binding sites on the $K^+ : Cl^-$ cotransporter in low K^+ sheep red cells. Plotted are the rate constants of ouabain-resistant $K^+ : Cl^-$ cotransport, $(^ok_K^{OR})_{\Delta Cl}$, as function of the logarithm of the Me^{2+} concentrations, $[Metal^{2+}]_o$, in presence of A23187. *A* = control. *B* = NEM-treated (note the change in the scale of the right hand abscissa). Bars are SE of at least four experiments. In the absence of 6 μM A23187, $(^ok_K^{OR})_{\Delta Cl}$ was 0.016 hr^{-1} in controls and 0.221 in NEM-treated cells showing a >10-fold stimulation by NEM. In the presence of A23187 (all other data) $(^ok_K^{OR})_{\Delta Cl}$ of controls was activated sixfold in the presence of EGTA (infinity point on *x*-axis). When instead of EGTA Me^{2+} were added in increasing concentrations, the following sequences of inhibitory potencies for $(^ok_K^{OR})_{\Delta Cl}$ were established in controls: $Mn^{2+} \gg Ca^{2+} > Mg^{2+} \gg Sr^{2+}$ and in NEM-treated cells: $Ca^{2+} \gg Sr^{2+}$. Hence the Mn^{2+} and Mg^{2+} inhibitory effects on $(^ok_K^{OR})_{\Delta Cl}$ were abolished in NEM-treated LK sheep red cells [56].

NEM would interfere with such a redox system required for Mn^{2+} action through site *A*. A redox basis of function has been proposed for the Ca^{2+} activated K^+ channel [41].

Whatever the mechanism of Mn^{2+} action may be, its proven inhibitory function of $K^+ : Cl^-$ symport is physiologically of considerable interest. It is known that Mn^{2+} activates adenylate cyclases in a variety of cells such as *Neurospora crassa* [29, 30], baker's yeast [72, 91], fat cells [8], rat hepatocytes [73, 74], sheep thyroid glandular cells [14], rat brain cells [93] and turkey erythrocytes [70, 86]. In the latter cells Mn^{2+} can substitute for Mg^{2+} as enzyme activator, resulting in a loss of enzyme inhibition by Ca^{2+} [86]. This finding is significant in light of our observation that in NEM-treated cells Mn^{2+} prevented the inhibitory action of Ca^{2+} [63]. Although there is no evidence for an active adenylate cyclase in mature red cell membranes [75], the Mn^{2+} effects should be further studied in light of the metabolic dependence of both the NEM- and A23187-activated $K^+ : Cl^-$ cotransporter. It is also possible that the metabolic dependence of A23187-activated $K^+ : Cl^-$ flux signals involvement of more complex metabolic events. There is evidence that in colonic cells A23187 activates K^+ and Cl^- secretion through stimulation of acylhydrolases producing arachidonic acid and prostaglandins, a process blocked by indomethacin [85].

What is the nature of site *I* through which Ca^{2+} and Sr^{2+} inhibit $K^+ : Cl^-$ cotransport? Recent experiments from my laboratory have shown that in control as well as in NEM-treated cells Cd^{2+} and Zn^{2+} are also inhibitory in the presence of A23187 and at Me^{2+} concentrations not very different from those of Ca^{2+}. In human red cells Cd^{2+} and Zn^{2+} in the absence of A23187 enhance the Ca^{2+}-mediated K^+ flux [79]. Since site *I* is SH-independent, it is inviting to speculate whether perhaps calmodulin participates in this effect. Calmodulin lacks cystein and has been shown recently [18] to bind all those Me^{2+} whose ionic radii are close to that of Ca^{2+} (0.99 Å) such as Cd^{2+} (0.97 Å), Zn^{2+} (0.83 Å) and Sr^{2+} (1.13 Å), but not Ba^{2+} (1.35 Å) and Mg^{2+} (0.65 Å). Also, site *I*, which only in the presence of A23187, i.e., from the inside, reacts with these bivalent metal ions, may be on the $K^+ : Cl^-$ cotransporter itself. In contradistinction to a two-site model, there may be one large Me^{2+} binding site accommodating in a complex interactive manner any of the Me^{2+} tested.

