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Ionic Dependence of Bumetanide Binding to the Rabbit Parotid Na/K/Cl Cotransporter

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Summary. The Na/K/Cl-dependent component of the binding of the loop diuretic bumetanide to basolateral membrane vesicles from the rabbit parotid is studied. A Scatchard analysis indicates that this binding is due to a single high-affinity site with K_D = 3.2 ± 0.3 μM (n = 9) at 100 mM sodium, 100 mM potassium and 5 mM chloride. When KCl-dependent ^{22}Na transport and tracer [^3H]-bumetanide binding are monitored simultaneously as a function of (unlabeled) bumetanide concentration it is found that the $K_{0.5}$ for bumetanide inhibition of both processes are identical indicating that the high-affinity bumetanide binding site studied here is identical with a bumetanide-inhibitory site on the Na/K/Cl cotransport system previously identified in this preparation (R.J. Turner, J.N. George and B.J. Baum, *J. Membrane Biol.* **94:**143–152, 1986). High-affinity bumetanide binding exhibits a hyperbolic dependence on both [Na] and [K] consistent with Na/bumetanide and K/bumetanide binding stoichiometries of 1 : 1 and $K_{0.5}$ values of approximately 33 mM for sodium and 23 mM for potassium. In contrast, the dependence on [Cl] is biphasic, with bumetanide binding increasing from 0 to 5 mM chloride and decreasing toward baseline levels thereafter. Scatchard analysis of this latter inhibitory effect of chloride indicates a competitive interaction with bumetanide in agreement with earlier indications that bumetanide inhibits Na/K/Cl cotransport at a chloride site. However, studies of the effects of various anions on bumetanide binding and ^{22}Na transport show a poor correlation between the specificities of these two processes, suggesting that the inhibitory chloride site is not a chloride transport site.

Key Words loop diuretics · exocrine gland · fluid secretion · parotid · acinar cell · ion transport

Introduction

There is now considerable evidence indicating that a loop diuretic-sensitive Na/K/Cl cotransport system plays a major role in transepithelial chloride movements across a number of absorptive and secretory epithelia [4, 6, 12, 15, 16, 18]. In secretory epithelia such a transporter is thought to be localized to the basolateral membrane where it is responsible for driving chloride into the cell against its electrochemical gradient using the extracellular to intracellular gradient for sodium produced by Na, K-ATPase. This electrochemical gradient for chloride in turn provides the driving force for chloride movement from cytoplasm to lumen via an apical chloride channel. In absorptive epithelia a similar mechanism has been proposed, except that here the cotransporter and chloride channel are localized to the apical and basolateral membranes, respectively, and net chloride movement is from lumen to interstitium.

It has been suggested that the above model can account for transepithelial chloride fluxes in salivary acinar cells where fluid secretion is thought to follow transepithelial chloride movements [14, 18, 22]. We have, in fact, recently provided direct evidence for the existence of a Na/K/Cl cotransporter in a basolateral membrane vesicle (BLMV) preparation from rabbit parotid acinar cells [20]. In particular we demonstrated the presence of a KCl-dependent component of sodium flux in this preparation which was electroneutral, stimulated by both potassium and chloride chemical gradients, and inhibited by the loop diuretic furosemide. Our data were consistent with a Na/K/Cl coupling stoichiometry of 1 : 1 : 2 as reported for Na/K/Cl cotransport in several other tissues [7]. Our results also suggested that rabbit parotid acinar BLMV were a particularly rich source of this transporter and might thus provide a useful system for characterizing it in more detail.

In the present paper we study the binding properties of bumetanide, a potent inhibitor of Na/K/Cl cotransport systems, to rabbit parotid acinar BLMV. The use of similar high-affinity inhibitors of other coupled transporters as probes of carrier mechanism and structure is now well established [2, 21]. A bumetanide binding site whose properties correlate well with those of the cotransporter is identified and characterized. Particular emphasis is placed on the dependence of this binding site on the transported ions sodium, potassium and chloride.

