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Ionic Effects on Bumetanide Binding to the Activated Na/K/2Cl Cotransporter: Selectivity and Kinetic Properties of Ion Binding Sites

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Summary. The loop diuretic bumetanide binds specifically to the Na/K/2Cl cotransporter of many cell types including duck erythrocytes. Membranes isolated from these erythrocytes retain the ability to bind bumetanide when cells are exposed to cotransport-activity stimuli prior to membrane isolation. An extensive study of the effects of ions on specific [^{3}H]bumetanide binding to such membranes is presented here and compared to the activity of these ions in supporting transport function in intact cells. Both Na^+ and K^+ enhanced bumetanide binding in a saturable manner consistent with a single-site interaction. The K_m for each ion was dependent on the concentration of the other cation suggesting heterotropic cooperative interactions between the Na^+ and K^+ binding sites. Na^+ and K^+ were partially replaceable, with the selectivity of the Na^+ site being $Na^+ > Li^+ > NH_4^+$; N-methyl-D-glucamine$^+$, choline$^+$ and tetramethylammonium$^+$ also supported a small amount of specific binding when substituted for Na^+. The selectivity of the K^+ site was $K^+ \approx Rb^+ > NH_4^+ > Cs^+$; N-methyl-D-glucamine$^+$, choline$^+$ and tetramethylammonium$^+$ were inactive at this site. The results of transport experiments revealed a slightly different pattern. Li^+ could partially substitute for Na^+ in supporting cotransport, but other monovalent cations were completely inactive. The order of potency at the K^+ site was $NH_4^+ > K^+ \approx Rb^+ > Cs^+ >>$ other monovalent cations. The effect of Cl^- on bumetanide binding was biphasic, being stimulatory at low [Cl^-] but inhibitory at high [Cl^-]. As this implies the existence of two Cl^- binding sites (termed Cl_H and Cl_L for the "high-" and "low-" affinity sites, respectively) each phase was examined individually. Cl^- binding to Cl_H could be described by a rectangular hyperbola with a K_m of 2.5 mM, while kinetic analysis of the inhibition of bumetanide binding at high [Cl^-] revealed that it was of a noncompetitive type (K_i = 112.9 mM). The selectivity of anion binding to the two sites was distinct. Cl_H was highly selective with $Cl^- > SCN^- > Br^-$; F^-, NO_3^-, ClO_4^-, $MeSO_4^-$, gluconate$^-$ and SO_4^{2-} were inactive. The efficacy of anion inhibition of binding to Cl_L was $ClO_4^- > I^- > SCN^- > NO_3^- > Cl^-$; F^-, $MeSO_4^-$, gluconate$^-$, and SO_4^{2-} were inactive. Thus, Cl_H is much more selective than Cl_L and largely accounts for the specificity of the system with respect to anion transport. SO_4^-, NO_3^-, I^-, SCN^- and ClO_4^- did not support cotransport when bound to Cl_L and the latter three anions were inhibitory. Mg^{2+} was found to stimulate binding at a narrowly defined peak around 1.5 mM, but was inhibitory at higher concentrations. Other divalent cations caused a similar inhibition of bumetanide binding but did not exert a stimulatory effect at 1.5 mM. Divalent cations have little effect on cotransport in intact cells at concentrations up to 20 mM, suggesting that their effects on diuretic binding reflect interactions at internally disposed sites. Bumetanide binding was optimal at a pH of 7.8–8.1 and declined sharply as the pH was lowered towards 6. The titration curve correlated well with the effect of pH on cotransport in intact cells; the inhibitory effect of low pH suggests that protonation of the cotransporter may inhibit its function.

Key Words cation selectivity · anion selectivity · loop diuretics · divalent cations · pH effects

Introduction

Diuretics of the 5-sulfamoylbenzoic acid class are potent inhibitors of salt reabsorption in the thick ascending limb of Henle's loop and can affect salt transport in many different cells and tissues. Over the last decade the site of action of these agents has been shown to be the Na/K/2Cl cotransporter (Palfrey, Feit & Greengard, 1980; Schlatter, Greger & Weidtke, 1983), a secondary active system with a widespread distribution in mammalian tissues (for reviews *see* Geck & Heinz, 1986; O'Grady, Palfrey & Field, 1987*b*; Haas, 1989). Substantial evidence indicates that diuretics such as bumetanide and its derivatives bind directly to the cotransporter (Forbush & Palfrey, 1983; Haas & Forbush, 1986; O'Grady, Palfrey and Field, 1987*a*; Haas, 1989; Turner & George, 1990; Pewitt et al., 1990*a*,*b*). Our previous studies with [^{3}H]bumetanide have shown that, (i) the affinity of binding agrees well with the inhibitory potency of bumetanide on cotransport, (ii) binding is displaced by other diuretics in agreement with their potency as cotransport inhibitors and (iii) binding is dependent on the presence of each of the cotransported ions (Forbush & Palfrey, 1983; Pewitt et al., 1990*b*). Moreover, specific [^{3}H]bumetanide binding to intact cells appears to be dependent on the state of activation of the cotransport system (Haas & Forbush, 1986; O'Donnell, 1989; Franklin, Turner & Kim, 1989). The most striking example of this

occurs in the avian erythrocyte, where specific binding appears only if cells are pretreated with cotransport-activating stimuli such as catecholamines or cAMP (Haas & Forbush, 1986).

We recently showed that enhanced specific binding of bumetanide is retained in membranes isolated from duck erythrocytes exposed to various cotransport-activating stimuli. Furthermore, bumetanide binding in isolated membranes was shown to correlate exactly with the state of activation of cotransport in the intact cell under a number of conditions. (Pewitt et al., 1990*a,b*). Bumetanide is thus a useful probe of a conformation of the Na/K/2Cl cotransporter that is involved in transport function. The availability of such a membrane preparation has allowed us in this study to examine [^{3}H]bumetanide binding as a function of ionic conditions.

An important facet of Na/K/2Cl cotransport is the high selectivity of the system with respect to transportable ions. For instance, Cl^- and, to a lesser extent, Br^- are the only anions capable of supporting cotransport activity in avian erythrocytes (Palfrey & Greengard, 1981). In addition, bumetanide binding is modulated in avian erythrocytes as well as in other cells by Na^+, K^+, and Cl^- (e.g., Palfrey et al., 1980; Forbush & Palfrey, 1983; Haas & McManus, 1983; Haas & Forbush, 1986; Pewitt et al., 1990*b*). Binding to cells as well as membranes is enhanced in a saturable manner as [Na^+] and/or [K^+] is increased (Forbush & Palfrey, 1983; Haas & Forbush, 1986). However, the dependence of bumetanide binding on [Cl^-] has been shown to be biphasic in a number of systems, with a stimulatory phase at low [Cl^-] that gives way to inhibition as [Cl^-] increases (e.g., Forbush & Palfrey, 1983; Haas & Forbush, 1986; O'Grady et al., 1987*b*; Turner & George, 1988; O'Donnell, 1989, Wiener & van Os, 1989). The sensitivity of bumetanide binding to ions offers the unique opportunity of comparing the ionic selectivity of binding with that of transport itself. This is particularly important at the anion binding sites as it is difficult to evaluate the selectivity of the two sites on the basis of transport experiments alone. The data presented in this study allow us to distinguish the effects of anions at both sites. Furthermore, an examination of the effects of divalent cations and pH on bumetanide binding provides important information concerning the nature of possible modulatory influences of nontransported ions on the Na/K/2Cl cotransporter.

