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Na-K-2Cl Cotransport in Winter Flounder Intestine and Bovine Kidney Outer Medulla: [^{3}H] Bumetanide Binding and Effects of Furosemide Analogues

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Summary. The effects of several sulfamoyl benzoic acid derivatives on Na-K-Cl cotransport were investigated in winter flounder intestine. The relative efficacy (IC_{50} values) and order of potency of these derivatives were benzmetanide, 5×10^{-8}M > bumetanide 3×10^{-7}M > piretanide 3×10^{-6}M > furosemide 7×10^{-6}M > amino piretanide 1×10^{-5} 3-amino-4-penoxy-5-sulfamoyl benzoic acid. Binding of [^{3}H] bumetanide was studied in microsomal membranes from winter flounder intestine and compared to that in bovine kidney outer medulla. Binding was also studied in brush-border membranes from winter flounder intestine. The estimated values for K_d and number of binding sites (n) were: bovine kidney, $K_d = 1.6 \times 10^{-7}$, $n = 10.5$ pmol/mg protein; winter flounder intestine, K_d 1.2×10^{-7}, $n = 7.3$ pmol/mg protein, and brush-border membranes from winter flounder, $K_d = 5.3 \times 10^{-7}$, $n = 20.4$ pmol/mg protein. The estimated K_d for bumetamide binding to winter flounder brush-border membranes derived from association and dissociation kinetics was 6.8×10^{-7}M. The similarity in magnitudes of IC_{50} and K_d for bumetanide suggests that the brush-border cotransporter is ordinarily rate-limiting for transmural salt absorption and that bumetanide specifically binds to the cotransporter. Measurement of bumetanide binding at various concentrations of Na, K and Cl showed that optimal binding required all three ions to be present at about 5 mM concentrations. Higher Na and K concentrations did not diminish binding but higher Cl concentrations (up to 100 mM Cl) inhibited bumetanide binding by as much as 50%. Still higher Cl concentrations (500 and 900 mM) did not further inhibit bumetanide binding. Scatchard analysis of bumetanide binding at 5 and 100 mM Cl concentrations showed that both K_d and n were lower at the higher Cl concentration (5 mM Cl: $K_d = 5.29 \times 10^{-7}$M, $n = 20.4$ pmol/mg protein; 100 mM Cl: $K_d = 2.3 \times 10^{-7}$M, $n = 8.8$ pmol/mg protein). These data suggest two possibilities: that bumetanide and Cl binding are not mutually exclusive (in contrast to pure competitive inhibition) and that they each bind to separate sites or that two distinct bumetanide binding sites exist, only one of which exhibits Cl inhibition of binding. This inhibition would then be consistent with a competitive interaction with Cl.

Key Words Na-K-2Cl cotransport · loop diuretics · winter flounder · bumetanide · furosemide · epithelial transport

Introduction

Na-K-2Cl cotransport can be distinguished from other transport processes on the basis of its sensitivity to "loop" diuretics such as furosemide and its insensitivity to amiloride and thiazide diuretics [6, 7, 9, 17, 19, 23]. Recent studies with avian erythrocytes [18] and shark rectal gland [20] have described the effects of a series of sulfamoyl benzoic acid derivatives (benzmetanide, bumetanide, piretanide, etc.) which inhibit Na-K-2Cl cotransport with greater specificity and affinity than furosemide. These compounds range in potency from 5×10^{-8} to 10^{-3}M which suggested that the relative efficacy and order of potency of these drugs is a useful tool in assessing the similarities of cation-dependent Cl transport in different tissues [19]. Furthermore, development of [^{3}H] analogs of bumetanide and benzmetanide (the most potent drugs of this class of diuretics) has made it possible to use them as a means of identifying putative Na-K-2Cl cotransport elements in membranes from cells expressing this transport function [3, 11].

The intestinal epithelium of the winter flounder has been previously shown to possess a Na-K-2Cl cotransport system in the brush-border membrane [2, 4, 5, 14, 16]. In this tissue, the cotransporter comprises the major apical permeability mechanism for Cl and the short-circuit current (I_{sc}) is directly dependent upon a functional cotransport system [2, 4, 14, 16]. The cotransporter can be inhibited by mucosal addition of sulfamoyl benzoic acid diuretics such as furosemide and bumetanide but the relative affinities and order of potency of these compounds have not previously been established.

In the present study we characterized the pharmacological properties of the Na-K-2Cl cotransport system from flounder intestine with respect to the relative affinities and order of potency of several sulfamoyl benzoic acid derivatives. In addition the dissociation constants obtained from [^{3}H] bumetanide binding studies in bovine kidney outer medulla and flounder intestine were measured and compared with IC_{50} values from dose-response measurements in intact mucosa.