Materials and Methods

Vesicle Preparation

Basolateral membrane vesicles were prepared from rabbit parotid by a Percoll© gradient method as previously described [20]. Relative to the starting tissue homogenate the activity of the basolateral membrane marker K-stimulated *p*-nitrophenyl phosphatase is enriched 9 to 12 times in this membrane vesicle preparation while the activity of dipeptidyl peptidase IV, a luminal membrane marker, is enriched two times [20]. Owing to the pyramidal shape of parotid acinar cells the total area of the basolateral membrane is approximately an order of magnitude greater than that of the apical membrane [3]. Thus the absolute contamination of the BLMV preparation by apical membranes is expected to be very small.

Freshly prepared vesicles were suspended in Buffer A (10 mM Tris/HEPES plus 100 mM mannitol) containing 100 mM KCl and 1 mM EDTA at a protein concentration of approximately 5 mg/ml. Aliquots (0.75 mg protein) of BLMV were fast frozen and stored above liquid nitrogen.

Binding and Uptake Measurements

On the day of the experiment 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 48,000 × *g* for 20 min. The resulting pellets were taken up in the same medium at a protein concentration of approximately 2 mg/ml.

In control experiments (*not shown*) we have established that high-affinity bumetanide binding to rabbit parotid acinar BLMV is time dependent with $T_{1/2} \sim 10$ min at 1 μM [^{3}H]-bumetanide and 23°C. Accordingly, equilibrium bumetanide binding was measured as follows. At time zero a 20-μl aliquot of vesicles was combined with a 20-μl aliquot of incubation medium consisting of Buffer A plus [^{3}H]-bumetanide (approximately 10 μCi/ml) and other constituents as required. After 60 min of incubation at 23°C the reaction was terminated by the addition of 1.5 ml of ice-cold stop solution (*see below*) and the vesicles were applied to a Millipore filter (HAWP 0.45 μm) under light suction. The filter, which retained the BLMV, 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, Ill.) containing 0.1 ml glacial acetic acid and counted for radioactivity along with samples of the incubation medium and appropriate standards.

The stop solution was Buffer A containing 100 mM NaCl plus 100 mM KCl. From control experiments in which the time the vesicles were left in the stop solution was prolonged we have established that no significant binding or loss of [^{3}H]-bumetanide occurs during the stopping and washing procedure.

All data were corrected for low-affinity binding and nonspecific trapping of [^{3}H]-bumetanide by the membranes and filter by subtracting the [^{3}H]-bumetanide binding measured in the presence of 1 mM unlabeled bumetanide. Nonspecific binding and trapping measured in this way was 5.65 ± 0.92 pmol/mg protein at 1 μM [^{3}H]-bumetanide (n = 17).

^{22}Na uptake measurements were carried out in essentially the same manner as [^{3}H]-bumetanide binding (*see* ref. 20 for details). Uptakes were measured after 15 sec of incubation and represent initial uptake rates [20].

The detailed composition of the various media used in each experiment is given in the Table and Figure legends. All experiments were carried out in triplicate or quadruplicate. The errors shown in the Table and Figures are standard deviations. Unless otherwise indicated the results of single representative experiments are shown.

Protein was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond Calif.) with bovine gamma globulin as the standard.

Calculations

In least-squares fits to the data, points were weighted according to their relative experimental errors. The errors quoted in the text on least-squares parameters are standard deviations.

Materials

^{22}Na was from New England Nuclear (Boston, Mass.), *n*-methyl-D-glucamine (NMDG) was from Aldrich (Milwaukee, Wis.) and unlabeled bumetanide was a gift from Hoffman-LaRoche (Nutley, N.J.). All other chemicals were from standard commercial sources and were reagent grade or the highest purity available.

[^{3}H]-bumetanide (24.8 Ci/mmol), the generous gift of Drs. B. Forbush III and R.W. Mercer, was prepared as described in ref. 5 using 3-amino-4-phenoxy-5-sulfamoylbenzoic acid, supplied by Dr. P.W. Feit (Leo Pharmaceuticals, Ballerup, Denmark), as the starting material. The purity of the radioactive material was determined by thin-layer chromatography (Silica Gel 60F run with chloroform/methanol/acetic acid/cyclohexane 80 : 2.5 : 10 : 1) where it was found that >90% of the label comigrated with authentic bumetanide (*data not shown*).

