J. Membrane Biol. 113, 203–210 (1990)

The Journal of
Membrane Biology

Solubilization and Partial Purification of the Rabbit Parotid Na/K/Cl-Dependent Bumetanide Binding Site

R. James Turner and Janet N. George
Clinical Investigations and Patient Care Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892

Summary. We demonstrate that the high affinity bumetanide binding site of the rabbit parotid acinar cell can be extracted from a basolateral membrane fraction using relatively low concentrations (0.07%, wt/vol; 1 mg membrane protein/ml) of the nonionic detergent Triton X-100. This extracted site cannot be sedimented by ultracentrifugation at 100,000 × g × 1 hr. Bumetanide binding to this site retains the ionic characteristics of bumetanide binding to native membranes but shows a fivefold increase in binding affinity (K_d = 0.57 ± 0.15 μM *vs.* K_d = 3.3 ± 0.7 μM for native membranes). Inactivation of the extracted bumetanide binding site observed at detergent/protein ratios >1 can be prevented or (partially) reversed by the addition of exogenous lipid (0.2% soybean phosphatidylcholine). When the 0.07% Triton extract is fractionated by sucrose density gradient centrifugation in 0.24% Triton X-100, 0.2% exogenous lipid and 200 mM salt, the high affinity bumetanide binding site sediments as a single band with $S_{20,w}$ = 8.8 ± 0.8 S. This corresponds to a molecular weight ~200 kDa for the bumetanide binding protein-detergent-lipid complex and represents a sevenfold purification of this site relative to the starting membrane fraction. In contrast to previous attempts to purify Na/K/Cl cotransport proteins and their associated bumetanide binding sites, the present method avoids harsh detergent treatment as well as direct covalent modification (inactivation) of the transporter itself. As a consequence, one can follow the still active protein through a series of extraction and purification steps by directly monitoring its bumetanide binding properties.

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

Introduction

Considerable evidence now indicates that salt and water movement across many secretory and absorptive epithelia is due to transepithelial chloride fluxes [9, 18, 27, 34]. In a number of tissues these chloride movements are driven by a Na/K/Cl cotransport system which is responsible for concentrative chloride entry [6, 9, 18, 25, 27, 34]. Na/K/Cl cotransporters have also been identified in a variety of nonepithelial tissues [10] where they are involved in volume regulatory ion fluxes and possibly in nonrenal potassium homeostasis. Studies from a number of laboratories indicate that such a transporter is responsible for driving the bulk of the acinar chloride secretion which results in salivary fluid production [23, 24, 27, 29, 34]. We have, in fact, provided direct evidence for the existence of a Na/K/Cl cotransporter in parotid acinar basolateral membrane vesicles [33] and argued that the parotid may provide a particularly rich source of this transport protein [32].

Owing to their obvious physiological importance, a considerable amount of recent experimental effort has been devoted to characterizing Na/K/Cl cotransporters [e.g., 2, 6, 8, 10, 12, 16, 18, 19, 25, 26, 32, 33]. Much of this work has made use of the loop diuretic bumetanide, a potent specific inhibitor of these systems. Several laboratories [8, 12, 26], including our own [32], have identified high affinity Na/K/Cl-dependent bumetanide binding sites whose properties correlate well with the bumetanide inhibitory sites associated with Na/K/Cl cotransporters in the same tissue.

An essential step in our eventual understanding of the mechanism of all membrane transport phenomena is the identification and characterization of the relevant molecular constituents. A number of groups have employed bumetanide and its analogues in attempts to label or purify putative components of Na/K/Cl cotransporters. Jorgensen et al. [17] identified a 34,000-Da polypeptide which was photolabeled with [^{3}H]-bumetanide when pig renal outer medullary membranes, preincubated with the labeled drug, were irradiated with light at 345 nm (an absorptive maximum for bumetanide). Using similar techniques with radiolabeled bumetanide analogues, DiStephano et al. [5] have photolabeled a 24-kDa protein in isolated pig kidney thick ascending limbs of Henle's loop, and Haas and For-

bush have photolabeled 150-kDa proteins in both dog renal medullary membranes [13] and duck red blood cells [14]. More recently, Pewitt et al. [28] have demonstrated that, when solubilized calf renal outer medullary membranes are passed over, an affinity chromatography column consisting of 4-*p*-aminobumetanide coupled to Affigel-10, a 160-kDa protein is retained and can be subsequently eluted with excess bumetanide. Feit et al. [7] have also used affinity chromatographic techniques with a bumetanide analogue to identify proteins with molecular weights ~76 and ~38 kDa in membranes from Ehrlich ascites tumor cells. At present it is not clear whether the above lack of consensus regarding the molecular weight of the Na/K/Cl cotransporter is due to the identification of various subunits or degradation products of the protein, to differences in the cotransporter between tissues, or to methodological difficulties.

In the present paper we have used a somewhat different approach to the purification of the Na/K/Cl cotransporter from the rabbit parotid. This approach was dictated by our desire to ultimately isolate the transporter in a still functional form. Since our preliminary studies indicated that the bumetanide binding activity of rabbit parotid basolateral membranes was quite labile in detergent solution, in this report we first investigate the conditions necessary to extract and stabilize the high affinity bumetanide binding site. We then demonstrate that a partial purification of the functional binding site is possible using these conditions and sucrose density gradient centrifugation.

