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Solubilization of Na/K/Cl Cotransporter in Rabbit Parotid Acini and Its Purification-Purification by Sucrose Density Gradient

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The high affinity bumetanide binding site of the rabbit parotid acinar cell can be extracted from a basolateral membrane fraction using relative low concentration (0.07%, 1 mg membrane protein/ml) of the non-ionic detergent Triton x-100. This extracted site can not 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. Inactivation of the extracted bumetanide binding site observed at detergent/protein ratios>1 can be partially prevented by the addition of 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 bumetanide binding site sediments as a single band with $S_{20,w}=8.8\pm0.8$ S. This corresponds to a molecular weight ~200 kD for the bumetanide binding protein-detergent-lipid complex and represents a sevenfold purification of this site relative to the starting membrane fraction. Consequently, it may be suggested that partial purification of bumetanide binding site can be able to obtained using the 0.07% Triton X-100 and sucrose density gradient centrifugation.

Key words: Na/K/Cl cotransporter, parotid acini, sucrose density gradient, non-ionic detergent, Triton X-100, loop diuretics

Introduction

Na/K/Cl cotransporter is known to be responsible for the chloride entry in the absorptive and secreting epithelium as well as nonepithelial cells (Epstein *et al.*, 1983; O'Grady *et al.*, 1987). Considerable evidence now indicates that salt and water movement across many secretory and absorptive epithelia is due to transepithelial chloride fluxes (Frizzel *et al.*, 1979; Petersen and Maruyama, 1984; Kinne *et al.*, 1985; Young *et al.*, 1987). In a number of tissues these chloride movements are driven by a Na/K/Cl cotransporter system which is responsible for concentrative chloride entry. Na/K/Cl cotransporters have also been identified in a variety of nonepithelial tissue (Geck and Heinz, 1986) where they are involved in volume regulatory ion fluxes and possibly in non-renal 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 (Petersen and Maruyama, 1984; Nauntofte and Poulsen, 1986; Melvin *et al.* 1987; Pirani *et al.*, 1987; Young *et al.*, 1987). In fact direct evidence provides that Na/K/Cl cotransporter exists in parotid acinar basolateral membrane vesicle. According to the previous results in salivary parotid acini, the electrochemical gradient for Na/K^+-ATPase causes Cl^- to be driven into the acinar cell against its electrochemical gradient via the basolateral cotransporter. Cl^- then diffuse down its electrochemcal gradient into the acinar lumen via the apical chloride conductance, and Na^+ follows, presumably by leaking through the tight junctions between the cells, in order to preserve electrical neutrality. Mentioned the above, beginning of salivary secretion is triggered by the

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Cl^- entry which is driven through the Na/K/Cl cotransporter system.

Owing to Na/K/Cl cotransporter's obvious physiological importance, a considerable amount of recent experimental effort has been devoted to characterizing Na/K/Cl cotransporter (Epstein *et al.*, 1983; Forbush and Palfrey, 1983; Brown and Murer, 1985; Geck and Heinz, 1986; Hoffman *et al.*, 1986; Turner an George, 1988; Corcelli and Turner, 1991). Much of this work has made use of the loop diuretic bumetanide, a potent specific inhibitor of this cotransporter. Other labaratories (Forbush and Palfrey, 1983; Haas and Forbush, 1987; O'Grady *et al.*, 1987) 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 attemps to label or purify putative components of Na/K/Cl cotransporters. Jorgensen *et al.* (1984) 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. Using similiar techniques with radiolabeled bumetanide analogues, DiStephanno *et al.*, (1986) have photolabeled 24 kD protein in isolated pig kidney thick ascending of Henle's loop, and Haas and Forbush have photolabeled 150 kD protein in both dog renal medullary membranes (Haas and Forbush, 1986) and duck red blood cells (Haas and Forbush, 1988). More recently, Pewitt *et al.* (1988) have demonstrated that an affinity chromatography column consisting of 4-p-aminobumetanide coupled to Affigel-10, a 160 kD protein is retained and can be subsequently eluted with excess bumetanide. Feit *et al.* (1988) have also used affinity chromatographic techniques with a bumetanaide analogue to identify proteins with molecular weights ~76 and ~38 kD 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 methodological difficulties.