Abbreviations

10 mM Tris/HEPES: 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffered with Tris to pH 7.4; EDTA: ethylenediaminetetraacetic acid; NMDG: N-methyl-D-glucamine.

Results

Initial Characterization of the Parotid BLMV Bumetanide Binding Site

In preliminary experiments we established that bumetanide binding to parotid acinar BLMV was markedly dependent on the presence of sodium, potassium and chloride in the incubation medium. This behavior is illustrated in Fig. 1 where we compare binding measured in the presence of 100 mM sodium, 100 mM potassium and 5 mM chloride (100%) to binding measured when one or more of these ions are replaced by NMDG or gluconate. Under all conditions where sodium and/or potassium were replaced by NMDG, bumetanide binding was reduced by 90 to 95%, but when chloride alone

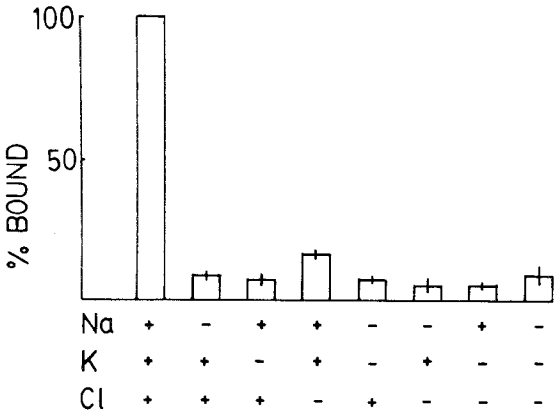

Fig. 1. Dependence of bumetanide binding to parotid BLMV on the presence of sodium, potassium and chloride. The binding of 1.2 μM [^{3}H]-bumetanide was measured in the presence of 100 mM sodium, 100 mM potassium, 5 mM chloride and 195 mM gluconate (100%) or with one or more of sodium, potassium and chloride replaced with NMDG or gluconate as indicated

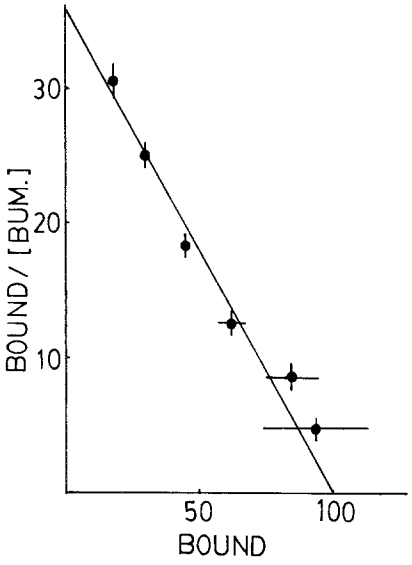

Fig. 2. Dependence of bumetanide binding to parotid BLMV on bumetanide concentration. Binding was measured over the bumetanide concentration range 0.625 to 20 μM in the presence of 100 mM sodium, 100 mM potassium, 5 mM chloride and 195 mM gluconate. The line drawn through the data points is a least-squares fit given by K_D = 2.8 ± 0.3 μM and N_O = 101 ± 6 pmol/mg protein with r = 0.985

was replaced (by gluconate) binding was approximately twice the level observed in the absence of sodium or potassium.

Figure 2 shows the concentration dependence of bumetanide binding plotted according to Scatchard. The experimental points lie on a good straight line