Materials and Methods

Vesicle Preparation

Basolateral membrane vesicles (BLMV) were prepared from rabbit parotid by a Percoll gradient method as previously described [33]. Relative to the starting tissue homogenate the activity of the basolateral membrane marker K-stimulated *p*-nitrophenyl phosphatase is enriched 9–12 times in this membrane vesicle preparation. Freshly prepared BLMV were suspended in Buffer A (10 mM Tris/HEPES plus 100 mM mannitol) containing 1 mM EDTA and 100 mM KCl at a protein concentration of approximately 5 mg/ml. Aliquots (0.75 mg protein) of BLMV were fast frozen and stored above liquid nitrogen.

On the day of the experiment an appropriate number of aliquots of frozen BLMV were thawed for 30 min at room temperature, diluted 100 times with Buffer A containing 1 mM EDTA (Buffer A/EDTA) and centrifuged at 48,000 × *g* for 20 min. The resulting pellets were taken up in Buffer A/EDTA at a protein concentration of 2 mg/ml.

Vesicle Treatment with Triton X-100

BLMV in Buffer A/EDTA (2 mg protein/ml) were diluted 1 : 1 with Triton X-100 (Sigma T-6878) stock solutions prepared in the same buffer. These vesicles were then left on ice for 30 min before experimental use. In most cases the resulting material was centrifuged for 5 min at 150,000 × *g* in a Beckman Airfuge. The supernate from this spin is referred to as the vesicle "extract." The extract obtained from treatment with 0.07% Triton X-100 (final concentration, wt/vol) is used in a number of experiments described here and is referred to as the "0.07% Triton extract."

Binding Measurements

Nitrocellulose Filtration Assay

Equilibrium bumetanide binding was measured using a nitrocellulose filtration assay as previously described [32]. Briefly, a 20-μl aliquot of sample was combined with the same volume of incubation medium consisting of Buffer A plus [^{3}H]-bumetanide and other constituents as required (*see below*). After 60 min of incubation 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). 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) containing 1% glacial acetic acid (vol/vol) and counted for radioactivity along with samples of the incubation medium and appropriate standards.

Unless otherwise stated, bumetanide binding was determined in Buffer A containing 100 mM sodium gluconate, 95 mM potassium gluconate, 5 mM KCl, 0.5 mM EDTA and other constituents as indicated. In previous experiments we have established that these concentrations of sodium, potassium and chloride yield near optimal conditions for high affinity bumetanide binding to rabbit parotid BLMV [32]. All data have been corrected for nonspecific retention of [^{3}H]-bumetanide by the filters. All binding studies were carried out at 23°C in duplicate (Fig. 6) or triplicate (all others). The errors shown in the figures and quoted in the text are standard deviations. Unless otherwise indicated, the results of single experiments, representative of three or more studies yielding similar results, are shown.

The above rapid filtration method was used to measure [^{3}H]-bumetanide binding to both native and detergent-treated BLMV. It is well known that nitrocellulose filters of the type employed here adsorb proteins from dilute solutions with high efficiency [11, 31]; in fact, they are used extensively for this purpose in blotting and immunoassays [31]. In order to verify that BLMV protein was retained by the filters under the conditions employed in these studies, vesicles were treated with 0.2 or 0.24% Triton X-100 (final protein concentration, 1 mg protein/ml) for 30 min on ice then centrifuged for 5 min at 150,000 × *g*. The resulting extracts (approx. 0.7 mg protein/ml, *see* Results) were diluted 20 times with stop solution (*see above*) then passed through Millipore HAWP filters. The filtrate was collected in 500 μl aliquots and assayed for protein content. These experiments showed that <7% of the protein in the first 1000 μl of diluted vesicle extract appeared in the filtrate. Thus virtually all of the protein in 50 μl of undiluted extract, or approximately 35 μg, can be retained by the filter. This quantity of protein is well in excess

of the amount typically employed in our [^{3}H]-bumetanide binding experiments (≤0.14 μg).

Gel Filtration Assay

In some cases [^{3}H]-bumetanide binding was also determined by gel filtration as follows: Sephadex G-75 (Pharmacia) was equilibrated overnight in stop solution containing 1% (wt/vol) bovine serum albumin (3.8 g gel/100 ml), rinsed with 4 × 100 ml of stop solution without albumin and loaded into disposable polystyrene columns (Pierce #29920) using approximately 3 ml gel/column. Just before use these columns were packed by placing them in 15-ml disposable plastic tubes (Falcon #2095) and centrifuging at 1500 × g × 1.5 min. An aliquot of sample plus incubation medium (typically 70 μl total volume; incubations were carried out as described above for the filtration method) was applied to the top of the packed gel column followed by 100 μl of stop solution. The column was then centrifuged again at 1500 × *g* for 2 min and the effluent, containing [^{3}H]-bumetanide bound to material eluted in the void volume of the column, was collected and transferred to a scintillation vial for counting. In control experiments where incubation medium without sample was applied to the gel, no detectable [^{3}H]-bumetanide was found in the effluent.

Protein Determinations

Except where indicated protein was measured by the method of Bradford [1] using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond CA) with bovine gamma globulin as the standard. All protein values quoted were determined using this assay. Where appropriate, corrections for interference by detergent and/or lipid were made by assaying protein standards in the same solution as the samples. It should be noted that the more traditional protein determination method of Lowry [21], using bovine serum albumin as the standard, yields protein values for the BLMV which are approximately 35% lower than those obtained with the above procedure (*data not shown*).

