Na/K/Cl CO-TRANSPORT AND ITS REGULATION

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SUMMARY

NaCl and Na/K/Cl co-transport systems in many cell types show a number of similarities, among which are sensitivity to 'loop' diuretic inhibition and extremely high anion selectivity. Avian erythrocytes possess a $Na + K + 2Cl$ co-transporter that is stimulated by agents raising intracellular cAMP. The system is also inhibited following ATP-depletion of the cells, in agreement with results in other tissues; this nucleotide may exert a regulatory role on the operation of the co-transporter. A Na/K/Cl co-transport system is also present in flounder intestine, where it plays a central role in salt absorption at the luminal border of the tissue. In contrast to the avian erythrocyte this system is inhibited by raising intracellular cyclic nucleotide content, cGMP being more effective than cAMP. From these results it is concluded that second messenger regulation of Na/K/Cl co-transport processes is heterogeneous and may exhibit some tissue specificity.

INTRODUCTION

It is now clear that the linkage between Cl and cation transport in the form of NaCl and/or Na/K/Cl‡ and KCl transport systems is a widespread phenomenon in animal tissues (for reviews see Frizzell, Field & Schultz, 1979a; Palfrey & Greengard, 1981). In epithelia, these systems serve as important components in transcellular salt transport, whereas in non-epithelial cells similar systems appear to be involved in volume regulation (Kregenow, 1981). What is less certain, at present, is the relationship between these processes in different tissues. Are they homologous entities, with similar properties, or are there a family of transporters with analogous, but distinct properties? This question cannot yet be addressed at the molecular level, because of the unknown nature of the membrane components involved in such systems, but similarities are beginning to emerge at the physiological and pharmacological level between NaCl or Na/K/Cl co-transport mechanisms from various sources. It is as yet unclear whether these two mechanisms are distinguishable, or whether all NaCl co-transport systems involve K. Recently, a number of epithelial transport systems that had previously been thought to involve only Na and Cl (Frizzell *et al.* 1979a) have been shown to be K-dependent (e.g. flounder intestine: Musch *et al.* 1982; TALH:

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‡ Abbreviations: 'Na/K/Cl' co-transport is retained as a general term when stoichiometry is not specified; 'Na + K + 2Cl' is used when this specific stoichiometry is indicated. TALH: thick ascending limb of Henle's loop.
■ Key words: Cyclic nucleotides, avian erythrocytes, flounder intestine.

Greger & Schlatter, 1981; amphibian distal tubule: Oberleithner, Guggino Giebisch, 1983). On the other hand, NaCl co-transport in *Necturus* gallbladder may not involve K (Ericson & Spring, 1982). Similarly, Cl has been implicated as a co-transported ion in the avian erythrocyte 'Na+K' system only recently (Kregenow & Caryk, 1979; Palfrey & Greengard, 1981; Haas, Schmidt & McManus, 1982). It seems likely that such a convergence reflects a true similarity, and that the avian erythrocyte can serve as a useful model system for defining some of the characteristics of Na/K/Cl co-transport in more complex tissues such as epithelia. Other cells have been shown to possess similar Na/K/Cl co-transporters (squid axon: Russell, 1980, 1983; MDCK cells: Rindler, McRoberts & Saier, 1982; McRoberts, Erlinger, Rindler & Saier, 1982; and Ehrlich ascites cells: Geck *et al.* 1980) and the phenomenon may be widespread in cultured cells (Aiton *et al.* 1981). In contrast, Na-independent, KCl co-transport processes, which appear to operate in a number of cell types (e.g. Ellory, Dunham, Logue & Stewart, 1982; Greger & Schlatter, 1983; Lauf, 1983), seem to exhibit properties distinct from those of the NaCl or Na/K/Cl mechanisms (Ellory *et al.* 1982).

A major common feature of NaCl and Na/K/Cl co-transporters is inhibition by 'loop' diuretics such as furosemide, bumetanide and related compounds (Palfrey & Greengard, 1981). Comparison of the relative potencies of a series of such agents (as inhibitors of Na + K + 2Cl co-transport in avian erythrocytes, as diuretics in dogs and as inhibitors of salt secretion in shark rectal gland) yields strong correlations (Palfrey, Alper & Greengard, 1980*a*; Palfrey, Feit & Greengard, 1980*b*; Palfrey, Silva & Epstein, 1983). Moreover, the higher potency of bumetanide relative to that of furosemide (about 50–100: 1) has been observed in a number of other tissues (e.g. McGahan, Yorio & Bentley, 1977; Schlatter, Greger & Weidtke, 1983; Rindler *et al.* 1982). A further observation linking these co-transport systems is that of extreme anion specificity. Cl can be replaced only partially by Br (about 50–60 %) but other anions are inactive (Palfrey & Greengard, 1981; Geck *et al.* 1980; McRoberts *et al.* 1982; Solomon *et al.* 1977). These two characteristics alone are sufficient to distinguish such co-transport systems from other modes of cation and anion permeation in most cells. For example, directly coupled NaCl or Na/K/Cl co-transport can be differentiated from putative 'parallel exchanger' systems, that have been proposed to mediate net salt transport in certain tissues (e.g. Liedtke & Hopfer, 1982). The latter process, involving indirectly coupled Na/H and Cl/OH or HCO_3 exchangers, would be expected to be susceptible to inhibition by amiloride and disulphonic stilbenes (e.g. 'SITS' or 'DIDS'), agents that are ineffective against NaCl or Na/K/Cl co-transporters (e.g. Palfrey & Greengard, 1981; Rindler *et al.* 1982; Schlatter *et al.* 1983). Moreover, the stilbene-sensitive anion-exchanger of the red cell, presumably analogous to systems in other tissues, has a relatively broad anion specificity (Knauf, 1979).

