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IDENTIFICATION OF A Na^+, K^+, Cl^--COTRANSPORT PROTEIN OF M_r 34000 FROM KIDNEY BY PHOTOLABELING WITH [^{3}H]BUMETHANIDE

THE PROTEIN IS ASSOCIATED WITH CYTOSKELETON COMPONENTS

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A polypeptide of M_r 34000 is photolabeled with [^{3}H]bumethanide after binding of this drug to membranes from the outer renal medulla and irradiation at 345 nm, a wavelength where bumethanide has an absorption maximum. Our data show that the polypeptide of M_r 34000 is a component of the $Na^+/K^+/Cl^-$-cotransport system. The [^{3}H]bumethanide binding protein is not extracted by concentrations of the nonionic detergent $C_{12}E_8$ that solubilizes 67% of the protein of the membranes including ($Na^+ + K^+$)-ATPase. This step increases the capacity for binding of [^{3}H]bumethanide to 681 pmol/mg protein. Extraction of the binding protein requires high ionic strength suggesting that the $Na^+/K^+/Cl^-$-cotransport protein is associated with cytoskeleton components. This association may be important for control of the entry of NaCl into the cytoplasm and for cellular regulation of the rate of active transport of NaCl across the tubule cells in the thick ascending limb of Henles loop.

Active transcellular transport of NaCl in the thick ascending limb of Henles loop (TAL) consists of primary active transport of Na^+ and secondary active transport of Cl^- [1,2]. A $Na^+/K^+/Cl^-$-cotransport system that mediates Na^+-gradient-driven secondary active transport of Cl^- across the luminal membrane of the tubule cells in the thick ascending limb can be identified in kidney cell membranes [3,4] and in avian erythrocytes [5] by high-affinity binding of bumethanide, a substituted 3-aminobenzoic acid derivative that was first synthesized by Feit [6]. The potent diuretics furosemide and bumethanide act by inhibiting this secondary active $Na^+/K^+/Cl^-$-cotransport process. The mechanism of the Na^+-gradient-driven energy transfer processes is unknown and none of the proteins involved in secondary active transport processes have been purified and characterized.

The present work is part of an attempt at identification and purification of the $Na^+/K^+/Cl^-$-cotransport system. In order to identify protein components of the transporter, [^{3}H]bumethanide was bound to membrane preparations from the outer renal medulla [7] and the complexes were exposed to light at wavelengths (345 nm and 278 nm) where bumethanide has absorption maxima. To isolate the transporter we examined solubilization of the bumethanide binding protein in nonionic detergents in media of different ionic strengths.

The data show that a protein of M_r 34000 forms at least a part of the $Na^+/K^+/Cl^-$-cotransport system. This protein is covalently pho-

Abbreviations: $C_{12}E_8$, dodecyl octaethyleneglycol monoether; Tes, 2-((2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)-ethanesulphonic acid.