Sucrose Density Gradient Sedimentation [3, 22]

The following "clarified lipid solution" formed the basis of our sucrose density gradients. Soybean phosphatidylcholine (Sigma, Type II-S), evaporated from chloroform as a thin film, was taken up in a buffer made up of 10 mM Tris/HEPES, 5% sucrose, 100 mM sodium gluconate, 95 mM potassium gluconate, 5 mM KCl, 1 mM EDTA and 0.24% Triton X-100 to give a final lipid concentration of 0.2% (wt/vol). The resulting cloudy solution was sonicated for 1 min in a Branson B 12 ultrasonic cleaner, passed through a 25 then a 30 gauge needle, and centrifuged for 60 min at 48,000 × *g*. A portion of the supernate from this centrifugation was made up to 25% sucrose (wt/vol) and this, with the remaining 5% sucrose supernate was used to pour 4.4 ml linear 5–25% sucrose gradients. A 600 μl-aliquot of 0.07% Triton extract was applied to each gradient and these were centrifuged at 116,000 × *g* for 16 hr in the SW55Ti rotor of a Beckman L8-M ultracentrifuge at 4°C. The gradients were fractionated by puncturing the bottom of the tube with a 25-gauge needle and collecting 8 drop (~0.25 ml) fractions.

The following marker proteins were used to calibrate the sucrose gradients: porcine thyroglobulin ($s_{20,w}$ = 19.0 S), bovine IgG ($s_{20,w}$ = 7.2 S) and bovine serum albumin ($s_{20,w}$ = 4.7 S). The positions of these markers in the fractions from the gradients were determined by direct protein assay (thyroglobulin) or by liquid scintillation counting using ^{14}C-labeled proteins (IgG and bovine serum albumin).

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

N-methyl-D-glucamine (NMDG) was from Aldrich (Milwaukee, WI), porcine thryoglobulin was from from Sigma Chemical (St. Louis MO) and unlabeled bumetanide was a gift from Hoffman-LaRoche (Nutley, NJ). All other chemicals were from standard commercial sources and were reagent grade or the highest purity available.

[^{14}C]-bovine serum albumin and [^{14}C]-bovine IgG were from NEN. [^{3}H]-bumetanide (66.2 Ci/mmol, radiochemical purity 98%) was custom synthesized for us by Amersham (Arlington Heights, IL) from the precursor 3-amino-4-phenoxy-5-sulfamoylbenzoic acid, generously supplied by Dr. P.W. Feit (Leo Pharmaceuticals, Ballerup, Denmark). In preliminary experiments for this study we used [^{3}H]-bumetanide kindly given to us by Drs. B. Forbush, III, and R.W. Mercer, also prepared by Amersham from starting material supplied by Dr. Feit.

Results

Effect of Triton X-100 on Bumetanide Binding to BLMV

In the experiment illustrated in Fig. 1*A*, BLMV were treated with the concentrations of Triton X-100 indicated, then [^{3}H]-bumetanide binding was measured either in this detergent-treated material or in the "extract" obtained by centrifuging this material at 150,000 × *g* for 5 min. To allow direct comparison of binding to vesicles and extract, the data are presented as binding per milliliter of sample. This experiment shows several interesting results. First, at Triton concentrations ≤0.1% there is an apparent "activation" of bumetanide binding over that found in the native vesicles ([Triton X-100] = 0). This effect is investigated in more detail below. Second, as the Triton concentration increases above its critical micelle concentration (0.02%), there is a dramatic increase in bumetanide binding