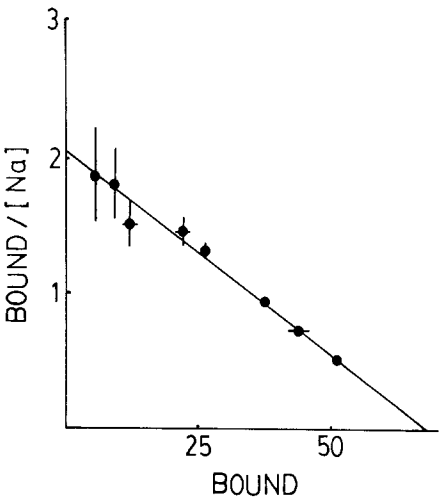

Fig. 3. Dependence of bumetanide binding to parotid BLMV on [Na]. The binding of 1 μM [^{3}H]-bumetanide was measured in the presence of 100 mM potassium, 5 mM chloride, 195 mM gluconate and 0 to 100 mM sodium with sodium replaced isosmotically by NMDG. The sodium-dependent component of bumetanide binding is illustrated. The line drawn enough through the data points is a least-squares fit given by $K_{0.5}$ = 33 ± 2 mM and x-intercept 68 ± 2 pmol/mg protein with r = 0.993

indicating the existence of a single dominant bumetanide binding site with K_D = 2.8 μM (*see* least-squares fit in Figure caption).

Dependence of Bumetanide Binding on [Na], [K] and [Cl]

The sodium dependence of bumetanide binding to parotid BLMV is shown in Fig. 3. Here binding was measured as a function of [Na] at constant [K] and [Cl] (100 and 5 mM, respectively). The data have been plotted in a Scatchard-like format. The linearity of this plot indicates that bumetanide binding is a hyperbolic function of [Na] consistent with a Na/bumetanide binding stoichiometry of 1 : 1 [19] and a $K_{0.5}$ for sodium of 33 mM. A similar experiment for potassium (at 100 mM Na and 5 mM Cl), illustrating a hyperbolic dependence of binding on [K] with $K_{0.5}$ = 23 mM, is shown in Fig. 4.

In contrast to the above results for sodium and potassium, the dependence of bumetanide binding on [Cl] is biphasic (Fig. 5) suggesting the involvement of at least two chloride binding sites in the bumetanide binding event—a high-affinity stimulatory site and a lower-affinity inhibitory site. At large [Cl] bumetanide binding is inhibited to levels below that observed in the absence of chloride and close to that observed in the absence of sodium and/or potassium (*cf.* Figs. 1 and 5).

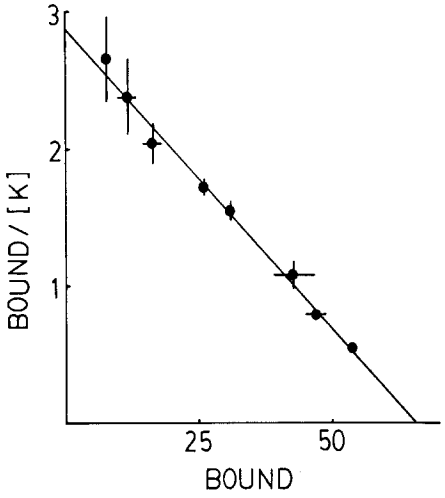

Fig. 4. Dependence of bumetanide binding to parotid BLMV on [K]. The binding of 1 μM [^{3}H]-bumetanide was measured in the presence of 100 mM sodium, 5 mM chloride, 195 mM gluconate and 0 to 100 mM potassium with potassium replaced isosmotically by NMDG. The potassium-dependent component of binding is illustrated. The line drawn through the data points is a least-squares fit given by $K_{0.5} = 23 \pm 1$ mM and x-intercept 66 ± 1 pmol/mg protein with $r = 0.997$

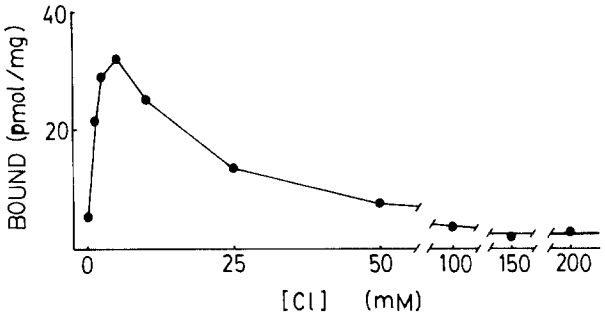

Fig. 5. Dependence of bumetanide binding to parotid BLMV on [Cl]. The binding of 1 μM [^{3}H]-bumetanide was measured in the presence of 100 mM sodium, 100 mM potassium and 0 to 200 mM chloride with chloride replaced isosmotically by gluconate