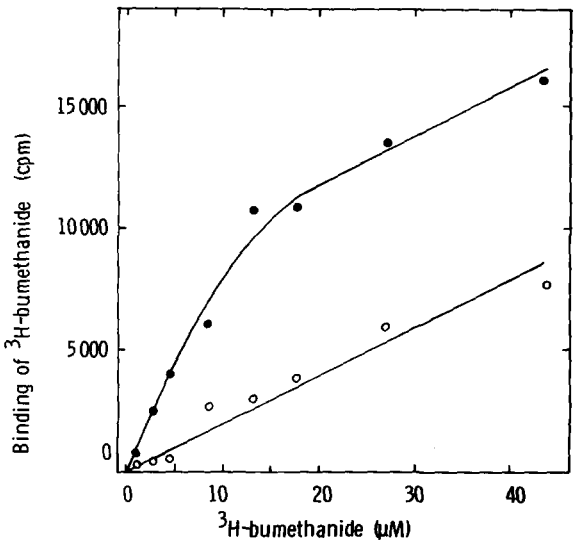

Fig. 1. [^{3}H]Bumethanide binding to $C_{12}E_8$ pellet (●), the fraction remaining after extraction of crude membranes with $C_{12}E_8$ as described in Table I; (○) represent retention of [^{3}H]-bumethanide in presence of 2 mM bumethanide. Aliquots containing 300 μg protein were mixed with [^{3}H]bumethanide in 340 μl 130 mM sodium acetate/20 mM potassium acetate/0.6 mM magnesium acetate/10 mM Tes (pH 7.5) with 0.9–50 μM [^{3}H]bumethanide without or with 2 mM unlabeled bumethanide. After incubation for 30 min at 20 °C aliquots of 160 μl were filtered through 0.22 μm GSWP Millipore filters of 22 mm diameter with glass microfiber Whatman GF/C prefilters of 10 mm diameter. The filters were washed twice with 2 ml ice-cold 10 mM Tris-acetate (pH 7.5) and counted. The specific radioactivity of [^{3}H]bumethanide (306 μCi/μg) was estimated from fluorescence measurements as in Fig. 3 using unlabeled bumethanide as standard. The fluorimetric measurements were in agreement with spectrophotometric analysis using $E_{268} = 9200$ and $E_{340} = 4000$ [4]. Both [^{3}H]bumethanide and unlabeled bumethanide appeared as single peaks at 63–65% ethanol when eluted from a 5 μm Nucleosil C-18 reverse phase liquid chromatography column at 43 °C with a linear gradient from 0.1% trifluoroacetic acid/5% ethanol to 0.02% trifluoroacetic acid/80% ethanol.

tolabeled with [^{3}H]bumethanide when this compound is bound to the membranes and exposed to monochromatic light of wavelength 345 nm or to a broader spectrum of ultraviolet light. The bumethanide binding protein remains in the pellet after extraction of the membranes with $C_{12}E_8$ in concentrations that are sufficient to extract most of the membrane proteins including the ($Na^+ + K^+$)-ATPase. Extraction of the bumethanide-binding protein requires the presence of KCl suggesting that this protein is associated with the cytoskeleton. This association may be important for regulation of the rate of entry of Cl^-, Na^+ and K^+ into the cell and thus for the cellular regulation of the transcellular transport of NaCl.

The concentration dependence of [^{3}H]bumethanide binding to the $C_{12}E_8$ pellet was examined as shown in Fig. 1. A hyperbolic curve of specific binding was observed with $K_{1/2} = 5.6$ μM, while unspecific binding in the presence of 2 mM unlabeled bumethanide was linearly related to

TABLE I

BINDING OF [^{3}H]BUMETHANIDE TO CRUDE MEMBRANES AND TO THE PELLET AFTER EXTRACTION OF MEMBRANE PROTEINS WITH $C_{12}E_8$

Crude membrane fraction was prepared from the outer renal medulla of pig kidneys as previously described [7], except that chloride ions were replaced with acetate ions in all buffer solutions. For solubilization with nonionic detergent [8] the crude membranes were sedimented for 30 min at 30000 rpm in the Beckman 65 rotor and resuspended to 1.5 mg protein per ml in 20 mM Tes/2 mM EDTA/2 mM dithiotreitol/300 mM potassium acetate (pH 7.5). Equal volumes of $C_{12}E_8$ 10 mg/ml or 20 mg/ml were added. The mixture was incubated for 30 min at 20 °C and centrifuged for 30 min at 30000 rpm in the Beckman 65 rotor. Binding was measured as in Fig. 1 using a concentration of [^{3}H]bumethanide of 10 μM.