Materials and Methods

Materials

Benzmetanide, piretanide, amino-piretanide and 3-amino-4-phenoxy-5-sulfamoyl benzoic acid were a gift from Leo Pharmaceuticals (P.W. Feit). Bumetanide was obtained as a gift from Hoffmann-LaRoche, Inc. Furosemide was purchased from Hoechst. *p*-Nitrophenyl phosphate was purchased from Sigma. [^{3}H] $NaBH_4$ (77 Ci/mmol) was purchased from New England Nuclear.

Methods

Animals

Winter flounder (*Pseudopleuronectes americanus*) were obtained from Southampton College Marine Station and held at the John G. Shedd Aquarium. These animals were maintained at 15 ± 2°C and initially fed chopped pieces of fish twice a week. After a three week acclimation period the flounder were starved for 1 week and then used for experiments. At Columbia University flounder were held in a 200-gal seawater aquarium at 5°C and starved for 1 week before use. Fresh bovine kidneys were obtained from a local slaughter house.

[^{3}H] Bumetanide Synthesis

[^{3}H] Bumetanide was prepared by reduction of the Schiff base adduct of 3-amino-4-phenoxy-5-sulfamoyl benzoic acid and butyraldehyde with [^{3}H] $NaBH_4$. Details of the synthesis have been previously described [3]. Briefly 35 μmol of butyraldehyde was added to 75 μmol of 3-amino-4-phenoxy-5-sulfamoyl benzoic acid in 75 μl of dimethyl sulfoxide (DMSO). After 30 min at 25°C, 7 μmol of [^{3}H] $NaBH_4$ (in 120 μl 0.1 N NaOH) was added and after 1 hr 3 M acetic acid (1.2 ml) and water (5 ml) were added to dilute the reaction. Products were extracted into methylene chloride (4 times, 3 ml each). The organic phase was pooled and evaporated under nitrogen. The residue was taken up in 1 ml methanol and purified on a reverse-phase HPLC column (Whatman Partisil ODS III, 10 μM) with 30 MeOH, 20 CH_3CN, 0.2 CH_3COOH, and 50 H_2O as solvent (2 ml/min). Product purity was found to be greater than 90% as determined by liquid scintillation counting of samples which comigrated with authentic bumetanide on TLC plates. (Silica gel 60F; 80 $CHCl_3$, 2.5 MeOH, 10 CH_3COOH, and 1 cyclohexane as solvent system.) The estimated specific activity was 1 Ci/mmol.

Isolation of Microsomal and Brush-Border Membranes

Bovine kidney outer medulla was dissected, finely minced and washed three times in ice-cold homogenization buffer containing (in mM) 250 sucrose, 5 Tris, 1 EGTA, 1 DTT, 1 $KHCO_3$, pH 7.4. The tissue was homogenized in the same buffer (10% wt/vol) in Teflon® glass homogenizer. The homogenate was centrifuged at 5000 × *g* at 4°C for 15 min to pellet mitochondria and cell debris. The supernatant was removed and centrifuged at 48,000 × *g* (4°C) for 1 hr. The final pellet was suspended in 100 mM mannitol, 2 mM Tris-HEPES, 0.1 mM PMSF, pH 7.4, and stored at −70°C until use.

Winter flounder microsomal membranes were prepared from intestinal scrapings which were homogenized (10% wt/vol, 10 strokes in a Teflon glass homogenizer) in ice-cold buffer containing 50 mM mannitol, 1 mM Tris-HEPES (pH 7.8) and 0.1 mM PMSF. The homogenate was centrifuged at 5000 × *g* (4°C for 15 min). The supernatant fraction was removed and centrifuged at 48,000 × *g* (4°C) for 1 hr. The final pellet was resuspended in 100 mM mannitol, 2 mM Tris-HEPES, 0.1 mM PMSF, pH 7.8, and stored at −70°C until use.

Winter flounder brush-border membranes were prepared from intestinal scrapings that were incubated in 100 ml cell separation buffer (140 mM NaCl, 5 mM Na-EDTA, 20 mM Tris base, pH 7.5, with HCl) for 30 min at 4°C. The cells were centrifuged at 400 × *g* for 5 min and resuspended in vesicle buffer (290 mM mannitol, 20 mM HEPES, 2 mM EGTA, pH 7.3, with Tris base) at 4°C. Cells were washed three times with this buffer. The cell suspension was then frozen in 10 ml volumes with liquid nitrogen then thawed in water at 4°C. After thawing, $MgCl_2$ (10 mM final concentration) was added and the solution kept at 4°C with constant stirring for 20 min. The cell lysate was centrifuged at 5000 × *g* for 15 min at 4°C. The supernatant was then centrifuged at 27,000 × *g* for 30 min at 4°C. The pellet from this step was resuspended in vesicle buffer and the two centrifugation steps described above were repeated. The final brush-border membrane pellet was suspended in vesicle buffer. Estimated enrichment in alkaline phosphatase activity (*p*-nitrophenyl phosphate as substrate; OD_{405}) ranged from 10- to 15-fold above the lysate. Protein concentrations were determined by the Lowry assay [12].