Na + K + 2Cl CO-TRANSPORT IN THE AVIAN ERYTHROCYTE

The general characteristics of Na + K + 2Cl co-transport in the avian erythrocyte have been reviewed previously (McManus & Schmidt, 1978; Kregenow, 1978; Palfrey & Greengard, 1981). Three aspects will be briefly covered here: interactions of

and diuretics with the system, inhibition of the system by metabolic depletion, and regulation by various extracellular stimuli. The ion kinetics of Na/K/Cl co-transport are only beginning to be elucidated. The system in avian erythrocytes is evidently electroneutral (i.e. Na + K + 2Cl, e.g. Haas *et al.* 1982), but some discrepancies exist between tracer flux and net (chemical) flux studies. Thus, the K:Na ratio measured with isotopes yields values >1 (Kregenow, 1978; Palfrey & Greengard, 1981) whereas net flux measurements yield a ratio of unity (McManus & Schmidt, 1978; Haas *et al.* 1982). Variable K:Na stoichiometry has led one investigator to postulate separate NaCl and KCl systems with some kind of coupling between them (Kregenow, 1981); but this neglects the sigmoid dependence of co-transport on Cl (Palfrey & Greengard, 1981; see also McRoberts *et al.* 1982; Greger, 1981; Musch *et al.* 1983 for other tissues) that suggests that 2Cl must bind to a single transporter for it to function. In contrast, the Na and K dependencies of transport are hyperbolic in character (Kregenow, 1978; McManus & Schmidt, 1978). Whatever the precise stoichiometry of co-transport, it is probable that ion binding to the system will not be independent (i.e. the binding of one ion may affect the binding of a second one). This has been conclusively demonstrated in the Na + K + 2Cl co-transport

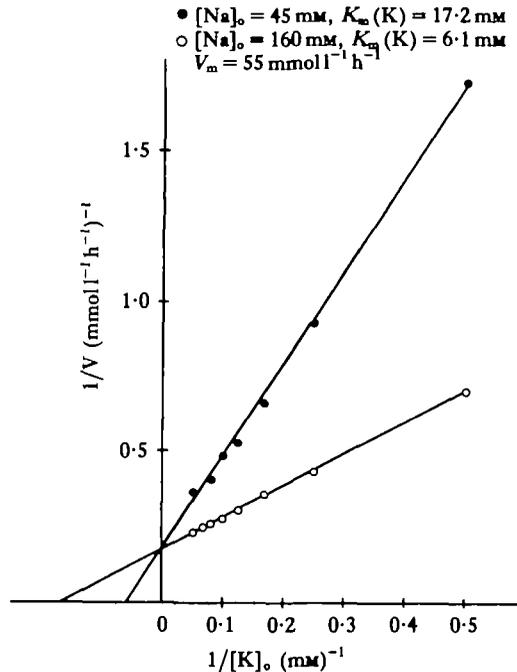Fig. 1. Interaction between Na and K sites in the Na + K + 2Cl co-transport system of turkey erythrocytes. Erythrocytes were prepared as described in Palfrey *et al.* (1980a) and suspended in media containing the Na concentrations indicated and various concentrations of K (1–20 mM); isotonicity was maintained with choline-Cl. Cells were then stimulated with 1 mM-8-Br-cAMP and the co-transport-mediated influx of ^{86}Rb as a tracer for K measured as described (Palfrey *et al.* 1980a). The figure shows Lineweaver-Burke plots of the $[K]_o$ dependence of tracer uptake at suboptimal (50 mM) Na_o (approximately half-maximal in these cells) and saturating (160 mM) Na_o concentrations. Note that V_m does not change, but that the K_m for K is dramatically reduced when the Na_o concentration is saturating.

system of MDCK cells (Rindler *et al.* 1982; McRoberts *et al.* 1982). Here, ◼ saturation of one cation binding site (e.g. the K site) increases the affinity of the second cation (e.g. Na) for its binding site. An analogous phenomenon takes place in avian red cells (see Fig. 1); increasing $[Na]_o$ from a suboptimal value of 50 mM to a saturating value of 160 mM increases the apparent K_m of K_o for the system from 17·2 mM to 6·1 mM. Such apparently cooperative interactions are also reflected in the effects of ions on 'loop' diuretic inhibition of co-transport. Thus, the IC_{50} value for bumetanide inhibition of $Na + K + 2Cl$ co-transport is shifted to lower values when extracellular cations are increased from suboptimal to optimal concentrations (Palfrey *et al.* 1980*b*). The K_m values of Na and K for this effect are close to the K_m values for these cations for the transport system itself (Haas & McManus, 1982) strongly suggesting that the cation effects are caused by their binding to the transporter and that the diuretics bind to another site on the transporter. This site may well be one of the Cl sites. The effective 'loop' diuretics are all negatively charged at physiological pH (Palfrey *et al.* 1980*b*) and increasing concentrations of $[Cl]_o$ are able to diminish the effectiveness of 'loop' diuretics as inhibitors, possibly by a direct competitive mechanism (Haas & McManus, 1983; Forbush & Palfrey, 1983).