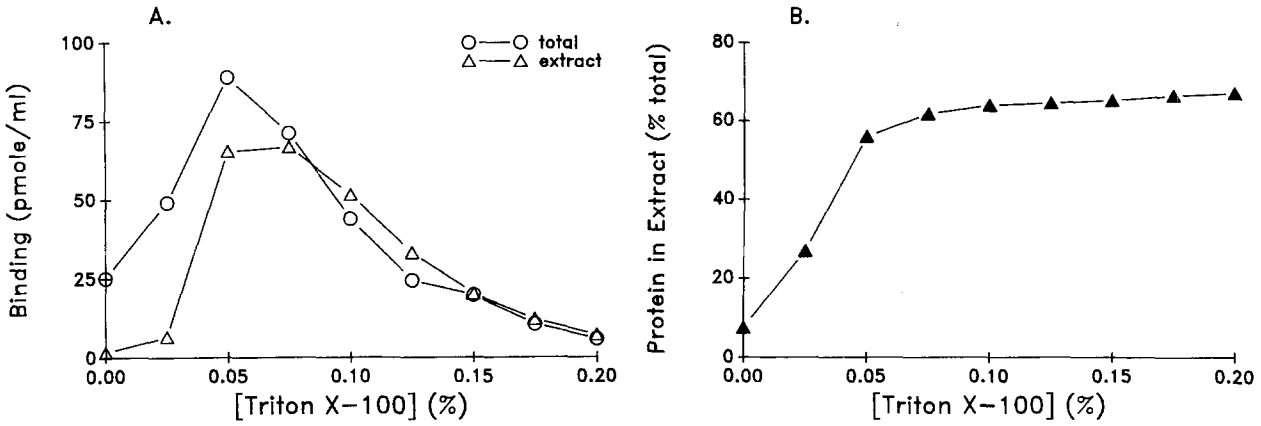

Fig. 1. (*A*) Effect of Triton X-100 on bumetanide binding to rabbit parotid basolateral membrane vesicles. BLMV (1 mg protein/ml) in Buffer A/EDTA were treated for 30 min on ice with Triton X-100 at the concentrations indicated (wt/vol). The binding of 1 μM [^{3}H]-bumetanide was then assayed in this material (○) or in the extract obtained by centrifuging this material at 150,000 × *g* for 5 min (△). The data illustrated were obtained using the nitrocellulose filtration assay described in Materials and Methods. (*B*) Protein content of the vesicle extracts obtained in *A*, expressed as a percentage of the total vesicle protein (1 mg/ml). The BCA protein assay (Pierce) was used here for convenience since it is insensitive to Triton X-100 over this concentration range

activity in the vesicle extract, and at higher detergent concentrations (≥0.075%) all of the bumetanide binding activity observed is associated with the extract. In control experiments (*not shown*) we have verified that there is, in fact, no detectable bumetanide binding in the pellets obtained after centrifuging the BLMV following treatment with Triton concentrations ≥0.075%. Finally, at Triton concentrations >0.05% there is a progressive loss of bumetanide binding activity with increasing detergent concentration. As already indicated in Materials and Methods, we have verified that the nitrocellulose filters used in this experiment are capable of adsorbing virtually all of the BLMV protein employed in our bumetanide binding assays. However, we have nevertheless confirmed that the same pattern observed in Fig. 1*A* for the vesicle extract is also obtained when bumetanide binding is determined with the gel filtration assay described in Materials and Methods (*data not shown*). Thus the loss of binding activity at higher Triton X-100 concentrations illustrated in Fig. 1*A* is not due to a selective failure of the nitrocellulose filter to retain the bumetanide binding protein. Owing to its relative convenience we have used the nitrocellulose filtration assay in most of our experiments, and only those results are presented in the studies which follow.

Figure 1*B* shows the protein content of the vesicle extracts obtained at various concentrations of Triton X-100. Note that little additional protein is extracted from the BLMW as the Triton X-100 concentration is increased above 0.075%.

We have chosen the vesicle extract obtained at 0.07% Triton X-100 as the starting point for the studies which follow. In additional experiments (*not shown*) we have demonstrated that <10% of the bumetanide binding activity found in this extract is lost after centrifugation at 116,000 × *g* × 1 hr. Thus this material appears to represent a reasonable compromise between a partial purification and solubilization of the bumetanide-binding protein and its inactivation by higher concentrations of Triton X-100. The results presented below support this conjecture.

Characteristics of the Extracted Bumetanide Binding Site

We have previously demonstrated that high affinity bumetanide binding to parotid BLMV requires the presence of sodium, potassium and chloride [32]. In the experiment illustrated in Fig. 2 we compare bumetanide binding to the 0.07% Triton extract measured in the presence of 100 mM sodium, 100 mM potassium and 5 mM chloride (100%) to binding measured in the absence of each of these ions. It is clear from Fig. 2 that optimal bumetanide binding requires the presence of all three of these ions. The pattern seen in this figure is virtually identical to that observed with native BLMV [32].

High-affinity bumetanide binding to BLMV also shows a biphasic dependence on chloride concentration; at 100 mM sodium and potassium, binding increases from 0 to 5 mM chloride and decreases thereafter [32]. The experiment shown in Fig. 3 illustrates that the same biphasic behavior is found in the 0.07% Triton extract. Thus the bumetanide

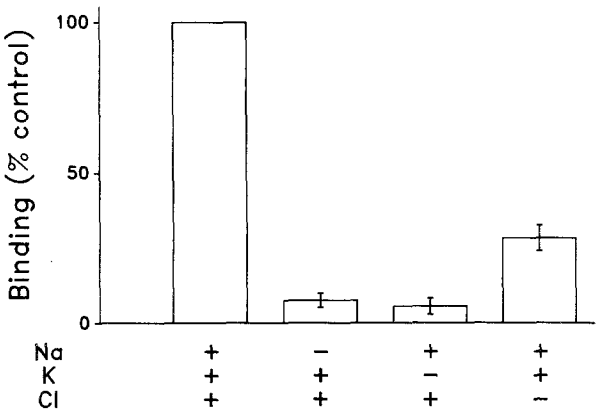

Fig. 2. Dependence of bumetanide binding to the 0.07% Triton extract on the presence of sodium, potassium and chloride. The binding of 1 μM [^{3}H]-bumetanide was measured in the presence of 100 mM sodium, 100 mM potassium, 5 mM chloride and 195 mM gluconate, or with sodium replaced by NMDG (n = 6), potassium replaced by NMDG (n = 6), or chloride replaced by gluconate (n = 4). The results of the number of experiments indicated have been normalized to the binding observed in the presence of all three ions and averaged to produce the figure