Diuretic Dose-Response Relationships

Flounder intestine was stripped of longitudinal and circular muscle layers and mounted in Ussing chambers (membrane surface area = 0.63 cm^2). Tissues were bathed on both sides with identical Ringer's solutions containing (in mM) 170 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 3 NaH_2PO_4, 5 EPPS, 20 glucose, pH 8.0 at 12°C. Tissue short-circuit current (I_{sc}) was allowed 60 min to stabilize. Diuretic drugs were added to the mucosal solution beginning with the lowest concentration. After each addition the I_{sc} was allowed to stabilize (usually 15 min) before the next higher concentration of drug was tested. This procedure was continued until maximum inhibition of I_{sc} was obtained.

Diuretic Binding Studies

For the determination of K_d and number of binding sites for [^{3}H] bumetanide, microsomal or brush-border membrane vesicles were diluted 1 : 1 with binding buffer which contained (in mM) 5 K_2HPO_4, 5 NaCl, 5 NaH_2PO_4, 2 mM Tris HEPES, pH 7.8 (100 μl final binding volume), 50–100 μg protein/assay). The concentration of [^{3}H] bumetanide was varied from 2.5×10^{-8} to 1×10^{-6} M. For determination of nonspecific binding 1×10^{-5} M unlabeled bumetanide was added. Reactions were carried out at 4°C for 60 min. In preliminary experiments it was established that equilibrium binding was achieved at 4°C within 60 min. Samples were filtered using microsep 0.45 μM cellulosic filters presoaked in wash buffer (100 mM Na_2HPO_4, pH 7.4), and binding was determined by scintillation counting. Specific binding was estimated

by subtraction of nonspecific binding from total binding. The K_d and number of binding sites were determined by Scatchard analysis.

To examine the effects of different Na, K and Cl concentrations on bumetanide binding a constant ionic strength (0.15) was maintained by substituting N-methyl-D-glucamine for Na or K and gluconate for Cl. Optimal binding was achieved using 5 mM Na, 5 mM K and 5 mM Cl. For these experiments 5×10^{-7} M [^{3}H] bumetanide was used.

Association and Dissociation Time Course of Bumetanide Binding to Winter Flounder BBMV

Brush border membrane vesicles (BBMV) were diluted 1 : 1 with binding buffer (5 mM Na_2HPO_4, 10 mM NaCl, 5 mM K_2HPO_4, 2 mM Tris HEPES, pH 7.8) with 5×10^{-7}M ^{3}H bumetanide in the presence and absence of excess (100-fold) unlabeled bumetanide. For the determination of the association time course of bumetanide specific binding to flounder BBMV, the binding reaction was initiated at time = 0 and at various times afterwards (2, 5, 10, 30 and 60 min), and 150-μl samples were removed and diluted into 2.0 ml of ice-cold wash buffer (100 mM Na_2HPO_4, pH 7.4) and filtered onto microsep 0.45-μm cellulosic filters followed by a second 2-ml wash (time required for filtration ~ 10 to 15 sec). Specific binding was determined by subtraction of nonspecific binding from total binding. The dissociation time course was determined after incubation of membranes and [^{3}H] bumetanide ± unlabeled bumetanide for 1 hr at 4°C. At time zero, 5×10^{-5}M unlabeled bumetanide was added to the tube in which total bumetanide binding was being determined and 150-μl aliquots of this reaction mixture were removed at various times and filtered as described above. Determination of the K_d from the rate constants is described in the Results section.