	Protein remaining (%)	[^{3}H]Bumethanide binding (pmol/mg protein)	[^{3}H]Bumethanide binding capacity (%)
Crude membranes	100	226 ± 14 (4)	100
$C_{12}E_8$ pellet (5 mg/ml)	37 (2)	681 (2)	115 (2)
$C_{12}E_8$ pellet (10 mg/ml)	33 ± 2 (4)	595 ± 5 (4)	108 ± 12 (4)
0.6 M KCl	14 (2)	705 (2)	50 (2)
1.2 M KCl	12 (2)	631 (2)	39 (2)

the concentration of [^{3}H]bumethanide. As shown in Table I the maximum capacity of the $C_{12}E_8$ pellet for [^{3}H]bumethanide binding was 3-fold higher than for the crude membrane fraction due to the removal of membrane protein upon extraction with $C_{12}E_8$. The apparent affinity for binding of [^{3}H]bumethanide was the same with $K_{1/2}$ in the range 5–7 μM for both preparations. At constant ionic strength we found maximum levels of binding in acetate media. Addition of Cl^- had only inhibitory effects on the binding in contrast to previous observations [4], and we did not observe stimulation by addition of Cl^- to the acetate media. The fact that binding is retained in the pellet after treatment with excess $C_{12}E_8$ excludes the possibility that the binding is affected by uptake of radioactivity in vesicles.

It is seen from Table I that the binding sites for [^{3}H]bumethanide were quantitatively recovered in the pellet after extraction with $C_{12}E_8$ in conditions where 2/3 of the protein in the crude membrane fraction was rendered soluble. Enzymatic assay showed that the extraction with $C_{12}E_8$ rendered all ($Na^+ + K^+$)-ATPase soluble as previously demonstrated for the pure membrane bound enzyme [8]. The electrophoretic analysis in Fig. 2 shows that the α-subunit and β-subunit of ($Na^+ + K^+$)-ATPase were absent from the pellet after extraction with $C_{12}E_8$. In addition to the bumethanide-binding protein the pellet contained a protein forming an intense band near the top of the gel with M_r close to 200000. This protein could be myosin or an actin-crosslinking protein. The other intense band with molecular weight close to 43000 could be actin [10], but the nature of the proteins

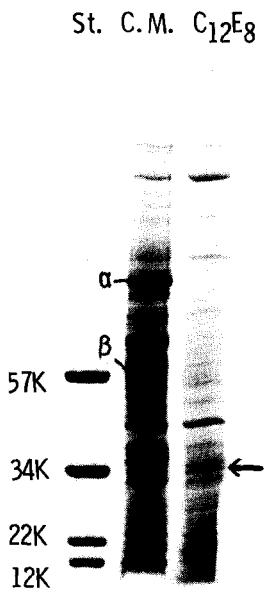

Fig. 2. Electrophoretograms of crude membranes (C.M.), $C_{12}E_8$ pellet and molecular weight standards (St.): pyruvate kinase (57 K), lactate dehydrogenase (34 K), trypsin inhibitor (22 K) and cytochrome *c* (12 K); (α) and (β) identifies the α-subunit and β-subunit of ($Na^+ + K^+$)-ATPase. Aliquots containing 30–50 μg protein were dissolved in 2% SDS/1% mercaptoethanol and applied on 5–15 T% linear gradient gels prepared according to Laemmli [9]. Arrow points at the band with M_r 34000 which is covalently labeled with [^{3}H]bumethanide in Fig. 4.

