Detergent Solubilization, Functional Reconstitution, and Partial Purification of Epithelial Amiloride-Binding Protein†

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ABSTRACT: The amiloride-binding protein from cultured toad kidney cells (A6) was solubilized in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), functionally reconstituted into liposomes, and partially purified. The specific binding of [^{3}H]methylbromoamiloride ([^{3}H]CH_3BrA) was measured in intact A6 epithelia, A6 cell homogenate (H), apical plasma membrane vesicle (V_1), and CHAPS-solubilized V_1 and on material obtained after affinity chromatography of CHAPS-solubilized plasma membrane vesicles on agarose-immobilized wheat germ agglutinin (WGA). Specific [^{3}H]CH_3BrA binding to H, V_1, and WGA material reached equilibrium after 10 min. Scatchard analysis of [^{3}H]CH_3BrA binding to V_1 and WGA material revealed a homogeneous class of binding sites with K_D's of 130 and 128 nM, respectively. These K_D values were similar to the apparent inhibitory dissociation constant determined from amiloride inhibition of $^{22}Na^+$ influx in both intact A6 epithelia and V_1. The total number of specific binding sites was 4 pmol/mg of V_1 protein, which represented a 10-fold enrichment compared to H, and 66.6 pmol/mg of WGA material (a 148-fold enrichment). From association/displacement kinetic studies of specific [^{3}H]CH_3BrA binding to V_1, the rate constants of association (k_a) and dissociation (k_d) were calculated to be 3.6×10^5 M^{-1} s^{-1} and 49.5×10^{-3} s^{-1}, respectively. These values yield an equilibrium dissociation constant of 138 nM. In solubilized V_1 protein, binding activity was enriched approximately 20-fold over H and was markedly dependent upon the relative concentrations of detergent and phospholipid. CHAPS solubilization of V_1 resulted in an average 44% recovery of protein with 90% retention of the total number of specific [^{3}H]CH_3BrA binding sites. After WGA chromatography 2.7% of the applied protein and 46% of the specific binding sites were recovered. Amiloride-sensitive $^{22}Na^+$ uptake was measured after reconstitution of the solubilized V_1 protein into phosphatidylcholine vesicles, indicating preservation of transport function. Initial parallel studies were performed with bovine kidney papilla as starting material. Comparable results were obtained.

Amiloride-sensitive Na^+ channels are found in Na^+-transporting tissues displaying high transepithelial electrical resistance and are involved in many physiological processes, including salt and water balance. The electrophysiology of the channel has been widely studied (Macknight et al., 1980; Lindemann, 1984; Sariban-Sohraby & Benos, 1986), but the molecular mechanisms underlying the Na^+ transport function as well as the primary structure of the channel are unknown. A potential approach to investigating these problems has been made apparent by the availability of specific probes having reasonably high specific radioactivities (Kleyman et al., 1983; Garvin et al., 1986; Lazorick et al., 1985), and by the characterization of a cultured cell line (A6) that can be confidently substituted for naturally occurring tissues (Sariban-Sohraby et al., 1983). Amiloride, an acylguanidine-substituted pyrazine ring based diuretic drug, specifically and reversibly inhibits Na^+ ion flux in amphibian tissues (Benos, 1982), cultured A6 epithelia, (Sariban-Sohraby et al., 1983), A6 apical plasma membrane vesicles (Sariban-Sohraby et al., 1984a), and single epithelial Na^+ channels incorporated into planar lipid bilayers (Sariban-Sohraby et al., 1984c). Radiolabeled analogues of amiloride such as [^{3}H]methylbromoamiloride ([^{3}H]CH_3BrA; Lazorick et al., 1985) that bind with high affinity to the Na^+-transporting protein provide convenient molecular tags to identify this protein and serve as markers during purification.

In this paper we report the successful detergent solubilization and partial purification of the amiloride-binding receptor associated with epithelial sodium channels. Amiloride-sensitive Na^+ transport activity is retained after reconstitution of the solubilized proteins into phospholipid vesicles. The observation that drug binding and channel function can be preserved even after detergent treatment indicates a close association between these two parameters, and that enrichment of binding activity can be potentially used as an assay for biochemical purification of functional Na^+ channels. In this regard, because the ultimate purification of these sodium channels will require abundant starting material, we have begun in addition to characterize the $^{22}Na^+$ transport function and specific [^{3}H]-CH_3BrA binding properties of bovine kidney papilla before and after detergent treatment.

