Anion dependence of bumetanide binding and ion transport by the rabbit parotid $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transporter: evidence for an intracellular anion modifier site

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The anion dependence of [^3H]bumetanide binding and $^{22}\text{Na}^+$ transport by the rabbit parotid $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transporter was studied in acinar basolateral membrane vesicles (BLMV). Cl^- , Br^- and NO_3^- have a biphasic effect on binding consistent with the presence of two anion sites associated with the bumetanide binding event, a high-affinity stimulatory site and a lower-affinity inhibitory site. We show that formate shares only the stimulatory site and SO_4^{2-} only the inhibitory site. The initial rate of [^3H]bumetanide binding was stimulated by formate or low $[\text{Cl}^-]$ and inhibited by SO_4^{2-} or high $[\text{Cl}^-]$, but the rate of [^3H]bumetanide dissociation was not affected by the presence of these anions in the dissociation medium. However, when [^3H]bumetanide was bound to BLMVs in the presence of formate its rate of dissociation was more than four times faster than when

binding took place in the presence of Cl^- . These observations indicate that the binding of bumetanide and the stimulatory anion are ordered such that the anion must necessarily bind first and subsequently cannot dissociate until after bumetanide dissociates. In zero-trans-flux experiments, extravesicular SO_4^{2-} and formate had no effect on $^{22}\text{Na}^+$ transport via the co-transporter [Turner and George (1988) *J. Membr. Biol.* **102**, 71–77]. Thus neither of the anion sites associated with bumetanide binding is a Cl^- transport site. However, we show here that SO_4^{2-} inhibits transport when present in the intravesicular space. Since the BLMV preparation is predominantly oriented cytosolic-side-in, this observation indicates the existence of an inhibitory cytosolic anion modifier site. Our data suggest that this site is identical to the inhibitory anion site associated with bumetanide binding.

INTRODUCTION

Salt and water movements across many epithelial tissues have been shown to be due to net transepithelial fluxes of Cl^- driven by a $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transporter localized to either the apical (absorptive epithelia) or basolateral (secretory epithelia) membrane [1,2]. In the kidney this transporter is the site of action of the commonly prescribed loop diuretic furosemide and its more potent and specific analogue bumetanide. Recent work from several laboratories [3,4], including our own [5], has provided strong evidence that the $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transporter plays a major role in salivary fluid secretion. Consistent with these observations we have demonstrated the presence of $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transport activity in basolateral membrane vesicles (BLMV) prepared from the rat and rabbit parotid [6,7]. In addition we have identified a high-affinity ($K_d \sim 3 \mu\text{M}$) bumetanide binding site in rabbit parotid basolateral membranes and provided convincing evidence that this site is identical with the bumetanide inhibitory site associated with the $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transporter in this preparation [8]. Based on these bumetanide binding studies and more recent estimates of the molecular mass of the co-transporter (135 kDa; [9]) we calculate that this protein accounts for $>1\%$ of the total rabbit parotid basolateral membrane protein, making this tissue one of the richest sources of the $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transporter identified to date.

As for most other tissues studied, the $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transporter of the rabbit parotid has a transport coupling stoichiometry of 1 Na^+ :1 K^+ :2 Cl^- [7]. As also typically found, bumetanide binding to the parotid co-transporter is dependent on the simultaneous presence of Na^+ , K^+ and Cl^- in the medium

[8]. In our hands, maximal [^3H]bumetanide binding to rabbit parotid BLMVs was found at approximately 100 mM Na^+ , 100 mM K^+ and 5 mM Cl^- [8]. In these earlier experiments we observed that bumetanide binding increased hyperbolically with both $[\text{Na}^+]$ and $[\text{K}^+]$, consistent with a binding stoichiometry of 1 Na^+ :1 K^+ :1 bumetanide. However, the dependence of binding on $[\text{Cl}^-]$ was found to be biphasic, rising dramatically (~ 6 -fold) as the medium $[\text{Cl}^-]$ was increased from 0 to 5 mM (at $[\text{Na}^+] = [\text{K}^+] = 100 \text{ mM}$), then falling back to levels observed in the absence of Cl^- with increasing $[\text{Cl}^-]$. These results indicate that there are at least two Cl^- binding sites involved in the bumetanide binding event, i.e. a high-affinity stimulatory site ($K_d \approx 1 \text{ mM}$) and a lower-affinity inhibitory site ($K_d \approx 20 \text{ mM}$). Qualitatively similar observations have been made in bumetanide binding studies with plasma membrane fractions from a number of other tissues [10–14], suggesting that this biphasic dependence on $[\text{Cl}^-]$ is a common feature of $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transporters.

