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Photoaffinity labelling of a 150 kDa (Na + K + Cl)-cotransport protein from duck red cells with an analog of bumetanide

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We have used a radiolabelled, benzophenone analog of bumetanide, 4-[^{3}H]benzoyl-5-sulfamoyl-3-(3-thenyloxy)benzoic acid ([^{3}H]BSTBA) to photolabel plasma membranes from duck red blood cells. BSTBA, like bumetanide, is a loop diuretic and a potent inhibitor of (Na + K + Cl) cotransport, and [^{3}H]BSTBA binds to intact duck red cells with a high affinity similar to that of [^{3}H]bumetanide ($K_{1/2} \cong 0.1$ μM). We incubated duck red cells with [^{3}H]BSTBA, then lysed the cells and exposed the ghosts to ultraviolet light. The ghosting and photolysis was done at 0 °C to prevent dissociation of the [^{3}H]BSTBA. The ghosts were then sonicated to remove the nuclei and run on SDS-polyacrylamide gels. Analysis of H_2O_2-digested gel slices revealed [^{3}H]BSTBA to be incorporated into a protein of approx. 150 kDa. This is the same molecular weight we obtain for a protein from dog kidney membranes which is photolabelled by [^{3}H]BSTBA in a manner highly consistent with labelling of the (Na + K + Cl) cotransporter (Haas and Forbush (1987) Am. J. Physiol. 253, C243–C252). Several lines of evidence strongly suggest that the 150 kDa protein from duck red cell membranes is an integral component of the (Na + K + Cl)-cotransport system in these cells: (1) Photolabelling of this protein by [^{3}H]BSTBA is blocked when 10 μM unlabelled bumetanide is included in the initial incubation medium with [^{3}H]BSTBA; (2) Photoincorporation of [^{3}H]BSTBA into the 150 kDa protein is markedly increased when the initial incubation medium is hypertonic or contains norepinephrine, conditions which similarly stimulate both (Na + K + Cl) cotransport and saturable [^{3}H]bumetanide binding in duck red cells; (3) The photolabelling of this protein shows a saturable dependence on [^{3}H]BSTBA concentration, with a $K_{1/2}$ (0.06 μM) similar to that for the reversible, saturable binding of [^{3}H]BSTBA and [^{3}H]bumetanide to duck red cells; and (4) [^{3}H]BSTBA photoincorporation into the 150 kDa protein, like saturable [^{3}H]bumetanide binding to intact cells, requires the simultaneous presence of Na^+, K^+, and Cl^- in the medium containing the radiolabelled diuretic.

Introduction

Duck red blood cells exhibit a specific pathway for the coupled, electrically neutral transport of (Na + K + 2Cl) across their plasma membrane [1]. This pathway is stimulated by cell shrinkage and by β-adrenergic catecholamines [1–4], and is inhibited by the loop diuretics bumetanide and furosemide [5,6]. (Na + K + Cl)-cotransport systems are also present in a wide variety of cell types including the epithelial cells of the thick ascending limb of Henle's loop (TALH) of mammalian kid-

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ney [7], other reabsorptive and secretory epithelia [8–11], cultured epithelial and nonepithelial cell lines [12–15], excitable cells [16,17], Ehrlich ascites tumor cells [18], and red blood cells from mammalian and other avian species [5;19–23]. In most cases, the stoichiometry of the cotransported ions is 1Na : 1K : 2Cl, though there are exceptions (e.g., 2Na : 1K : 3Cl in squid axon; Ref. 16). However, in all cases (Na + K + Cl)-cotransport systems are inhibited by loop diuretics, with bumetanide being a more potent and specific inhibitor of these systems than furosemide [5,9,24].

Studies of the binding of radiolabelled loop diuretics to tissues exhibiting high levels of (Na + K + Cl) cotransport have produced insight into the mechanism by which these agents inhibit cotransport. [^{3}H]Bumetanide binds with high affinity to plasma membranes isolated from dog kidney outer medulla and cortex [25], and to intact duck red cells [26]. The binding of [^{3}H]bumetanide to duck red cells was found to directly correlate with its inhibition of (Na + K + Cl) cotransport, and binding was stimulated by both cell shrinkage and exposure of cells to norepinephrine [26]. Binding of [^{3}H]bumetanide to both dog kidney membranes [25] and duck red cells [26], and binding of *N*-[^{3}H]methylfurosemide to shark rectal gland membranes [27] requires the simultaneous presence of Na^+, K^+, and Cl^- in the incubation medium, suggesting that these diuretics bind preferably to the loaded form of the cotransporter, forming the stable, inhibited conformation (Na + K + Cl + diuretic).

Recently, we have used a radiolabelled analog of bumetanide, 4-[^{3}H]benzoyl-5-sulfamoyl-3-(3-thenyloxy)benzoic acid ([^{3}H]BSTBA) in photoaffinity labelling studies aimed at the identification of the (Na + K + Cl)-cotransport protein in dog kidney membranes [28,29]. BSTBA is a loop diuretic [30] which inhibits saturable [^{3}H]bumetanide binding to kidney membranes with an affinity similar to that of bumetanide itself [28], and is a potential photoaffinity label for the (Na + K + Cl) cotransporter because it contains a highly photoreactive benzophenone group [31]. We found that when plasma membranes from dog kidney cortex [28] and outer medulla [29] are exposed to ultraviolet light in the presence of [^{3}H]BSTBA, the label is incorporated into a 150 kDa protein. The photolabelling of this protein by [^{3}H]BSTBA closely resembles the reversible, saturable binding of [^{3}H]BSTBA and [^{3}H]bumetanide to dog kidney membranes with respect to apparent affinity ($K_{1/2} \cong 0.1$ μM), requirement for the simultaneous presence of Na^+, K^+, and Cl^- in the medium containing the labelled diuretic, and distribution among sucrose density gradient fractions [28], strongly suggesting this 150 kDa protein is an integral component of the (Na + K + Cl)-cotransport system.

In this study, we examine the photolabelling of duck red cell membranes by [^{3}H]BSTBA. We first show that BSTBA inhibits (Na + K + Cl) cotransport in duck red cells with an IC_{50} in the 0.1 μM range, and that [^{3}H]BSTBA binds to intact duck red cells with a $K_{1/2}$ in this same range. We then find that when cells are incubated with [^{3}H]-BSTBA, then lysed and the ghosts exposed to ultraviolet light, the label is incorporated into a protein of about 150 kDa. The photolabelling of this protein by [^{3}H]BSTBA resembles the reversible, saturable binding of [^{3}H]bumetanide to intact cells with respect to apparent affinity, stimulation by cell shrinkage and by norepinephrine exposure, and requirement for the simultaneous presence of Na^+, K^+, and Cl^-. These findings together demonstrate that this 150 kDa protein is a component of the (Na + K + Cl)-cotransport system. A preliminary report of this work has been presented in abstract form [32].

Materials and Methods

Materials

The [^{3}H]bumetanide, BSTBA, and [^{3}H]BSTBA used in these studies was the same material as was used in our previous binding and photolabelling studies [26,28,29]. [^{3}H]Bumetanide was from the same batch as used previously [26], and [^{3}H]-BSTBA was purified as previously described [28] within three months of use in all experiments presented. The specific activity of the purified [^{3}H]BSTBA was 0.5 Ci/mmol; this material was stored as a 200 μM solution in absolute ethanol at −70 °C. Unlabelled bumetanide was the gift of Hoechst Pharmaceuticals, Sommerville, NJ. Tetramethylammonium (TMA) chloride was obtained from Kodak, Rochester, NY, and routinely re-

crystallized from absolute ethanol and stored at −20°C. Sodium and potassium methylsulfate ($MeSO_4$) were obtained from ICN/K & K, Plainview, NY.