Materials and Methods

Materials

[^{3}H]Bumetanide (sp act 66 Ci/mmol) was the generous gift of R. James Turner (National Institutes of Health). The material was stored at −70°C, and its purity was periodically checked by thin-layer chromatography. Unlabeled bumetanide was the gift of Hoechst Pharmaceuticals (New Jersey). 4-Acetamido-4′-isothiocyano-stilbene-2,2′ disulfonic acid (SITS) was from Pierce and ^{86}Rb and ^{22}Na were from Amersham. Other reagents were of analytical quality. Ducks were purchased from a local poultry house and cells collected and washed as described previously (Pewitt et al., 1990*b*).

Preparation of Cells and Isolation of Membranes

These were carried out exactly as described previously (Pewitt et al., 1990*b*). Briefly, in all the experiments conducted here, cotransport was maximally stimulated by incubating washed duck erythrocytes (10% hematocrit in "transport buffer": 145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, pH 7.4, 0.2% glucose) with 10^{-5} M norepinephrine and 5 mM NaF for 10 min prior to membrane preparation. After incubation, cells were pelleted, cooled and lysed in 40 vol of ice-cold 10 mM Tris · HCl (pH 7.4), 5 mM $MgSO_4$. Membranes were prepared after sonication of washed ghosts as described previously (Pewitt et al., 1990*b*), flash-frozen in liquid N_2 at a concentration of 4–6 mg/ml and stored at −70°C.

Unidirectional ^{86}Rb Influx and ^{22}Na Measurements

These measurements were performed as previously described for ^{86}Rb (Pewitt et al., 1990*b*). In the case of ^{22}Na influx (*see* Fig. 3), the Na^+ content of transport buffer was reduced to 50 mM (choline replacement) to optimize the specific activity of the isotope while maintaining relatively high transport rates. In the pH titration and anion substitution experiments, cells were preincubated in a buffer containing 1 mM SITS in transport buffer for 30 min at room temperature prior to stimulation for 15 min. This was done to block anion exchange, thus minimizing alteration of the internal anion composition and mitigating the effects of changing external pH. After stimulation, cells were rapidly washed in transport buffer of different pH (using 10 mM Na · MES for pH 6–7, 10 mM Na · HEPES for pH 7–8 and 10 mM Tris · HCl for pH 8–9) or anion composition (*see* Fig. 7) just prior to transport assay (1 min influx).

Determination of [^{3}H]Bumetanide Binding to Membranes

These assays were performed as described previously (Pewitt et al., 1990*b*). Briefly, membranes were rapidly defrosted and added to incubation tubes containing 100 μg membrane protein, 10 mM HEPES, pH 7.4, 0.7 mM EGTA, 0.07 mM phenylmethylsulfonylfluoride, 120 nM [^{3}H]bumetanide (0.79 μCi) in the absence and presence of 10 μM cold bumetanide (to assess total and nonspecific binding, respectively), plus various salts; the ionic conditions in the binding assay were varied according to the particular experiment and are given in the figure legends. The buffer contained 0–150 mM Na^+, K^+ and Cl^-. Na^+ and K^+ were replaced by N-methyl-D-glucamine$^+$ ($NMDG^+$) or choline$^+$ and Cl^- was replaced by SO_4^{2-}. The total ionic strength was maintained at 150–190 mM and was constant in a given experiment. In the experiments presented here the maximal binding capacity of membranes derived from different animals varied in the range

0.7–1.7 pmol/mg protein. In order to avoid expressing data as percentages of maximal binding, we have presented typical data from binding experiments carried out in duplicate or triplicate with a variation of <10%.

Results

Monovalent Cation Requirements and Specificities for [^{3}H]Bumetanide Binding

Both Na^+ and K^+ must be present to obtain specific bumetanide binding to the Na/K/2Cl cotransporter in both whole duck erythrocytes or isolated membranes (Haas & Forbush, 1986; Pewitt et al., 1990*b*). We further examined the affinity and selectivity of the cation binding sites by varying either [Na^+] or [K^+] at constant [Cl^-]. Examples of typical binding isotherms for Na^+ and K^+ are shown in Fig. 1. Rectangular hyperbolic kinetics were obeyed with both cations; the calculated K_m values ranged from approximately 2–60 mM and 3–15 mM for Na^+ and K^+, respectively, dependent on the concentration of the other cation present (*data not shown*). Such cooperative interactions between the Na^+ and K^+ sites have previously been observed in transport experiments (Palfrey & Rao, 1983) as well as in bumetanide binding experiments on intact erythrocytes (Haas & Forbush, 1986). To investigate the selectivity of the two cation binding sites, Na^+ and K^+ were independently substituted with a series of monovalent cations at a constant concentration of K^+ and Na^+, respectively. Several cations were tried as inert substitutes for Na^+ including N-methyl-D-glucamine$^+$ (NMDG), choline$^+$ and tetramethylammonium$^+$. However, all these cations yielded a small amount of specific binding even in the complete absence of Na^+. $NMDG^+$ (Fig. 1) or choline$^+$ (Fig. 2) were used in the experiments shown here. The selectivity of the Na^+ binding site was found to be $Na^+ > Li^+ > NH_4^+ \approx$ NMDG at 10 mM cation and $Li^+ > Na^+ > NH_4 >$ NMDG (at 100 mM cation). The selectivity of the K^+ binding site was $Rb^+ \geq K^+ > NH_4^+ > Cs^+ >>$ $NMDG^+$ at 5 mM cation; at 50 mM cation K^+ was slightly better than Rb^+ (Fig. 2).

In order to correlate these results with the ability of various cations to be transported, we investigated cotransport-mediated ^{86}Rb and ^{22}Na influx as a function of different cationic conditions in the external medium. At a concentration of 145 mM, Li^+ was 37% as effective as Na^+ at supporting ^{86}Rb influx, while $NMDG^+$, choline$^+$ and TMA^+ were completely inactive (*data not shown*). Substitution at the K^+ site was investigated at 2.5 and 15 mM cation (Fig. 3). Here, the selectivity sequence was found to be: $NH_4^+ > K^+ \approx Rb^+ > Cs^+$, with $NMDG^+$ and other large cations being inactive. These findings indicate that both cation binding sites show slightly different specificities with respect to bumetanide