The above observations of 1 Na^+ , 1 K^+ and 2 Cl^- sites associated with both transport and bumetanide binding by the $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transporter make it tempting to speculate that the same ion binding sites are involved in both events. Indeed, a good correlation has been observed between the half maximal concentrations of Na^+ and K^+ required for co-transport activity and bumetanide binding in several tissues [1]. Also, in at least some tissues there is evidence for competition between Cl^- and bumetanide for a common site [8,13,15] consistent with the hypothesis that the inhibitory effect of bumetanide may be due to a direct interaction at a Cl^- transport site. However, in our previous studies of bumetanide binding to rabbit parotid BLMVs [8] we found that the ability of some anions to mimic the

inhibitory effect of Cl^- on bumetanide binding did not correlate well with their abilities to support co-transport activity in the same preparation, suggesting that the inhibitory Cl^- site associated with bumetanide binding may not be a transport site after all.

Since bumetanide and its analogues are not only powerful tools for the study of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter but also drugs of considerable clinical importance, the elucidation of their mode of interaction with this transport protein is obviously of great interest. In the present paper we continue our previous examination of the anionic dependence of bumetanide binding to the rabbit parotid $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter. Our data indicate that neither of the anion binding sites associated with bumetanide binding is, in fact, a Cl^- transport site. Indeed, we present evidence that one of these sites is an intracellular modifier site whose occupation results in inhibition of the co-transporter. The properties of this modifier site are consistent with it playing a significant regulatory role in co-transport activity under normal physiological conditions.

MATERIALS AND METHODS

Membrane preparation

BLMVs were prepared from rabbit parotid by a Percoll gradient method as previously described [7]. Freshly prepared vesicles were suspended in Buffer A (10 mM Hepes plus 100 mM mannitol buffered with Tris to pH 7.4) containing 100 mM KCl and 1 mM EDTA at a protein concentration of approximately 5 mg/ml. Aliquots (0.75 mg of protein) of BLMV were fast frozen and stored above liquid nitrogen.

Binding and uptake measurements

On the day of the experiment a suitable number of aliquots of frozen vesicles were thawed for 20 min at room temperature, diluted 100 times with Buffer A containing 1 mM EDTA, and spun at 48000 *g* for 20 min. The resulting pellets were taken up in the same medium at a protein concentration of approximately 2 mg/ml.

Equilibrium bumetanide binding was measured using a Millipore filtration assay as previously described [8]. Briefly, a 20 μl aliquot of BLMV was combined with the same volume of incubation medium consisting of Buffer A plus [^3H]bumetanide and other constituents as required (see Figure and Table captions). After 60 min of incubation at 25 °C the reaction was terminated by the addition of 1.5 ml of ice-cold Stop Solution (Buffer A containing 100 mM NaCl plus 100 mM KCl) followed by Millipore filtration (HAWP 0.45 μm pore size). The filter was then washed with a further 6.0 ml of Stop Solution, placed in a scintillation vial with 10 ml of ACS (Aqueous Counting Scintillant, Amersham, Arlington Heights, IL, U.S.A.) containing 1% acetic acid (v/v) and counted for radioactivity along with samples of the incubation medium and appropriate standards. All equilibrium binding data were corrected for low-affinity binding and non-specific trapping of [^3H]bumetanide by the membranes and filter by subtracting the binding measured in the presence of 1 mM unlabelled bumetanide. Non-specific binding and trapping measured in this way was 3.9 ± 0.3 pmol/mg of protein at 1 μM [^3H]bumetanide ($n = 10$). Previous work from our laboratory has shown that the resulting 'specific' component of binding is due to a single bumetanide binding site with $K_d \approx 3 \mu\text{M}$ at 100 mM Na^+ , 100 mM K^+ and 5 mM Cl^- [8]. Under these ionic conditions, at 1 μM bumetanide, binding is typically ≈ 30 pmol/mg of protein ([8] and Table 1 of the present paper). This value is ~ 30 -times larger than the amount of bumetanide

which will equilibrate with the intravesicular space (estimated to be $\approx 1.1 \mu\text{l}/\text{mg}$ of protein from the equilibrium uptake of $^{22}\text{Na}^+$, see, for example, legend of Figure 3 in this paper) and thus clearly represents bumetanide binding as opposed to uptake.