A feature of Na/K/Cl co-transport systems that has emerged recently is the possible ATP-dependence of the mechanism. Metabolic depletion of avian erythrocytes results in an inhibition of co-transport-mediated K flux, in parallel with a decline in ATP concentrations (Palfrey, 1983 and Fig. 2). Stimulation of $Na + K + 2Cl$ co-transport by either cAMP-dependent (e.g. isoproterenol or 8-Br-cAMP) or cAMP-independent (e.g. hypertonicity or NaF: Palfrey & Greengard, 1981) mechanisms is similarly affected by ATP depletion. The effects are fully reversible on repletion of ATP (Palfrey, 1983). Reduction in ATP levels has also been found to block Na/K/Cl co-transport in squid axon (Russell, 1980, 1983), Ehrlich ascites cells (Geck *et al.* 1980) and MDCK cells (Rindler *et al.* 1982). In squid axon, the effect appears to be specifically attributable to ATP, as removal of the nucleotide by internal dialysis results in a loss of co-transport (Russell, 1980). Moreover, neither the hydrolysable α,β-methylene nor the non-hydrolysable β,γ-methylene analogues of ATP could substitute for ATP in supporting co-transport in the axon. In addition, Geck *et al.* (1980) found no evidence for consumption of ATP by the $Na + K + 2Cl$ co-transport system of Ehrlich ascites cells. These results suggest that ATP may play a regulatory role in co-transport aside from its possible involvement in protein phosphorylation as discussed below. It is of interest that ATP has been shown to affect the activity of other transport systems (e.g. Na/Ca exchange: DiPolo & Beauge, 1983; glucose transport: Jacquez, 1983) and is known to bind at regulatory sites on other transporters (e.g. Na,K-ATPase: Glynn & Karlish, 1975; Ca,Mg-ATPase: Muallem & Karlish, 1979). A mandatory requirement for ATP in the operation of NaCl or Na/K/Cl co-transport could partly explain why difficulties have been encountered in finding a directly-coupled mechanism in brush-border vesicles from various absorptive epithelia (e.g. mammalian intestine: Liedtke & Hopfer, 1982 but compare Fan, Faust & Powell, 1983). Such subcellular particles may contain insufficient ATP to support a directly-coupled NaCl or Na/K/Cl process.

$Na + K + 2Cl$ co-transport is markedly stimulated by agents that raise cAMP lev◼
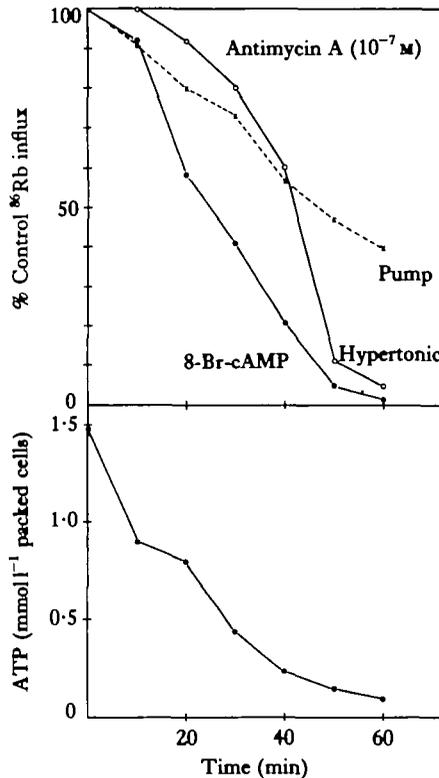
Fig. 2. Inhibition of Na + K + 2Cl co-transport by metabolic depletion of turkey erythrocytes. Red cells were washed into a glucose-free buffer (157·5 mM-NaCl, 2·5 mM-KCl, 10 mM-Hepes, pH 7·4) containing antimycin A (10^{-7} M). At various times thereafter, samples were taken for analysis of co-transport [stimulated either with 1 mM-8-Br-cAMP or 200 mM-sucrose (hypertonicity)] or 'pump' – (ouabain-sensitive) mediated ^{86}Rb uptake (Palfrey *et al.* 1980*b*). Simultaneously, samples were processed for ATP measurement using a luciferase assay. Note that 8-Br-cAMP-stimulated co-transport fluxes are slightly more sensitive to ATP depletion than hypertonicity-stimulated flux or 'pump' flux. These processes were reversible upon washing out the inhibitor and resynthesis of ATP in the presence of glucose, adenine, inosine and P, (Palfrey, 1983).

in avian erythrocytes (Riddick, Kregenow & Orloff, 1971; Rudolph, Schafer & Greengard, 1977; Palfrey & Greengard, 1981) and also by certain treatments which do not act *via* cAMP, e.g. hypertonicity (Kregenow, Robbie & Orloff, 1976), NaF and deoxygenation (Palfrey & Greengard, 1981). A role for protein phosphorylation has been proposed in the case of cAMP-dependent stimuli (Rudolph & Greengard, 1974; Palfrey *et al.* 1980*a*; Palfrey & Greengard, 1981) but the mechanism of action of cAMP-independent stimuli is at present unknown. Evidently the regulation of co-transport by second messengers in different tissues is heterogeneous: e.g. in the human erythrocyte cAMP may *inhibit* a Na/K/Cl process that is essentially identical to the avian erythrocyte (Garay, 1982); cGMP appears to be an intracellular inhibitor of Na/K/Cl co-transport in the flounder intestine (Rao, Nash & Field, 1983; see below). These findings suggest that the regulatory mechanisms controlling co-transport may be tissue-specific and caution must be exercised in extrapolating results from one cell type to another.