Materials and Methods

Drugs and Reagents. Carrier-free ^{22}NaCl was obtained from Amersham Corp. (Arlington Heights, IL). Tissue culture products were obtained from Gibco (Grand Island, NY), fetal calf serum from HyClone (Logan, UT), and Sephadex, Dowex, and the protease inhibitors from Sigma Chemical Co. (St. Louis, MO). Amiloride and bromoamiloride were gifts from Dr. E. J. Cragoe, Jr., of Merck, Sharp, and Dohme Research Laboratories (West Point, PA). All other chemicals were

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reagent grade, and all solutions were made up with distilled, deionized water and filter sterilized before use (Millipore filter, type GS, 0.22 μm, Millipore Corp., Bedford, MA).

Cell Culture. A6 cells from toad kidney were purchased from American Type Culture Collection. Stock cultures were carried on plastic Petri dishes (Falcon 1013) and subcultured onto filter-bottomed cups made of Millipore filters (HAWP, 0.45 μm) glued onto acrylic rings (11.4-cm diameter) as described in detail previously (Sariban-Sohraby et al., 1984a).

The growth medium was powdered Dulbecco's modified Eagle's medium (Gibco) with low NaCl (75 mM) and bicarbonate (8 mM) as required for amphibian cells. Fetal bovine serum (10% v/v), penicillin (100 units/mL), and streptomycin (100 μg/mL) were added to the growth medium, and cells were fed twice weekly. Cells were cultured at 28 °C with 1.3% CO_2 in air. Three hours before starting the membrane preparation, A6 cells were fed with 100 nM aldosterone on both the apical and basolateral sides. Only A6 cultures with initial transepithelial voltages exceeding 10 mV were used. Further, only cultures responding to aldosterone pretreatment with a minimum 2–3-fold increase in their transepithelial voltages were used. Voltage was measured by using sterile 2% agar–amphibian Ringer bridges placed in the fluid inside and outside the filter-bottomed cups and connected to a voltmeter via calomel electrodes.

Membrane Preparation. (A) A6 Cells. Apical plasma membrane vesicles were prepared as follows. Filter-bottomed cups (10–40) containing confluent epithelia were washed by dipping into ice-cold homogenization buffer (HM) consisting of 60 mM sucrose and 10 mM tris(hydroxymethyl)aminomethane (Tris)–*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes). Cells were scraped off the filters with a rubber policeman into HM supplemented with DNase (2 μg/mL), aprotinin (25 μg/mL), leupeptin (10 μg/mL), pepstatin (10 μg/mL), and phenylmethanesulfonyl fluoride (PMSF) (175 μg/mL) (2 mL per filter). The scraped material was homogenized on ice with a motor-driven Teflon pestle (10 strokes). After addition of $CaCl_2$ to a final concentration of 10 mM, the homogenate was spun for 10 min at 5000*g*. The resulting pellet was discarded, and the supernatant was then centrifuged for 30 min at 43000*g*. The pellet (designated V_1) was either resuspended in phosphate-buffered saline (PBS) for enzyme assays and methylbromoamiloride binding assays or solubilized (see below).

(B) Bovine Renal Papilla. Fresh bovine kidneys were collected early in the morning at a local abattoir and rushed to the laboratory in oxygenated, ice-cold PBS. Each lobe was split longitudinally, and papillary tips (white section) were excised and transferred to medium consisting of 250 mM sucrose and 10 mM triethanolamine, pH 7.6, supplemented with DNase and protease inhibitors as described above. The protocol for obtaining plasma membrane vesicles was adapted from the procedure described by Turner and Silverman (1981) for dog renal cortex. Homogenization was performed with a VirTis homogenizer (10 min at setting 40, followed by 4 min at setting 100). The homogenate was spun twice at 190*g* for 10 min, and the pellets were discarded. The supernatant was spun for 20 min at 16000*g*. The resulting pellet was resuspended in PBS (pH 7.4) to which 10 mM $CaCl_2$ was added and allowed to sit on ice for 15 min. Subsequently, this material was spun at 3000*g* for 15 min. The resulting supernatant was spun at 43000*g* for 20 min. The final pellet was either resuspended in PBS for binding and flux studies or detergent solubilized. All dissections and centrifugations were done at 4 °C.

Solubilization of A6 and Bovine Papillary Membrane Proteins. Membranes were resuspended in ice-cold NaH_2PO_4 (150 mM), PMSF (175 μg/mL), ethylenediaminetetraacetic acid (EDTA) (1 mM), and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (10 mM), pH 7.4 (to a final protein concentration of 5 mg/mL), and shaken at 4 °C for 60 min. This material was then spun for 60 min at 43000*g*. The supernatant containing the detergent-solubilized proteins was supplemented with 0.2% w/v egg phosphatidylcholine (2 mM) and stored at 4 °C until use.