The initial rate of [^3H]bumetanide binding was measured as described above except that the binding reaction was terminated (by the addition of Stop Solution) after 1 min of incubation. In control experiments (results not shown) we have established that [^3H]bumetanide binding is linear over this time interval and thus that a 1 min point is a measure of the initial rate of binding. These data were not corrected for low-affinity bumetanide binding but were corrected for non-specific trapping of [^3H]bumetanide by the membranes and filter by subtracting the binding measured at 'zero time' (1.77 ± 0.22 pmol/mg of protein at 1 μM [^3H]bumetanide, $n = 8$). Binding at 'zero time' was determined by adding the Stop Solution to the BLMVs before the incubation medium and filtering as usual.

The initial rate of bumetanide dissociation was measured by diluting 15 μl of BLMV pre-equilibrated (≥ 1 h) with [^3H]bumetanide under the conditions indicated into 1500 μl of dissociation medium consisting of Buffer A plus appropriate salts (see text and Figure legends for details). [^3H]Bumetanide still bound to the membranes at later times was then determined by Millipore filtration as described above. [^3H]Bumetanide bound at time zero was measured by diluting 15 μl of BLMVs directly into ice-cold Stop Solution and filtering as usual.

$^{22}\text{Na}^+$ uptake measurements were carried out in essentially the same manner as [^3H]bumetanide binding using 10 μl of vesicles and 40 μl of incubation medium (see [7] for details). Uptakes were measured after 15 s of incubation and represent initial uptake rates [7].

All binding and uptake studies were carried out in triplicate or quadruplicate; standard errors on these experimental points were typically $< 3\%$. Unless otherwise stated the results are averages $\pm$ standard errors of the number of independent experiments indicated (where no error bars are shown they lie within the symbols drawn). *P* values < 0.05 (Student's *t*-test) were taken to represent statistically significant differences. In some cases bumetanide binding results are averages $\pm$ range of duplicate experiments. The results of experiments only repeated twice are supported by additional studies (some of which are also shown) carried out under slightly different experimental conditions. Based on our previous [^3H]bumetanide binding and $^{22}\text{Na}^+$ uptake studies [7,8], as well as the results presented here, we have assumed that the non-physiological ions *N*-methyl-D-glucamine (NMDG) and gluconate are inactive substitutes for the various ions which interact with the parotid $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter.

Orientation of vesicle preparation

The orientation of the BLMV preparation (cytosolic-side-out, cytosolic-side-in and unvesiculated) was determined by measuring K^+ -dependent *p*-nitrophenyl phosphatase (KpNPPase) activity (hydrolysis of *p*-nitrophenyl phosphate by Na^+/K^+ ATPase) in the presence and absence of ouabain (1 mM) and digitonin (0.0075%). The rationale for the method is described in the text. KpNPPase activity was determined at 25 °C in Buffer A containing 10 mM KCl, 6 mM MgCl_2 , 2 mM EGTA, 0.33 mM EDTA, 6 mM *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate, Sigma Chemical Co., St. Louis, MO, U.S.A.) and 0.05 mg of BLMV protein/ml. The reaction volume was 600 μl and the assay was terminated by the addition of 2.0 ml of 0.2 M NaOH. *p*-Nitrophenyl concentrations were determined from the absorbance of the sample at 405 nm.

Materials

$^{22}\text{Na}^+$ was from New England Nuclear (Boston, MA, U.S.A.), NMDG was from Aldrich (Milwaukee, WI, U.S.A.) and unlabelled bumetanide was a gift from Hoffman-LaRoche (Nutley, NJ, U.S.A.). All other chemicals were from standard commercial sources and were reagent grade or the highest purity available.