Preparation of cells

Blood from white Pekin ducks was obtained on the day of each experiment. Following the removal of the plasma and buffy coat, the red cells were washed three times with an ice-cold solution, referred to below as NaCl + $NaMeSO_4$, comprised of 32 mM NaCl plus sufficient $NaMeSO_4$ to adjust the osmolality to 323 mosmol/kg (isotonic for ducks), or with ice-cold, isotonic NaCl or $NaMeSO_4$ (the latter only when complete removal of chloride was desired). For most experiments it was desirable to work at an external chloride concentration ($[Cl]_o$) of 32 mM, since saturable [^{3}H]bumetanide binding to duck red cells is maximal under these conditions [26]. To maintain a constant ratio of intracellular/extracellular chloride in these experiments, the cells were preincubated for two successive 30-min periods at 41°C, 3% hematocrit in a medium containing 23 mM NaCl, 9 mM KCl, 1 mM KH_2PO_4, 20 mM Na-Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, titrated to pH 7.4 at 41°C with NaOH), 5 mM glucose, and sufficient $NaMeSO_4$ to adjust the osmolality to 323 mosmol/kg. The cells were centrifuged and resuspended in fresh media following the first 30-min period. For experiments in which test incubation media contained either zero or 160 mM $[Cl]_o$, the same preincubation procedure was followed except that chloride salts were replaced by those of methylsulfate, or vice versa. Following the preincubations, the cells were washed once in the appropriate medium for [^{3}H]BSTBA or [^{3}H]bumetanide binding, [^{3}H]BSTBA photolabelling, or ^{24}Na influx studies, and then resuspended at the appropriate hematocrit in these media.

[^{3}H]BSTBA and [^{3}H]bumetanide binding determinations

All binding determinations were done in duplicate as previously described [26]. 0.075 ml of a 40% hematocrit suspension in 32 mM $[Cl]_o$ preincubation medium (see above) or in an otherwise similar all-chloride medium (158 mM Na, 10 mM K) was warmed in a water bath to 41°C. An equal volume of this medium (also at 41°C) containing 4 μM norepinephrine was added. One minute later, 0.15 ml of the same medium (41°C) containing [^{3}H]BSTBA or [^{3}H]bumetanide, with or without unlabelled bumetanide, was added. This final addition marked zero-time for the incubation; the final hematocrit was 10%. The final concentration of unlabelled bumetanide, when present, was always 10 μM. The cells were incubated with the radiolabelled diuretics for 15 min; the binding of these compounds after this period represents equilibrium binding (Ref. 26; Haas, M. and Forbush, B., III, unpublished observations).

After the 15-min incubations, the cell suspensions were diluted into 5 ml of ice-cold lysing buffer (20 mM Na-Hepes (pH 7.4) plus 1 mM $CaCl_2$) and immediately filtered onto a Millipore prefilter (AP25) and underlying glass microfiber filter (Whatman GF/A). The filters were then washed with two 5-ml aliquots of ice-cold lysing buffer and counted as previously described [26].

^{24}Na influx determinations

All ^{24}Na influx determinations were done in duplicate at 41°C, 10% hematocrit as previously described [26]. 0.3 ml aliquots of a 40% hematocrit suspension in a medium identical to the 32 mM $[Cl]_o$ preincubation medium (see above) except for the presence of 0.1 mM ouabain were warmed to 41°C. 0.3 ml of this same medium (also at 41°C) containing 4 μM norepinephrine was then added to each aliquot of cell suspension. One minute later, 0.6 ml of this same medium (41°C) containing one of various concentrations of BSTBA or 18.4 μM bumetanide (unlabelled) was added to each sample. The samples were incubated 5 min to allow for binding of the diuretic [26], after which 18 μCi of ^{24}Na (in a volume of 0.02 ml) was added to each incubation. Three minutes after ^{24}Na addition, 1.0 ml of each cell suspension was diluted into 7.5 ml of ice-cold, isotonic (NaCl + $NaMeSO_4$). The cells were then washed and lysed, and both cells and incubation media were counted in a gamma counter [26].

[^{3}H]BSTBA photolabelling experiments

1.0 ml of packed duck red cells, preincubated

as described above, was suspended to a hematocrit of 10% in the appropriate incubation medium (prewarmed to 41° C). Unless otherwise indicated in the figure legends, this medium was identical to the 32 mM $[Cl]_o$ preincubation medium (see above). When a hypertonic medium was required (Fig. 4), $NaMeSO_4$ was added to adjust the osmolality to 400 mosmol/kg. Where appropriate, norepinephrine (from a 1 mM aqueous stock solution) was added to the cell suspensions to yield a final concentration of 1 μM. One minute later, [^{3}H]BSTBA, and where appropriate unlabelled bumetanide, were added. Bumetanide was added from a 20 mM stock solution in dimethyl sulfoxide (DMSO) to yield a final concentration of 10 μM, and an equivalent volume of DMSO alone was added to control incubations. The final concentration of [^{3}H]BSTBA, except where varied (Fig. 5), was 0.2 μM. The cells were incubated with [^{3}H]BSTBA for 15 min at 41° C. Due to limitations in the photolysis method (see below), only two such incubations were done simultaneously. After this incubation period, the suspensions were centrifuged and the cells lysed with 40 volumes of high-Mg lysing solution (20 mM $MgCl_2$, 10 mM Na-Hepes (pH 8.0 at 0° C), 2 mM NaCl, 2 mM KCl). The ghosts were pelleted by centrifuging for 1 min at 12000 × *g* and 0° C, washed with 30 volumes of ice-cold high-Mg lysing solution, repelleted, and resuspended with 2 ml of this same solution at 0° C in preparation for photolysis. In some instances, 10 mM dithiothreitol (DTT) was added to the final suspension (see Fig. 7). This ghosting and washing procedure, done entirely at 0° C, requires about 20 min. As shown below (see Results), nearly all of [^{3}H]-BSTBA bound in a saturable manner to duck red cells during a 15-min incubation at 41° C remains bound following the above ghosting procedure.

The ghost suspensions were transferred to polystyrene dishes (uncovered) having a depth of 1.0 cm and a diameter of 3.5 cm. Two such dishes fit tightly under the window of the ultraviolet (UV) lamp used in these experiments (see below). The dishes were placed in a pan filled to a depth of approx. 0.5 cm with finely crushed ice and a small amount of water, and the pan was placed on an Orbit Shaker (Lab-Line Instruments, Chicago, IL). A hand-held ultraviolet lamp (Mineralight lamp model UVGL-25, multiband UV 254/366 nm, UVP, San Gabriel CA) was placed directly over the dishes containing the ghost suspensions, and the samples were exposed to ultraviolet light (long-wave setting) for 20 min while being mixed gently on the Orbit Shaker. After the photolysis, the samples were transferred to 25 ml glass Corex tubes, diluted to a volume of approx. 5 ml with high-Mg lysing solution, and sonicated (with the tubes immersed in ice) for 30 s using a probe sonifier (Heat Systems Co., Danbury, CT) to remove the nuclei from the ghosts [33]. The sonicated ghost suspensions were then diluted to a volume of approx. 30 ml with high-Mg lysing solution and centrifuged successively for 10 min at 2000 × *g* and 30 seconds at 12000 × *g*, both at 0° C. The pellets, containing the nuclei and mitochondria, were discarded, and the supernatants were centrifuged at 150000 × *g* and 0° C for 60 min to yield a pellet containing plasma membranes [33]. This final pellet was resuspended to a volume of 150–200 μl with ice-cold high-Mg lysing solution, and a known fraction of each sample was saved for protein determination [34]. The remainder of each sample was centrifuged in a Beckman airfuge (Beckman Instruments, Irvine, CA), and the pellets were solubilized in buffer containing 2% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol, and run on SDS-polyacrylamide gels (1.5 mm thick slab gels, 6.5% acrylamide). The gels were stained with Coomassie blue and then cut into 4 mm slices that were completely digested overnight at 78° C with 30% H_2O_2. The digested gel slices were counted for 10 min. Thus, for slices having 30–40 counts per minute (cpm) above background, or 70–80 total cpm (see Figs. 3, 4, and 6), the counting error is less than ±2 cpm.