Therefore, in an attempt to identificaton of purification of cotransporter system, different accesses were used in rabbit parotid. Firstly we used low concentration of Triton X-100 for minimizing the degradation of the intact materials which is cotransporter system. Then sucrose gradient centrifugation was adopted for isolation of Na/K/Cl cotransporter rich fraction which is identified by the [^{3}H]-bumetanide binding assay. Secondly, we employed the isolation technique using lectin, since previous evidence suggests the possibility that Na/K/Cl cotransporter protein might be glycosylated.

Materials and Methods

Basolateral Membrane Preparation

White rabbits were sacrified by decapitation (all subsquent steps were carried out at 4℃). the parotid gland were immediately removed, dissected free of lymph nodes, superficial fat and connective tissue and placed in 200 to 300 μl of Homogenization Medium (10 mM Tris/HEPES containing 10% sucrose, 1 mM EDTA and 0.1 mM PMSF). The glands were then minced into approximately 1 mm pieces using fine scissors and diluted in Homogenization Medium to a total volume of 10 ml. This parotid mince was left on ice for several minutes to allow any remaining fatty tissue to float to the top. The parotid mince was then homogenized by two 10 sec bursts at power level 5 in a Polytron. The resulting homogenates was spun at 2,500 g for 5 min and then combined supernatants were filtered through a fine nylon mesh (Nitex 155 μm, Tetko Inc., Elmsford NY) and spun at 22,000 g for 20 min.

The above supernatant was discarded and the pellet was suspended in a few ml of Buffer A/EDTA (10 mM Tris/HEPES with 100mM mannitol) containing 1 mM EDTA passed once through a 25 gauge needle and once through a 30 gauge needle, then diluted with same buffer. Basolateral mem-

Table 1. Enzymatic characterization of rabbit parotid basolateral membrane

Enzyme	Activity in homogenate (nmol/mg protein/hr)	Enrichment (vesicle/homogenate)	% Recovery (vesicle/homogenate)
K^+-stimulated p-nitrophenyl phosphatase	0.91± 0.26	10.4± 1.6	22± 1

Values are mean± SD for three indcpendent determinations.
Enzyme activity is given as micromoles substrate consumed per milligram protein per hour.

brane (BLM) were prepared by a Percoll gradient method as previously described (Turner and George, 1988). 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 vesicle membrane preparation (Table 1). Freshly prepared BLM 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 BLM were fast frozen and stored above liquid nitrogen.

On the day of the experiment an appropriate number of aliquots of frozen BLM were thawed 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.

Solubilization of Vesicle with Triton X-100

BLM 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 (Turner and George, 1988). 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 incubation the reaction was terminated by the addition of 1.5 ml of ice-cold stop solution (Buffer A containing 200 mM NaCl plus 100 mM KCl) followed millipore filtration (HAWP 0.45 μm). the filter was then washed with a further 6.0 ml of stop solution, placed in a Scintillant (Amersham, Arlington Heights) 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 concentration of sodium, potassium and chloride yield near optimal condition for high affinity bumetanide binding to rabbit parotid BLM (Turner and George, 1988). All data have been corrected for nonspecific retention of [^{3}H]-bumetanide by the filters. All binding studies were carried out at 23℃ 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 BLM. It is well known that nitrocellulose filters of the type employed here ad-

sorb proteins from dilute solutions with high efficiency (Towbin *et al.*, 1979; Gershoni and Palade, 1982): in fact, they are used extensively for this purpose in blotting and immunoassays (Towbin *et al.*, 1979). In order to verify that BLM protein was retained by the filters under the conditions employed in these stuides, 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 1,000 μl of diluted vesicle 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).