Results

Potency of 5-Sulfamoyl Benzoic Acid Derivatives as Na-K-2Cl Cotransport Inhibitors

Figure 1*A* shows the structures of various sulfamoyl benzoic acid diuretics used for the pharmacologic characterization of Na-K-2Cl cotransport in flounder intestine. The major structural difference in these drugs (except furosemide) is at position 3. Substitution at this position with benzylamino, butylamino or pyrrolidino groups results in compounds with a potency greater than that of furosemide in reducing I_{sc} in flounder intestine (Fig. 1*B*). The IC_{50} values and order of potency for these compounds are 1) benzmetanide 5×10^{-8}M, 2) bumetanide 3×10^{-7}M, 3) piretanide 3×10^{-6}M, 4) furosemide 7×10^{-6}M, and 5) amino-piretanide 10^{-5}M. Earlier studies with avian erythrocytes [18] and cultured human fibroblasts [16] showed similar IC_{50} values and orders of potency for these diuretics [e.g. turkey erythrocytes: benzmetanide (6.1×10^{-8}M) > bumetanide (2.5×10^{-7}M) > piretanide (2.8×10^{-6}M) > furosemide (2.3×10^{-5}M) > 3-amino-4-phenoxy-5-sulfamoyl benzoic acid (>10^{-3}M)] [19]. Less effective inhibition was observed in shark rectal gland [21] with benzmetanide (5×10^{-7}M), bumetanide (5×10^{-6}M) and furosemide (2×10^{-4}M). The order of potency, however, was the same as flounder, avian red cells and cultured fibroblasts.

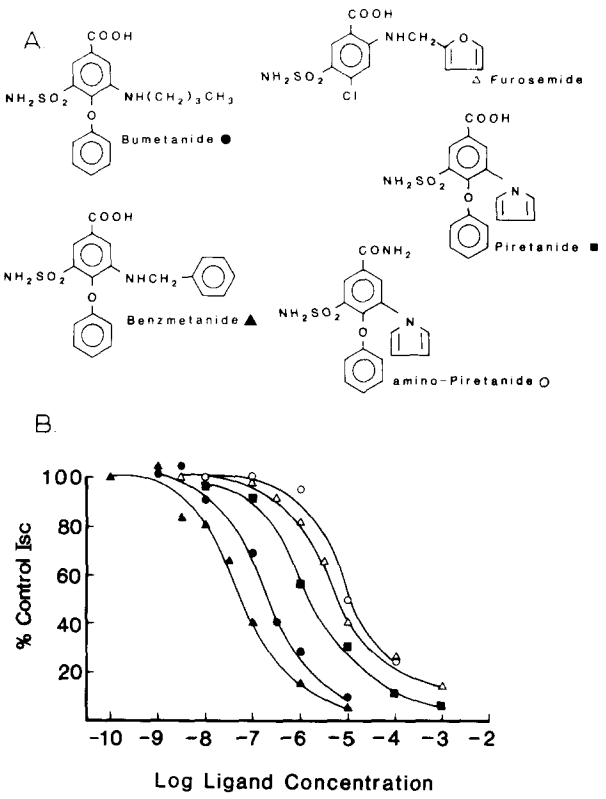

Fig. 1. (*A*) Structures of several 5-sulfamoyl benzoic acid derivatives which affect Na-K-2Cl cotransport in flounder intestine. (*B*) Dose-response curves of these derivatives on short-circuit current. In flounder intestine the order of potency is benzmetanide (5×10^{-8}M, $n = 4$) >, bumetanide (3×10^{-7}M, $n = 7$) >, piretanide (3×10^{-6}M, $n = 4$) >, furosemide (7×10^{-6}M, $n = 4$) >, amino piretanide (1×10^{-5}M, $n = 4$)

^{3}H Bumetanide Binding Studies

Bumetanide binding under equilibrium conditions was carried out using microsomal membranes from bovine kidney outer medulla and winter flounder enterocytes. In both tissues specific binding (i.e. that displaceable by excess unlabeled bumetanide) was 30 to 50% of the total binding. Specific binding of bumetanide was saturable and Scatchard analyses (Figs. 2 and 3) gave K_d values of 1.6×10^{-7}M in kidney microsomes and number of