Enzyme and Protein Assays. The enrichment in apical membrane enzyme markers in the membrane vesicle fraction was assessed by measuring the activity of alkaline phosphatase as described by Turner and Moran (1982), and γ-glutamyltransferase was assayed by using colorimetric kit no. 545 from Sigma. Mitochondrial contamination was assessed by measuring succinic dehydrogenase activity as described by Pennington (1961). Protein was measured by using the Coomassie blue binding technique of Bradford (1976).

Preparation of [^3H]Methylbromoamiloride ([^3H]CH_3BrA). [^{3}H]CH_3BrA was synthesized as described previously (Lazorick et al., 1985) by reacting tritiated methyl iodide with 6-bromoamiloride. Purification of the resulting molecule was performed by thin-layer chromatography. The final specific activity was 1.24 Ci/mmol. [^{3}H]CH_3BrA was used as a reversible blocker for the equilibrium binding studies.

[^3H]Methylbromoamiloride Binding Assay Using Rapid Gel Filtration. Binding was determined essentially by following the gel filtration method of Penefsky (1977). Sephadex G-25–150 (Sigma) was washed and swollen in NaCl (75 mM), KCl (2.5 mM), NaH_2PO_4 (2.0 mM), and KH_2PO_4 (0.5 mM), pH 7.4 (0.5 × PBS). The swollen gel was poured into 1-mL plastic syringes fitted with a disk of nylon mesh (74 μm) to retain the gel. Just before use, the columns were dried by a 1-min centrifugation in a clinical centrifuge (IEC, Needham Heights, MA) at setting 3. One hundred microliter samples containing between 0.6 and 100 μg of protein were incubated with 1 μL of [^{3}H]CH_3BrA at appropriate concentrations for 30 min on ice. The samples were then applied to the Sephadex columns and immediately spun in a clinical centrifuge for 2 min at setting 4. Protein and radioactivity were measured in the collected material. Background radioactivity was assessed by loading similarly packed Sephadex columns with 1 μL of the experimentally used concentration of [^{3}H]CH_3BrA diluted in 100 μL of 0.5 × PBS. Nonspecific binding was determined in separate samples in the presence of 0.1 mM amiloride and subtracted from total binding. Specific binding of detergent-solubilized material was unaffected by the incubation of 1.6 mM CHAPS in the resin-equilibrating solution. Protein recoveries averaged 52 ± 2.8% (n = 10) when nonsolubilized material (homogenate and fraction V_1) was used and 96 ± 2.0% (n = 5) when the protein was solubilized.

Preparation of Liposomes. The procedure for reconstitution of membrane proteins into phospholipid liposomes as described by Anholt et al. (1982) was followed. Egg phosphatidylcholine (PC) in chloroform (Sigma) was dried under nitrogen and resuspended in a 100 mM sucrose, 10 mM Tris–Hepes, pH 7.45, solution at a final concentration of 10.3 mM. CHAPS-solubilized V_1 material was mixed with PC liposomes in the presence of 50 mM CHAPS in a 3:5:2 volume ratio to yield a final concentration of 5.2 mM PC and 10 mM CHAPS. The mixture was incubated at 0 °C for 30–60 min, followed by dialysis against 500–1000 volumes of PBS for 24 h using Spectrapor 3 dialysis tubing.

$^{22}Na^+$ Fluxes into Papilla Membrane Vesicles and A6 Liposomes. $^{22}Na^+$ fluxes into V_1 and reconstituted liposomes were performed following the protocol described by Garty et al. (1983). In brief, $^{22}Na^+$ fluxes are measured against a large sodium electrochemical potential energy gradient (≥50 mM Na^+ inside the vesicles, 1 μM outside). The isotope will equilibrate according to the magnitude of the electrical diffusion potential set up across the membrane and will transiently accumulate inside the vesicles until the gradient dissipates.

External sodium was first removed from the A6 liposome mixture by passage over Sephadex G-25–150 columns prepared as described under [^{3}H]Methylbromoamiloride Binding Assay Using Rapid Gel Filtration. The eluted material was collected in a 95 mM sucrose solution, pH 7.4. One hundred fifty microliters of liposomes (~0.5 mg of protein/mL) was incubated with carrier-free $^{22}Na^+$ (10 μCi/mL; Amersham) in the absence or presence of 0.1 mM amiloride. At the end of the incubation period, the samples were applied to Sephadex G-25–150 columns and spun immediately in a clinical centrifuge at setting 4 for 2 min. Protein and radioactivity were measured in the collected samples.