Na / K / Cl CO-TRANSPORT IN FLOUNDER INTESTINE

Transepithelial salt and water transport in a variety of epithelia including the intestine involves a mechanism directly coupling the movement of Cl to that of Na into the cell such that intracellular [Cl] is above electrochemical equilibrium (Frizzell *et al.* 1979*a*). There are two possible sites for Na-coupled Cl entry in epithelial tissues: the basolateral membrane in secretory epithelia (e.g. trachea, shark rectal gland, cornea) and the apical membrane in absorptive epithelia (e.g. gallbladder, flounder intestine). Exposure of the serosal but not the luminal surface to 'loop' diuretics results in an inhibition of Cl secretion in a number of secretory epithelia (e.g. shark rectal gland: Palfrey *et al.* 1983; canine trachea: Widdicombe, Nathanson & Highland, 1983), suggesting the presence of a NaCl co-transport system; however, this awaits confirmation by direct (albeit technically difficult) measurements of Na and Cl influxes across this membrane. It remains to be established whether K plays a direct role in these NaCl coupled entry processes at the basolateral membrane; this possibility may not be easily testable as K is required at this surface for the operation of the Na/K pump. The regulation of this co-transport process is also uncertain: e.g. in canine trachea, secretory stimuli such as epinephrine (acting *via* cAMP) increase conductive Cl permeability of the apical membrane and decrease intracellular Cl activity (Shorofsky, Field & Fozzard, 1983*a,b*); however, it is not known whether this entails a direct stimulation of NaCl co-transport at the basolateral membrane.

In absorptive epithelia, such as the rabbit gallbladder, TALH and flounder intestine (Frizzell *et al.* 1979*a*), the NaCl coupled entry process is located on the luminal membrane. In these tissues, luminal but not serosal exposure to 'loop' diuretics results in an inhibition of Na and Cl influx into the epithelium, confirming an apical membrane localization for NaCl co-transport. Recent studies in both TALH (Greger & Schlatter, 1981) and flounder intestine (Musch *et al.* 1982) show that NaCl co-transport is K-dependent, indicating the presence of a Na/K/Cl co-transport system. In contrast to secretory epithelia, the ready accessibility of the luminal surface of absorptive epithelia makes it easier to study the regulation of transport processes occurring at this surface. Thus, it has been demonstrated that NaCl influx across the apical membrane is inhibited by cAMP in rabbit gallbladder (Frizzell *et al.* 1979*a*) and by both cAMP and cGMP in the flounder intestine (Frizzell, Smith, Vosburgh & Field, 1979*b*; Rao *et al.* 1983) and rabbit ileum (Guandalini, Rao, Smith & Field, 1982).

The flounder intestinal epithelium is less heterogeneous than its mammalian counterpart: it contains no crypts, and the enterocytes from the base to the apex of its mucosal folds are morphologically indistinguishable (Field *et al.* 1978). Another structural feature of interest is the presence of unusually long and intermittently constricted intercellular spaces; the average dimensions of the absorptive cells being $60\,\mu m \times 3\cdot5\,\mu m$; this indicates a complex paracellular pathway with a distributive resistance and compartment-like properties.

When bathed in Ringer's solution (pH 8) flounder intestinal mucosa (stripped of its underlying muscle and mounted in Ussing chambers) exhibits a serosa-negative potential difference (PD, 4–6 mV) with reference to the mucosa. Under conditions of chemical and electrical (short-circuited) equilibrium the tissue actively absorbs N

Cl and secretes K (Fig. 3) (Field *et al.* 1978; Musch *et al.* 1982). The short-circuit current (I_{sc}) is roughly equal to the sum of these net ion fluxes. The net flux of Cl is roughly three times that of Na ($J_{net}^{Cl} \sim 5$–$6 \cdot 0$ and $J_{net}^{Na} \sim 1 \cdot 5$–$2 \cdot 5$ mequiv h^{-1} cm^{-2} and K secretion is comparatively small ($0 \cdot 7$–$0 \cdot 8$ mequiv h^{-1} cm^{-2}). In the presence of 5 mM-K in both bathing media, measurements of influx of Na and Cl across the luminal surface indicate that a fraction of the two influxes is coupled and that this portion is blocked by furosemide (Frizzell *et al.* 1979b). Measurement of K/Rb (Rb can quantitatively substitute for K in this tissue) influx across the luminal border under various conditions reveals that the entry of K is coupled to that of Na and Cl, and is also inhibited by furosemide; thus there appears to be a single entry mechanism for all three ions (Musch *et al.* 1982). Furthermore, luminal K is required for both the influx and the transepithelial transport of Cl. The stoichiometry of this co-transport system is as yet unresolved. Using different approaches such as simultaneous ^{42}K and ^{36}Cl influxes in the presence and absence of furosemide, or by measuring ^{86}Rb influx as a function of extracellular Cl concentrations (a sigmoidal relation with a $K_{1/2}$ of 32 mM and a Hill coefficient of $2 \cdot 1$) it is evident that the stoichiometry of K:Cl entry is 1:2 (Musch *et al.* 1983). What is not clear is the number of Na ions involved; simultaneous ^{42}K:^{22}Na influxes in the presence and absence of furosemide suggest at least a 1:2 stoichiometry and earlier estimates of Na:Cl suggest a $1 \cdot 3$:1 stoichiometry (the large paracellular flux of Na makes the estimates of Na influx into the cell a rough approximation at best); these estimates
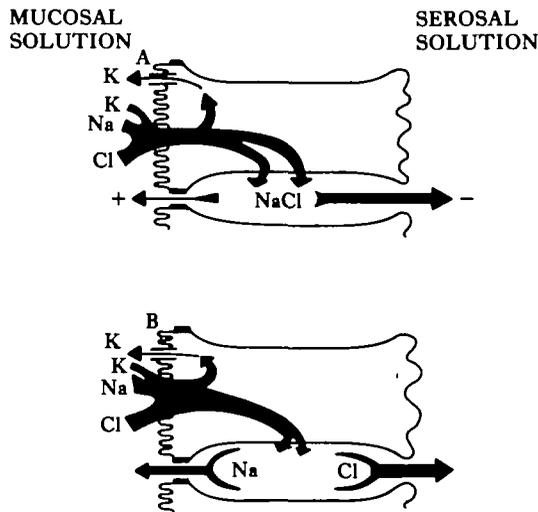