For photolabelling of membranes from dog kidney cortex with [^{3}H]BSTBA (Fig. 7), 80 μl of a membrane suspension (10 mg protein/ml in 0.25 M sucrose, 30 mM histidine, pH 7.2) was mixed with 80 μl of a medium containing 0.4 μM [^{3}H]BSTBA, 45 mM K, 45 mM Na, 10 mM Cl, and 40 mM SO_4, and incubated for 20 min at 22° C. Following this, the membranes were centrifuged in a Beckman airfuge, and the supernatant containing unbound [^{3}H]BSTBA removed by aspiration. The membranes with bound

[^{3}H]BSTBA were resuspended at 0 °C in a medium containing 22.5 mM K, 22.5 mM Na, 5 mM Cl, 20 mM SO_4, 0.125 M sucrose, 15 mM histidine (pH 7.2) with or without 10 mM DTT. The membrane suspension was then photolyzed for 15 min at 0 °C as described previously [28]. After centrifugation and removal of the photolysis medium, the membranes were solubilized and analyzed by SDS-polyacrylamide gel electrophoresis as described above.

Presentation of data

The lines accompanying the data points in Figs. 1, 2, and 5 are nonlinear least-squares curve fits; the nonlinear fit is to a single binding site model [26,28]. $K_{1/2}$ and IC_{50} values, as well as values for maximal saturable [^{3}H]BSTBA binding and photoincorporation noted in the text were derived from these fits.

Results

BSTBA is a potent inhibitor of (Na + K + Cl) cotransport in duck red cells, as illustrated by the experiment in Fig. 1. In this experiment, we examined the effect of different concentrations of BSTBA on bumetanide (10 μM)-sensitive ^{24}Na influx in the presence of norepinephrine. Under these experimental conditions, this influx is equivalent to (Na + K + Cl)-cotransport activity [1,26]. Fig. 1 shows that 10 μM BSTBA is as effective as 10 μM bumetanide in inhibiting ^{24}Na influx. Furthermore, BSTBA inhibits (Na + K + Cl) cotransport with an IC_{50} of 0.062 μM, a value very close to the IC_{50} for bumetanide in inhibiting (Na + K + Cl) cotransport in duck red cells under similar conditions (0.056 μM; Ref. 6).

The experiment illustrated in Fig. 2 shows that [^{3}H]BSTBA binds to duck red cells in a saturable manner. Cells were incubated with different [^{3}H]BSTBA concentrations in the presence of norepinephrine and the presence or absence of 10 μM unlabelled bumetanide. In the absence of bumetanide, the [^{3}H]BSTBA binding curve shows both a saturable and a linear component, and the linear component alone is observed in the presence of 10 μM bumetanide (Fig. 2). The saturable component, or the difference in [^{3}H]BSTBA binding in the absence and presence of 10 μM

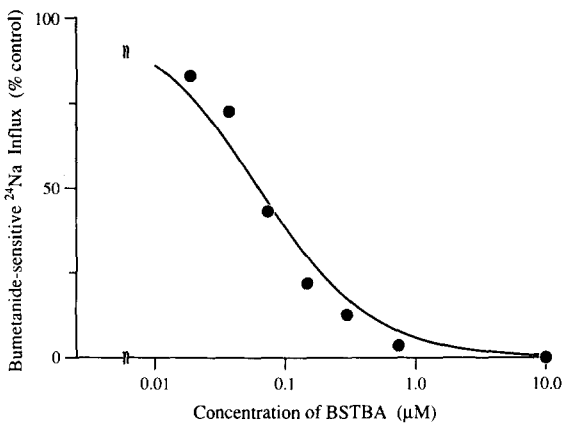

Fig. 1. Dose-response curve for BSTBA inhibition of bumetanide (10 μM)-sensitive ^{24}Na influx. All test incubations were done in duplicate and contained 1 μM norepinephrine. Bumetanide-sensitive influxes were calculated by subtracting the influx in the presence of 10 μM bumetanide (1.02 mmol Na/l cells per 3 min) from the influx at each concentration of BSTBA indicated by the abscissa, and are expressed as the percent of the bumetanide-sensitive influx in the absence of BSTBA (5.06 mmol Na/l cells per 3 min). The line accompanying the data points is a nonlinear least squares curve fit of the data to a single binding site model; the IC_{50} value noted in the text was derived from this fit.

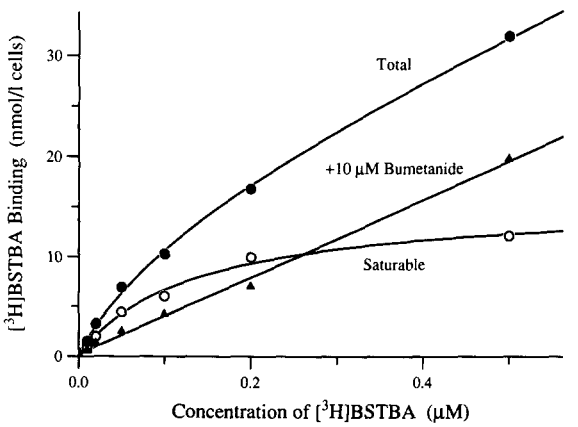

Fig. 2. Concentration dependence of [^{3}H]BSTBA binding to intact duck red cells. All incubations were done in duplicate in the presence of 1 μM norepinephrine, with or without 10 μM unlabelled bumetanide. Final concentrations of [^{3}H]BSTBA are given by the abscissa. The curve drawn for the saturable component of binding (see text) is a nonlinear least-squares fit of the data to a single binding site model; values for $K_{1/2}$ and maximal saturable binding given in the text were derived from this fit. The line drawn for the linear component of binding (+10 μM bumetanide) is a linear least-squares fit and has a slope of 38.40 nmol/l cells per μM, a y-intercept of 0.27 nmol/l cells, and a correlation coefficient of 0.998. The curve drawn for total binding is the sum of the saturable and linear binding curves.

bumetanide, is half-maximal ($K_{1/2}$) at 0.130 μM [^{3}H]BSTBA (see Fig. 2 legend). This $K_{1/2}$ is similar to the IC_{50} for BSTBA inhibition of (Na + K + Cl) cotransport (0.062 μM; Fig. 1), though the former is slightly higher (see Discussion). The $K_{1/2}$ for saturable [^{3}H]BSTBA binding to duck red cells (Fig. 2) is also within the range of $K_{1/2}$ values we found for saturable [^{3}H]bumetanide binding to duck red cells (0.068–0.150 μM; Ref. 26), and is very close to the $K_{1/2}$ values we obtained for [^{3}H]BSTBA binding to membranes from dog kidney cortex (0.050–0.125 μM; Ref. 28). Thus, based on affinity [^{3}H]BSTBA appears to bind to the site on the (Na + K + Cl) cotransporter which is responsible for inhibition of this pathway by BSTBA and bumetanide. From the maximal saturable [^{3}H]BSTBA binding in the experiment in Fig. 2 (14.1 nmol/l cells), and using a value of $1.4 \cdot 10^{-13}$ liter as the volume of a single cell [35], we calculate 1190 [^{3}H]BSTBA binding sites per cell, a value very close to the approx. 1000 [^{3}H]bumetanide binding sites per duck red cell which we previously reported [26].