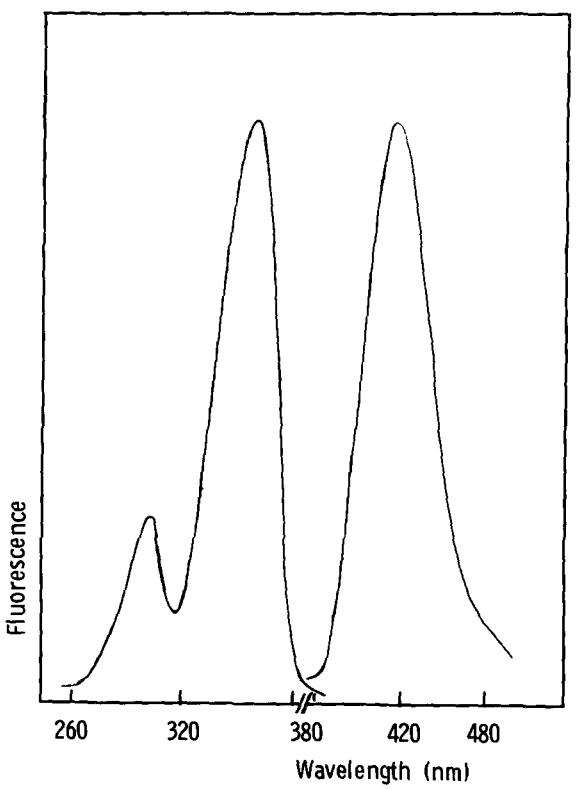

Fig. 3. Fluorescence spectrum of 10 μM [^{3}H]bumethanide in 10 mM Tes (pH 7.5). (Left) Excitation spectrum at emission 410 nm; (right) emission spectrum at excitation 345 nm using a Perkin-Elmer MPF 44 A spectrofluorometer with 5 nm slit widths on both monochromators.

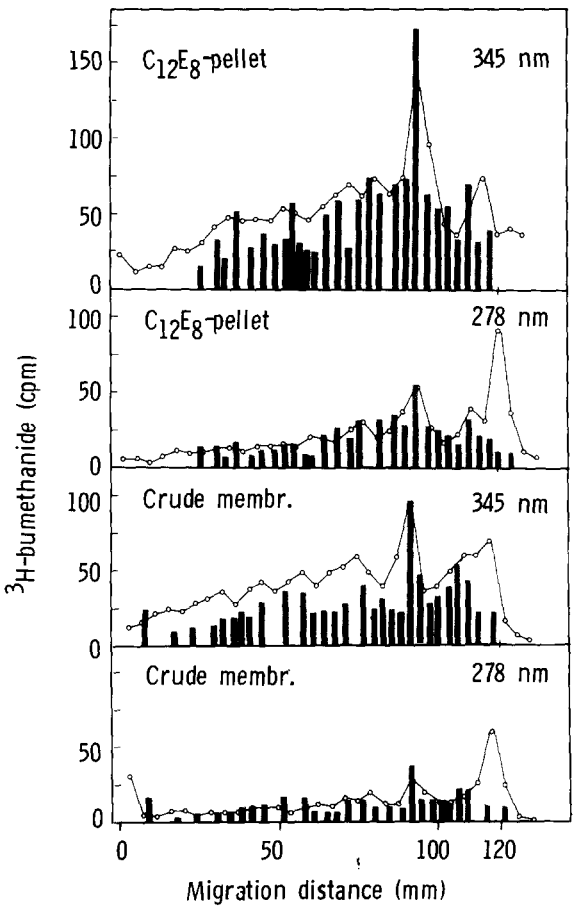

Fig. 4. Electrophoretic profile of [^{3}H]bumethanide incorporated after photolysis of $C_{12}E_8$ pellet or crude membranes at 345 nm or 278 nm; (○) radioactivity in slices before staining of gel; the bars show radioactivity in individual protein bands of gels that were sliced after staining with Coomassie blue and destaining. Aliquots of 3 ml containing 12 μM [^{3}H]bumethanide/130 mM sodium acetate/20 mM potassium acetate/0.6 mM magnesium acetate/10 mM Tes (pH 7.5) and 250–300 μg protein per ml were irradiated for 30 min at 278 nm or 345 nm in a Perkin-Elmer MPF 44 A fluorimeter in a continuously stirred 1 cm cuvette that was thermostatted at 10 °C using maximum slit of 20 nm on the excitation monochromator. After irradiation, unlabeled bumethanide was added to a final concentration of 2 mM. The mixture was centrifuged for 30 min at 40000 rpm in the Beckman 65 rotor. The pellet was resuspended in 2 ml 10 mM Tes/1 mM EDTA/130 mM sodium acetate/20 mM potassium acetate/0.6 mM magnesium acetate/2 mM bumethanide (pH 7.5) and centrifuged for 30 min at 40000 rpm. The pellet was resuspended in 150 μl 10 mM Tes/1 mM EDTA/2% SDS/1% mercaptoethanol and 80 μl were applied on linear 5–15 T% gradient slab gels prepared according to Laemmli [9]. After electrophoresis the gel slabs were divided in two halves. One half was stained with Coomassie blue and destained. The other half was cut in 4.5 mm slices, 1 ml 30% hydrogen peroxide (Merck) was added and the slices were dissolved by heating for 1–2 h at 80 °C and counted.