Relationship between Bumetanide Binding and Na/K/Cl Cotransport

In order to use bumetanide as a probe of the Na/K/Cl cotransporter it is important to establish that the high-affinity bumetanide binding site characterized above is indeed associated with the cotransporter. In Fig. 6 we have measured KCl-dependent ^{22}Na uptake (i.e., uptake via the cotransporter—*see* ref. 20) and tracer (0.2 μM) [^{3}H]-bumetanide binding as a function of unlabeled bumetanide concentration. Owing to our earlier observations (above) that bumetanide binding was dependent on both time and [Na], [K] and [Cl] this experiment was carried out by preincubating vesicles with various concentrations of bumetanide (plus tracer [^{3}H]-bumetanide) in the presence of these ions (at 5 mM each), then measuring initial ^{22}Na flux rates (*see* Fig. 6 caption). When vesicles were preincubated with bumetanide in this way the $K_{0.5}$ of bumetanide inhibition of KCl-dependent ^{22}Na flux (~30 μM) was found to be identical to the $K_{0.5}$ of bumetanide inhibition of tracer [^{3}H]-bumetanide binding (*see* open symbols in Fig. 6). On the other hand, when vesicles were preincubated with bumetanide in the absence of ions, little inhibition of ^{22}Na flux was observed (Fig. 6, closed symbols). This latter result is consistent with the time and ionic dependence of bumetanide binding documented earlier in this manuscript. In additional experiments (*not illustrated*), carried out under similar conditions to those used in Fig. 6 (5 mM each of sodium, potassium and chloride and 30 μM [^{3}H]-bumetanide), we have verified that the time course of bumetanide binding to parotid BLMV corresponds exactly to the time course of the inhibitory effect of bumetanide on the initial rate of KCl-dependent ^{22}Na transport ($T_{1/2}$ for both effects is approximately 5 min under these conditions). Taken together the above results provide convincing evidence that the high-affinity bumetanide binding site observed here is one and the same with the bumetanide inhibitory site on the Na/K/Cl cotransporter.

Chloride is a Competitive Inhibitor of Bumetanide Binding

It has been proposed on the basis of flux data that bumetanide and chloride compete for a common site on the Na/K/Cl cotransporter of the duck red blood cell [9], possibly for a chloride transport site. This hypothesis is explored in Fig. 7 for the rabbit parotid basolateral membrane Na/K/Cl cotransporter. In this experiment we have measured bumetanide binding as a function of bumetanide concentration at two chloride concentrations (5 and 20 mM), corresponding approximately to the peak stimulation and half-maximal inhibition of binding by chloride in Fig. 5. The results are plotted according to Scatchard and indicate that chloride increases the K_D of bumetanide binding without significantly changing the number of bumetanide binding sites (*see* Fig. 5 caption). This result is consistent with a competitive interaction between bumetanide and chloride.

Anion Specificity of Sodium Transport and Bumetanide Binding

In the Table we illustrate the effects of various anions on sodium transport by BLMV. When present as the only anion in the extravesicular medium (Ex-