[^3H]Bumetanide (66.2 Ci/mmol) was custom synthesized for us by Amersham (Arlington Heights, IL, U.S.A.) from the precursor 3-amino-4-phenoxy-5-sulphamoylbenzoic acid, generously supplied by Dr. P. W. Feit (Leo Pharmaceuticals, Ballerup, Denmark).

Protein was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with bovine γ globulin as the standard.

RESULTS AND DISCUSSION

Dependence of bumetanide binding on anions

As discussed above, in previously published work from our laboratory [8], using the same rabbit parotid BLMV preparation employed here, we have shown that there are at least two Cl^- sites associated with bumetanide binding to the rabbit parotid $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ co-transporter, i.e. a high-affinity stimulatory site and a lower-affinity inhibitory site. In these earlier studies we also found that the following anions mimic the inhibitory effect of Cl^- with the relative potencies indicated,

$\text{Br}^- > \text{Cl}^- > \text{SO}_4^{2-} \approx \text{NO}_3^-$ (formate, F^- , acetate and gluconate inactive) (1)

In the experiments summarized in Table 1 we examined the abilities of these same anions to produce the stimulatory effect of low $[\text{Cl}^-]$ on bumetanide binding. Each anion was tested at 2.5 mM and 25 mM. These data indicate the following specificity for stimulation of bumetanide binding,

$\text{Cl}^- \approx \text{Br}^- > \text{NO}_3^- \approx \text{formate}$ (SO_4^{2-} , F^- , acetate and gluconate inactive) (2)

Combining these results with those given in eqn. (1) we conclude that Br^- and NO_3^- share both the stimulatory and inhibitory Cl^-

Figure 1 Effect of $[\text{NO}_3^-]$, $[\text{formate}]$ and $[\text{SO}_4^{2-}]$ on bumetanide binding to rabbit parotid BLMVs

(a) The binding of $1 \mu\text{M}$ [^3H]bumetanide was measured as described in the Materials and methods section in the presence of 100 mM Na^+ , 100 mM K^+ , and 0 to 200 mM NO_3^- with NO_3^- replacing gluconate. The results are the averages of two independent experiments normalized to the binding observed at 25 mM NO_3^- . (b) The binding of $1 \mu\text{M}$ [^3H]bumetanide was measured in the presence of 100 mM Na^+ , 100 mM K^+ , and 0 to 200 mM formate with formate replacing gluconate. The results are the averages of three independent experiments normalized to the binding observed at 25 mM formate. (c) The binding of $1 \mu\text{M}$ [^3H]bumetanide was measured in the presence of 100 mM Na^+ , 100 mM K^+ , 5 mM Cl^- and 0 to 100 mM SO_4^{2-} with SO_4^{2-} replacing gluconate. The results are the averages of two independent experiments normalized to the binding observed in the absence of SO_4^{2-} .

Table 1 Abilities of various anions to stimulate bumetanide binding to rabbit parotid BLMV

The binding of $1 \mu\text{M}$ [^3H]bumetanide was measured as described in the Materials and methods section in the presence of 50 mM Na^+ , 50 mM K^+ (52.5 mM with SO_4^{2-}), 97.5 mM gluconate and 2.5 mM of the test anion, or in the presence of 100 mM Na^+ , 100 mM K^+ (125 mM with SO_4^{2-}), 175 mM gluconate and 25 mM of the test anion, as indicated. The results shown are the averages $\pm$ S.E.M. of three independent experiments. Values marked with an asterisk indicate binding significantly greater than observed with gluconate.

Anion	Equilibrium bumetanide binding (pmol/mg of protein)	
	2.5 mM anion	25 mM anion
Chloride	$29.9 \pm 2.8^*$	$13.5 \pm 1.0^*$
Bromide	$27.2 \pm 2.8^*$	7.6 ± 0.3
Formate	11.6 ± 1.5	$25.4 \pm 1.6^*$
Nitrate	12.5 ± 1.3	$20.5 \pm 1.8^*$
Fluoride	8.7 ± 1.7	8.1 ± 1.6
Sulphate	8.9 ± 0.8	5.2 ± 0.7
Acetate	8.3 ± 1.5	7.2 ± 0.7
Gluconate	9.9 ± 1.0	7.4 ± 1.6

sites, while formate shares only the stimulatory site, SO_4^{2-} shares only the inhibitory site, and F^- , acetate and gluconate are non-interacting.