in these bands remains to be established. When KCl in concentrations up to 1.2 M was added, the [^{3}H]-bumethanide binding capacity disappeared from the pellet, suggesting that the binding protein was solubilized. The resistance to solubilization by nonionic detergent and the release at high salt concentration is characteristic for proteins associated with cytoskeleton components [10].

Another indication that the [^{3}H]bumethanide binding $Na^+/K^+/Cl^-$-cotransport protein can be associated with the cytoskeleton is our observation that more than half of the [^{3}H]bumethanide binding protein in the crude membranes sediments to the pellet through a gradient of metrizamide from 1.05 to 1.18 g/ml that retains membrane vesicles, all ($Na^+ + K^+$)-ATPase activity and mitochondrial fragments (data not shown).

In a series of preliminary experiments we obtained consistent labeling of protein after irradiation of the crude membrane fractions or of the $C_{12}E_8$ extracted pellet using a Philips 150 W ultraviolet lamp. After slicing of the gels a peak of radioactivity was always observed in a peptide with M_r 34000. Counting without staining and destaining showed additional peaks of radioactivity at the position of the marker dye. This is interpreted as unspecific binding or aggregates of [^{3}H]bumethanide because the peak was eluted from the gel during destaining. No labeling of the protein with M_r 34000 was seen if cold bumethanide was added before irradiation or if [^{3}H]bumethanide was irradiated before addition of the crude membranes and unlabeled bumethanide (data not shown).

To increase the specificity of labeling, the samples were irradiated at wavelengths where bumethanide has absorption maxima. Bumethanide emits a strong blue fluorescence with maximum at 410 nm. As shown in Fig. 3, the excitation spectrum has absorption maxima at 280 nm and 345 nm. We therefore decided to compare the incorporation of [^{3}H]bumethanide after irradiation with monochromatic light at these two wavelengths. The data in Fig. 4 show that specific incorporation of [^{3}H]-bumethanide in a relatively

large peak was seen after irradiation at 345 nm. This band is labeled with an arrow in Fig. 2, where it is seen that the protein migrates together with lactate dehydrogenase of M_r 34000. After irradiation at 278 nm, labeling of the protein with M_r 34000 was smaller relative to background and unspecific labeling at the position of the marker dye was more pronounced than after irradiation at 345 nm. The covalent labeling with [^{3}H]bumethanide was increased relative to background when the concentration of sites for [^{3}H]bumethanide labeling was increased by extraction of membrane proteins with $C_{12}E_8$. The efficiency of photolabeling was about 3% of the [^{3}H]bumethanide binding capacity for both of the preparations tested in Fig. 4. The data in Fig. 5 shows that covalent incorporation of [^{3}H]bumethanide into the protein with M_r 34000 was prevented when excess cold bumethanide was present during photolysis. The data suggests that some of the labeling of proteins with higher molecular weights may also be specific, but well-defined peaks were not observed.