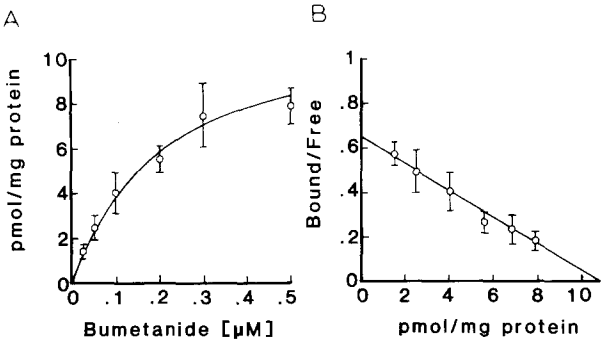

Fig. 2. (*A*) Michaelis plot of bumetanide specific binding to bovine kidney outer medulla microsomal membranes. A + 0.5 μM bumetanide specific binding represents approximately 30 to 35% of total bumetanide binding. Each point shows the mean ± SE of six experiments from membranes pooled from 10 bovine kidneys. Binding was carried out under equilibrium conditions (1-hr incubation at 4°C) in binding buffer which contained (100 μl final volume) 5 mM NaCl, 5 mM NaH_2PO_4, 5 mM K_2HPO_4, 50 mM mannitol, 2 mM Tris HEPES, pH 7.8 (final concentration) and 50 μg of microsomal membranes. The best-fit curve was determined from a nonlinear least-squares analysis of the data. The estimated K_d and number of binding sites were 1.7×10^{-7}M and 10.8 pmol/mg protein. (*B*) Scatchard analysis of bumetanide binding to microsomal membranes from kidney outer medulla. The K_d was 1.6×10^{-7}M and the number of binding sites was 10.5 pmol/mg protein. These values are in good agreement with the results from nonlinear least-squares analysis of specific binding reported in part *A*

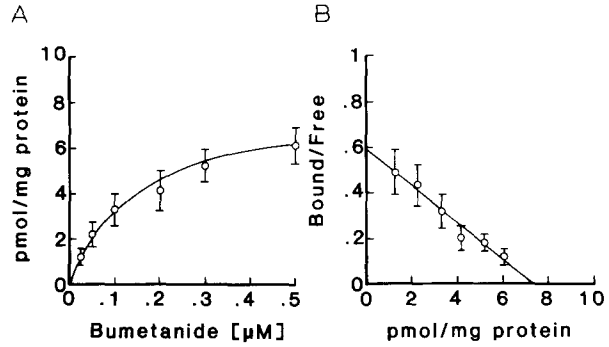

Fig. 3. (*A*) Michealis plot of bumetanide specific binding to winter flounder intestine microsomal membranes. At 0.5 μM bumetanide, specific binding represents approximately 30% of total bumetanide binding. Each point shows the mean of seven experiments from intestinal membranes pooled from 30 flounder. Binding was carried out under conditions described above (Fig. 1). The best-fit curve was calculated from a nonlinear least-squares analysis of the data. The estimated K_d and number of binding sites were 1.3×10^{-7}M and 7.5 pmol/mg protein. (*B*) Scatchard analysis of bumetanide binding to flounder intestinal microsomes. The K_d was 1.2×10^{-7} and the number of binding sites was 7.3 pmol/mg protein ($n = 7 \pm$ SE)

bumetanide bindings sites (n) as 10.5 pmol/mg protein (Fig. 2). For flounder microsomal membranes (Fig. 3) the K_d was 1.2×10^{-7}M and the number of binding sites was 7.3 pmol/mg protein. The linearity

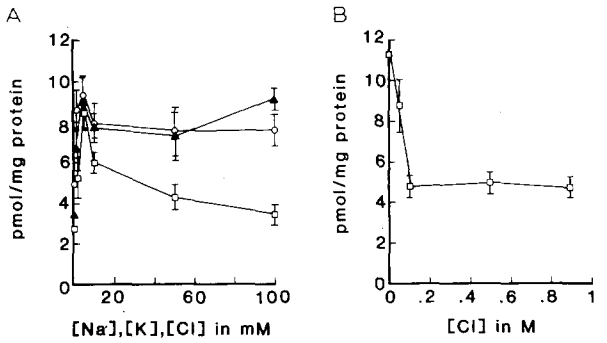

Fig. 4. (*A*) Effect of Na, K and Cl concentration on [^{3}H] bumetanide specific binding to winter flounder brush-border membranes. All reactions were carried out under constant ionic strength conditions using NMDG-gluconate to balance changes in cation or anion concentrations. Chloride concentration was varied in the presence of constant Na and K (5 mM) while Na and K concentrations were varied with constant Cl (5 mM) and 5 mM of either Na or K depending on whether the effects of Na or K were being tested. The [^{3}H] bumetanide concentration was kept constant at 5×10^{-7}M, with a 100-fold excess of cold bumetanide. Na (▲), K (○), Cl(□). (*B*) Effect of Cl concentration on bumetanide binding over an extended concentration range. The ionic strength was constant at 1.0 and had little effect on binding at the lower Cl concentrations

of the data from these Scatchard plots suggests that a single population of bumetanide binding sites exists over a range of bumetanide concentrations from (10^{-8} to 5×10^{-7}M). Measurements of bumetanide binding at concentrations greater than 2 μM were highly variable presumably because of the increased fraction of nonspecific binding [3]. Thus extrapolation of the number of binding sites may underestimate the true number of bumetanide binding sites in these two preparations. We interpret our results as representing the minimum number of binding sites for bumetanide in kidney outer medulla and winter flounder enterocytes.