Fig. 3. Model of apical events in ion transport in flounder intestine. A Na/K/Cl co-transport system and a conductive K channel are present in the luminal membrane. Most of the K entering *via* co-transporter recycles to the lumen *via* the channel. The basolaterally-located Na,-K pump maintains internal [Na] low, such that the inward-directed Na gradient powers Na/K/Cl co-transport. Cl exit at the basolateral membrane may involve both conductive and electroneutral processes. (A) Open-circuit conditions (modified from the model proposed for rabbit gall bladder: Machen & Diamond, 1969): a serosa negative potential difference is generated and maintained by a salt diffusion potential across the highly cation-selective tight junction. (B) Short-circuit conditions: the highly cation-selective tight junction allows three times as much Cl to be absorbed as Na and most of the transported Na recycles back to the lumen.

suggest an electrogenic 2Na:1K:2Cl entry mechanism. Conditions that dire█
inhibit the co-transporter (such as Na or Cl removal, cGMP or furosemide treatment)
cause a hyperpolarization of the apical membrane (Musch *et al.*1982). This would be
expected if the co-transporter were electrogenic [although alternative explanations,
e.g. a drop in intracellular Cl activity (Greger, Schlatter & Lang, 1983) could also
account for this hyperpolarization]. However, the hyperbolic relationship between
Rb influx and increasing luminal Na concentration ($K_{1/2}$: 3·8 mM; Hill coefficient:
1·1), observed when Na is replaced with N-methyl-D-glucamine, suggests a
1Na:1K:2Cl stoichiometry. Such an electroneutral mechanism would be more in
line with observations in other tissues (see above).

A number of agents appear to regulate ion transport in the flounder intestine and
the effects of some of these agents on the Na/K/Cl co-transport system (as measured
by their action on Na, Cl or K influx) have been examined (Frizzell *et al.* 1979*b*;
Musch *et al.* 1982; Rao *et al.* 1983). The effects of cyclic nucleotides are especially
interesting because, in contrast to the mammalian ileum where cAMP and cGMP are
equally effective in inhibiting furosemide-sensitive NaCl influx (Guandalini *et al.*
1982), in the flounder their effects differ (Rao *et al.* 1983). The phosphodiesterase-
resistant analogue 8-Br-cGMP is as effective as the 'loop' diuretics in inhibiting Rb
or Cl influx (Fig. 4) and the effects of the two inhibitors are not additive. The
nucleotide also abolishes I_{sc}, net Cl absorption and net Rb secretion without altering
the conductance or cation selectivity of the tight junction. 8-Br-cAMP on the other
hand causes at best a 60% inhibition of I_{sc} (that is not additive with cGMP or
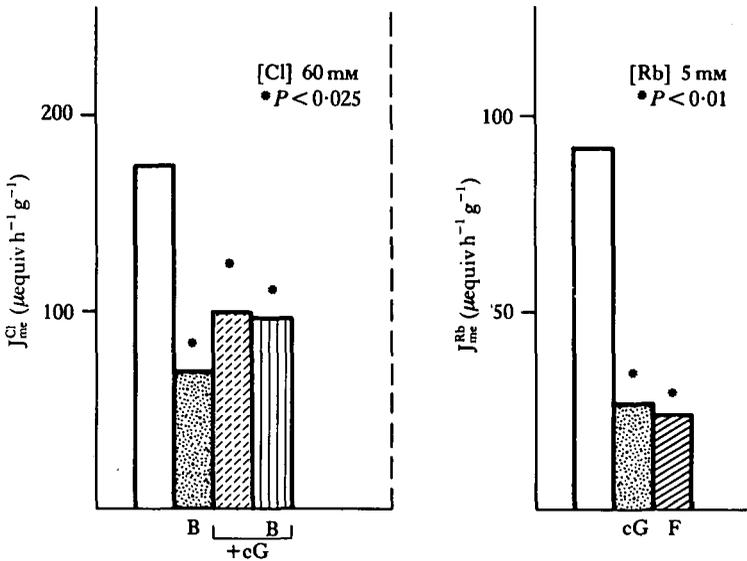bumetanide but can be further reduced by these agents) and only partially inhibits Rb

Fig. 4. Effects of 8-Br-cGMP and 'loop' diuretics on Cl and Rb influxes in flounder intestine: initial
rates of uptake of [36]Cl (30 s) and [86]Rb (90 s) were measured as described by Frizzell, Smith, Vosburgh
& Field (1979*b*) and Musch *et al.* (1982). 8-Br-cGMP (cG, 0·2 mM) was added to both sides of the
epithelium and bumetanide (B, 10 μM) and furosemide (F, 1 mM) were added only to the luminal
bathing solution. Values represent means of four paired experiments. J_{me}^{Cl} and J_{me}^{Rb} refer to the unidirec-
tional mucosa to epithelium fluxes of Cl and Rb respectively.