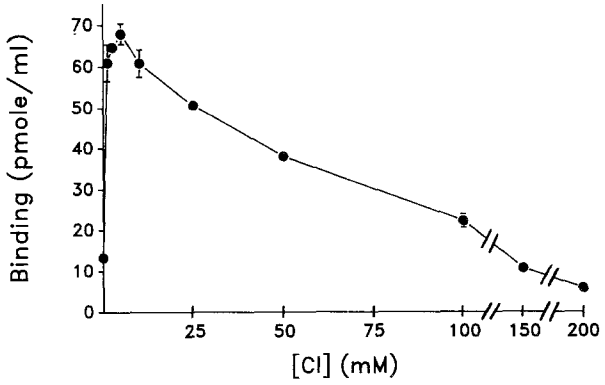

Fig. 3. Dependence of bumetanide binding to the 0.07% Triton extract on [Cl]. The binding of 1 μM [^{3}H]-bumetanide was measured in the presence of 100 mM sodium, 100 mM potassium and 0 to 200 mM chloride with chloride replaced isosmotically by gluconate

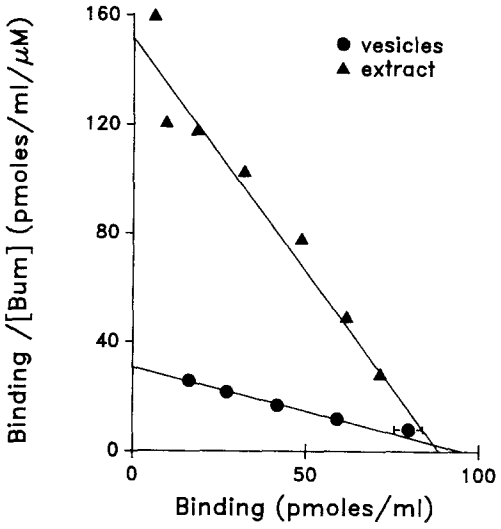

Fig. 4. Scatchard analysis of bumetanide binding to parotid BLMV (1 mg/ml) and to the 0.07% Triton extract from the same preparation. Binding was measured over the bumetanide concentration range 0.625 to 10 μM (●) or 0.04 to 2.5 μm (▲) in the presence of 100 mM sodium, 100 mM potassium, 5 mM chloride and 195 mM gluconate. The lines drawn through the data points are least squares fits given by K_d = 3.1 ± 0.2 μm, N_o = 95.0 ± 5.2 pmoles/ml, r = 0.991 (●) and 0.58 ± 0.04 μm, 88.5 ± 4.4 pmoles/ml, r = 0.988 (▲)

binding site found in the 0.07% Triton extract preserves the ionic characteristics of bumetanide binding to native basolateral membranes.

The nature of the "activation" of bumetanide binding observed in Fig. 1*A* at lower Triton concentrations is explored in the experiment illustrated in Fig. 4. Here the concentration dependence of bumetanide binding to native BLMV and to the 0.07% Triton extract are compared (again the data are presented as binding per milliliter of sample). The data are shown as a Scatchard plot. It is clear from this figure that the affinity of bumetanide binding in the extract is considerably greater than that found in the native BLMV. For three independent experiments identical to the one shown in Fig. 4, K_d = 0.57 ± 0.15 μM for the 0.07% Triton extract and K_d = 3.3 ± 0.7 μM for native BLMV. Note, however, that almost all (85 ± 15%, n = 3) of the high affinity bumetanide binding sites are preserved in the extract.

Stabilization of Bumetanide Binding in Triton X-100

In our early experiments with BLMV Triton extracts it quickly became clear that any maneuver which lead to an increase in the detergent : protein ratio also lead to a dramatic loss of bumetanide binding activity (e.g., Fig. 1*A*). Subsequent studies indicated that binding activity could be recovered by the addition of exogenous soybean phosphatidylcholine (Sigma, Type II-S); however, the extent of this recovery was reduced by the time and harshness of detergent treatment. For example, BLMV treated for 30 min with 0.3% Triton X-100 showed no detectable bumetanide binding activity (*cf.*, Fig. 1*A*), but the addition of of 0.2% soybean phosphatidylcholine restored binding to within ~80% of BLMV levels. If, on the other hand, the addition of

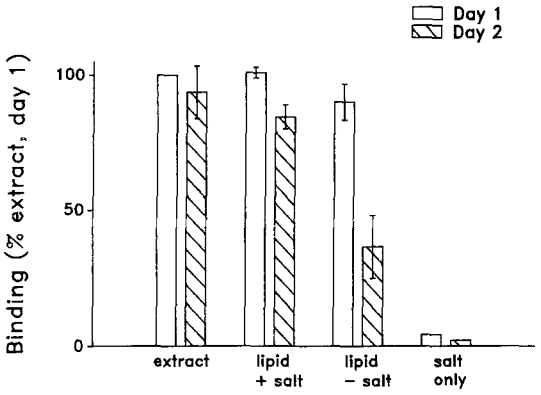

Fig. 5. Recovery of bumetanide binding following dilution of the 0.07% Triton extract with various detergent solutions. The 0.07% Triton extract diluted five times with either Buffer A/EDTA containing 0.12% Triton X-100, 5% sucrose, 100 mM sodium gluconate, 95 mM potassium gluconate, 5 mM KCl and 0.2% "clarified" (by centrifugation at 150,000 × *g* × 5 min) soybean lipid (lipid + salt), the same solution without salts (lipid − salt), or the same solution without lipid (salts only). The binding of 1 μM [^{3}H] bumetanide was then determined immediately (*Day 1*) or after leaving the material at 4°C overnight (*Day 2*). The data have been normalized to the total binding in the starting 0.07% Triton extract on day 1. The results shown are the averages of three independent experiments