TABLE I

[^{3}H]BUMETANIDE AND [^{3}H]BSTBA BINDING BEFORE AND AFTER GHOSTING

Fresh duck red cells were washed three times in isotonic NaCl and then incubated at 10% hematocrit, 41° C in media containing 9 mM KCl, 150 mM NaCl, 1 mM KH_2PO_4, 20 mM Na-Hepes (pH 7.4 at 41° C), 5 mM glucose, 1 μM norepinephrine, and 0.2 μM [^{3}H]bumetanide or [^{3}H]BSTBA, with or without 10 μM unlabelled bumetanide. After incubation for 15 min, four 0.3 ml aliquots of each cell suspension were removed. Two were immediately diluted into 5 ml of ice-cold lysing buffer (see text) and filtered to determine [^{3}H]bumetanide or [^{3}H]BSTBA bound to intact cells ('Cells'). The remaining two samples were each lysed in 20 ml of ice-cold high-Mg lysing solution (see text) and centrifuged for 1 min at 12000 × *g* and 0° C. The ghosts were then washed with 20 ml of this same ice-cold medium, centrifuged, resuspended in 5 ml of ice-cold lysing buffer, and filtered to determine bound [^{3}H]bumetanide or [^{3}H]BSTBA ('Ghosts'). Values shown are the average of these duplicate determinations for each experimental condition. Saturable binding (Δ) is defined as the difference in binding in the absence and presence of 10 μM unlabelled bumetanide (Ref. 26; also see Fig. 2).

Diuretic	Sample	Bumetanide (10 μM)	Binding (nmol/l cells)
[^{3}H]Bumetanide	Cells	−	11.61
		+	3.74
		Δ	7.87
	Ghosts	−	9.03
		+	2.06
		Δ	6.97
[^{3}H]BSTBA	Cells	−	12.43
		+	5.35
		Δ	7.08
	Ghosts	−	8.66
		+	1.80
		Δ	6.86

Since photolabelling experiments similar to those we performed with dog kidney membranes [28,29] are not practical using intact duck red cells, we examined whether such experiments could be done using ghosts. In a variety of experiments, we were unable to demonstrate high-affinity, saturable binding of [^{3}H]bumetanide or [^{3}H]BSTBA to duck red cell ghosts (data not shown). However, as shown in Table I, when duck red cells are first incubated at 41° C with norepinephrine and [^{3}H]bumetanide or [^{3}H]BSTBA, then ghosted at 0° C (see Materials and Methods), nearly all of the radiolabelled diuretic bound in a saturable manner to the intact cells remains bound to the ghosts. The procedure of ghosting and washing the ghosts does reduce nonspecific binding of [^{3}H]BSTBA and [^{3}H]bumetanide (binding in the presence of 10 μM unlabelled bumetanide; Table I). This is presumably because unbound [^{3}H]BSTBA and [^{3}H]bumetanide are removed when the ghosts are washed, and a significant fraction of nonspecific binding actually represents binding of free radiolabelled diuretic to the filters (particularly with [^{3}H]BSTBA; Ref. 28).

Ghosts prepared from cells incubated with [^{3}H]BSTBA were then used in photolabelling experiments, an example of which is illustrated in Fig. 3. In these experiments, the ghosts with bound [^{3}H]BSTBA were exposed to ultraviolet light, and the plasma membranes analyzed by SDS-polyacrylamide gel electrophoresis. The uppermost panel of Fig. 3 shows a densitometric scan of the Coomassie blue stained gel of the photolyzed plasma membranes, performed just prior to slicing the gel to determine the distribution of the tritium label, and a log molecular weight plot of the standards on the gel (see Fig. 3 legend). When the initial incubation of intact cells with [^{3}H]BSTBA

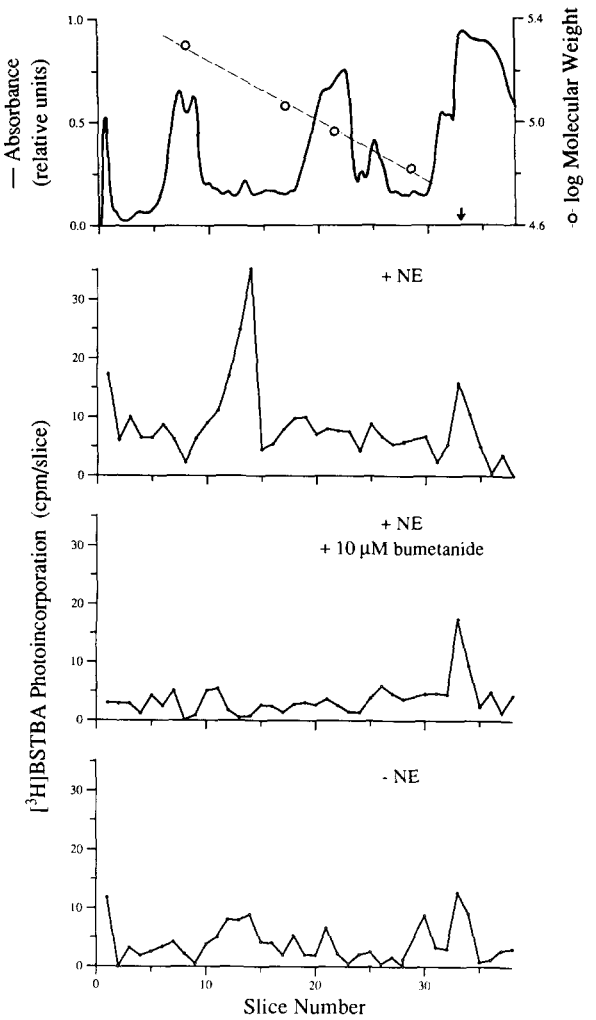

Fig. 3. Photoincorporation of [^{3}H]BSTBA into duck red cell membranes. Cells were incubated for 15 min with 0.2 μM [^{3}H]BSTBA in the presence or absence of 1 μM norepinephrine and in the presence of norepinephrine and 10 μM bumetanide (unlabelled). The cells were then lysed, the ghosts were photolyzed and sonicated, and the plasma membranes run on a SDS-polyacrylamide gel (6.5% acrylamide) as described in Materials and Methods. All three samples were run on the same slab gel. The top panel shows both a densitometric scan of a lane of the Coomassie blue stained gel done immediately before the gel was cut into 4 mm slices and a plot of log molecular weight versus slice number (i.e., distance migrated) for the molecular weight standards on the gel. The location of these standards (Bio-Rad Laboratories, Richmond, CA) is indicated by the open circles (from left to right: 200, 116, 92 and 66 kDa); the dashed line is a linear least-squares fit of the data. The arrow indicates the location of the tracking dye on the gel. The lower three panels show the distribution of [^{3}H]BSTBA in H_2O_2-digested gel slices; membranes run on the gel were derived from cells incubated with [^{3}H]BSTBA in the presence (+NE) or absence (−NE) of norepinephrine, or in the presence of norepinephrine and 10 μM unlabelled bumetanide (+NE, +10 μM bumetanide). The amount of protein loaded onto the gel for each sample was: +NE, 0.91 mg; +NE, +bumetanide, 1.11 mg; −NE, 1.02 mg; these amounts each represent loading of two adjacent lanes on the gel (see text). Data shown are counts per minute (cpm) in each slice after subtraction of background of 40 cpm (the counts obtained from H_2O_2-digested slices of gels not containing tritium). From the linear least-squares fit of the log molecular mass plot of the standards (see above), it is calculated that the peak of [^{3}H]BSTBA incorporation (slices 11.5–14.5) extends from 161 to 137 kDa.

is done in the presence of norepinephrine, we find one major peak of [^{3}H]BSTBA photoincorporation centered at molecular mass $\approx$ 150 kDa (panel labelled + NE; Fig. 3). This peak of [^{3}H]BSTBA photoincorporation is broad, extending over four 4 mm gel slices comprising the approximate molecular mass range 135–160 kDa (see legends to Figs. 3–7). As illustrated in Fig. 3, this peak is not detectable when the initial incubation medium either lacks norepinephrine (−NE) or contains both norepinephrine and 10 μM unlabelled bumetanide (+NE, +10 μM bumetanide). A second, smaller peak at the location of the tracking dye on the gel, which we observe in some experiments, is seen in Fig. 3 to be unaffected by the presence of either norepinephrine or 10 μM bumetanide in the initial incubation medium.