⬛etion and Cl absorption (Rao *et al.* 1983). Earlier studies have shown that cAMP in combination with theophylline can partially inhibit the furosemide-sensitive portion of Cl and Rb influx (Frizzell *et al.* 1979*a,b*; M. W. Musch & M. Field, unpublished observations); however, theophylline increases both cAMP as well as cGMP concentrations. Thus the involvement of cAMP in the regulation of the co-transport process remains to be established. Like the cyclic nucleotides, Ca has been shown to inhibit absorption and stimulate secretion in a variety of epithelia (Frizzell *et al.* 1979*a*). In the flounder intestine, Ca agonists such as A23187 and serotonin inhibit net Cl absorption (Donowitz *et al.* 1982) but it remains to be determined if this occurs by a direct inhibition of the co-transporter. It is possible that the second messengers act by stimulating the phosphorylation of specific proteins, since specific kinases and substrates for all three mediators can be detected in this tissue (M. C. Rao, unpublished observations).

Irrespective of the stoichiometry of the co-transport system, a portion or most of the serosa-negative potential difference appears to be due to a diffusion potential across the highly cation-selective tight junction (Field *et al.* 1978; Krasny, Madara, DiBona & Frizzell, 1983). In addition to being a less complete inhibitor of Na/K/Cl co-transport than cGMP, 8-Br-cAMP disrupts the permselectivity of this epithelium by specifically increasing anion permeability (Rao *et al.* 1983; Krasny *et al.* 1983). It is as yet uncertain whether the location for this action of cAMP is in the cell or in the paracellular pathway. The apical membrane also contains a Ba-sensitive K-conductive channel (Krasny, Halm & Frizzell, 1982) which contributes to net K secretion by this tissue (Musch *et al.* 1982). Thus most of the K entering *via* the co-transporter appears to recycle back to the lumen *via* this channel. Similar K recycling mechanisms have been suggested in other absorptive epithelia (e.g. TALH: Greger, 1981).

FUTURE PERSPECTIVES

The recent discovery that coupled NaCl transport in a number of tissues is dependent on K establishes the fact that Na/K/Cl co-transport systems exist in a wide variety of cells. The similarities of such systems at the molecular level must await the development of suitable reagents for identification of the membrane constituents involved in transport. An initial step in this direction has recently been made with the introduction of [^{3}H] bumetanide as a reversible probe for the 'loop' diuretic binding site on the co-transporter from canine kidney membranes (Forbush & Palfrey, 1983). This compound exhibits saturable binding to these membranes with an affinity comparable with that found in co-transport inhibition studies; also binding is competed for by other diuretics in accordance with their potencies as transport inhibitors. Moreover, specific [^{3}H] bumetanide binding is manifest only in the presence of all three transported ions and appears to be inhibited at high [Cl], in good agreement with other observations. This radiolabel may thus be useful for the identification of co-transport systems in other tissues and in the purification of the co-transporter from various membranes.

The regulation of Na/K/Cl co-transport by second messengers exhibits some ⬛ue specificity. Avian erythrocytes, and possibly some secretory epithelia, exhibit

accelerated co-transport as a result of cAMP elevation. However, co-transpor~~~~ human erythrocytes has been reported to be inhibited by cAMP, and cGMP inhibits co-transport in flounder intestine. As all these events may be mediated by protein phosphorylation, it is clear that the phosphorylation events must also differ from tissue to tissue. It is anticipated that the specific nature of these events will become clearer when the components of the co-transport system are identified.

REFERENCES

AITON, J. F., CHIPPERFIELD, A. R., LAMB, J. F., OGDEN, P. & SIMMONS, N. L. (1981). Occurrence of passive furosemide-sensitive transmembrane K transport in cultured cells. *Biochim. biophys. Acta* **646**, 389–398.

DIPOLO, R. & BEAUGE, L. (1983). The Ca pump and Na/Ca exchange in squid axons. *Ann. Rev. Physiol.* **35**, 313–324.

DONOWITZ, M., BATTISTI, L., MADARA, J., TRIER, J., CUSOLITO, S., CARLSON, S. & FIELD, M. (1982). Calcium and active transport in intestine of the winter flounder, *Pseudopleuronectes americanus. Bull. Mt. Desert Island biol. Lab.* 21, 22–26.

ELLORY, J. C., DUNHAM, P. B., LOGUE, P. J. & STEWART, G. W. (1982). Anion-dependent cation transport in erythrocytes. *Phil. Trans. R. Soc. Ser. B* **299**, 483–495.

ERICSON, A. C. & SPRING, K. (1982). Coupled NaCl entry into *Necturus* gall bladder epithelial cells. *Am. J. Physiol.* **243**, C140–C145.

FAN, C.-C., FAUST, R. G. & POWELL, D. W. (1983). Coupled sodium-chloride transport by rabbit ileal brush border membrane vesicles. *Am. J. Physiol.* **244**, G375–G385.