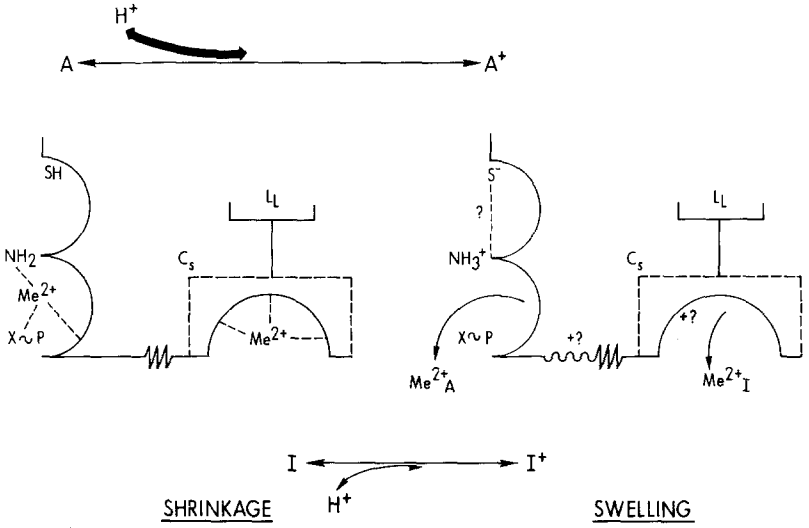

Fig. 9. Hypothetical activation of $K^+ : Cl$ cotransport by protonation of chemical groups which control Me^{2+} binding, at least to site *A*. Protonation, occurring during cell swelling, of sites *A* to A^+ and *I* to I^+ bumps off Me^{2+}, leading to a conformational change in the $K^+ : Cl^-$ cotransport molecule (-complex) and $K^+ : Cl^-$ flux activation. Deprotonation upon cell shrinkage is accompanied by Me^{2+} binding to amino-acid contact points which, in the protonated state, may prevent Me^{2+} binding by ion-pair formation. The situation is conjectured to be analogue to the "relaxed-tense" state transition in the hemoglobin molecule

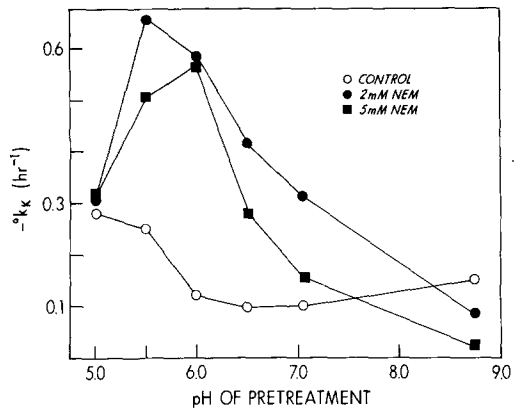

Fig. 10. Effect of two different NEM concentrations during pretreatment of LK sheep red cells at different pH values on the K^+ efflux rate constants. Fluxes were measured at pH 7.4 in isosmotic Cl^- media. (Reproduced from reference [5] with permission of publisher)

Chemical Groups and Cell Volume Sensing: An Integrated View and Consequences

The work with NEM and ionophores has provided a glimpse at the possible mechanism regulating the activity of $K^+ : Cl^-$ cotransport in LK sheep red cells. Although most of what has been said about the regulating mechanism is still very conjectural and has its basis in the work on a genetic curiosity, the LK sheep red cell, I hope that nature's tendency to conserve important mechanisms will prove to be helpful in uncovering similarities in the $K^+ : Cl^-$ cotransport machinery of other cell types. Based on this confidence I propose that all three stimuli, i.e., cell swelling, NEM, and A23187 and EGTA-mediated Me^{2+} removal affect the same transport molecule through which $K^+ : Cl^-$ flux is activated.

Figure 9, utilizing the data obtained with NEM and A23187 and the features of Fig. 7, proposes the existence of an equilibrium between a deprotonated and a protonated state of the $K^+ : Cl^-$ cotransporter, determining its activity through Me^{2+} binding. An additional key element leading to the model proposed was our finding that NEM reacts preferentially with low pK_a SH-groups as shown in Fig. 10 [5]. At $pH > 6$, less and less NEM effect on $K^+ : Cl^-$ cotransport is seen [5]. This effect may be due to either inhibition by NEM through inhibitory SH-groups [5, 71] or due to deprotonation of an amino group such as an ε-amino group or an imidazol-nitrogen in close proximity to the NEM-reactive SH-group. Independent chemical evidence favors the last of the two possibilities.

The main features of the model in Fig. 9 are as follows. Consistent with the Donnan distributions of small ions, osmotic cell swelling causes cell pH to be lowered leading to protonation of both site *A* and *I* to A^+ and I^+, respectively. For example, ion pair formation in site A^+ between S^- and NH_3^+ or N^+-imidazol, known to occur in other proteins such as papain [69], may perturb the pocket into which Me^{2+} (Mn^{2+}, Mg^{2+}) bind, with or without help of a metabolite, then leading to Me^{2+} release and activation of $K^+ : Cl^-$ cotransport. This mechanism is defacto identical to both the reversible Me^{2+} removal by A23187 and the irreversible alteration by NEM of site A^+. The physiologic importance of the *I* to I^+ equilibrium shift in the process of $K^+ : Cl^-$ transport inhibition requires more studies. In a more general way, the interaction of H^+ and Me^{2+} with the transport molecule, at site *A*, may be described by the following equation:

$$\underset{\text{Activation}}{Me^{2+} + A \cdot H^+} \longleftrightarrow \underset{\text{Inhibition}}{Me^{2+} \cdot A + H^+}. \qquad (3)$$