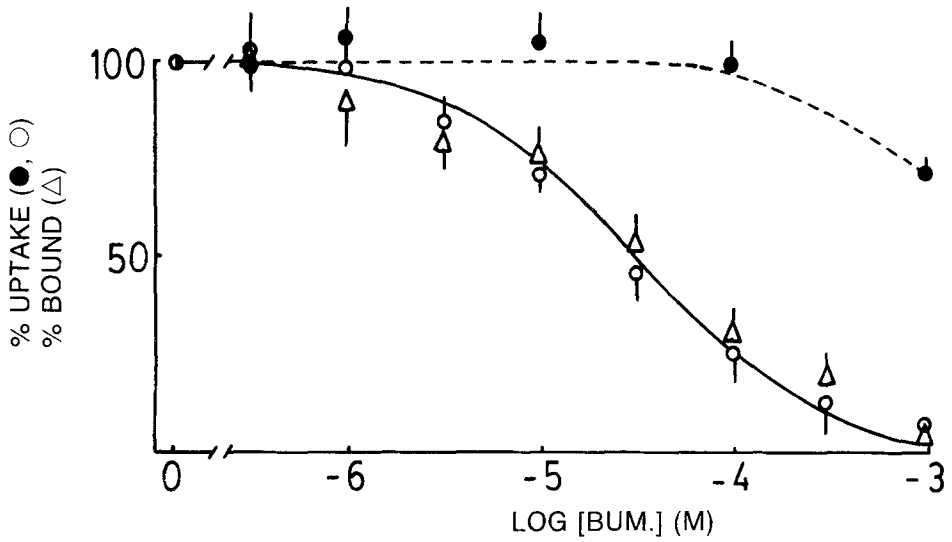

Fig. 6. Dependence of KCl-dependent ^{22}Na flux and tracer (0.2 μM) [^{3}H]-bumetanide binding on [bumetanide]. Rabbit parotid BLMV were preincubated for at least one hour in Buffer A plus 1 mM EDTA, 0.2 μM [^{3}H]-bumetanide and the concentration of unlabeled bumetanide indicated, with (open symbols) or without (closed symbols) 5 mM KCl and 5 mM Na methylsulfate. Following this preincubation period the initial rate (15 sec) of ^{22}Na uptake (○, ●) was measured by combining 10 μl of vesicles with 40 μl of incubation medium containing sufficient salts to yield final extravesicular concentrations of 1 mM Na methylsulfate and 100 mM KCl or K methylsulfate. The KCl-dependent component of ^{22}Na flux was calculated by subtracting the flux measured when chloride was replaced by methylsulfate. Fluxes have been normalized to the values observed in the absence of bumetanide. [^{3}H]-bumetanide binding to vesicles preincubated with ions (△) has been normalized to its value in the absence of added unlabeled bumetanide. [^{3}H]-bumetanide binding to vesicles preincubated in the absence of ions was too small for accurate determination and has not been illustrated

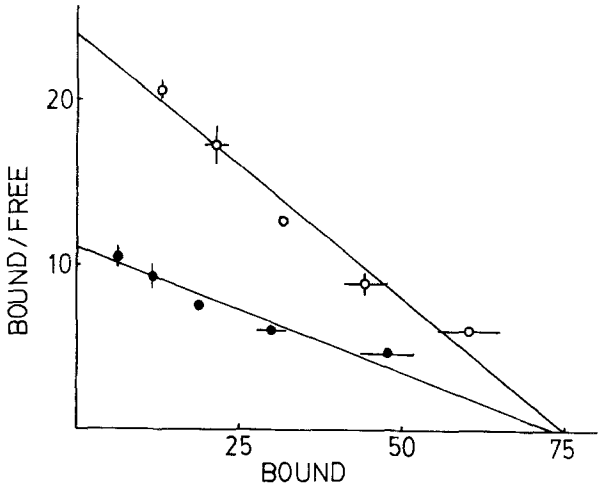

Fig. 7. Dependence of bumetanide binding to parotid BLMV on bumetanide concentration in the presence of 5 and 20 mM chloride. Binding was measured in the presence of 100 mM sodium, 100 mM potassium and either 5 mM chloride with 195 mM gluconate (○) or 20 mM chloride and 180 mM gluconate (●). The lines drawn through the data points are least-squares fits given by K_D = 3.1 ± 0.3 μM and 75 ± 4 pmol/mg protein with r = 0.986 (○), and 6.6 ± 1.1 μM and 72 ± 9 pmol/mg protein with r = 0.958 (●)

periment A) chloride, bromide, fluoride and nitrate stimulate ^{22}Na uptake ($P < 0.002$ relative to gluconate), while formate, sulfate, acetate and gluconate are without effect. Since there are at least two chloride transport sites associated with the Na/K/Cl cotransporter [20], and since a given anion may react preferentially with one of these, in Experiment B of the Table we have also examined the effects of the same anions in the presence of chloride. Again chloride, bromide, fluoride and nitrate show significant stimulatory effects ($P < 0.02$ relative to gluconate) while formate, sulfate and gluconate are nonreacting; however, in absolute terms, a more dramatic effect of nitrate is seen in Experiment B than in Experiment A, indicating that nitrate is a significantly poorer substrate for one of the chloride sites than the other(s). A similar conclusion was reached by Brown and Murer for the Na/K/Cl cotransporter in the renal epithelial cell line LLC-PK$_1$ [1] and by Kinne et al. in the rabbit kidney outer medulla [13].