The above effects of NO_3^- , formate and SO_4^{2-} are explored in more detail in Figure 1. In the upper panel of Figure 1 we illustrate that bumetanide binding to rabbit parotid BLMVs does, indeed, have a biphasic dependence on $[\text{NO}_3^-]$ as previously

Table 2 Effects of ions on the initial rate of bumetanide association to rabbit parotid BLMV

The initial rate of [^3H]bumetanide binding was measured as described in the Materials and methods section in the presence of the ions and compounds indicated. Na^+ and K^+ were replaced with the non-interacting cation NMDG [8] and anions replaced gluconate. The results are the averages of three independent experiments normalized to the initial rate of binding observed under 'control' conditions (100 mM sodium gluconate, 95 mM potassium gluconate plus 5 mM KCl; the absolute rate of binding under 'control' conditions was 4.9 ± 0.65 pmol/min per mg of protein, $n = 3$).

Experimental condition				Initial rate of binding (rate/control)
[Na^+]	[K^+]	[Cl^-]	Other	
100	100	5	—	1.00 (control)
100	100	5	1 mM bumetanide	0.49 ± 0.08
0	100	5	—	0.38 ± 0.02
100	0	5	—	0.44 ± 0.07
100	100	0	—	0.50 ± 0.08
100	100	25	—	0.71 ± 0.03
100	100	0	100 mM formate	3.19 ± 0.54
100	100	5	50 mM SO_4^{2-}	0.74 ± 0.03

observed for Cl^- [8], although as expected from eqns. (1) and (2), NO_3^- is less potent than Cl^- in both its stimulatory and inhibitory effects ($K_{\frac{1}{2}}$ values for the stimulatory and inhibitory effects of Cl^- are ≈ 1 mM and ≈ 20 mM, respectively). The dependence of bumetanide binding on [formate] is illustrated in Figure 1(b). Stimulation of bumetanide binding by formate is half-maximal at approximately 10 mM and no evidence for a significant inhibitory effect is evident over the concentration range studied here (0–200 mM). In Figure 1(c) the concentration dependence of inhibition of binding by SO_4^{2-} is illustrated. A half-maximal effect of SO_4^{2-} is seen at approximately 65 mM. Analysis of these data on a Dixon plot (not shown) yields a good straight line, indicating that the effect of SO_4^{2-} is consistent with its interaction at a single inhibitory site. In additional experiments (not shown) we have also confirmed that F^- has no significant effect on bumetanide binding over the concentration range 0–200 mM (same experimental conditions as formate and NO_3^- in Figure 1).

In studies of the bumetanide binding properties of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter of duck erythrocytes Hegde and Palfrey [14] found similar results for Br^- and F^- ; however, in contrast to our results they found that NO_3^- shared only the inhibitory Cl^- site (with a higher potency than Cl^-) and that SO_4^{2-} shared neither site in this tissue. In similar experiments with dog outer medullary plasma membranes Forbush and Palfrey [10] found that NO_3^- shared both Cl^- sites. These data clearly indicate the existence of significant species-to-species or tissue-to-tissue differences in the anion specificity of bumetanide binding. Some of these differences can almost certainly be accounted for by the recent evidence from molecular cloning studies that there are at least two isoforms of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter, one expressed in secretory epithelia [16,17] and the other in absorptive epithelia [18,19]. Whether there is yet another isoform in non-epithelial tissues remains to be determined.

Ionic dependence of bumetanide association and dissociation

The effects of the ionic composition of the medium on the initial rate of bumetanide binding to rabbit parotid BLMVs are illustrated in Table 2. The data demonstrate a marked dependence

Figure 2 Effects of the association medium on bumetanide dissociation from rabbit parotid BLMVs

[^3H]bumetanide dissociation was measured as described in the Materials and methods section by diluting (1:100) BLMV pre-equilibrated with $0.1 \mu\text{M}$ [^3H]bumetanide in the presence of 100 mM sodium gluconate, 95 mM potassium gluconate and 5 mM KCl (○); 100 mM sodium gluconate, 75 mM potassium gluconate and 25 mM potassium formate (▽); or 50 mM Na_2SO_4 , 95 mM potassium gluconate and 5 mM KCl (●) into dissociation media containing 100 mM sodium gluconate, 95 mM potassium gluconate and 5 mM KCl. [^3H]bumetanide binding at each time point has been expressed as a percentage of binding before dilution. The results are the averages $\pm$ S.E.M. of three independent experiments.