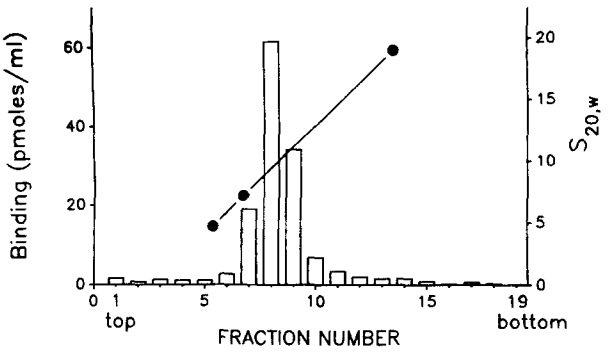

Fig. 6. Sucrose density gradient sedimentation of the 0.07% Triton extract. A 600-μl aliquot of the 0.07% Triton extract was run on a 5–25% sucrose gradient (116,000 × *g* × 16 hr) prepared as described in Materials and Methods. The [^{3}H]-bumetanide binding activity (1 μM) of the gradient fractions is illustrated by the vertical bars. In addition the sedimentation positions of three marker proteins, porcine thyroglobulin ($s_{20,w}$ = 19.0 S), bovine IgG ($s_{20,w}$ = 7.2 S) and bovine serum albumin ($s_{20,w}$ = 4.7 S), determined on independent but otherwise identical gradients is shown (●)

lipid was delayed for two additional hours, ~40% of this recoverable binding was lost (*data not illustrated*). Accordingly, we attempted to establish conditions that would preserve bumetanide binding activity throughout a series of purification and concommitant dilution steps in detergent-containing media. The experiment shown in Fig. 5 illustrates the efficacy of the conditions we have found. Here we have taken the 0.07% Triton extract and diluted it 1:5 into three Triton X-100 solutions, one containing "clarified" soybean phosphatidylcholine (*see* figure caption) and salt (100 mM sodium gluconate, 95 mM potassium gluconate and 5 mM KCl), one containing lipid without added salt, and one containing salt only. When bumetanide binding was measured shortly after dilution (day 1, open bars), binding activity was hardly detectable in the extract diluted into lipid free medium, whereas almost all binding activity was recovered in the extract diluted into media containing soybean lipid. After being left overnight at 4°C (day 2, hatched bars), however, it is clear that the lipid solution containing salt was considerably more effective in stabilizing bumetanide binding activity in the diluted extract.

Sucrose Density Gradient Sedimentation

Figure 6 illustrates the results of an experiment where the proteins in the 0.07% Triton extract were separated by sucrose density gradient (5–25%) sedimentation. The figure shows the bumetanide binding activity of the fractions of the gradient obtained after centrifugation at 116,000 × *g* × 16 hr. Also shown are the positions at which three proteins with known sedimentation coefficients appear (*see* figure caption). Bumetanide binding activity appears on the gradient as a single band with a sedimentation coefficient of 8.8 ± 0.8 S (n = 8). In control experiments (*not shown*) we have demonstrated that the K_d for bumetanide binding to the peak fractions obtained from the sucrose gradient (K_d = 0.51 ± 0.09, n = 3) is the same as that obtained for the 0.07% Triton extract (Fig. 4), confirming that this band corresponds to the purified high affinity bumetanide binding site.

On average the total bumetanide binding activity recovered from the sucrose gradient is 65.1 ± 16.0% (n = 4) of that loaded, and approximately one half of this activity (29.4 ± 6.4% of that loaded, n = 6) is found in the peak fraction (fraction 8 in Fig. 6). The protein concentration of this fraction is 0.148 ± 0.006 mg/ml (n = 3). Assuming that lost bumetanide binding activity is due to inactivation and that these inactivated proteins copurify with active proteins on the sucrose gradient, we calculate that this corresponds to an enrichment of the high affinity bumetanide binding site of approximately sevenfold relative to the starting BLMV.

Discussion

We have previously characterized a high affinity bumetanide binding site on rabbit parotid BLMV and provided convincing evidence that this is the bu-

metanide inhibitory site on the acinar Na/K/Cl cotransporter [32]. In the experiments presented here we demonstrate that this binding site can be extracted from BLMV by a relatively gentle treatment with the non-ionic detergent Triton X-100 and partially purified in a still functional state. For practical reasons (limited amounts of tissue) we have used relatively low concentration of membranes (1 mg protein/ml) in our experiments and consequently were able to work with low detergent concentrations as well. Our results indicate that the high affinity bumetanide binding site is essentially totally extracted from the BLMV at 0.07% Triton X-100 (Figs. 1 and 4) and that this extracted site retains the ionic characteristics of bumetanide binding to native membranes (Figs. 2 and 3). At this detergent : protein ratio (approximately 1 : 1) membranes typically disperse into large, slowly sedimenting, macromolecular lipid-protein-detergent complexes with molecular weights in the order of 0.5 to 1 million [15]. Consistent with this expectation, we find that the bumetanide binding site is not sedimented from the 0.07% Triton extract by centrifugation at 116,000 $\times$ g $\times$ 1 hr (*see* Results). The fivefold increase in affinity of the extracted bumetanide binding site relative to native membranes (Fig. 4) may be simply due to reduced steric hindrance for binding as a consequence of the disruption of normal membrane structure.