Figure 4*A* shows the effects of changes in Na, K and Cl concentration on specific bumetanide binding to flounder intestinal brush-border membranes. At constant K (5 mM) and Cl (5 mM) concentrations, bumetanide binding was shown to increase from about 3 to 9 pmol/mg protein from zero to 5 mM Na with no significant effects on binding from 5 to 100 mM Na. The same pattern was also true for K when Na (5 mM) and Cl (5 mM) were held constant. Cl, however, showed a biphasic response. Under conditions of constant K and Na (5 mM each), bumetanide binding was low at zero Cl, peaked at 5 mM Cl and decreased back to zero Cl levels at 100 mM Cl. In a separate series of experiments (Fig. 4*B*) the effects of Cl on bumetanide specific binding were determined over an extended range of concentra-

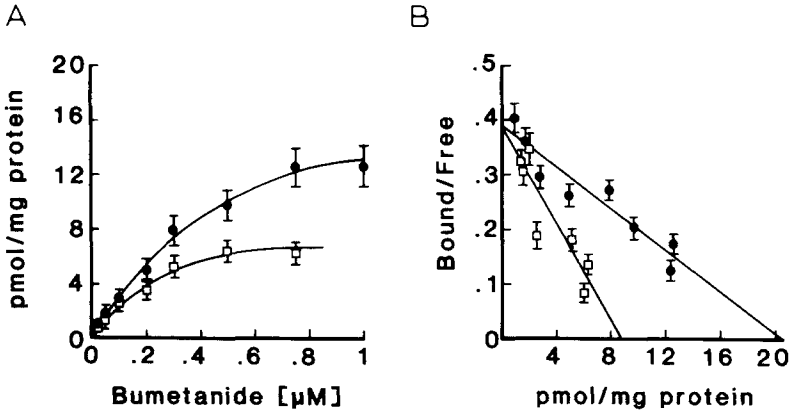

Fig. 5. (*A*) Michaelis plot of bumetanide specific binding to flounder brush-border membranes at 5 mM Cl (as NaCl) and 100 mM Cl (as 5 mM NaCl + 95 mM NMDG-Cl) concentrations. Reactions were performed using constant ionic strength conditions (0.15). Na and K (K-gluconate) were maintained at 5 mM with NMDG-gluconate used to balance the ionic strength. Each point represents the mean ± SE of four experiments. Nonlinear least-squares analysis of the data results in estimates of K_d for 5 and 100 mM Cl concentrations of 5.2 × 10^{-7}M and 2.6 × 10^{-7}M, respectively, and the number of binding sites equal to 19.9 and 8.9 pmol/mg protein, respectively. (*B*) Scatchard analysis of bumetanide specific binding at 5 mM Cl (○) and 100 mM Cl (●). The K_d and number of sites at 5 mM was 5.3 × 10^{-7}M and 20.4 pmol/mg protein. At 100 mM [Cl] the K_m was 2.3 × 10^{-7}M and the number of binding sites equal to 8.8 pmol/mg protein (n = 4 ± SE)

tions. Maximal binding of bumetanide appears to depend on the presence of Na, K and Cl (each at 5 mM). High Cl concentrations (>50 mM) significantly decreased specific binding of bumetanide by as much as 50 to 60%. Increases in Cl concentration above 100 mM, however, had little further effect on bumetanide binding.

Figure 5 shows differences in specific binding to flounder brush-border membranes at various bumetanide concentrations when measured at 5 and 100 mM Cl. Scatchard analysis of bumetanide binding at 5 mM Cl gives a K_d of 5.3 × 10^{-7}M and 20.4 pmol/mg protein for the number of binding sites (a threefold increase over the number of sites obtained from microsomal membranes, Fig. 3. When binding is measured over the same range of bumetanide concentrations in the presence of 100 mM Cl a shift in K_d and number of binding sites was noted (K_d = 2.3 × 10^{-7}M; n = 8.8 pmol/mg protein). Both the K_d and the number of binding sites decreased by the same factor (K_d 5 mM Cl/K_d 100 mM Cl = 2.31; n 5 mM Cl/n 100 mM Cl = 2.33).

The association and dissociation kinetics of bumetanide binding to flounder brush-border membranes was also evaluated (Fig. 6). An estimate of the K_d for bumetanide binding was determined using the following equation:

$$k = ka[^3\text{H-bumetanide}] + kd$$

(assuming pseudo first-order reaction kinetics: k is the rate constant of association, ka is the second-order rate constant of association and kd is the first-order rate constant of dissociation). From the slope of the semi-logarithmic plot of the association time course, k was estimated to be 3.6 × 10^{-4}/sec. The rate constant for dissociation (kd) was determined from the slope of the semi-logarithmic plot describing the displacement of ^{3}H bumetanide with excess unlabeled drug. The kd value was 2.1 × 10^{-4}/sec. The value of ka was calculated from the equation above. Kd was determined from $Kd = kd/ka$ = 6.8 × 10^{-7}M. This value is in close agreement to that obtained from equilibrium bumetanide binding to flounder brush-border membranes.