on Na^+ , K^+ and Cl^- . Relative to measurements carried out in the presence of 100 mM Na^+ , 100 mM K^+ and 5 mM Cl^- ('control'), the initial rate of binding is reduced to non-specific levels (seen in the presence of 1 mM unlabelled bumetanide) in the absence of any one of these ions. Furthermore, when Cl^- levels are raised to 25 mM, where we expect significant occupancy of the Cl^- inhibitory site (see above), the initial rate of binding is also reduced relative to that seen with 5 mM Cl^- . Consistent with the specificity of the Cl^- stimulatory and inhibitory sites documented above we find that bumetanide binding is inhibited when 50 mM SO_4^{2-} is added in the presence of 5 mM Cl^- and markedly stimulated when 5 mM Cl^- is replaced with 100 mM formate.

In contrast to the above results, the rate of bumetanide dissociation was found to be independent of the composition of the dissociation medium. Briefly stated, in these experiments (results not shown) BLMVs pre-equilibrated with $1 \mu\text{M}$ [^3H]bumetanide in the presence of 100 mM sodium gluconate, 95 mM potassium gluconate and 5 mM KCl were diluted 1:100 into the same medium or into Na^+ -free, K^+ -free, Cl^- -free, high- Cl^- (200 mM), high-formate (200 mM) or high- SO_4^{2-} (100 mM) modifications of this medium (with NMDG replacing cations, and gluconate replacing, or replaced by anions). None of these media yielded [^3H]bumetanide dissociation rates significantly different from one another (average dissociation rate = 3.3 ± 0.1 %/min).

The above data are consistent with an ordered binding scheme in which all of the various ionic sites associated with the bumetanide binding event (the Na^+ , K^+ and Cl^- stimulatory sites and the Cl^- inhibitory site) are accessible before bumetanide binds (thus their occupancy affects the rate of bumetanide association) but are inaccessible after bumetanide binds (thus the presence or absence of these ions in the dissociation medium has no effect on the rate of dissociation). An experiment which addresses this point further for the stimulatory and inhibitory anion sites is shown in Figure 2. Here we have preincubated BLMVs with [^3H]bumetanide in the presence of Na^+ and K^+ and

either Cl^- (○), formate (▽) or Cl^- plus SO_4^{2-} (●), then monitored dissociation after dilution into our standard solution containing 100 mM Na^+ , 100 mM K^+ and 5 mM Cl^- . No significant difference was observed between dissociation rates for vesicles preincubated in the presence of Cl^- alone or Cl^- plus SO_4^{2-} ; however, [^3H]bumetanide dissociated significantly faster (> 4-fold) from vesicles preincubated in the presence of formate. This latter observation shows clearly that the rate of bumetanide dissociation is highly dependent on the identity of the stimulatory anion present during the bumetanide binding event. Taken together with the above results, this observation provides strong evidence that the stimulatory anion remains present at its binding site even after its removal from the medium (by dilution) and that this anion cannot be replaced by external anions after bumetanide is bound.

An interpretation consistent with these data is that Na^+ , K^+ and Cl^- bind to their respective stimulatory sites on the protein thus generating the high-affinity bumetanide binding site. Bumetanide, upon binding to this site, occludes the occupied ion binding sites and prevents dissociation from them until bumetanide itself dissociates. A similar suggestion has been made in a preliminary study of the $\text{Na}^+/\text{K}^+-2\text{Cl}^-$ co-transporter of the shark rectal gland [20].

The observations that (i) the initial rate of bumetanide binding is reduced at high chloride concentration or in the presence of added SO_4^{2-} (Table 2), and (ii) that bumetanide dissociation is not affected by these conditions or by the presence of SO_4^{2-} during the bumetanide binding event (Figure 2), are consistent with the hypothesis that the occupation of the inhibitory anion site and the bumetanide binding site are mutually exclusive.

Are the anion sites associated with bumetanide binding also chloride transport sites?