Lectin Chromatography

Vesicle was treated with 0.6% Triton X-100 in order to extract the cotransporter protein. then after reaction of this material on ice for 30 min, sample was spinned for 5 min using Airfuge (30 psi). Taking the supernatant as soon as possible, 0.2% lipid (soybean phophatidylcholine, Sigma) were added to the supernatant and sonified with Vortex for 10 sec. Lipidated sample was eluted through the lectin column (BioRad) which is packed in 10 ml Econo-column and Eppendorf tube. First lectin column was washed with Buffer I (Buffer C; Buffer A/EDTA+100 mM Na+100 mM K+5 mM Cl, 0.3 % Triton X-100, 0.02% sodium dodecyl sulfate), Buffer II (Buffer C, 0.3% Triton X-100, 1% bovine serum albumin), Buffer III (Buffer C, 0.3% Triton X-100, 300 mM N-acetylglucosamine, 0.2% clarified lipid) and Buffer IV (Buffer C, 0.3% Triton X-100, 0.2% clarified lipid) in order. The volume of each Buffer was 50 fold volume relative to lectin amount, finally after three times of passing with Buffer III (1 ml) sample was eluted through the conditioned lectin column. Bumetanide binding assay and protein measurement in this eluted sample were conducted.

Protein Determination

Except where incubated protein was measured by the method of Bradford (1976) 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.

Sucrose Density Gradient Sedimentation

The following "clarified lipid solution" formed the basis of our sucrose density gradients. Soybean phosphatidylcholine (Sigma, Type-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 ffom 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 SW 55Ti rotor of a Beckman L8-M ultracentrifuge at 4℃. 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, bovine IgG and bovine serum albumin. The position 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).

Results

Effect of Triton X-100 on Bumetanide Binding to BLM

BLM 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 comparision of binding to vesicles and extract, the data are presented as binding per milliliter of sample. As results of these studies, First, at Triton concentrations <0.1% there is an apparent "activation" of bumetanide binding over that found in native vesicles. 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 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 it has been verified that there is, in fact, no detectable bumetanide binding in the pellets obtained after centrifuging the BLM 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 reported, it has been verified that the nitrocellulose filters used in this experiment are capable of adsorbing virtually all of the BLM protein employed in our bumetanide binding assays. However, in this regards it was confirmed that the same pattern observed in Fig. 1A for the vesicle extract is also obtained when bumetanide binding is determined with the gel filtration assay described in previous report. Thus the loss of binding activity at higher Triton X-100 concentrations illustrated in Fig. 1A is not due to a selective failure of nitrocellulose filter to retain the bumetanide binding protein. Owing to its relative convenience we used the nitrocellulose filtration assay in most of our experiments, and only those results are presented in the studies which follow.

Fig. 1B 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 BLM as the Triton X-100 concentration is increased above 0.075%.

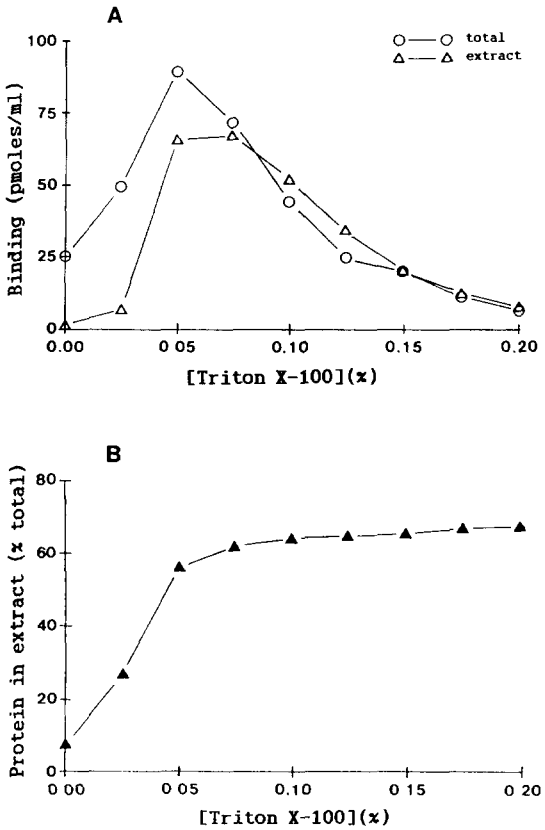

Fig. 1. A. effect of Triton X-100 on bumetanide binding to rabbit parotid basolateral membrane vesices BLM (1 mg protein/ml) in Buffer A/EDTA were treated for 30 min on ice with Triton X-100 at the concentrations indicated (w/v). B. Protein content of the vesicle extracts obtained in A, expressed as a percentage of the total protein (1 mg/ml).