In the experiment shown in Fig. 3, as well as in other photolabelling experiments (e.g., see Figs. 4 and 6), the number of counts in the gel slices comprising the peak of [^{3}H]BSTBA photoincorporation at approx. 150 kDa is low (30–50 cpm above background in peak slices). This is due both to the low number of [^{3}H]BSTBA binding sites (approx. 1200 sites/cell; Fig. 2) and the low specific activity of our [^{3}H]BSTBA (0.5 Ci/mmol). However, with our gels loaded heavily (for each sample we load two lanes with 0.3–0.6 mg protein/lane), the peak of [^{3}H]BSTBA photoincorporation at approx. 150 kDa has been a reproducible finding in each of over fifteen experiments employing cells from five different ducks.

Since (Na + K + Cl) cotransport and saturable [^{3}H]bumetanide binding in duck red cells are stimulated by cell shrinkage as well as by catecholamines, we also examined whether ex-

posure of cells to [^{3}H]BSTBA under hypertonic conditions in the absence of norepinephrine promotes [^{3}H]BSTBA photoincorporation into the 150 kDa protein or other proteins. Fig. 4 shows that when cells are initially incubated with [^{3}H]BSTBA in a 400 mosmol/kg medium without norepinephrine, then lysed and the ghosts photolyzed and analyzed as described above, incorporation of [^{3}H]BSTBA in the 150 kDa region is observed. Again, no such photoincorporation is seen when the initial incubation with [^{3}H]BSTBA is done in an isotonic (323 mosmol/kg), catecholamine-free medium, conditions under which saturable [^{3}H]bumetanide binding [26] and (Na + K + Cl)-cotransport activity [4] are minimal.

Fig. 5 shows that [^{3}H]BSTBA photoincorporation into the 150 kDa protein has a saturable dependence on [^{3}H]BSTBA concentration. In this experiment, the initial incubation medium contained norepinephrine and concentrations of [^{3}H]BSTBA between 0.025 and 0.50 μM.

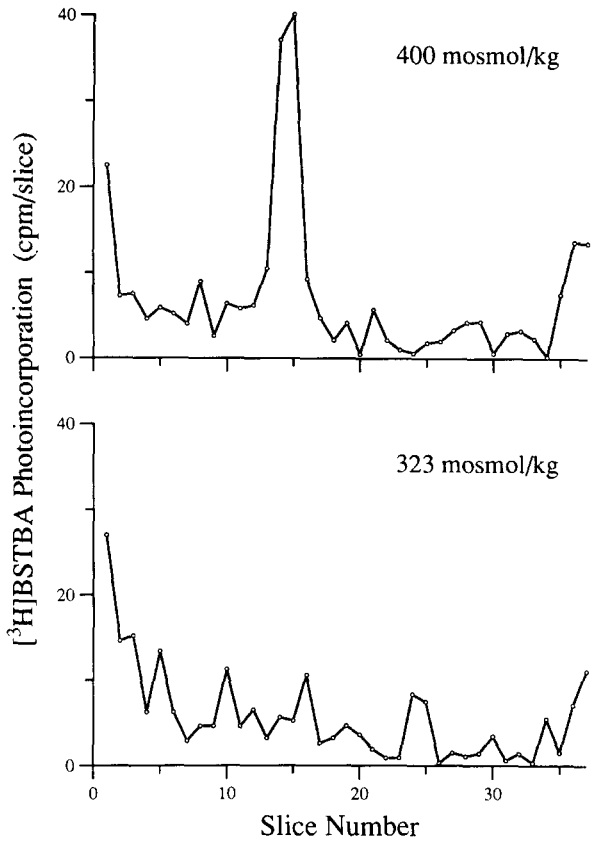

Fig. 4. Effect of hypertonicity on [^{3}H]BSTBA photoincorporation into duck red cell membranes. Cells, preincubated as described in Materials and Methods, were incubated for 15 min at 41° C in media containing 0.2 μM [^{3}H]BSTBA, 23 mM NaCl, 9 mM KCl, 1 mM KH_2PO_4, 5 mM glucose, 20 mM Na-Hepes (pH 7.4 at 41° C), and sufficient $NaMeSO_4$ to adjust the osmolality to either 323 mosmol/kg (isotonic) or 400 mosmol/kg. Norepinephrine was not added to either incubation. The cells were then lysed and the ghosts photolyzed, sonicated, and run on a SDS-polyacrylamide gel (6.5% acrylamide). The gel was stained with Coomassie blue, then cut into 4 mm slices which were digested with H_2O_2 and counted. Molecular weight standards on the gel corresponded to the following slices: 200 kDa, slices 10–11; 116, slices 17–18; 92, slice 22; 66, slice 28; tracking dye, slice 32. The peak of [^{3}H]BSTBA incorporation (400 mosmol/kg, slices 13–16) corresponds to the molecular mass range 162–136 kDa. The amount of protein loaded onto the gel for each sample was: 400 mosmol/kg, 1.08 mg; 323 mosmol/kg, 1.02 mg. Data shown are counts per minute (cpm) in each slice after subtraction of background of 40 cpm.

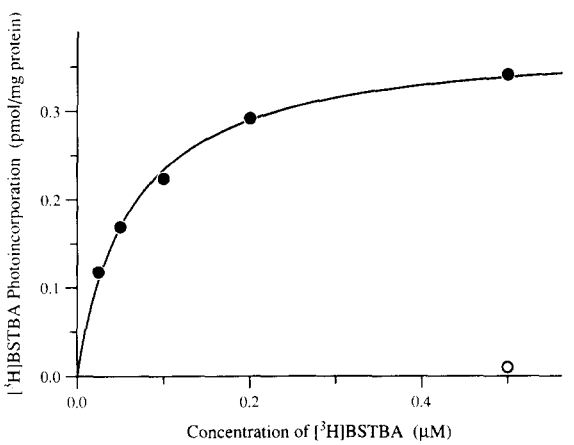

Fig. 5. Concentration dependence of [^{3}H]BSTBA photoincorporation into the 150 kDa region. Cells were incubated for 15 min at 41° C in the presence of 1 μM norepinephrine and the concentration of [^{3}H]BSTBA indicated by the abscissa. The photolyzed membranes from these cells were then run on SDS-polyacrylamide gels (6.5% acrylamide), which were stained with Coomassie blue and then cut into 4 mm slices. Data shown are the counts per minute (cpm) above background (40 cpm/slice) incorporated into the four slices (13–16 of 37) corresponding to the molecular weight range 160–132 kDa. The open symbol at 0.5 μM [^{3}H]BSTBA represents counts incorporated into these slices when the initial incubation medium contained both 0.5 μM [^{3}H]BSTBA and 10 μM unlabelled bumetanide. The line accompanying the data points was derived from a nonlinear least-squares fit of the data to a single binding site model; values for $K_{1/2}$ and maximal [^{3}H]BSTBA photoincorporation noted in the text were derived from this fit. Data are adjusted for a 20% decrease in [^{3}H]BSTBA counting efficiency resulting from heating overnight with 30% H_2O_2 (Ref. 28).