In previously published studies [8] we found that the relative abilities of anions to stimulate $^{22}\text{Na}^+$ fluxes via the $\text{Na}^+/\text{K}^+-2\text{Cl}^-$ co-transporter under 'zero trans' conditions were given by

$\text{Br}^- > \text{Cl}^- > \text{F}^- > \text{NO}_3^-$ (formate, SO_4^{2-} , acetate and gluconate inactive) (3)

when the anion was present alone (the extravesicular medium contained 1 mM $^{22}\text{Na}^+$ and 100 mM of the K^+ salt of the anion), and by

$\text{Br}^- > \text{Cl}^- > \text{NO}_3^- > \text{F}^-$ (formate, SO_4^{2-} , acetate and gluconate inactive) (4)

when the anion was present along with Cl^- (the extravesicular medium contained 1 mM $^{22}\text{Na}^+$, 50 mM KCl and 100 mM of the K^+ salt of the anion). Briefly stated, these results indicate that Br^- , F^- and NO_3^- share both Cl^- transport sites while formate, SO_4^{2-} , acetate and gluconate are non-interacting, i.e. they neither stimulate nor inhibit $^{22}\text{Na}^+$ fluxes via the co-transporter. The differences in relative specificities for NO_3^- and F^- in the presence and absence of Cl^- (cf. eqns. 3 and 4) may indicate that NO_3^- has a low affinity for one transport site and thus that its effectiveness is enhanced when this site is occupied by Cl^- .

Comparison of the abilities of various anions to support $^{22}\text{Na}^+$ transport via the co-transporter under zero trans conditions (eqns. 3 and 4) with their relative specificities for the inhibitory and stimulatory anion sites associated with bumetanide binding (eqns. 1 and 2, respectively) reveal several differences. For example, both F^- and NO_3^- can support transport in the absence of Cl^- , indicating that they share both Cl^- transport sites, but F^- has no stimulatory or inhibitory effect on bumetanide binding under the same experimental conditions where NO_3^- mimics the

biphasic effects of Cl^- (Table 1 and Figure 1). Also, SO_4^{2-} inhibits and formate stimulates bumetanide binding, each with effects comparable to NO_3^- (Table 1 and Figure 1); however, neither SO_4^{2-} nor formate has any significant effect on zero trans $^{22}\text{Na}^+$ flux under conditions where NO_3^- is clearly effective (eqns. 3 and 4 and [8]). Taken together, these observations strongly suggest that the anion sites associated with bumetanide binding are not Cl^- transport sites (note that thermodynamic constraints require that a transported substrate be active from both sides of the membrane). Nevertheless, since formate and SO_4^{2-} clearly interact strongly with the bumetanide binding site it seems strange that neither of these anions should have any effect on transport. It occurred to us a possible explanation of this puzzling behaviour might lie in a difference between the methods we have employed to measure bumetanide binding and $^{22}\text{Na}^+$ transport. More specifically, we noted that eqns. (1) and (2) are based on bumetanide binding studies carried out at ionic equilibrium (after 1 h of incubation) whereas eqns. (3) and (4) are based on $^{22}\text{Na}^+$ fluxes measured under zero trans conditions where the anions tested were only present in the extravesicular space. Accordingly we wondered if the effects of SO_4^{2-} or formate might occur at the intravesicular side of the membrane.

We first carried out experiments to determine the orientation of the BLMV preparation. BLMVs KpNPPase activity was measured (as described in the Materials and methods section): (a) in the presence of digitonin (0.0075 %), (b) in the presence of ouabain (1 mM), (c) in the presence of digitonin plus ouabain, and (d) in the absence of both digitonin and ouabain.