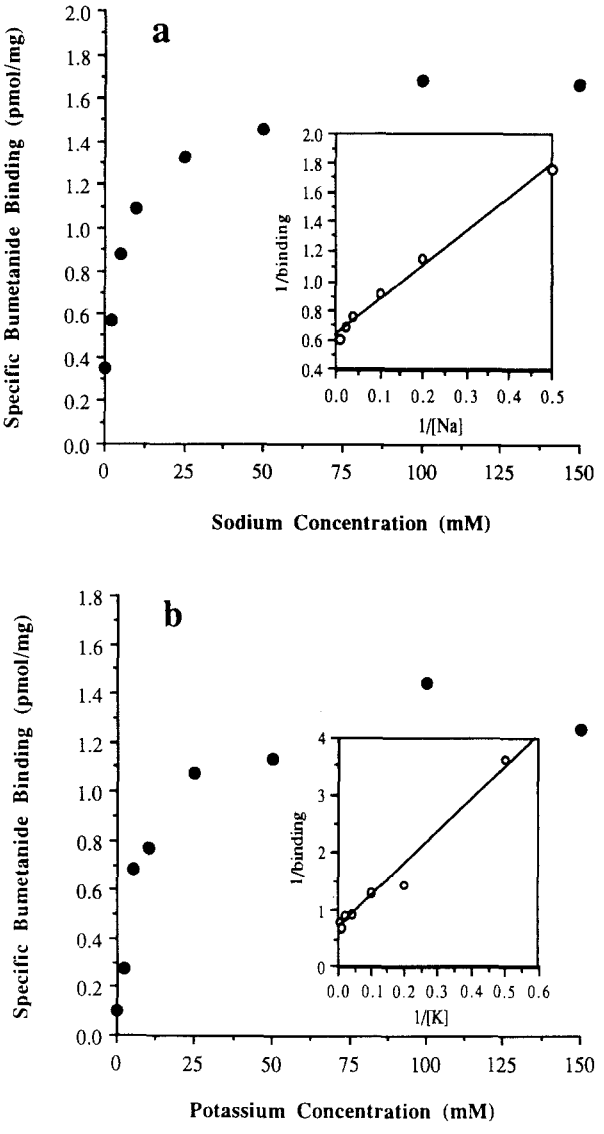

Fig. 1. Dependence of bumetanide binding on [Na^+] and [K^+]. Specific [^{3}H]bumetanide binding to membranes was measured in the presence of various [Na^+] and [K^+] as shown. (*a*) Na^+ was varied between 0–150 mM with isotonic replacement by $NMDG^+$, and [K^+] was kept constant at 40 mM. Note that specific binding in zero Na^+ is not completely eliminated because $NMDG^+$ substitutes weakly at this site. (*b*) K^+ was varied between 0–150 mM and replaced with $NMDG^+$; [Na^+] was kept constant at 20 mM. In both experiments, [Cl^-] and [SO_4^{2-}] were maintained at 20 and 85 mM, respectively. The total ionic strength in this experiment was 190 mM. Insets show double-reciprocal plots of the same data. In this experiment the graphically determined K_m for Na^+ was 3.6 mM, and that for K^+ was 8.3 mM. The results are the average of duplicate samples. This experiment was repeated four times on different batches of membranes with similar results.

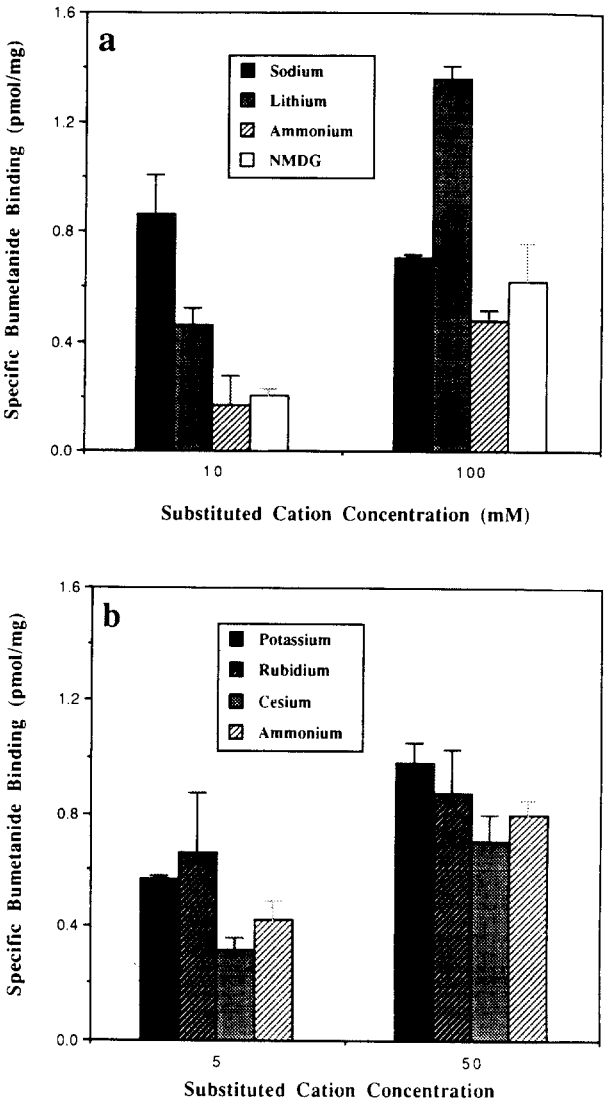

Fig. 2. Monovalent cation selectivity at the Na^+ and K^+ sites. (*a*) Na^+ was replaced by various cations at two different concentrations (10 and 100 mM) with $[K^+]$ = 40 mM, choline$^+$ was used as the substitute cation with $[Cl^-]$ = 20 mM and $[SO_4^{2-}]$ = 85 mM. (*b*) K^+ was replaced by various cations at two different concentrations with $[Na^+]$ = 20 mM. $NMDG^+$ was used as the substitute cation with $[Cl^-]$ and $[SO_4^{2-}]$ as in *a*. The total ionic strength was 190 mM. Results are expressed as means (±SE) of triplicate samples. This experiment was repeated three times on different batches of membranes with similar results.

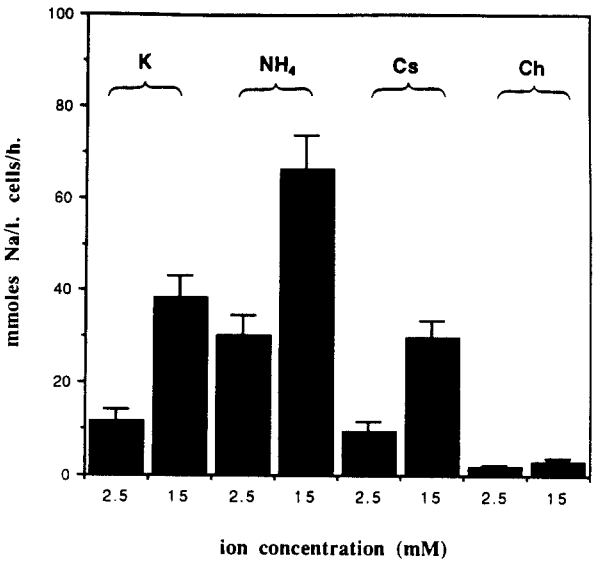

Fig. 3. Monovalent cation selectivity of the K^+ site in Na/K/2Cl cotransport. K^+, NH_4^+, Cs^+ and choline$^+$ (*Ch*) were compared at 2.5 and 15 mM in $^{22}Na^+$ influx assays. Red cells were preincubated at 5% hematocrit in 10^{-5} M norepinephrine for 10 min prior to isotope addition. 0.5 ml aliquots were dispensed into triplicate tubes containing $^{22}NaCl$ (0.5 μCi) and influx was allowed to continue for 4 min. Cells were then washed three times in ice-cold choline chloride (160 mM)/HEPES (10 mM) and the cell pellets counted in a gamma spectrometer. The transport buffer contained 50 mM NaCl and either 95 mM (low K^+) or 82.5 mM (high K^+) NMDGCl. Results are expressed as means ± SEM. Rb^+ at 2.5 and 15 mM gave results that were statistically indistinguishable from those with K^+ (*not shown*). Other large cations such as TMA^+ behaved like choline$^+$.

binding and cotransport, at least under the conditions used here.