[^{3}H]BSTBA photoincorporation into the 150 kDa region is half-maximal at 0.063 μM (see Fig. 5 legend), a value close to those determined above for the $K_{1/2}$ for saturable, reversible binding of [^{3}H]BSTBA to intact duck red cells (0.130 μM; Fig. 2) and the IC_{50} for inhibition of (Na + K + Cl) cotransport by BSTBA (0.062 μM; Fig. 1). At 0.50 μM [^{3}H]BSTBA, photoincorporation of [^{3}H]BSTBA into the 150 kDa protein is again seen to be completely blocked when the initial incubation medium contains 10 μM unlabelled bumetanide (open symbol, Fig. 5). The maximal [^{3}H]BSTBA photoincorporation in the experiment shown in Fig. 5 is 0.382 pmol/mg membrane protein. This value is close to that obtained with membranes from dog kidney cortex (0.303 pmol/mg; Ref. 28), where we also observe saturable photoincorporation of [^{3}H]BSTBA in the 150 kDa region with a similar $K_{1/2}$ (0.094 μM; Ref. 28). Using the density of [^{3}H]BSTBA binding sites as determined in Fig. 2 (1190 sites/cell), a surface area for one duck red cell of 160 μm^2 [36], and assuming $3 \cdot 10^{-12}$ mg membrane protein/μm^2 (human red cell ghosts; Ref. 37), we calculate there are 4.1 pmol of [^{3}H]BSTBA binding sites per mg membrane protein. Thus, the efficiency of [^{3}H]BSTBA photolabelling of the 150 kDa protein in our duck red cell experiments is approx. 10%, just as it is in our experiments with dog kidney membranes [28].

The above findings suggest that the 150 kDa protein photolabelled by [^{3}H]BSTBA is an integral part of the (Na + K + Cl)-cotransport system. If this is indeed the case, this labelling should require the simultaneous presence of Na^+, K^+, and Cl^- in the medium containing [^{3}H]BSTBA. Fig. 6 shows an experiment in which cells were initially incubated in media containing 1 μM norepinephrine and 0.2 μM [^{3}H]BSTBA from which either Na^+, K^+, or Cl^- was omitted in the continued presence of the other two ions. The cells were then lysed, and the ghosts photolyzed and treated as described above. As is evident in Fig. 6, the peak of [^{3}H]BSTBA photoincorporation at about 150 kDa seen when the cells are incubated with [^{3}H]BSTBA in the presence of Na^+, K^+, and Cl^- (upper left panel, Fig. 6) is not observed when either Na^+, K^+, or Cl^- is omitted from the incubation medium. Thus, just as is the case with

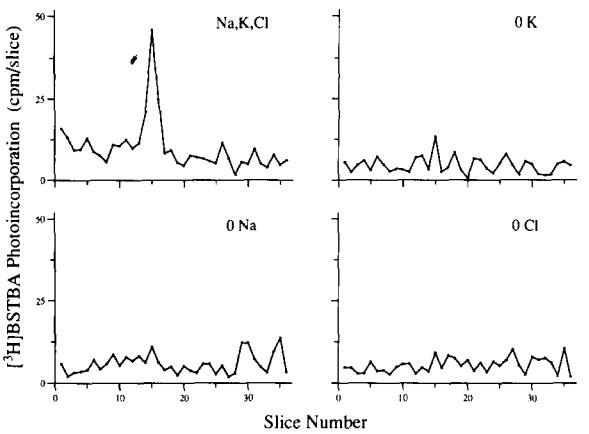

Fig. 6. Effect of removal of Na^+, K^+, and Cl^- on [^{3}H]BSTBA photoincorporation into duck red cell membranes. Cells were preincubated as described in Materials and Methods in media containing either 160 mM or zero $[Cl]_o$, then incubated for 15 min at 41°C in the presence of 1 μM norepinephrine and 0.2 μM [^{3}H]BSTBA. In the experiment illustrated in the upper left panel (Na, K, Cl), this incubation medium contained 10 mM K, 158 mM Na, and 160 mM Cl. In the experiments represented by the panels labelled 0 Na, 0 K, and 0 Cl, sodium, potassium, and chloride, respectively, were omitted from the [^{3}H]BSTBA-containing incubation medium in the continued presence of the other two ions. Cells preincubated in the zero $[Cl]_o$ medium were used only in the 0 Cl experiment. TMA was used to substitute for sodium and potassium, and $MeSO_4$ substituted for chloride. Following the incubations with [^{3}H]BSTBA, the cells were lysed and the ghosts photolyzed, sonicated, and run on a SDS-polyacrylamide gel (6.5% acrylamide) as described in Materials and Methods. The gel was stained with Coomassie blue, then cut into 4 mm slices which were digested with H_2O_2 and counted. Molecular weight standards on the gel corresponded to the following slices: 200 kDa, slice 10; 116, slices 17–18; 92, slices 21–22; 66, slices 27–28; tracking dye, slice 31. The peak of [^{3}H]BSTBA incorporation (Na, K, Cl; slices 14–16) corresponds to the molecular mass range 154–135 kDa. The amount of protein loaded onto the gel was between 0.73 and 0.78 mg for each sample. Data shown are counts per minute (cpm) in each slice, after subtraction of background of 40 cpm/slice.

reversible, saturable [^{3}H]bumetanide binding [26], [^{3}H]BSTBA photoincorporation into the 150 kDa protein requires the simultaneous presence of Na^+, K^+, and Cl^- in the medium containing the radiolabelled diuretic.

As noted above (see Introduction), we previously identified a protein in the 150 kDa region that is photolabelled when dog kidney membranes are exposed to ultraviolet light in the presence of [^{3}H]BSTBA [28]. Fig. 7 illustrates an experiment