The idea here was to take advantage of the facts that *p*-nitrophenyl phosphate and ouabain interact with Na^+/K^+ ATPase at the intracellular and extracellular sides of the membrane respectively, and that permeabilization by digitonin would allow access to intravesicular sites. In preliminary experiments (results not shown) we found that KpNPPase activity increased with digitonin concentrations up to 0.003 % and then remained constant over the concentration range 0.003–0.01 %. These data indicate that a digitonin concentration of 0.0075 % results in permeabilization of BLMVs without inhibition of KpNPPase activity. Total BLMV ouabain-sensitive KpNPPase activity is then given by the difference between the activities measured under conditions (a) and (c) above. By similar arguments the activity due to unvesiculated membrane fragments is given by (d) minus (b), that due to cytosolic-side-out vesicles by (b) minus (c), and that due to cytosolic-side-in vesicles by (a) minus (d). The averaged results of four experiments of this type indicate that our rabbit parotid BLMV preparation is oriented 30 ± 4 % cytosolic-side-in and 10 ± 2 % cytosolic-side-out with 60 ± 5 % of the membranes unvesiculated. Thus the vesiculated membrane fragments, which are the only ones which contribute to $^{22}\text{Na}^+$ flux studies, are predominantly (75 %) oriented cytosolic-side-in.

In the experiments shown in Figure 3 we have studied the effects of loading BLMVs with formate or SO_4^{2-} on initial $^{22}\text{Na}^+$ uptake rates. Loading with 25 mM formate had no significant effect on $^{22}\text{Na}^+$ flux; however, loading with 50 mM SO_4^{2-} significantly reduces $^{22}\text{Na}^+$ uptake ($P < 0.002$) relative to gluconate-loaded controls. Neither of these experimental manoeuvres had any effect on equilibrium $^{22}\text{Na}^+$ uptake (see legend to Figure 3), indicating that the reduced flux seen with SO_4^{2-} loading is not due to differences in degree of vesiculation in the various loading buffers. The facts that (i) our BLMV preparation is predominantly oriented cytosolic-side-in and (ii) we have previously shown that SO_4^{2-} has no significant effect on $^{22}\text{Na}^+$ uptake when added to the extravesicular medium (eqns. 3 and 4 and Table 1 of [8]) indicate that the effect of SO_4^{2-} seen in Figure 3 is due to a cytosolic modifier site on the $\text{Na}^+/\text{K}^+-2\text{Cl}^-$ co-

Figure 3 Effects of intravesicular formate and SO_4^{2-} on the initial rate of $^{22}\text{Na}^+$ uptake into rabbit parotid BLMVs

$^{22}\text{Na}^+$ fluxes were measured as described in the Materials and methods section using vesicles pre-equilibrated in Buffer A containing 1 mM EDTA and either 100 mM NMDG-gluconate ('Gluc.'), 25 mM NMDG-formate plus 75 mM NMDG-gluconate ('For.'), or 50 mM $\text{NMDG}_2\text{-SO}_4^{2-}$ plus 50 mM mannitol (' SO_4 '). The uptake medium contained (final concentrations) 1 mM $^{22}\text{Na}^+$, 100 mM KCl and 1 mM EDTA in Buffer A. Non-specific trapping of $^{22}\text{Na}^+$ by the membranes and filters has been subtracted from all fluxes. The results are the averages of four determinations normalized to the uptake observed in gluconate-loaded vesicles (1.46 ± 0.06 nmol/min per mg of protein). Equilibrium $^{22}\text{Na}^+$ uptakes measured after 2 h of incubation were 1.07 ± 0.04 nmol/mg ('Gluc.'), 1.15 ± 0.06 nmol/mg ('For.') and 1.19 ± 0.06 nmol/mg (' SO_4 '), $n = 4$.

transporter. Since this inhibitory effect of cytosolic SO_4^{2-} is seen in the same SO_4^{2-} concentration range as its inhibitory effect on bumetanide binding (Figure 1) we hypothesize that the inhibitory anion site associated with bumetanide binding is identical with the intracellular inhibitory anion site associated with transport.

The existence of a cytosolic anion modifier site as demonstrated directly here has been suggested in several tissues to explain the apparent down-regulation of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transport activity at high intracellular Cl^- levels [21–24]. Indeed, recently published results from rat parotid acinar cells indicate that a drop in intracellular Cl^- concentration is required to activate ion fluxes

via the co-transporter [25]. In parotid acini such a modifier site would serve a useful physiological role since intracellular Cl^- levels are typically high in the resting cell (≥ 60 mM) but fall dramatically (30–40%) due to secretagogue-induced salt loss. Thus the co-transporter would be down-regulated in the resting cell, with a concomitant saving of metabolic energy, but active following secretagogue activation when it is required to drive cellular Cl^- entry to support salt and water secretion [3–5].

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