The 0.07% Triton X-100 was chosen as the starting point for the studies which follow. In additional experiments 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.

Characteristics of the Extracted Bumetanide Binding Site

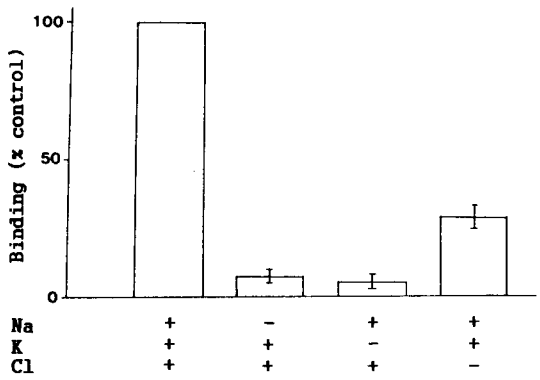

Fig. 2. Dependence of bumetanide binding to the 0.07 % Triton extract on the presence of sodium, potassium, and chloride. The binding of 1 μM [^{3}H]-bumetanide was measured in the presence of 100 mM sodium, 100 mM potassium, 5 mM chloride and 195 mM gluconate, or with sodium, replaced by NMDA, potassium by NMDG, or chloride replaced by gluconate.

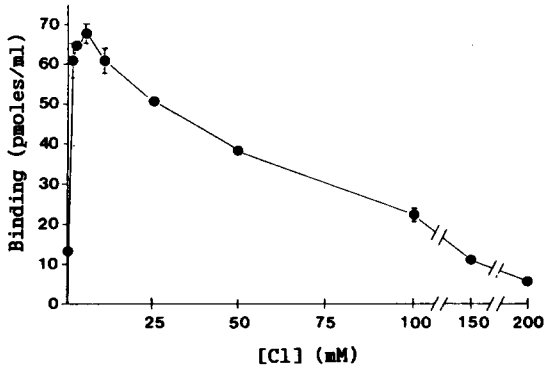

Fig. 3. Dependence of bumetanide to the 0.07% Triton extract on Cl. the binding of 1 μM [^{3}H]-bumetanide was measured in the presence of 100 mM sodium, 100 mM potassium and 0 to 200 mM chloride with chloride replaced isosmotically by gluconate.

It has been known that high affinity bumetanide binding to parotid BLM requires the presence of sodium, potassium and chloride. In the experiment illustrated in Fig. 2 we compared bumetanide binding to 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 BLM.

High-affinity bumetanide binding to BLM also shows a biphasic dependence on chloride concentration; at 100 mM and potassium, binding increases from 0 to 5 mM chloride and decreases thereafter. the experiment shown in Fig. 3 illustrates that the same biphasic behavior is found in the 0.07% Triton extract. Thus the bumetanide binding site found in the 0.07% Triton extract preserves the ionic characteristics of bumetanide binding to native basolateral membranes.

Stabilization of Bumetanide Binding in Triton X-100

In our preliminary experiments with 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. Subsequent studies indicated that binding activity could be recovered by the addition of exogenous soybean phophatidylcholine (Sigma, Type II-S); however, the extent of this recovery