At higher Triton concentrations the bumetanide binding site is inactivated (Fig. 1). This is presumably due to delipidation since this inactivation can be prevented or (partially) reversed by the addition of exogenous lipid (Fig. 5 and text). In this regard it has been demonstrated that the presence of lipid is required to preserve the function and structure of a number of membrane-bound proteins in detergent solutions [15, 20]. In our hands prolonged incubation of the extracted bumetanide binding site at the higher detergent : protein ratios required for complete membrane dissociation as well as for most commonly used protein separation procedures resulted in irreversible inactivation (*see* text). Accordingly, we adopted the approach of devising a buffer that would allow us to preserve bumetanide binding activity throughout the protein purification process. The buffer which we report and employ here contains soybean lipid and a relatively high concentration of salt (200 mM) in addition to Triton X-100. The inclusion of both salt and lipid are important for retaining bumetanide binding (Fig. 5).

When the 0.07% Triton extract is fractionated by sucrose density gradient centrifugation in 0.24% Triton X-100 in the above buffer, the high affinity bumetanide binding site appears as a single band with a sedimentation coefficient of 8.8 S (Fig. 6). An estimate of the molecular weight of this band can be obtained from the formula [30]

$$(S_{20,w})_1/(S_{20,w})_2 = (M_1/M_2)^{2/3}$$

which gives an approximate relationship between the molecular weights (M_i) and sedimentation coefficients ($S_{20,w}$) of two proteins. From the known sedimentation coefficients and molecular weights of porcine thyroglobulin (19.0 S, 660 kDa) and bovine IgG (7.2 S, 150 kDa) we calculate that the molecular weight corresponding to the solubilized bumetanide binding site in Fig. 6 is $\approx$205 $\pm$ 30 kDa. However, it should be emphasized that this is the molecular weight of the protein-detergent-lipid complex corresponding to the bumetanide binding site and can only be considered as an approximate upper limit on the molecular weight of the actual bumetanide binding protein.

Little is known about the detergent binding properties of membrane transport proteins. To our knowledge, the only determination of this type that has been carried out is for Na/K ATPase, which binds 0.28 g of Triton X-100 per g of transporter [3]. Although this may represent low level of detergent binding relative to other membrane proteins [3], using this estimate for the bumetanide binding site identified here yields a molecular weight of 160 $\pm$ 23 kDa for the detergent free bumetanide binding protein. This value is in remarkably good agreement with values obtained by Haas and Forbush using photolabeling techniques in the dog renal medulla and in duck red blood cells [13, 14] as well as with the results of Pewitt et al. [28] using bumetanide affinity chromatography of solubilized calf renal medulla. In this regard we would like to also emphasize that in the present studies we have been able to associate a purified protein fraction directly with a functional marker—high affinity bumetanide binding. This is in marked contrast to all previous attempts to purify Na/K/Cl cotransport proteins and their associated bumetanide binding sites. In many of these studies covalent modification (inactivation) of the transporter precludes a direct measurement of its functional properties [5, 13, 14, 17]. In the remainder [7, 28], the function of the final purified protein fraction has not yet been demonstrated.

Another point which remains to be clarified is whether the bumetanide binding site purified in the present study is the complete Na/K/Cl cotransporter or only a portion of it. We have recently reported a successful reconstitution of bumetanide binding activity starting from the 0.07% Triton extract studied here [4]. However, we have not as yet been able to demonstrate the presence of Na/K/Cl cotransport activity in the resulting proteolipo-

somes. Whether this is due to the insensitivity of our flux measurements or to an actual dissociation of bumetanide binding activity from Na/K/Cl cotransport is currently under investigation.

We would like to thank Drs. Ronald Weiner, William Harris, Suresh V. Ambudkar, Peter Maloney and Bruce J. Baum for helpful discussions and suggestions during the course of this work.