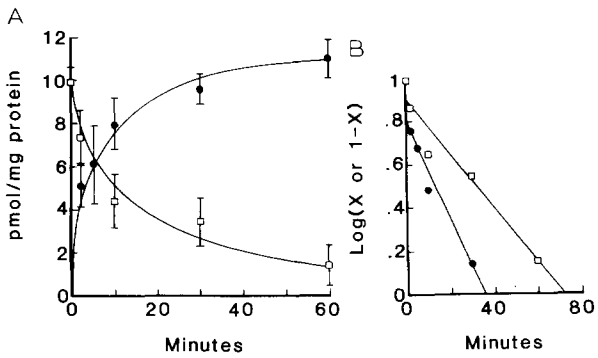

Fig. 6. (*A*) Association and dissociation time courses of bumetanide specific binding to brush-border membranes from winter flounder intestine. Reactions were carried out at 4°C in binding buffer which contained 5 mM Cl, 10 mM Na, 10 mM K, 50 mM mannitol and 2 mM Tris, pH 7.8 (n = 4 ± SE). (*B*) Semi-logarithmic plot of association and dissociation time courses shown in 6*a*. The slope of the association time course was 3.6/sec = k and the dissociation time course was 2.1/sec = kd. The value of ka was determined from $k = ka[^3\text{H-bumetanide}] + kd$ and the K_d from the ratio of kd/ka as outlined in the Results section

Discussion

Structure-Activity Relationships of Sulfamoyl Benzoic Acid Diuretics

From earlier studies of diuretic activity in avian erythrocytes [21] and cortical TALH [24] substitutions at positions 1, 3 and 4 all have effects on the potency of these drugs. Hydrophobic substitutions such as benzyl amino or butyl amino groups at position 3 result in highly potent derivatives whereas hydrophilic substitutions such as an amino group or a methoxy-benzylamino group have very little diuretic activity ($IC_{50} > 10^{-3}$) [19, 21, 24]. Substitution at position 4 of bumetanide with a phenylthio group causes a slight increase in potency (8.7×10^{-8}M) while anilino substitution has no effect in comparison to bumetanide (2.4×10^{-7}M *vs.* 2.5×10^{-7}M). However, 4-chloro substitution causes a large decrease in potency (3.9×10^{-5}M). Thus a certain degree of hydrophobicity at this position also seems necessary for effective inhibition of cotransport. Substitution at position 1 (the anionic site) with either a sulfonyl or carboxylate group (pK < 4 for each) resulted in potent diuretic activity for both compounds (4.8×10^{-6}M; 2.5×10^{-7}M) while an amino group at this position has essentially no diuretic activity ($IC_{50} > 10^{-3}$M). This result suggests that an anionic group at this position is important for optimal diuretic activity of these derivatives [6, 7, 19, 24].

Our results with winter flounder intestine show the same diuretic activity and order of potency as the avian erythrocyte and other systems, suggesting that Na-K-2Cl cotransport is pharmacologically similar in many cell types including human fibroblasts [17], shark rectal gland [21], canine trachea [26], and cortical thick ascending limb of Henle's loop [24]. Differences, however, do exist in the mechanisms of regulation by cyclic nucleotides in these tissues. For example, cAMP has a stimulating effect on cotransport in avian erythrocytes [19] and shark rectal gland [21] whereas cAMP has little or no effect on cotransport in flounder intestine but cGMP has been shown to be inhibitory [15, 23].

Diuretic Binding Studies

[^{3}H] Bumetanide has been synthesized and used in equilibrium binding studies to determine the dissociation constants and number of binding sites in canine and porcine kidney microsomal membranes. Estimates of bumetanide binding constants for canine kidney microsomes were $K_d = 45$ nM and number of sites = 4.7 pmol/mg protein [3]. In a study on porcine kidney in which much lower specific activity bumetanide was used, a $K_d = 5.6$ μM, and number of sites equal to 226 pmol/mg protein was determined [11]. The K_d for bovine kidney determined in this study was 3.6 times greater than that obtained for canine kidney but nearly 35 times lower than that obtained for porcine kidney. The number of sites were similar to those in canine kidney (10.5 pmol/mg protein *vs.* 4.7 pmol/mg protein) but nearly 45-fold lower than that reported for porcine kidney. Measurement of high affinity binding sites in porcine kidney may have been precluded, however, because of the lower specific activity bumetanide used in that study. The low affinity found for bumetanide binding in porcine kidney differs by a factor of 28 from the IC_{50} value obtained for bumetanide inhibition of I_{sc} in isolated, perfused rabbit TALH [24]. The latter value of 2×10^{-7}M agrees well with the value for bumetanide binding to bovine kidney microsomes reported here. The density of Na-K-2Cl cotransport sites in flounder and bovine kidney (assuming 1 : 1 correspondence between bumetanide binding sites and the number of cotransporters) is similar to that for Na-dependent glucose transporter in rabbit intestine (13.8 pmol/mg protein) [25] and somewhat lower than that of the Na-Cl coupled GABA transporter in rat brain membranes (between 30 to 40 pmol/mg protein, based on a 0.07 to 0.1% estimate of the fraction of membrane protein and a molecular weight of 24,000) [22]. An important conclusion of this study is that, in flounder intestine, the K_d of binding closely agrees with the IC_{50} for transport inhibition by bumetanide. This strongly suggests that binding of bumetanide is specifically associated with the functional Na-K-2Cl cotransporter in this system.