Anion Requirement and Specificity for [^{3}H]Bumetanide Binding

As previously described in a variety of systems (e.g., Forbush & Palfrey, 1983; Haas & Forbush, 1986; O'Grady et al., 1987*b*), bumetanide binding to duck erythrocyte membranes was found to exhibit a biphasic dependence on $[Cl^-]$. The initial stimulatory phase and subsequent inhibitory phase have been suggested to correspond to two putative Cl^- binding sites, one of high affinity (that we term here Cl_H) and another of low affinity (Cl_L) (Haas & Forbush, 1986; O'Grady et al., 1987*b*). Therefore, it was of interest to independently assess the affinity and selectivity of these two sites. Stimulation of bumetanide binding at low $[Cl^-]$ was fit by a rectangular hyperbola with a calculated K_m of 2.5 mM (Fig. 4*b*). Kinetic analysis of the inhibition of bumetanide binding by high $[Cl^-]$ revealed that this was of the noncompetitive type (Fig. 5). In these experiments SO_4^{2-} was used as a truly inert replacement anion as it failed to interact with either Cl_L or Cl_H (*see below*).

Various anions were able to substitute for Cl^- in the stimulation and inhibition of specific bumetanide binding, but the selectivity at each site differed. The efficacy of anions in stimulating binding was: $Cl^- > SCN^- > Br^-$; F^-, I^-, NO_3^-, $MeSO_4^-$, gluconate$^-$,

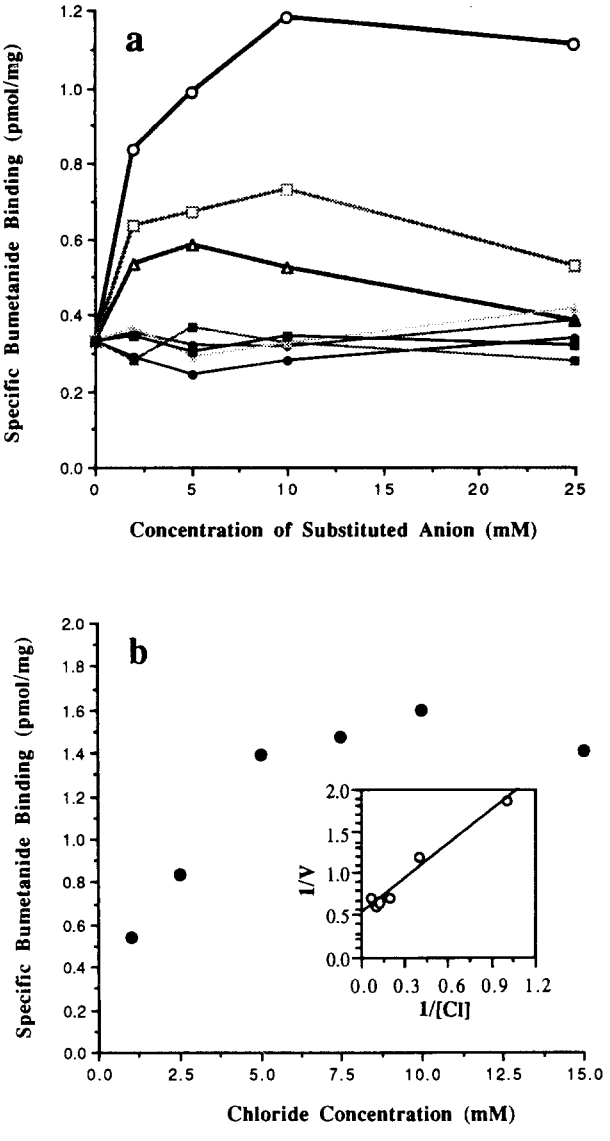

Fig. 4. Stimulation of bumetanide binding by monovalent anions. (*a*) The ability of various anions to substitute for Cl^- was assessed by replacement in the range of 0–25 mM. In each case, $[Na^+]$ and $[K^+]$ were held constant at 110 and 40 mM, respectively, and SO_4^{2-} was used as the inert anion substitute. The total ionic strength was 160 mM. (*b*) Kinetic analysis of the Cl^- stimulatory phase of bumetanide binding. The data can be fit by a rectangular hyperbolic isotherm as indicated in the double reciprocal plot (inset) with K_m for Cl^- of 2.5 mM. The ionic conditions for this experiment were the same as in *a*. The results are the average of duplicate samples from a single experiment, repeated four times. (○) Chloride, (▒) thiocyanate, (△) bromide, (▒) nitrate, (◆) methylsulfate, (●) fluoride, (■) gluconate, (▪) iodide.

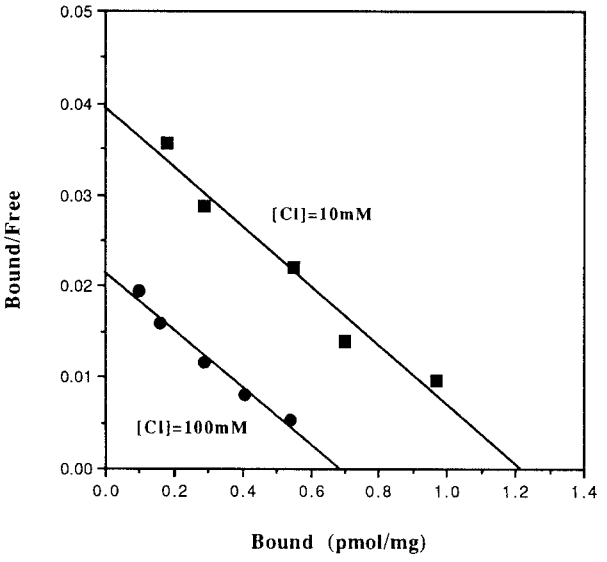

Fig. 5. Noncompetitive nature of high $[Cl^-]$ inhibition of $[^3H]$bumetanide binding. Bumetanide binding curves were constructed in the range 0.01–1 μM at two different $[Cl^-]$ of 10 mM (optimal) and 100 mM (inhibited). The data are plotted in the manner of Rosenthal-Scatchard where the intercept on the abscissa = B_{max} (1.21 and 0.68 pmol/mg protein, respectively) and the slope is $-1/K_D$ (K_D = 0.031 and 0.032 μM, respectively). The reduction in B_{max} with no change in K_D is indicative of a noncompetitive interaction. A similar conclusion emerged from plotting the data in double-reciprocal format or in computer-fitting lines to data plotted in rectangular hyperbolic format. This experiment was repeated three times on different batches of membranes with similar results.

ClO_4^- and SO_4^{2-} were essentially inactive (Fig. 3*a*). The inhibitory potency of various anions was tested in the presence of 10 mM Cl^- to saturate Cl_H (Fig. 6*a–d*). The efficacy of anions in inhibiting binding was found to be: $ClO_4^- > I^- > SCN^- \approx Br^- > NO_3^- > Cl^-$; F^-, $MeSO_4^-$, and gluconate$^-$ and SO_4^{2-} were inactive (Table). Dixon plots all intersected the *y*-axis at approximately 0.8 $pmol^{-1}$, suggesting that each anion interacts with the system at the same site as Cl^- (i.e., Cl_L); the K_i value for Cl^- at this site derived from such a plot was 112.9 mM. Analysis of I^- and SCN^- inhibition of bumetanide binding also showed noncompetitive kinetics (*data not shown*). The apparent K_i's obtained from these plots are compared in the Table.