References

1. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72:**248–254
2. Brown, C.D.A., Murer, H. 1985. Characterization of a Na : K : 2Cl cotransport system in the apical membrane of a renal epithelial cell line (LLC-PK_1) *J. Membrane Biol.* **87:**131–139
3. Clarke, S. 1975. The size and detergent binding of membrane proteins. *J. Biol. Chem.* **250:**5459–5469
4. Corcelli, A., Turner, R.J. 1989. Reconstitution of the rabbit parotid basolateral membrane Na/K/Cl dependent bumetanide binding site. *Ann. N.Y. Acad. Sci. (in press)*
5. Distephano, A., Wangemann, P., Friedrich, T., Burckhardt, G., Okonomopoulos, R., Englert, H. C., Lang, H. J., Greger, R. 1986. Photoaffinity labelling of the Na-2Cl-K carrier. *Pfluegers Arch.* **406:**R59
6. Epstein, F.H., Stoff, J.S., Silva, P. 1983. Mechanism and control of hyperosmotic NaCl-rich secretion by the rectal gland of *Squalus acanthias*. *J. Exp. Biol.* **106:**25–41
7. Feit, P.W., Hoffman, E.K., Schiødt, M., Kristensen, P., Jessen, F., Dunham, P.B. 1988. Purification of proteins of the Na/Cl cotransporter from membranes of Ehrlich ascites cells using a bumetanide-sepharose affinity column. *J. Membrane Biol.* **103:**135–147
8. Forbush, B., Palfrey, H.C. 1983. [^{3}H]Bumetanide binding to membranes isolated from dog kidney outer medulla. *J. Biol. Chem.* **258:**11787–11792
9. Frizzell, R.A., Field, M., Schultz, S.G. 1979. Sodium coupled chloride transport by epithelial tissues. *Am. J. Physiol.* **236:**F1–F8
10. Geck, P., Heinz, E. 1986. The Na-K-2Cl cotransport system. *J. Membrane Biol.* **91:**97–105
11. Gershoni, J.M., Palade, G.E. 1982. Electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to a positively charged membrane filter. *Anal. Biochem.* **124:**396–405
12. Haas, M., Forbush, B. 1986. [^{3}H]Bumetanide binding to duck red cells. *J. Biol. Chem.* **261:**8434–8441
13. Haas, M., Forbush, B. 1987. Photolabeling of a 150-kDa (Na + K + Cl) cotransport protein from dog kidney with a bumetanide analogue. *Am. J. Physiol.* **253:**C243–C250
14. Haas, M., Forbush, B. 1988. Photoaffinity labelling of a 150-kDa (Na + K + Cl) cotransport protein from duck red blood cells with an analogue of bumetanide. *Biochim. Biophys. Acta* **939:**131–144
15. Hjelmeland, L.M., Chrambach, A. 1984. Solubilization of functional membrane proteins. *Meth. Enzymol.* **104:**305–318
16. Hoffman, E.K., Schiodt, M., Dunham, P. 1986. The number of chloride-cation cotransport sites on Ehrlich ascites cells measured with [^{3}H]bumetanide. *Am. J. Physiol.* **250:**C688–C693
17. Jorgensen, P.L., Petersen, J., Rees, W.D. 1984. Identification of a Na,K,Cl cotransport protein of M_r 34,000 from kidney by photolabeling with [^{3}H]bumetanide. *Biochim. Biophys. Acta* **775:**105–110
18. Kinne, R., Hannafin, J.A., Konig, B. 1985. Role of the NaCl-KCl cotransport system in active chloride absorption and secretion. *Ann. N.Y. Acad. Sci.* **456:**198–206
19. Kinne, R., Kinne-Saffran, E., Scholermann, B., Schutz, H. 1986. The anion specificity of the sodium-potassium-chloride cotransporter in rabbit kidney outer medulla: Studies on medullary plasma membranes. *Pfluegers Arch.* **407:**S168–S173
20. Klausner, R.D., von Renswoulde, J., Rivnay, B. 1984. Reconstitution of membrane proteins. *Meth. Enzymol.* **104:**340–347
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275
22. Martin, R.G., Ames, B.N. 1961. A method for determining the sedimentation behaviour of enzymes: Application to protein mixtures. *J. Biol. Chem.* **236:**1372–1379
23. Melvin, J.E., Kawaguchi, M., Baum, B.J., Turner, R.J. 1987. A muscarinic agonist-stimulated chloride efflux pathway is associated with fluid secretion in rat parotid acinar cells. *Biochem. Biophys. Res. Commun.* **145:**754–759
24. Nauntofte, B., Poulsen, J.H. 1986. Effects of Ca^{2+} and furosemide on Cl^- transport and O_2 uptake in rat parotid acini. *Am. J. Physiol.* **251:**C175–C185
25. O'Grady, S.M., Palfrey, H.C., Field, M. 1987. Characteristics and functions of Na-K-Cl cotransport in epithelial tissues. *Am. J. Physiol.* **253:**C177–C192
26. O'Grady, S.M., Palfrey, H.C., Field, M. 1987. Na-K-2Cl cotransport in winter flounder intestine and bovine kidney outer medulla: [^{3}H]-bumetanide binding and effects of furosemide analogues. *J. Membrane Biol.* **96:**11–18
27. Petersen, O.H., Maruyama, Y. 1984. Calcium-activated potassium channels and their role in secretion. *Nature (London)* **307:**693–696
28. Pewitt, B., Palfrey, H.C., Burnham, C.E. 1988. Affinity purification of a bumetanide binding protein possibly related to the Na/K/Cl cotransporter. *FASEB J.* **2:**A1725
29. Pirani, D., Evans, A.R., Cook, D.I., Young, J.A. 1987. Intracellular pH in the rat mandibular salivary gland: The role of Na-H and Cl-HCO_3 antiports in secretion. *Pfluegers Arch.* **408:**178–184
30. Schachman, H.K. 1953. Ultracentrifugation in biochemistry. Academic, New York
31. Towbin, H., Staehelin, T., Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76:**4350–4354
32. Turner, R.J., George, J.N. 1988. Ionic dependence of bumetanide binding to the rabbit parotid Na/K/Cl cotransporter. *J. Membrane Biol.* **102:**71–77
33. Turner, R.J., George, J.N., Baum, B.J. 1986. Evidence for a Na^+/K^+Cl^- cotransport system in basolateral membrane vesicles from the rabbit parotid. *J. Membrane Biol.* **94:**143–152
34. Young, J.A., Cook, D.I., van Lennep, E.W., Roberts, M.L. 1987. Secretion by the major salivary glands. *In:* Physiology of the Gastrointestinal Tract. (2nd ed.) L. Johnson et al., editors. Raven, New York

Received 7 June 1989; revised 13 September 1989