The data therefore allow the conclusion that the protein with M_r 34000 forms a part of the $Na^+/K^+/Cl^-$-cotransport system which is involved in binding of the specific inhibitor bumethanide. This

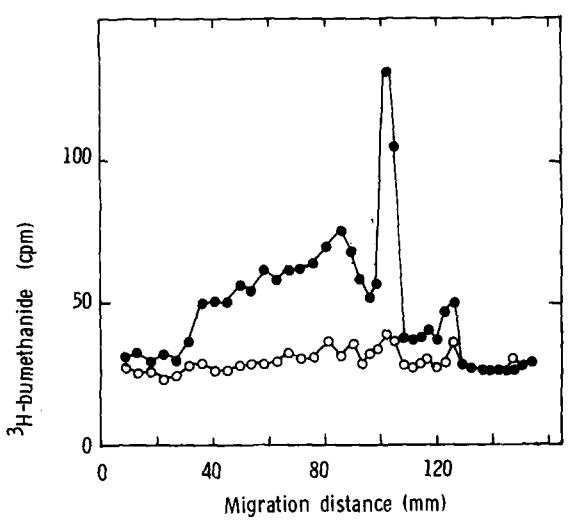

Fig. 5. Displacement of covalent labeling with [^{3}H]bumethanide from the protein with M_4 34000 by the presence of 2 mM unlabeled bumethanide during photolysis. Photolabeling of $C_{12}E_8$ pellet at 345 nm was done as in Fig. 4 without (●) or with (○) 2 mM bumethanide in the medium. The gels were sliced before staining and counted as in Fig. 4.

does not exclude the possibility that other polypeptides can be components of the transporter, since they may escape labeling with the inhibitor in the conditions of our experiments. Identification of the entire $Na^+/K^+/Cl^-$-cotransport protein can only be achieved when all components of the transporter have been isolated and reconstituted in a functional system.

If one assumes that the transport molecule that binds one molecule of [^{3}H]bumethanide contains only one protein unit of M_r 34000, it can be calculated that the maximum capacity for [^{3}H]bumethanide binding in a 100% pure preparation would be 25–30 nmol/mg protein. The measured capacities for binding by the crude membranes and the $C_{12}E_8$ pellet in Table I then corresponds to purities of < 1% and 2–3%, respectively. These values are in reasonably good agreement with the relative intensities of the protein with M_r 34000 in the gels in Fig. 2.

In agreement with previous data [4] we found that binding of [^{3}H]bumethanide was stimulated by Na^+ and K^+ and inhibited by Cl^- (not shown), but the binding capacity of the crude membrane fraction was much higher and the apparent affinity for [^{3}H]bumethanide binding was much lower than the data obtained by Forbush and Palfrey [4]. The specific radioactivity of [^{3}H]bumethanide from Leo Pharmaceutical Company that was used in the present study is much lower than that of the compound that was synthesized by Forbush and Palfrey [4]. Other explanations for the discrepancies than the different properties of these two preparations cannot be offered at present. Our binding data for the crude membranes are in better agreement with the affinities and capacities for binding of methylfurosemide to plasma membranes of shark rectal gland [11].

Covalent incorporation of a specific inhibitor after irradiation by ultraviolet light is a well known phenomenon. Previous examples include the covalent labeling of the α-subunit of $(Na^+ + K^+)$-ATPase by direct photolysis with [^{3}H]ouabain [12] and labeling with cytochalasin B of the glucose carrier of the erythrocyte membrane [13]. The nature of the chemical reaction for covalent attachment of [^{3}H]bumethanide is not known, but the observation that labeling was most effective at 345 nm suggests that the reaction involves the benzene

ring structures of the molecule.

The association of [^{3}H]bumethanide binding protein to cytoskeleton components can explain the resistance to extraction with nonionic detergent at low ionic strength and the sedimentation of the binding protein through iodinated gradient media of high density. The composition of the cytoskeleton of the thick ascending limb of Henles loop and its associations with the $Na^+/K^+/Cl^-$-cotransport protein in the luminal membrane of the tubule cells remain to be examined. It is tempting to suggest that binding of cytoskeleton components to the transporter may serve to mediate regulatory functions. This may include both control of the rate of transcellular transport of NaCl and a role of the cotransport system in regulation of cell volume that has been demonstrated in other cell systems [14].

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