The relationship between the anion dependence of bumetanide binding in membranes, described above, and cotransport activity in intact cells was assessed by ^{86}Rb influx experiments wherein (*a*) the *inhibitory potency* of various anions (75 mM) at a fixed $[Cl^-]_o$ of 75 mM was determined and (*b*) the ability of other anions to *support transport* when bound to Cl_L (i.e., in the presence of 10 mM Cl^- to saturate Cl_H) was studied. In the former situation, the inhibitory potency of various anions was found to follow the sequence: $SCN^- > ClO_4^- > I^- > NO_3^-$; SO_4^{2-} and other anions were inactive (Fig. 7*a*). In *b* we found no evidence that ions other than Cl^- or

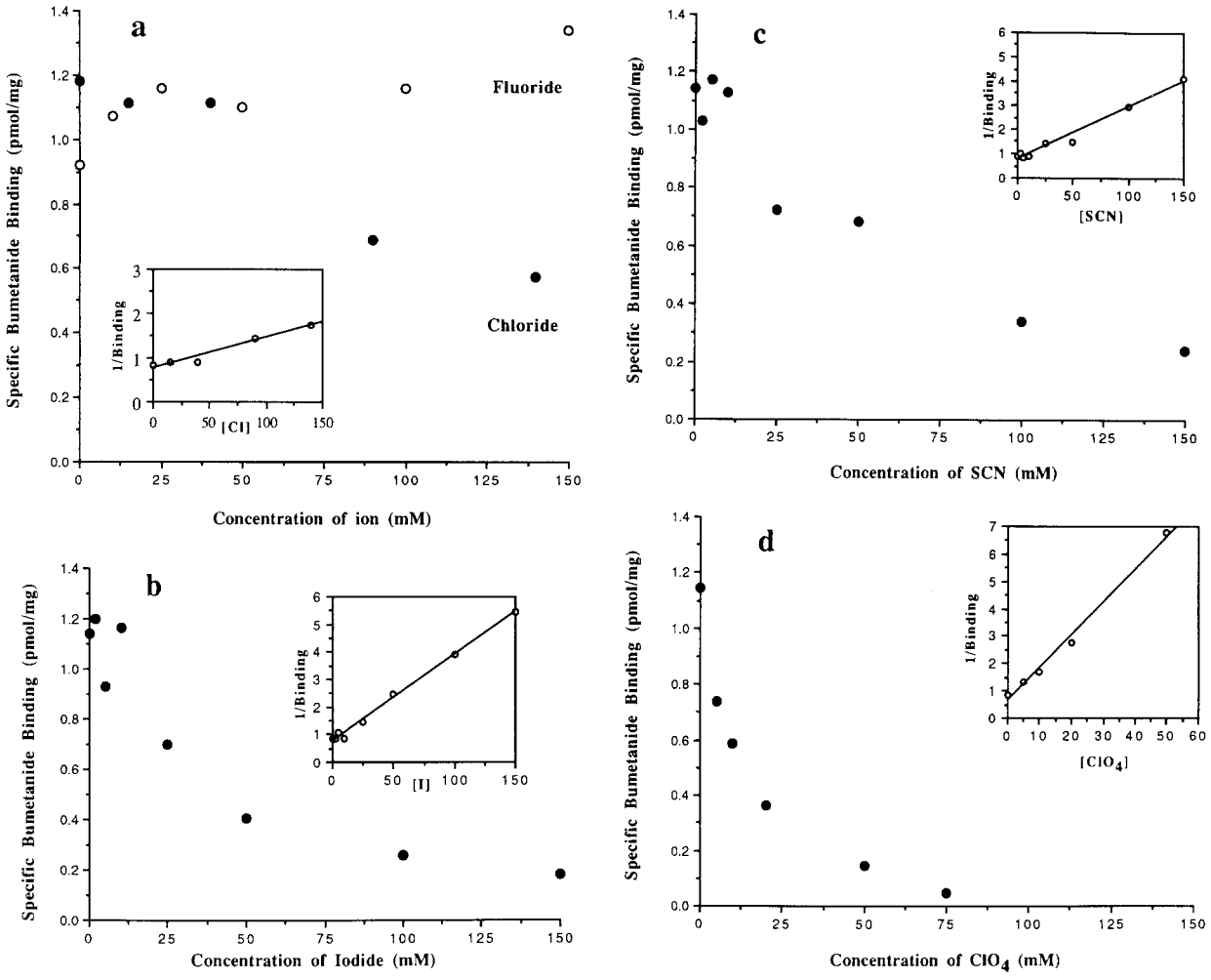

Fig. 6. Inhibition of [^{3}H]bumetanide binding by monovalent anions. Bumetanide binding was performed in a solution containing (in mM) 110 Na^-, 40 K^+ and 10 Cl^-. Various concentrations of the anion to be tested were present as the Na^+ and/or K^+ salt, using SO_4^{2-} as an inert anion substitute. The total ionic strength was 160 mM. Four representative experiments with increasing (*a*) Cl^-, (*b*) I^-, (*c*) SCN^-, and (*d*) ClO_4^- are shown. The inert anion F^- is included in *a* for comparison. Insets show Dixon plots of the data from which the apparent K_i values shown in the Table were obtained. The results are the mean of duplicate samples from a single set of experiments performed on the same batch of membranes. Similar results were obtained with three other membrane preparations.

Table. Inhibitory potencies of various anions on [^{3}H]bumetanide binding

Anion	K_i (mM)[a]
ClO_4^-	5.5
I^-	24.6
SCN^-	34.8
Br^-	34.8
NO_3^-	55.2
Cl^-	112.9
F^-	—
$MeSO_4^-$	—
Gluconate$^-$	—

[a] Values of K_i for above anions were obtained graphically from Dixon plots of data in Fig. 6. F^-, $MeSO_4^-$, and gluconate$^-$ were found to be inert.

Br^- could support transport when bound at Cl_L (Fig. 7*b*).