The model proposed originates from biochemical findings and the use of ionophores, Me^{2+}, and chelators. Hence, drawbacks are inescapable and caveats are indicated. First, it must be shown that $K^+ : Cl^-$ outward cotransport can be activated by lowering pH_i. Such experiments are not easy since only minute volume changes will alter $K^+ : Cl^-$ cotransport. Also, in human red cells $Na^+ : K^+ : 2Cl^-$ cotransport was inactivated at low pH_i [32]. However, this finding never has been put into perspective regarding cell volume and pH_i changes. Furthermore, other electroneutral K^+ transporters are turned off at more acid pH [16, 21]. Second, although it is inviting to assume a simple equilibrium as defined by the mass action law to explain regulation of $K^+ : Cl^-$ cotransport activity, other more complex mechanisms involving Me^{2+} have to be considered. It should not be overlooked that sheep red cells have a low level of glucose-6-phosphate dehydrogenase compared with other red cells, although they are equally resistant to oxidants [10]. Third, the presence of *several* versus *one,* large Me^{2+} binding site requires further proof. Work on analogue model proteins binding Me^{2+} such as calmodulin (site *I*) and metallothionein [87] (site *A*) has to be consulted. Fourth, the relationship of metabolism to any site needs to be studied, in particular in light of the presence of red cells of at least parts of the phosphatidyl-inositol-oligophosphate cascade [7].

Conclusion: Relation to Other Systems, and the Problem of Molecular Verifications

Ouabain-resistant $K^+ : Cl^-$ cotransport belongs in the group of Cl^--dependent cation transport systems reviewed extensively more recently [61]. Most of the evidence speaks for a molecular entity different from that involved in $Na^+ : K^+ : 2Cl^-$ or $Na^+ : Cl^-$ cotransport, although the final word needs to be said. Although its virtual absence in HK but presence in LK cells would argue for an inverse coupling to high Na^+/K^+ pump activity in HK and vice versa in LK cells, molecular identity of Cl^--dependent, furosemide-inhibitable cation fluxes with the ouabain-resistant Na^+/K^+ pump transport has been ruled out [45]. Nevertheless, the transitions from HK precursor cells to mature LK red cells may involve Na^+/H^+ exchange as recently proposed [61]. Clearly, NEM or MMTS react with protein SH-group. The sites through which Me^{2+} act have also been assumed to be on the transport protein or on the latter and the ubiquitous calmodulin. Alternatively, lipid-Me^{2+} interaction may be of crucial importance.

Based on what is known about the activation mechanisms there is no obvious relationship between electroneutral $K^+ : Cl^-$ cotransport (Fig. 1*A*), K^+/H^+ antiport (Fig. 1*B*) and K^+ fluxes through channels (Fig. 1*C*), since the latter two regimens require Me_i^{2+} to be activated. This has been shown for *Amphiuma* red cells [17], lymphocytes [37], and Ehrlich cells [42]. On the other hand, the fact that A23187 by removal of Mg^{2+} activates K^+/H^+ antiport in mitochondria [21, 24] suggests that not all K^+/H^+ antiporters are alike, or that we simply do not know enough about Me^{2+} activation of these systems. Other divalent ions have not yet been tested with exception of Cu^{2+} which in Ehrlich cells counteracts RVD in Na^+ media by permitting Na^+ to enter the cells [51].

The Ehrlich cells is a peculiar model since it may be capable to RVD via independent K^+ and Cl^- channels [42] or by electroneutral $K^+ : Cl^-$ cotransport [3, 88]. When treated with NEM, $K^+ : Cl^-$ cotransport is activated in cells supposed to have independent K^+ and Cl^- channels [47]. This finding needs to be further analyzed quantitatively before establishing dogmatically that there is no relationship between passive, ouabain-resistant K^+ transporters which volume-regulate.

In lymphocytes NEM wipes out the capability to RVD by K^+ and Cl^- electrogenic transport [4] and this effect has been confirmed [84]. Apparently NEM acts on both the K^+ and Cl^- pathways [84].

Molecular verification by radiolabeled NEM of $K^+ : Cl^-$ cotransport is difficult since there are millions of SH-groups per membrane and perhaps only a few hundred or so $K^+ : Cl^-$ transport proteins. Identification with radiolabeled bumetanide or furosemide is similarly problematic since the affinity of the carriers for these loop diuretics is low and other transporters may be labeled.

Hence, as in so many other examples of transport physiology and biochemistry, chemical modification will remain for quite some time the prime appoach to shed further light on the mystery of regulatory activation and deactivation of $K^+ : Cl^-$ cotransport.

Among the technicians who, during my last years as Professor of Physiology at Duke University Medical Center, helped me to execute the experiments leading to the evolution of the working model presented here, were Ms. B.E. Theg, Ms. D. Dietrich, Mr. J. Franklin, and Ms. K. Huber. I am indebted to Dr. N.C. Adragna for patient listening and discussing the development of my working concepts and for carefully editing the manuscript. I thank Gay Blackwell for typing the manuscript. This work was supported by Public Health Grant NIH AM 28.236/AM-GM.

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