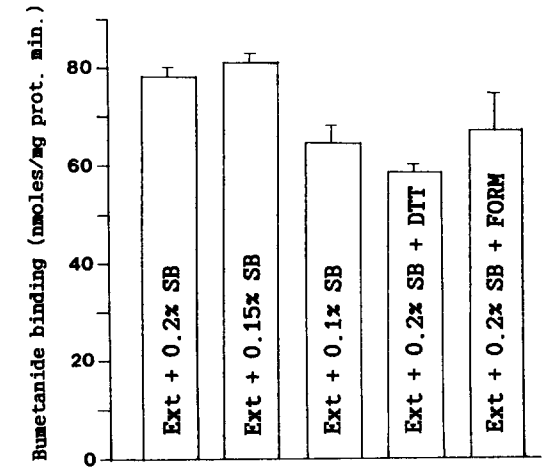

Fig. 4. The stability of the bumetanide binding by the addition of the soybean (SB) at the different concentration, dithiothreitol (DTT), and formate (FORM). 0.1%, 0.15%, 0.2% SB, DTT and FORM was added to 0.07% Triton extract (Ext), and then each bumetanide binding activity was measured.

was reduced by the time and harshness of detergent treatment, for example, BLM treated for 30 min with 0.3% Triton X-100 showed no detectable bumetanide binding activity, but the addition of 0.2 % soybean phosphatidylcholine restored binding to within ~80% of BLM levels, if, on the other hand, the addition of lipid was delayed for two additional hours, ~40% of this recoverable binding was lost. Accordingly, we attempted to establish conditions that would preserve bumetanide binding activity throughout a series of purification and concomitant dilution steps in detergent-containing media. The experiment shown in Fig. 4 illustrates the efficacy of the conditions we have found. Here we have taken the 0.07% Triton extract (designated by Ext) and diluted it 1:5 into Triton X-100 solutions with different conditions (Triton extract only, 0.2% soybean, 0.15% soybean, 0.1% soybean, 0.2% soybean +dithiothreitol and 0.2% soybean+formate) to test the effect of the addition of soybean and other reducing agents on the stability of bumetanide binding protein. This results show that 0.2% soybean (or 0.15% soybean) is more effective to preserve the stabilized bumetanide binding protein. From this experimental result, we used the 0.2% soybean for activating the bumetanide binding protein. Moreover, another test was done to confirm the stability of bumetanide binding protein under the condition of time (after 1 and 2 days). When bumetanide binding was measured (day 1 and day 2), binding activity in day 1 was higher than in day 2, however, a statistically significant difference was not found between them. Also the existence of solution containing "clarified" soybean phophatidylcholine and salt (100 mM sodium gluconate, 95 mM potassium gluconate, and 5 mM KCl) increased the stability of bumetanide binding protein (Fig. 5). In the extract diluted into lipid free medium, there was some loss in bumetanide binding activity, whereas almost all binding activity was recovered in the extract diluted into media containing soybean lipid. After being left overnight at 4℃ (day 2, hatched bars), however, it is clear that lipid solution containing salt was considerably contributed to the stabilizing bumetanide binding activity in diluted extract.

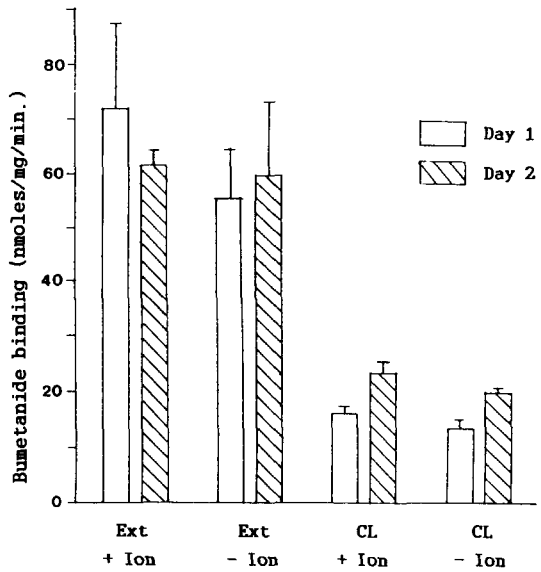

Fig. 5. The changes in bumetanide binding in basolateral membrane in the course of time. The stability of bumetanide binding protein in the course of time (after 1 and 2 days) was tested under the different solution. 0.7% Triton extract was diluted into clarified lipid (CL, 0.2% soybean) containing 100 mM sodium gluconate, 95 mM potassium gluconate, and 5 mM KCl, while for the comparision bumetanide binding activity was measured in only clarified lipid. The binding of 1 μM [^{3}H]-bumetanide was determined immediately (day 1) or after leaving the material at 4℃ overnight (day 2).