In the experiment illustrated in Fig. 8 we examine the anion specificity of the inhibitory effect of chloride on bumetanide binding. Here binding was measured in the presence of 5 mM chloride and 95 mM test anion (47.5 mM in the case of SO_4). Examination of these results indicates that bromide, nitrate and sulfate mimic the inhibitory effect of chloride on binding while formate, fluoride, acetate and gluconate are without effect. Comparison of these results with those shown in the Table indicates a poor correlation between the effects of fluoride and sulfate on bumetanide binding and sodium flux; namely, that fluoride can apparently substitute for

Table. Effect of various anions on sodium uptake by parotid BLMV[a]

	Anion	Relative uptake	*n*
Experiment A (without Cl):[b]	chloride	1.00	
	bromide	1.31 ± 0.22	4
	formate	0.08 ± 0.02	4
	nitrate	0.14 ± 0.03	4
	fluoride	0.35 ± 0.13	5
	sulfate	0.06 ± 0.01	3
	acetate	0.07 ± 0.03	3
	gluconate	0.07 ± 0.02	8
Experiment B (with Cl):[c]	chloride	3.14 ± 0.10	4
	bromide	3.70 ± 0.28	3
	formate	1.02 ± 0.08	3
	nitrate	1.83 ± 0.05	3
	fluoride	1.21 ± 0.12	4
	sulfate	1.01 ± 0.02	3
	acetate	0.89 ± 0.04	3
	gluconate	1.00	

[a] Vesicles were prepared in Buffer A containing 1 mM EDTA.
[b] *Experiment A.* The initial rate of 1 mM ^{22}Na uptake was measured in the presence of 100 mM of the potassium salt of the anion indicated. The results of nine independent experiments have been combined. For each experiment, uptakes were normalized to the uptake observed in the presence of KCl. The averages of these normalized values are shown in the Table. The average sodium uptake in the presence of KCl for all nine experiments was 2.6 ± 0.7 pmol/mg protein/min.
[c] *Experiment B.* The initial rate of 1 mM ^{22}Na uptake was measured in the presence of 50 mM KCl and 100 mM of the potassium salt of the anion indicated. The results of seven independent experiments have been combined. For each experiment, uptakes were normalized to the uptake observed in the presence of gluconate as test anion. The averages of these normalized values are shown in the Table. The average sodium uptake with gluconate as test anion for all seven experiments was 1.1 ± 0.3 pmol/mg protein/min.

chloride in the transport process but does not show any inhibitory effect on bumetanide binding, while sulfate inhibits binding but neither stimulates nor inhibits flux. These specificity differences suggest that the anion inhibitory site associated with bumetanide binding is not a chloride transport site.

Discussion

In the experiments reported here we study the ionic dependence of the binding of the loop diuretic bumetanide to basolateral membrane vesicles from the rabbit parotid. A component of binding which requires the simultaneous presence of sodium and potassium and is further stimulated by the presence of low concentrations of chloride is identified (Fig. 1). A Scatchard analysis of this component of binding is consistent with the existence of a single high-affinity site with K_D = 3.2 ± 0.3 μM (n = 9) at 100