FIELD, M., KARNAKY, K. J., JR., SMITH, P. L., BOLTON, J. E. & KINTER, W. B. (1978). Ion transport across the isolated intestinal mucosa of the winter flounder, *Pseudopleuronectes americanus.* I. Functional and structural properties of cellular and paracellular pathways for Na and Cl. *J. Membr. Biol.* **41**, 265–293.

FORBUSH, B. F., III & PALFREY, H. C. (1983). [³H] Bumetanide binding to canine kidney membranes: relationship to the Na/K/Cl cotransport system. *J. biol. Chem.* (in press).

FRIZZELL, R. A., FIELD, M. & SCHULTZ, S. G. (1979a). Sodium-coupled chloride transport by epithelial cells. *Am. J. Physiol.* **236**, F1–F8.

FRIZZELL, R. A., SMITH, P. L., VOSBURGH, E. & FIELD, M. (1979b). Coupled sodium-chloride influx across brush border of flounder intestine. *J. Membr. Biol.* **46**, 27–39.

GARAY, R. (1982). Inhibition of Na/K cotransport system by cyclic AMP and intracellular Ca in human red cells. *Biochim. biophys. Acta* **688**, 786–792.

GECK, P., PIETRZYK, C., BURCKHARDT, B.-C., PFEIFFER, B. & HEINZ, E. (1980). Electrically silent cotransport of Na, K and Cl in Ehrlich cells. *Biochim. biophys. Acta* **600**, 432–447.

GLYNN, I. M. & KARLISH, S. J. D. (1975). The sodium pump. *Ann. Rev. Physiol.* **37**, 13–55.

GREGER, R. (1981). Coupled transport of Na and Cl in the thick ascending limb of Henle's loop of rabbit nephron. *Scand. Audiol. Suppl.* **14**, 1–15.

GREGER, R. & SCHLATTER, E. (1981). Presence of luminal K, a prerequisite for active NaCl transport in the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pflügers Arch. ges. Physiol.* **392**, 92–94.

GREGER, R. & SCHLATTER, E. (1983). Properties of the basolateral membrane of the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pflügers Arch. ges. Physiol.* **396**, 325–334.

GREGER, R., SCHLATTER, E. & LANG, F. (1983). Evidence for electroneutral NaCl cotransport in the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pflügers Arch. ges. Physiol.* **396**, 306–314.

GUANDALINI, S., RAO, M. C., SMITH, P. L. & FIELD, M. (1982). cGMP modulation of ileal ion transport: *in vitro* effects of *Escherichia coli* heat stable enterotoxin. *Am. J. Physiol.* **243**, G36–G41.

HAAS, M. & MCMANUS, T. J. (1982). Bumetanide inhibition of Na+K+2Cl cotransport and K/Rb exchange at a Cl site in duck red cells: modulation by external cations. *Biophys. J.* **37**, 214a.

HAAS, M. & MCMANUS, T. J. (1983). *Am. J. Physiol.* (in press).

HAAS, M., SCHMIDT, W. F., III & MCMANUS, T. J. (1982). Catecholamine-stimulated ion transport in duck red cells. Gradient effects in electrically-neutral Na+K+2Cl cotransport. *J. gen. Physiol.* **80**, 125–147.

JACQUEZ, J. A. (1983). Modulation of glucose transport in human red blood cells by ATP. *Biochim. biophys. Acta* **727**, 367–378.

KNAUF, P. A. (1979). Erythrocyte anion-exchange and the Band III protein: transport kinetics and molecular structure. *Curr. Top. Membr. Transport* **12**, 249–363.

SNY, E. J., JR., HALM, D. R. & FRIZZELL, R. A. (1982). Apical membrane potassium conductance in flounder intestine: relation to chloride absorption. *Fedn Proc. Fedn Am. Socs exp. Biol.* 41, 1261.

KRASNY, E. J., JR., MADARA, J., DiBONA, D. & FRIZZELL, R. A. (1983). Cyclic AMP regulates tight junction permselectivity in flounder intestine. *Fedn Proc. Fedn Am. Socs exp. Biol.* 42, 1100.

KREGENOW, F. M. (1978). An assessment of the cotransport hypothesis as it applies to the norepinephrine and hypertonic responses. *Alfred Benzon Symposium* XI, 379–391.

KREGENOW, F. M. (1981). Osmoregulatory salt transporting mechanisms: control of cell volume in anisotonic media. *Ann. Rev. Physiol.* 43, 493–505.

KREGENOW, F. M. & CARYK, T. (1979). Cotransport of cations and Cl during the volume regulatory responses of duck erythrocytes. *The Physiologist* 22, 73.

KREGENOW, F. M., ROBBIE, D. E. & ORLOFF, J. (1976). Effect of norepinephrine and hypertonicity on K influx and cAMP in duck erythrocytes. *Am. J. Physiol.* 231, 306–312.

LAUF, P. K. (1983). Thiol-stimulated passive KCl transport in sheep red cells. I. Dependence on Cl and external K(Rb) ions. *J. Membr. Biol.* 73, 237–246.

LIEDTKE, C. M. & HOPFER, U. (1982). Mechanism of Cl⁻ translocation across small intestinal brush-border membrane. I. Absence of Na-Cl cotransport. *Am. J. Physiol.* 242, G263–G271.

MACHEN, T. E. & DIAMOND, J. E. (1969). An estimate of the salt concentration in the lateral intercellular spaces of rabbit gall bladder during maximal fluid transport. *J. Membr. Biol.* 1, 194–213.