Sucrose Density Gradient Sedimentation

Fig. 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 at 116,000×g×16 hr. Also shown are the positions at which three proteins with known sedimentation coefficients appear. Bumetanide binding activity appears on the gradient as a single band with a sedimentation coefficient of 8.8± 0.8 S (n=8), confirming that this band corresponds to the purified high affinity bumetanide binding site.

On the 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).

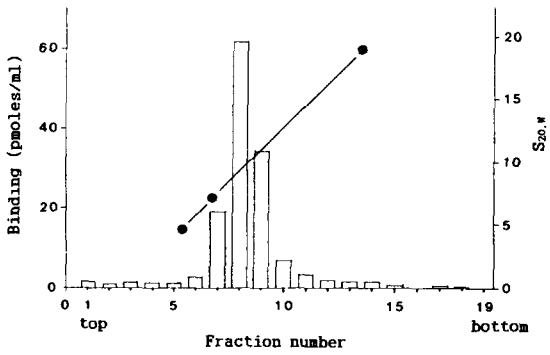

Fig. 6. The sucrose density gradient sedimentation of the 0.07% Triton extract. A 600 μl aliquot of the 0.07% Triton extract was run on a 5-25% sucrose gradient. In addition the sedimentation positions of three marker proteins, porcine thyroglobulin ($S_{20,w}$=19.0 S), bovine IgG ($S_{20,w}$=7.2 S), and bovine serum albumin ($S_{20,w}$=4.7 S) determined on the independent but otherwise identical gradients is shown (●)

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 copurified 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 BLM.

Discussion

In the previous results, a high affinity bumetanide binding site in the rabbit parotid BLM was characterized and it provided convincing evidence that there is the bumetanide inhibitory site on the acinar Na/K/Cl cotransporter (Turner and George, 1988). In the experiments presented here we demonstrate that this binding site can be extracted from BLM 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 havested relatively low concentration of membrane (1 mg/ml) in our experiments and consequently were able to work with low detergent concentration as well. Our results indicate that the high affinity bumetanide binding site is essentially totally extracted from the BLM at 0.07% Triton X-100 (Figs. 1 and 4) and that this extracted site retains the ionic chracteristics 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. Consistent with this expectation, we find the bumetanide binding site is not sedimented from the 0.07% Triton extract by centrifugation at 116,000×g×1 hr. 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 reversed by the addition of exogenous lipid (Fig. 4 and 5). 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 (Hjelmeland and Chrambach, 1984; Klausneer *et al.*, 1984). 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. Accordingly, we adopted the approach of devising a buffer that allow us to preserve bumetanide binding activity throughout the 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 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 if this band van be obtained from the formula (Schachman, 1953)

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

which gives an approximation relationship between the molecular weights (M_1) 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 kD) we calculated that the molecular weight corresponding to the solubilized bumetanide binding site in Fig. 6 is $\sim 205 \pm 30$ kD. 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/Cl ATPase, which binds 0.28 g of the Triton X-100 per g of transporter. Although this may represent low level of detergent binding relative to other membrane proteins (Clarks, 1975), using this estimate for the bumetanide binding site identified here yield a molecular weight of 160 ± 23 KD 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 (Haas and Forbush, 1987, 1988) as well as with the results of Pewitt *et al.* (1988) 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 cotransporter proteins and their associated bumetanide binding sites. On the other hand, we tried to separate the Na/K/Cl cotransporter protein lectin chromatography, since it has been suggested that this protein might be glycosylated. However, we failed to get a good elution of bumetanide binding protein through the lectin column. In many of these studies covalent modification (inactivation) of the transporter precludes a direct measurement of its functional properties (Jorgensen *et al.*, 1984; Distephano *et al.*, 1986; Haas and Forbush, 1988). In the remainder, 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.

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