Effect of Divalent Cations on Bumetanide Binding

In preliminary experiments, we noted that isolation of membranes in buffers of differing [Mg^{2+}] led to different amounts of bumetanide binding in the final membrane preparation. To investigate this phenomenon in more detail, we examined the effect of divalent cation chelation, as well as that of Mg^{2+} and other divalent cations, on bumetanide binding, under optimal monovalent ion conditions. Addition of EDTA and/or EGTA in a nominally divalent cation-

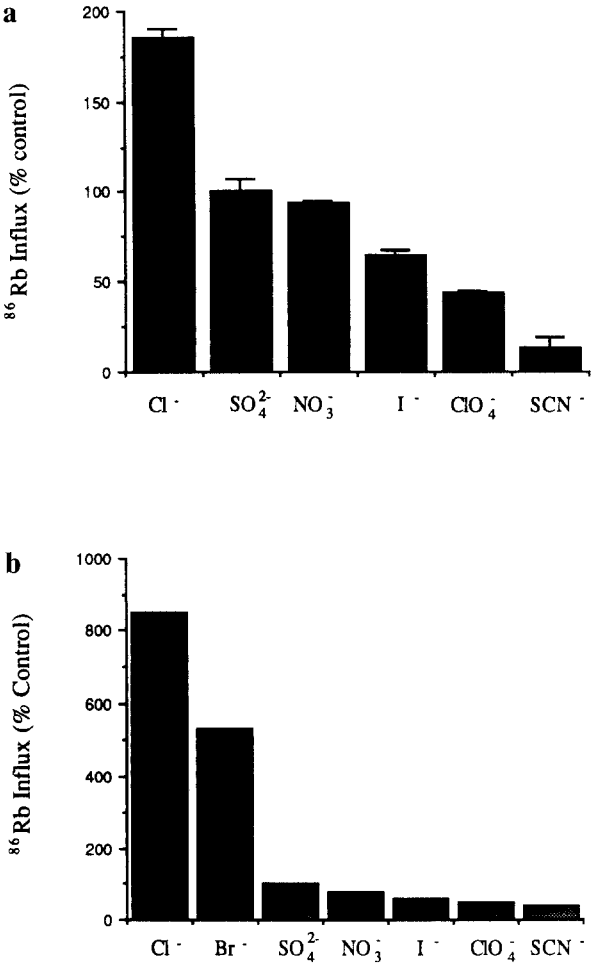

Fig. 7. Effects of various anions on cotransport activity. (*a*) Inhibitory effects of various anions were tested at a concentration of 75 mM (except for SO_4^{2-} at 37.5 mM) in a solution containing (in mM): 145 Na^+, 2.5 K^+ and 75 Cl^-. Cotransport was measured as the bumetanide-sensitive unidirectional influx of ^{86}Rb as described in Materials and Methods, and a representative experiment is shown (results are means ± SEM). (*b*) The ability of anions that interact at Cl_L to support cotransport activity was tested at 75 mM of each anion in a solution containing (in mM): 145 Na^+, 2.5 K^+ and 10 Cl^- (the residual anion was SO_4^{2-}). Cotransport was measured as in *a*. The results are from a single experiment carried out in duplicate. The values with SO_4^{2-} as substituting anion are taken as 100% in both experiments; absolute values were 110 mmol K equivalents/liter cells/hr in *a* and 23.8 mmol/liter cells/hr in *b*.

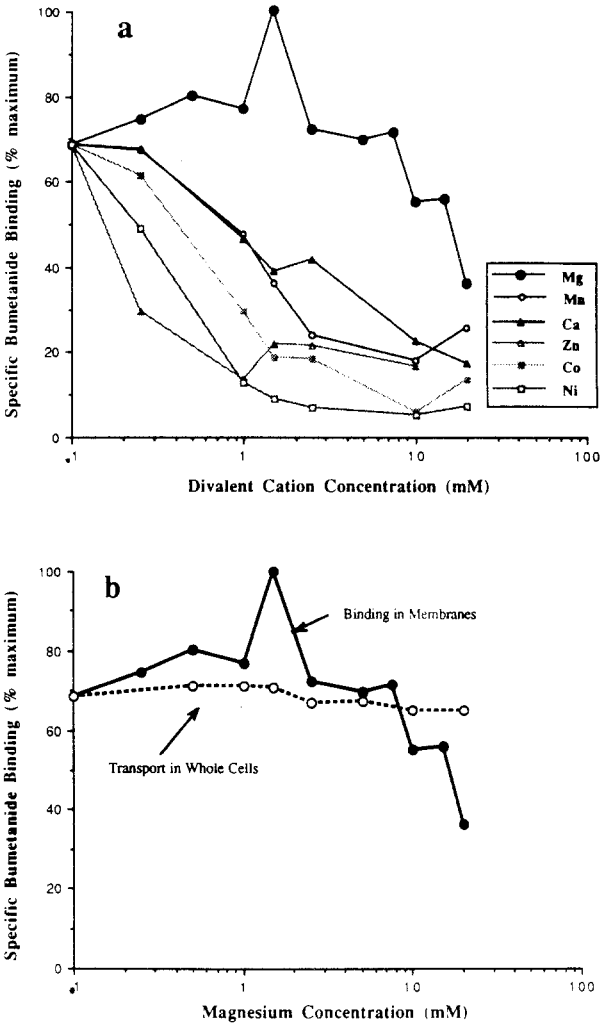

Fig. 8. Effect of divalent cations on [^{3}H]bumetanide binding. (*a*) The ability of various divalent cations (added as sulfate salts) to inhibit bumetanide binding was tested in the range 0–20 mM. The binding assays were performed under optimal monovalent ionic conditions (70–110 mM Na^+, 40 mM K^+, 10 mM Cl^-, 70 mM SO_4^{2-}). The experiments were repeated four times on different batches of membranes with identical results, and the peak at $[Mg^{2+}] = 1.5$ mM was highly reproducible; a representative experiment is shown. (*b*) The effect of external $[Mg^{2+}]$ on Na/K/2Cl cotransport in whole cells was tested. Unidirectional ^{86}Rb flux measurements were performed as indicated in the methods, with various $[Mg^{2+}]_o$ as indicated above. The binding data from *a* is superimposed for comparison. The transport data represent the average of duplicate samples and are expressed here in arbitrary units.

free buffer had little or no effect on bumetanide binding. Increasing $[Mg^{2+}]$ stimulated binding with a sharp, highly reproducible, optimum at approximately 1.5 mM followed by inhibition at higher concentrations, with binding reduced by >50% at 20 mM (Fig. 8*a*). Other divalent cations also inhibited binding, but did not show the narrowly defined peak at 1.5 mM, and appeared to be more potent inhibitors than Mg^{2+}. The order of inhibitory potency was: $Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} \approx Ca^{2+} > Mg^{2+}$ (Fig. 8*a*). The inhibitory effects of divalent cations were similar to those of high anion concentrations in that they were noncompetitive with respect to bumetanide (*data not shown*).

It was of interest to determine if divalent cations exerted effects on Na/K/2Cl cotransport as this