McGAHAN, M. C., YORIO, T. & BENTLEY, P. J. (1977). The mode of action of bumetanide: inhibition of chloride transport across the amphibian cornea. *J. Pharm. exp. Ther.* 203, 97–102.

McMANUS, T. J. & SCHMIDT, W. F. (1978). Ion and co-ion transport in avian red cells. In *Membrane Transport Processes*, Vol. 1, (ed. J. F. Hoffmann), pp. 79–106. New York: Raven Press.

McROBERTS, J. A., ERLINGER, S., RINDLER, M. J. & SAIER, M. H., JR. (1982). Furosemide-sensitive salt transport in MDCK cell line. *J. biol. Chem.* 257, 2260–2266.

MUALLEM, S. & KARLISH, S. J. D. (1979). Is the red cell calcium pump regulated by ATP? *Nature, Lond.* 277, 238–240.

MUSCH, M. W., ORELLANA, S. A., KIMBERG, L. S., FIELD, M., HALM, D. R., KRASNY, E. J., JR. & FRIZZELL, R. (1982). Na/K/Cl cotransport in a salt-absorbing epithelium. *Nature, Lond.* 300, 531–533.

MUSCH, M. W., RAO, M. C., HALM, D., FIELD, M. & FRIZZELL, R. A. (1983). Potassium influx across flounder intestinal brush border: Na and Cl dependence and effect of ouabain. *Fedn Proc. Fedn Am. Socs exp. Biol.* 42, 988.

OBERLEITHNER, H., GUGGINO, W. & GIEBISCH, G. (1983). The effect of furosemide on luminal Na, Cl and K transport in the early distal tubule of *Amphiuma* kidney. *Pflügers Arch. ges. Physiol.* 396, 27–33.

PALFREY, H. C. (1983). Na/K/Cl transport in avian red cells: reversible inhibition by ATP depletion. *J. gen. Physiol.* (abstr.) (in press).

PALFREY, H. C., ALPER, S. L. & GREENGARD, P. (1980a). Protein phosphorylation and the regulation of cation cotransport. *J. exp. Biol.* 89, 103–115.

PALFREY, H. C., FEIT, P. W. & GREENGARD, P. (1980b). cAMP-stimulated cation cotransport in avian erythrocytes: inhibition by "loop" diuretics. *Am. J. Physiol.* 238, C139–C148.

PALFREY, H. C. & GREENGARD, P. (1981). Hormone-sensitive ion transport systems in erythrocytes as models for epithelial ion pathways. *Ann. N.Y. Acad. Sci.* 372, 291–308.

PALFREY, H. C., SILVA, H. & EPSTEIN, F. H. (1983). Sensitivity of cAMP-stimulated salt secretion in the shark rectal gland to "loop" diuretics. *Am. J. Physiol.* (in press).

RAO, M. C., NASH, N. T. & FIELD, M. (1983). Different roles of cGMP and cAMP in regulating ion transport and permeability in flounder intestine. *Fedn Proc. Fedn Am. Socs exp. Biol.* 42, 1280.

RIDDICK, D. H., KREGENOW, F. M. & ORLOFF, J. (1971). The effect of norepinephrine and dibutyryl cAMP on cation transport in duck erythrocytes. *J. gen. Physiol.* 57, 752–766.

RINDLER, M. J., McROBERTS, J. A. & SAIER, M. H., JR. (1982). (Na⁺, K⁺)-cotransport in MDCK cell line. *J. biol. Chem.* 257, 2254–2259.

RUDOLPH, S. A. & GREENGARD, P. (1974). Regulation of protein phosphorylation and membrane permeability by β-adrenergic agents and cAMP in the avian erythrocyte. *J. biol. Chem.* 249, 5684–5687.

RUDOLPH, S. A., SCHAFER, D. E. & GREENGARD, P. (1977). Effects of cholera enterotoxin on catecholamine-stimulated changes in cation fluxes, cell volume, and cyclic AMP levels in the turkey erythrocyte. *J. biol. Chem.* 252, 7132–7139.

RUSSELL, J. M. (1980). Anion transport mechanisms in neurons. *Ann. N.Y. Acad. Sci.* 341, 510–523.

RUSSELL, J. M. (1983). Cation-coupled chloride influx in squid axon. Role of K and stoichiometry of the transport process. *J. gen. Physiol.* 81, 909–925.

SCHLATTER, E., GREGER, R. & WEIDTKE, C. (1983). Effect of "high ceiling" diuretics on active salt transport in the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pflügers Arch. ges. Physiol.* 396, 210–217.

SHOROFSKY, S. R., FIELD, M. & FOZZARD, H. (1983a). Electrophysiology of chloride secretion in canine trachea. *J. Membr. Biol.* 72, 105–115.

SHOROFSKY, S. R., FIELD, M. & FOZZARD, H. (1983b). Mechanism of Cl secretion in canine trachea: changes intracellular chloride activity with secretion. *J. Membr. Biol.* (in press).

SOLOMON, R., SILVA, P., STEVENS, A., EPSTEIN, J., STOFF, J., SPOKES, K. & EPSTEIN, F. H. (19⬛⬛
Mechanism of Cl transport in the rectal gland of *Squalus acanthias*: ionic selectivity. *Bull. Mt Desert Island
biol. Lab.* 17, 59–63.

WIDDICOMBE, J. H., NATHANSON, I. T. & HIGHLAND, E. (1983). Effects of "loop" diuretics on ion transport
by dog tracheal epithelium. *Am. J. Physiol.* (in press).