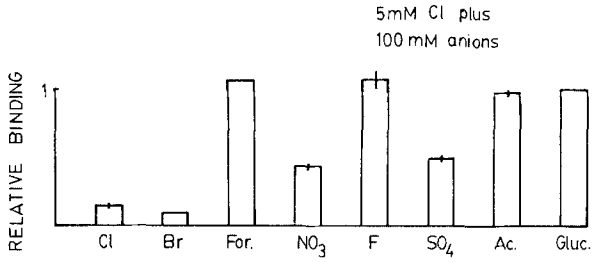

Fig. 8. Effect of various anions on the Na/K/Cl-dependent component of bumetanide binding to parotid BLMV. The binding of 1.2 μM [^3H]-bumetanide was measured in the presence of 50 mM sodium, 50 mM potassium, 5 mM chloride and 95 mM of the test anion (47.5 mM in the case of sulfate). Binding has been normalized to that observed in the presence of gluconate as the test anion. The results of a single representative experiment are shown

mM sodium, 100 mM potassium and 5 mM chloride (*cf.* Fig. 2). Measurements of the dependence of bumetanide binding on [Na] and [K] yield a hyperbolic relationship in each case (Figs. 3 and 4) indicating Na/bumetanide and K/bumetanide binding stoichiometries of 1 : 1 [19] and $K_{0.5}$ values of approximately 33 mM for sodium and 23 mM for potassium. In contrast, the dependence on [Cl] is biphasic, with bumetanide binding increasing from 0 to 5 mM chloride and decreasing thereafter (Fig. 5). This latter observation indicates the involvement of both stimulatory and inhibitory chloride sites associated with the bumetanide binding event, with the stimulatory site(s) presumably being of somewhat higher affinity.

When KCl-dependent ^{22}Na transport and high-affinity [^3H]-bumetanide binding are monitored simultaneously as a function of (unlabeled) bumetanide concentration it is found that the $K_{0.5}$ for bumetanide inhibition of both processes are identical (Fig. 6). In addition, the time course for high-affinity bumetanide binding corresponds directly to the time course of bumetanide inhibition of KCl-dependent ^{22}Na transport (*see* Results). Taken together these results provide strong evidence that the high-affinity bumetanide binding site studied here is intimately associated with the Na/K/Cl cotransport system we have previously identified in this preparation [20].

A sodium-, potassium- and chloride-dependent component of [^3H]-bumetanide binding has been reported in studies carried out on membrane preparations from dog kidney outer medulla [5] and flounder intestine [17]—both absorptive epithelial—as well as in studies using duck red blood cells [8]. These bumetanide binding sites are also thought to be related to endogenous plasma membrane Na/K/Cl cotransport systems [5, 8, 17]. In these studies a biphasic dependence of bumetanide binding on

chloride concentration, qualitatively similar to Fig. 5, was also observed. The inhibition of bumetanide binding by chloride was investigated in some detail by O'Grady et al. in flounder intestine [17]. However, in contrast to our observation that chloride is a competitive inhibitor of bumetanide binding (Fig. 7), these authors found that both the K_D and the number of bumetanide binding sites were reduced by increased chloride concentration. Whether this observation is due to a mixed-type inhibition of bumetanide binding by chloride, or the existence of two high-affinity bumetanide binding sites in the flounder intestine has not yet been established [17].

In all of the binding studies referenced above [5, 8, 17] the K_D for bumetanide was found to be at least an order of magnitude smaller than observed here for parotid BLMV. However, bumetanide binding studies on Ehrlich ascites tumor cells [10] and pig renal outer medullary membranes [11] report K_D values in closer agreement to the one reported here (9 and 6 μM, respectively). These data may indicate significant differences in Na/K/Cl cotransporters or their local membrane environments in various species and/or tissues.

Our demonstration that chloride can act as a competitive inhibitor of bumetanide binding (Fig. 7) is in agreement with the transport data of Haas and McManus from the duck red blood cell [9] indicating that bumetanide inhibits Na/K/Cl cotransport at a chloride site. However, our observations that sulfate can mimic the inhibitory effect of chloride on bumetanide binding but neither stimulates nor inhibits sodium transport via the cotransporter, and that fluoride stimulates transport but has no inhibitory effect on binding (Table and Fig. 8), suggest that the chloride site involved is distinct from the chloride transport site(s). Further studies are obviously required to clarify the interactions of anions and loop diuretics with the Na/K/Cl cotransporter.

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