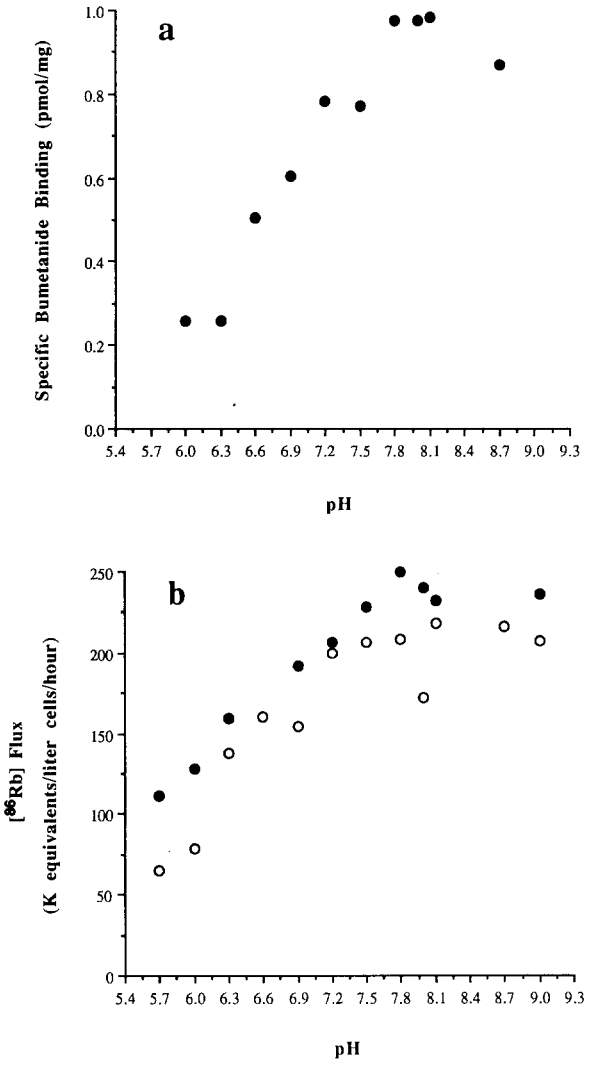

Fig. 9. Effects of pH on [^{3}H]bumetanide binding to membranes and Na/K/2Cl cotransport. (*a*) Membranes (1 mg/ml) isolated from maximally stimulated cells were resuspended in identical solutions of various pH buffered with 10 mM of (MES (pH 6.0–6.9), HEPES (pH 6.9–8.1), or Tris-HCl (pH 8.1–9.0)), and specific [^{3}H]bumetanide binding was measured. The results are the average of duplicate samples. (*b*) The effect of external pH on unidirectional ^{86}Rb influx (1 min influx) in whole cells was assessed as described in Materials and Methods and Fig. 3 (legend). The cells were prestimulated for 15 min with either 10 μM norepinephrine (●) or 10 mM NaF (○). The experiment was repeated three times on different batches of cells with similar results.

might suggest that these ions were binding at an externally disposed site. However, no inhibitory effects were observed even at divalent cation concentrations as high as 20 mM (Fig. 8*b*). The stimulatory effect of Mg^{2+} does not seem to be related to a possible involvement of nucleotides in avian erythrocyte cotransport (Palfrey & Rao, 1983) as addition of various adenine or guanine nucleotides had no effect on bumetanide binding in this preparation (*cf.* Altamirano, Watts & Russell, 1990, who observed a stimulatory effect of ATP and AMP · PNP on bumetanide binding to squid optic lobe microsomes).

Effects of pH on Bumetanide Binding and Ion Transport

Preliminary experiments in turkey erythrocytes had indicated that Na/K/2Cl cotransport was strongly pH dependent at pH < 7 (H.C. Palfrey, *unpublished results*). These findings were confirmed in the present study with duck erythrocytes. Cells were pretreated with 1 mM SITS to block the anion exchanger and minimize effects on cotransport due to shifts in internal pH. Under these conditions, the pH of the external medium was varied and unidirectional ^{86}Rb influx was measured in cells that had been maximally prestimulated at pH 7.4 with norepinephrine or NaF (thus obviating any possible effects of external pH on effector systems for these stimuli). Transport was progressively inhibited as the pH approached 6 (Fig. 9*a*) whichever stimulus was used. Similarly, specific bumetanide binding to isolated membranes was inhibited within the same range (Fig. 9*b*). The inhibition of binding at low pH was reversed on raising the pH above 7, suggesting that protein denaturation was not responsible for the effect.

Discussion

Bumetanide binding has proven to be a powerful tool in the study of the Na/K/2Cl cotransporter, because it is exquisitely sensitive to the conformational state of the system. Recent affinity labeling evidence strongly suggests that bumetanide and its congeners bind to an $M_r \sim 150{,}000$ species of the avian erythrocyte membrane that is the putative Na/K/2Cl cotransport protein (Haas & Forbush, 1988; Pewitt et al., 1990a; *see* Haas (1989) for review). Our purpose here was to use bumetanide binding to probe the cation and anion binding sites on the cotransporter and attempt to provide a biochemical correlate of the physiological selectivity of the cotransporter.

Kinetic studies have revealed that the Na/K/2Cl cotransporter probably binds ions in the sequence Na-Cl-K-Cl (Miyamoto et al., 1986) and apparently transports the complex across the membrane with "glide" symmetry, at least in the avian erythrocyte (Lytle, 1988). The dependence of transport activity on [Na^+] and [K^+] is hyperbolic (Schmidt & McManus, 1977) and suggests the presence of single

binding sites for these cations. The cation binding sites appear to interact as reflected in the dependence of the affinity of one cation on the concentration of the other cation in the extracellular solution (e.g., Palfrey & Rao, 1983). Such heterotropic cooperative interactions suggest conformational changes upon binding of cations to the system. In the present experiments, we observe similar effects in the dependence of bumetanide binding on monovalent cations; e.g. increasing $[K^+]$ from suboptimal to saturating values decreases the K_m for Na^+. As previously described in whole-cell binding studies (Haas & Forbush, 1986) the apparent K_m's of Na^+ and K^+ in transport experiments are in good agreement with the ability of these ions to stimulate bumetanide binding. In the present work we show that the selectivity of the Na^+ and K^+ sites correlates qualitatively well with the ability of cations to support transport activity. However, quantitative differences are apparent. For example, Li^+ is a poor transport substitute for Na^+, but at high concentrations promotes bumetanide binding to a level above that of Na^+. Moreover, in the binding assay Na^+ can also be less effectively substituted by other larger cations such as $NMDG^+$ and $choline^+$ that are completely inactive in transport. Rb^+ substitutes well for K^+ in transport and binding assays, whereas Cs^+ was a poorer substitute and large cations inactive. NH_4^+, on the other hand, exhibited slightly different behavior in transport and binding experiments. This cation was superior to K^+ in transport experiments, but was inferior in binding assays. Such discrepancies may reflect differences in the precise conditions used in the two assays and the fact that in binding experiments both sides of the membrane are exposed to solutions containing the same ionic constitutents while in intact cells ionic composition is asymmetric. Nevertheless, it seems likely that the Na^+ site can recognize, but not transport, large cations such as $NMDG^+$ and $choline^+$.

The dependence of Na/K/2Cl cotransport on external $[Cl^-]$ is more complex than that of Na^+ or K^+ because two Cl ions must bind to form the complete transported species. In several studies the dependence on $[Cl^-]_o$ has been described as "sigmoid" (e.g., Palfrey & Greengard, 1981; Brown & Murer, 1985; Miyamoto et al., 1986; Kort & Koch, 1989), but when studied carefully the Cl^- titration curves have different shapes depending on the substitute anion used (e.g., Palfrey, 1984; Brown & Murer, 1985; Owen & Prastein, 1985; Miyamoto et al., 1986; O'Grady et al., 1987*a*). These studies, as well as preliminary evaluations of the effects of different anions on bumetanide binding in canine kidney (Forbush & Palfrey, 1983), avian erythrocyte (Haas & Forbush, 1986) and shark rectal gland (Forbush & Haas, 1989), suggest that other anions may interact with one or both of the putative Cl^- sites on the cotransporter. However, as the cotransporter has a very narrow anion selectivity in terms of transport activity it is difficult to evaluate the two Cl^- binding sites separately using transport measurements alone. Indeed, in only two studies has it been proposed that the two Cl^- binding sites have a different affinity based on analysis of transport data (Brown & Murer, 1985; Miyamoto et al., 1986).