The effects of Na, K and Cl concentrations on bumetanide binding in flounder intestine were similar to the effects reported in canine kidney microsomal membranes [3]. All three ions are required for optimal binding of bumetanide but increases in Cl concentration above 5 mM caused a significant inhibition of bumetanide binding (~50% at 100 mM Cl).

This suggests that Cl binding to one of its sites on the cotransporter facilitates bumetanide binding whereas binding of Cl to a second site inhibits binding. This clearly argues for nonidentity of two Cl binding sites, possibly with different affinities for Cl (*see also* Reference [1]). We examined the effect of Cl at two higher Cl concentrations (500 and 900 mM) and found that inhibition of bumetanide binding appeared to be already maximal at 100 mM Cl. Previous studies regarding the nature of this Cl effect on the ability of loop diuretics such as furosemide and bumetanide to inhibit NaCl cotransport in toad

cornea [13] and avian erythrocytes [8] have suggested that Cl is a competitive inhibitor of the binding of these diuretics. Pure competitive inhibition implies that the interaction of Cl and bumetanide with the carrier is mutually exclusive. This can occur by interaction with a common binding site, steric interference between two distinct binding sites or a conformational change brought about by binding of either ligand which in turn excludes the binding of the other to a separate site on the molecule. In any of these cases, binding of bumetanide should continually decrease as more Cl is added. Thus one interpretation of the observed maximal inhibitory effect of Cl on bumetanide binding is that Cl is not a competitive inhibitor in this tissue. A second possible explanation is that two distinct bumetanide binding sites are present in winter flounder brush-border membranes, but only one site is Cl dependent. Under these conditions Cl-independent bumetanide binding would be equal to that observed at zero Cl, in which case, the extent of inhibition at 100 mM Cl would be close to that level and consistent with competitive inhibition of bumetanide binding. A similar explanation has been proposed by Halm et al. [10] to explain bumetanide and cGMP-inhibitable but K-independent NaCl uptake across flounder intestinal brush border: the existence of 2 NaCl transport systems in the same membrane, a Na-K-2Cl cotransport and a NaCl cotransport.

In Fig. 5 we compare bumetanide binding at two different Cl concentrations (5 and 100 mM). The Scatchard plots show that the K_d and number of binding sites decrease to the same extent. Assuming, however, that the results from Fig. 4 describe the ionic dependency of a single Na-K-2Cl carrier mechanism, such an effect of Cl on both the K_d and number of binding sites could reflect mixed type inhibition in which the Cl-induced change in affinity for bumetanide is coincidentally equal to the change in the total number of bumetanide binding sites. If, however, two distinct bumetanide-inhibitable carriers are present, then the affinities for bumetanide indicated by the K_d values differ by a factor of two and the number of binding sites at high Cl concentrations are approximately half of those observed at 5 mM Cl.

In a recent report by Jorgensen et al. [11] a 34,000 mol wt protein was identified by photoaffinity labeling as the bumetanide binding component of the Na-K-2Cl cotransport system in porcine kidney. Comparison of photoincorporation with and without unlabeled bumetanide (2 mM) revealed a major peak at 34,000 daltons. However, a concentration of 2 mM bumetanide is high enough to significantly quench the UV signal. This is suggested by the fact that all of the nonspecific peaks are also eliminated when 2 mM bumetanide is present during photolysis. At present we are attempting to identify the specific bumetanide binding protein associated with Na-K-2Cl cotransport in flounder intestine. Our preliminary results suggest that a much lower concentration of unlabeled bumetanide (50 μM) can be used to block specific photoincorporation of bumetanide in this system. The nature of the protein(s) that specifically bind bumetanide in flounder intestine remains to be established.

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