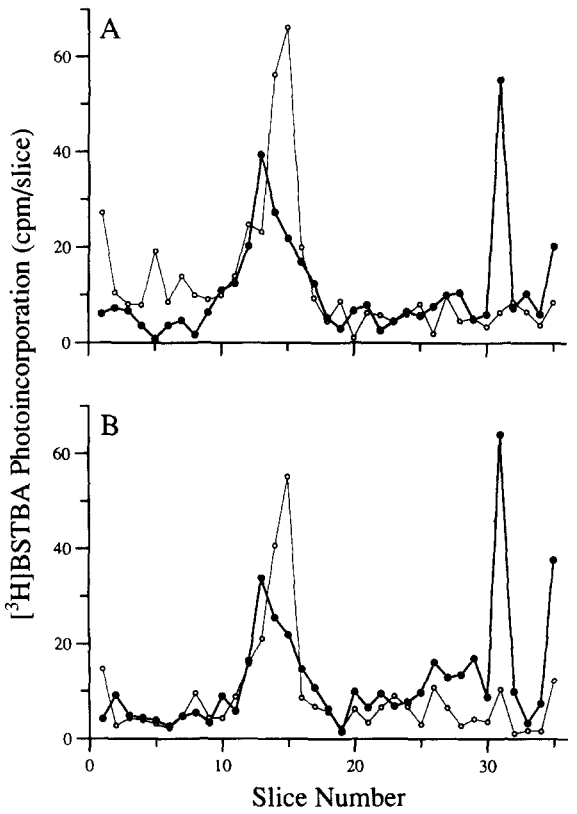

Fig. 7. Photolabelling of duck red cell membranes (open symbols, thin lines) and membranes from dog kidney cortex (closed symbols, thick lines) with [^{3}H]BSTBA, in the absence (panel A) or presence (panel B) of 10 mM dithiothreitol (DTT). Prior to photolysis, intact duck red cells were incubated with 0.2 μM [^{3}H]BSTBA and 1 μM norepinephrine, and dog kidney membranes were incubated with 0.2 μM [^{3}H]BSTBA as described in Materials and Methods. The duck red cell ghosts and dog kidney membranes with bound [^{3}H]BSTBA were each divided into duplicate samples which were photolyzed in the absence and presence of DTT, respectively. All four samples of membranes were then run on the same SDS-polyacrylamide gel, which was cut into 4 mm slices to determine the distribution of [^{3}H]BSTBA incorporated into the membrane proteins of each sample. Data shown are counts per minute (cpm) in each gel slice, after subtraction of background of 40 cpm/slice. Molecular mass standards (kDa) on the gel corresponded to the following slices: 200, slices 9–10; 116, slices 17–18; 92, slice 21; 66, slices 27–28; tracking dye, slice 31. Slices 12–16 correspond to the range 169–132 kDa. For each sample of duck red cell membranes, 1.15 mg of protein was loaded onto the gel; for each kidney membrane sample 0.80 mg was loaded. The amount of [^{3}H]BSTBA incorporated into the 150 kDa region (slices 12–16, average of panels A and B) was 0.44 pmol/mg protein for duck red cell membranes and 0.41 pmol/mg for dog kidney membranes.

in which both duck red cells and membranes from dog kidney cortex were incubated with 0.2 μM [^{3}H]BSTBA. The red cell and kidney membranes with bound [^{3}H]BSTBA were then photolyzed and run on the same SDS-polyacrylamide gel. The [^{3}H]BSTBA-containing medium for duck red cells contained 1 μM norepinephrine, and the photolysis of both membrane types was performed in the absence (panel A, Fig. 7) or presence (panel B) of 10 mM DTT. Fig. 7 illustrates that in both red cell and kidney membranes, a peak of [^{3}H]BSTBA photoincorporation is observed which extends between gel slices 12 and 16, though the peak shapes are not identical. This region of the gel corresponds to a molecular mass range of 169–132 kDa (see Fig. 7 legend). The somewhat sharper peak of [^{3}H]BSTBA incorporation into duck red cell membranes (Fig. 7) also contains more counts than the corresponding kidney membrane peak, however the ratio of counts in the two peaks is in proportion to the amount of protein loaded onto the gel for each membrane type. In each case, approx. 0.4 pmol/mg membrane protein was incorporated into the peak centered at about 150 kDa (see Fig. 7 legend). As previously reported [28], we also find (Fig. 7) a peak of [^{3}H]BSTBA photoincorporation in the region of the tracking dye on the gel of dog kidney membranes (see Discussion).

In the experiment illustrated in panel B of Fig. 7, 10 mM DTT was added to the photolysis media to test if inhibition of potential cross-linking of sulfhydryl groups during photolysis by this reagent alters the pattern of [^{3}H]BSTBA photoincorporation on our gels. Comparison of panels A and B of Fig. 7 shows that addition of 10 mM DTT to the photolysis medium does not affect [^{3}H]BSTBA photoincorporation into the 150 kDa region for either duck red cell or dog kidney membranes. DTT does reduce the amount of [^{3}H]BSTBA incorporated into material at the extreme top (slice 1) of the gel of duck red cell membranes.

Discussion

We have used a radiolabelled, benzophenone analog of bumetanide, [^{3}H]BSTBA, in experiments aimed at identifying the (Na + K + Cl)-

cotransport protein in duck red cell membranes by photoaffinity labelling. When cells are incubated with [^{3}H]BSTBA, lysed, and the ghosts exposed to ultraviolet light, we find the label to be incorporated into a 150 kDa protein. The studies presented in this paper provide several pieces of evidence which together demonstrate that this protein is a component of the (Na + K + Cl)-cotransport system: (1) Photoincorporation of [^{3}H]BSTBA into the 150 kDa protein is completely inhibited if the incubation medium containing [^{3}H]BSTBA also contains 10 μM unlabelled bumetanide. This concentration of bumetanide is approx. 100-times its IC_{50} for inhibition of (Na + K + Cl) cotransport in avian red cells [5,6], and completely blocks saturable [^{3}H]bumetanide [26] and [^{3}H]BSTBA (Fig. 2) binding to duck red cells. (2) Photoincorporation of [^{3}H]BSTBA into the 150 kDa protein shows a saturable dependence on [^{3}H]BSTBA concentration, with a $K_{1/2}$ (0.063 μM; Fig. 5) very similar to the IC_{50} for inhibition of (Na + K + Cl) cotransport in these cells by BSTBA (0.062 μM; Fig. 1). These values are also close to the $K_{1/2}$ we obtained for saturable binding of [^{3}H]BSTBA to intact duck red cells (0.130 μM; Fig. 2). Though the latter value is slightly higher, the experiments were performed with red cells from different ducks. The observed difference in $K_{1/2}$ values in the experiments in Figs. 2 and 5 appears to be well within the range of expected inter-animal variability, noting that the $K_{1/2}$ for saturable [^{3}H]bumetanide binding to duck red cells varied over a similar range (0.068–0.150 μM; Ref. 26) in cells from different ducks. (3) [^{3}H]BSTBA photoincorporation into the 150 kDa protein is not detectable when cells are exposed to [^{3}H]BSTBA in isotonic media without catecholamines, just as saturable [^{3}H]bumetanide binding to intact cells is barely detectable under these conditions [26]. However, when the cells are exposed to [^{3}H]BSTBA under hypertonic conditions or in the presence of norepinephrine, conditions which stimulate both (Na + K + Cl) cotransport [1–4] and saturable [^{3}H]bumetanide binding [26] in duck red cells, [^{3}H]BSTBA photoincorporation into the 150 kDa protein is reproducibly observed. (4) Photolabelling of the 150 kDa protein by [^{3}H]BSTBA requires the simultaneous presence of Na^+, K^+, and Cl^- in the [^{3}H]BSTBA-containing medium. This is strong evidence that this protein is an integral part of the (Na + K + Cl) cotransporter, since all three ions are required for high-affinity [^{3}H]bumetanide binding to both duck red cells [26] and dog kidney membranes [25], and all three ions must be present in the extracellular medium for bumetanide to exert its maximal potency as an inhibitor of (Na + K + Cl) cotransport efflux in duck red cells (Ref. 38; Lytle, C.Y. and McManus, T.J., personal communication).