It has been shown previously that Cl^- has a biphasic effect on bumetanide binding (e.g., Forbush & Palfrey, 1983; Haas & Forbush, 1986; O'Grady et al., 1987*b*; Turner & George, 1988) and we have confirmed and quantitated the phenomenon in this study. Analysis of the stimulatory phase at low $[Cl^-]$ revealed a hyperbolic relationship yielding a K_m value for Cl^- binding to the putative high affinity site (Cl_H) of 2.5 mM. Examination of the inhibitory phase at high $[Cl^-]$ indicated that Cl^- acts as a noncompetitive inhibitor of bumetanide at the putative low-affinity (Cl_L) site, with a K_i of 112.9 mM. Such a result implies that bumetanide interacts with a site that is exposed when the cotransporter binds Na^+, K^+ and a high affinity Cl^- (forming a cotransporter · Na · Cl · K · bumetanide complex) but that the bumetanide binding site is distinct from Cl_L. The apparent affinities of Cl_H and Cl_L using this approach are in the same range as those calculated by analysis of $[Cl^-]$ titration curves from transport experiments in vesicles by Brown and Murer (1985) for the two Cl^- sites in the Na/K/2Cl cotransporter of the kidney cell line LLC-PK1 (5 and 55 mM, respectively). Mixed inhibition (reduction of both K_D and B_{max}) was found in previous studies by O'Grady et al., (1987*b*) for the effects of high $[Cl^-]$ on bumetanide binding to the Na/K/2Cl cotransporter of flounder intestine, whereas Turner and George (1988) have concluded that Cl^- competitively inhibits bumetanide binding to rabbit parotid membranes. Haas and McManus (1983) also surmised, on the basis of transport inhibition measurements, that bumetanide and Cl^- compete for the same site on the duck erythrocyte cotransporter. At present we are unable to explain the discrepancy between transport and binding data in the avian erythrocyte. However, it should be noted that NO_3^- was used as a Cl^- substitute in the transport experiments and, as shown here, that anion is not inert with respect to bumetanide binding. In view of the fact that Cl_L has a selectivity profile similar to that found in many other anion-binding sites in biological systems, if bumetanide did indeed bind to such a site it would not explain its relatively high specificity for the Na/K/2Cl cotransporter.

The marked disparity in the affinities of the two

anion-binding sites for Cl strongly suggests that they must differ chemically from each other. An expectation, therefore, is that the two sites should exhibit different selectivities for anions. We have confirmed this prediction here. Confining the analysis to halides alone, at Cl_H: $Cl^- > Br^-$ with F^- and I^- being inactive; at Cl_L: $I^- > Br^- > Cl^-$ with F^- again being inactive. Moreover, SCN^- interaction with Cl_H and Cl_L, respectively, both stimulated and inhibited bumetanide binding. NO_3^- and ClO_4^- were inactive at Cl_H but inhibited bumetanide binding when bound to Cl_L. The larger anions SO_4^{2-}, $MeSO_4^-$, gluconate$^-$ and glutamate were inert at both sites. To a large extent these findings explain why the cotransporter is very specific for Cl^- and Br^- and probably account for the high degree of specificity found in those transepithelial anion transport pathways where Na/K/2Cl cotransport is involved. It is evident that Cl_H determines the high selectivity of the transport process. Other anions such as I^-, NO_3^- and ClO_4^- bind only to Cl_L and are appropriately not transported while F^- and several larger monovalent anions fail to bind at either site and are similarly unable to support cotransport. SCN^- is apparently anomalous in that it binds both to Cl_L and Cl_H but is not transported; however, this result may explain why SCN^- is the most potent inhibitor of cotransport among the several anions tested here (Fig. 7*b*; *cf.* Owen & Prastein, 1985). The failure of F^- to bind to the cotransporter is interesting in view of its ability to stimulate cotransport activity in avian erythrocytes (Palfrey & Greengard, 1981). It seems likely that F^- activates an unidentified regulatory process that stimulates cotransport (Pewitt et al., 1990*a*).

The present findings differ somewhat from those of Turner and George (1988) who examined the effects of various anions on bumetanide binding in rabbit parotid. In that study, while NO_3^- and F^- behaved in a manner similar to that described here, SO_4^{2-} was found to inhibit bumetanide binding, in contrast to its lack of effect in the avian erythrocyte system. It is interesting to note that the affinity of bumetanide in rabbit parotid is at least an order of magnitude lower than that in the avian erythrocyte, so subtle differences between cotransporters in different tissues and species might well exist. Further differences may occur in the canine kidney and shark rectal gland where there is some evidence for NO_3^- stimulation of bumetanide binding (Forbush & Palfrey, 1983; Forbush & Haas, 1989).

The rank order of potency of anions at Cl_L is similar to the Hofmeister lyotropic series or selectivity sequence 1 of Wright and Diamond (1977). This corresponds to a weak field-strength site found in a number of biological systems, where anions bind in accordance with the inverse order of their hydration energies. For example, many anion channels exhibit a selectivity profile of this type [*see* Frizell (1987) for references]. In contrast, Cl_H is much more selective and corresponds to a higher field-strength site [e.g., sequence 4 or 5 of Wright and Diamond (1977)]. This type of specificity is found more rarely in biological systems. The mammalian erythrocyte KCl cotransporter (Lauf, 1985) and one type of Cl^- channel, found in *Torpedo* electroplax (Miller & White, 1980), are also highly selective for Cl^- and Br^-. The erythrocyte anion exchanger also transports $Cl > Br$ but differs from Cl_H in that it also recognizes NO_3^-, I^-, etc., albeit with lower affinity.

Divalent cations are shown here for the first time to affect bumetanide binding. While all divalents inhibited binding, the Mg^{2+} curve was clearly biphasic, with enhancement at low concentrations followed by inhibition at higher concentrations (Fig. 8*a*). The complexity of the curve may reflect superimposition of low $[Mg^{2+}]$ stimulation at a site which is very Mg^{2+} selective upon high $[Mg^{2+}]$ inhibition at a site that may accept several divalent cations. Neither site appears to be on the external face of the membrane as addition of divalent cations to intact duck erythrocytes had little effect on cotransport activity. Instead, the finding of a putative stimulatory Mg^{2+} site may be related to recent reports of the dependence of cotransport on internal Mg^{2+} in ferret (Flatman, 1988), avian (Starke & McManus, 1990) and human (Mairbaurl & Hoffman, 1990) red cells. In these systems the effective $[Mg^{2+}]$ is in the range of the stimulation seen here. In contrast, divalent cations have an exclusively inhibitory effect on KCl cotransport in mammalian erythrocytes [*see* Lauf (1985) for review]. Whether Mg^{2+} is a physiological regulator of Na/K/2Cl cotransport, as has been suggested, remains to be seen.

The effects of pH on bumetanide binding and cotransport activity seemed to be well correlated. These results suggest the presence of titratable group(s) in the cotransporter that may be accessible from the external face of the membrane. The apparent pK_a of the titratable residue is above 6 and thus may represent a histidine residue or a carboxylate in an unusual environment. Whether the effects of pH are related to one of the ion binding sites remains to be tested. It is hoped that future chemical modification studies may shed light on the specific groups involved.

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