We previously found that a 150 kDa protein in membranes from dog kidney cortex [28] and outer medulla [29] is specifically photolabelled by [^{3}H]BSTBA in a manner consistent with labelling of the (Na + K + Cl) cotransporter both with respect to apparent affinity and requirement for the simultaneous presence of Na^+, K^+, and Cl^- in the [^{3}H]BSTBA-containing photolysis medium [28,29]. In Fig. 7 of this paper, it is demonstrated that the proteins of duck red cell and dog kidney membranes which are specifically photolabelled by [^{3}H]BSTBA do in fact have similar molecular mass ($\approx$ 150 kDa) on the same SDS-polyacrylamide gel, thus suggesting some degree of structural similarity between the (Na + K + Cl) cotransporters of duck red cells and dog kidney TALH. Furthermore, the finding that similar molecular weight proteins in two different tissues from very different species are specifically photolabelled by [^{3}H]BSTBA is additional strong evidence that these proteins do in fact represent a component of the (Na + K + Cl)-cotransport system in each respective tissue. As is the case with the red cell experiments presented in this paper, the efficiency of [^{3}H]BSTBA photolabelling of the 150 kDa protein in dog kidney membranes is approx. 10% [28]. The only presently detectable difference in the duck red cell and dog kidney 150 kDa proteins with regard to photolabelling by [^{3}H]BSTBA is that the latter comprises a somewhat broader peak on SDS-polyacrylamide gels (Fig. 7). Based on the width of the peak of [^{3}H]BSTBA photoincorporation in gels of dog kidney membranes, we postulated [28] that the 150 kDa protein in these membranes into which [^{3}H]BSTBA is specifically photoincorporated is most likely post-translationally modified (e.g., glycosylated). The peak of [^{3}H]BSTBA photoincorporation at approx. 150

kDa in gels of duck red cell membranes, while sharper than the corresponding peak in gels of dog kidney membranes, still comprises three to four 4 mm gel slices (Figs. 3, 4, 6, and 7). Thus, it is likely that this red cell membrane protein is also post-translationally modified, though perhaps differently than the corresponding 150 kDa protein in dog kidney membranes.

Our previous photolabelling studies with dog kidney membranes also identified lower molecular weight proteins which are photolabelled by [^{3}H]BSTBA: one of approx. 50 kDa and other(s) running with the tracking dye on the gels [28,29]. As [^{3}H]BSTBA photoincorporation into these proteins was only slightly inhibited when 10 μM unlabelled bumetanide was present in the [^{3}H]BSTBA-containing photolysis medium, and increased linearly with [^{3}H]BSTBA concentration up to 1.0 μM [28], we feel these proteins represent low-affinity [^{3}H]BSTBA binding sites which are not part of the (Na + K + Cl)-cotransport system. Direct photolabelling of protein(s) in the 34–50 kDa range with [^{3}H]bumetanide has been observed by Jørgensen and co-workers [39] and by ourselves [29]. However, the affinity of such photolabelling is also much lower ($K_{1/2}$ in the 5–10 μM range) than would be expected for the active (Na + K + Cl)-cotransport system. In our duck red cell experiments, we do not detect [^{3}H]BSTBA photoincorporation in the $\approx$ 50 kDa region, and observe only a small amount of incorporation in the region of the tracking dye on our 6.5% acrylamide gels in some experiments. This latter peak persists when 10 μM bumetanide is added to the [^{3}H]BSTBA-containing medium and is unaffected by the presence of norepinephrine in this medium (Fig. 3).

In our duck red cell experiments, the ghosts containing bound [^{3}H]BSTBA were washed prior to photolysis, so that the majority of [^{3}H]BSTBA present during photolysis was presumably bound to the inhibitory site on the (Na + K + Cl) cotransporter. This conclusion is supported by our finding that [^{3}H]BSTBA photoincorporation into the 150 kDa protein is appreciable only when the cells are exposed to [^{3}H]BSTBA under conditions favoring high-affinity, saturable binding of the diuretic (i.e., in hypertonic media or in the presence of norepinephrine). Thus, nonspecific photolabelling was not a problem in our red cell experiments. In our previous photolabelling experiments with dog kidney membranes [28], [^{3}H]BSTBA was present in the photolysis medium, and therefore only a small fraction of the total [^{3}H]BSTBA present during photolysis was specifically bound to the (Na + K + Cl)-cotransport pathway. These latter conditions allow photolabelling of low-affinity, low-specificity [^{3}H]BSTBA binding sites, which we feel the proteins in the $\approx$ 50 kDa and tracking dye regions represent. Indeed, if we preincubate dog kidney membranes with [^{3}H]BSTBA, then centrifuge the membrane suspension and remove the medium containing unbound [^{3}H]BSTBA prior to photolysis (e.g., Fig. 7), we find that [^{3}H]BSTBA photoincorporation into the $\approx$ 50 kDa region is at most barely detectable and incorporation into the region of the tracking dye is reduced, while incorporation of the label into the 150 kDa region is the same as in experiments where [^{3}H]BSTBA is present in the photolysis medium (Haas, M. and Forbush, B., III, unpublished experiments).

In addition to those noted above, other lower molecular weight proteins have been identified which are photolabelled with radiolabelled loop diuretics. These include a $\approx$ 24 kDa protein from rabbit kidney TALH segments which was labelled with an azido derivative of piretanide [40], and a $\approx$ 5 kDa peptide from bovine kidney outer medulla that was directly photolabelled with [^{3}H]bumetanide [41]. In both cases, photolabelling was inhibited when 1–5 μM unlabelled diuretic was present during photolysis. We have not identified proteins of these molecular weights from either dog kidney or duck red cell membranes which are specifically photolabelled by [^{3}H]BSTBA, either on our standard 6.5–7.5% acrylamide gels or in single experiments with each membrane type using 14% acrylamide SDS gels, which provide far better resolution of lower molecular weight proteins. However, even with these latter gels it is quite possible that we could have missed labelling of proteins of very low molecular weight, particularly those of less than 10 kDa. In addition, the fact that we identify only a single (150 kDa) protein which is specifically photolabelled with [^{3}H]BSTBA does not preclude the possibility that the (Na + K + Cl) cotransporter is comprised of multiple subunits.

We previously calculated, based on 1000 [^{3}H]bumetanide binding sites per cell and assuming a molecular mass of 100 kDa for the (Na + K + Cl) cotransporter, that this cotransporter comprises only about 0.03% of the total protein of duck red cell membranes [26]. Using a molecular weight of 150 kDa, this fraction is increased to approx. 0.05%, however even this amount would not be likely to be visible on our Coomassie blue stained gels. Thus, we conclude that a Coomassie blue stained band at $\approx$ 150 kDa (upper panel, Fig. 3) which comprises $\approx$ 2.5% of the membrane protein (based on the area under the corresponding peak on the densitometric scan in Fig. 3) does not represent the (Na + K + Cl) cotransporter. The fraction of the total membrane protein comprised by the (Na + K + Cl) cotransporter in duck red cells is similar to that determined for sucrose gradient purified membranes from dog kidney cortex [28]. In kidney membranes, however, it is possible that we are underestimating the density of cotransporters, since a sizeable fraction of such transporters in our membrane preparations could be in an 'inactive' form incapable of binding [^{3}H]bumetanide or [^{3}H]BSTBA with high affinity. In contrast, since our [^{3}H]bumetanide and [^{3}H]BSTBA binding assays and [^{3}H]BSTBA photolabelling studies with duck red cells were done in the presence of norepinephrine, conditions under which cotransport activity and diuretic binding are maximal [4,26], it is safe to assume that the (Na + K + Cl)-cotransport system is only a minor component of the duck red cell membrane.

In summary, we have identified a 150 kDa protein from duck red cell membranes which is specifically photolabelled with [^{3}H]BSTBA, and have provided several independent pieces of evidence which together demonstrate that this protein is a component of the (Na + K + Cl)-cotransport system of these cells. The molecular weight of this protein is also similar to that of a protein from dog kidney plasma membranes which is photolabelled by [^{3}H]BSTBA in a manner highly consistent with labelling of the (Na + K + Cl)-cotransport system of the TALH [28,29]. It is hoped that future studies will enable us to isolate one or both of these 150 kDa proteins, and further define their similarities and putative role in the (Na + K + Cl)-cotransport process.

Acknowledgements

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