For the bovine papilla V_1 samples, external Na^+ was removed prior to tracer uptake studies by first passing the vesicles over Dowex 50W-X8 columns (Tris form). The columns were prepared in 1-mL tuberculin syringes. The resin was extensively washed with 1 M Tris base, followed by 5 bed volumes of 175 mM sucrose and 10 mM Tris–Hepes, pH 7.4, containing 1 mg/mL bovine serum albumin (BSA). Three hundred microliters of V_1 (~2 mg/mL) was applied to the column and collected by centrifugation as described above. The collected vesicles were diluted to 1 mL by addition of 175 mM sucrose and 10 mM Tris–Hepes, pH 7.4. The experiment was initiated by the addition of 50 μL of $^{22}Na^+$ (final specific activity = 10 μCi/mL; $[Na^+]$ = 0.93 μM). One hundred fifty microliter samples were taken in triplicate at various times, and the uptake was stopped by passing the samples through either Dowex or Sephadex columns. The eluate was saved for later determination of protein and radioactivity. All results in this paper are presented as mean values of *N* independent determinations plus or minus 1 standard deviation (SD).

Wheat Germ Affinity Chromatography. Wheat germ affinity columns were prepared as described by Hedo (1984). Wheat germ agglutinin–agarose (WGA, Vector Laboratories) was poured into a Bio-Rad 10-mL glass Econo-Column (7 mg in 4 mL). The column was washed successively with 50 bed volumes of buffer I [NaCl (150 mM), Hepes (50 mM), CHAPS (1.6 mM), and sodium dodecyl sulfate (SDS) (0.01%), pH 7.6], 10 bed volumes of buffer II [NaCl (150 mM), Hepes (50 mM), CHAPS (1.6 mM), *N*-acetylglucosamine (300 mM), pH 7.6], and 100 bed volumes of buffer III [NaCl (150 mM), Hepes (50 mM), CHAPS (1.6 mM), pH 7.6]. The CHAPS-solubilized sample (≤2 mg of protein per mL of WGA) was applied to the column and recycled 6 times. Next, the column was washed with 50 bed svolumes of buffer III. Two to three bed volumes of buffer II were used for specific elution. The collected material (~10 mL) was filtered on Amicon microconcentrators to reduce the volume to 2–3 mL and passed through a Sephadex column to remove salts and free detergent. WGA columns were stored in buffer II containing 0.01% sodium azide at 4 °C.

Results

Starting Material. We have previously observed that the lack of transepithelial voltage across A6 monolayers was associated with the absence of amiloride-sensitive $^{22}Na^+$ influx in both intact cultured A6 cells and apical plasma membrane vesicles (V_1) prepared from them (Sariban-Sohraby et al., 1984a). Similarly, while enzymatic activities of alkaline phosphatase and γ-glutamyltransferase were usually enriched 10-fold in V_1 compared to cell homogenate (Sariban-Sohraby et al., 1984a), we observed only marginal increases (2–3-fold) in the activity of these apical membrane markers when the transepithelial voltage was less than 2 mV. Therefore, only preparations showing initial transepithelial voltages in excess of 10 mV and voltage increases in response to aldosterone were used in all work reported in this paper. Aldosterone has been shown to stimulate amiloride-sensitive $^{22}Na^+$ uptake in A6 cells and plasma membrane vesicles (Sariban-Sohraby et al., 1983, 1984b) as well as in native amphibian tissues (Palmer et al., 1982). Aldosterone treatment was thus used as a functional test for the existence of amiloride-sensitive Na^+ transport in A6 epithelia under our growth conditions.

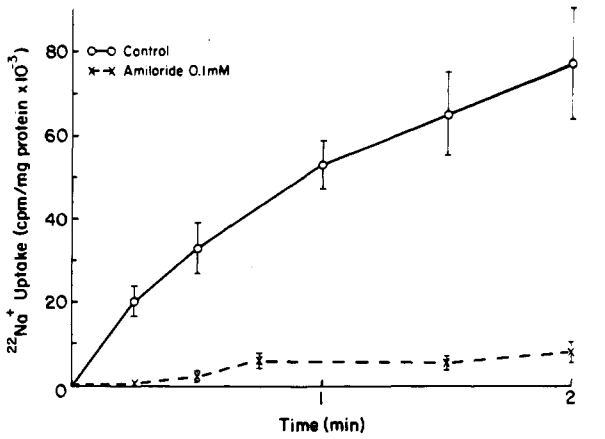

FIGURE 1: Time course of $^{22}Na^+$ uptake into bovine kidney papilla apical plasma membrane vesicles (V_1). One hundred fifty microliters of V_1 (protein concentration, ~0.3 mg/mL) was incubated at room temperature with carrier-free $^{22}Na^+$ (10 μCi/mL) for appropriate times in the absence and presence of 0.1 mM amiloride. Uptake is expressed as cpm/mg of protein. Means ± SD of four independent experiments carried out in triplicate are shown.

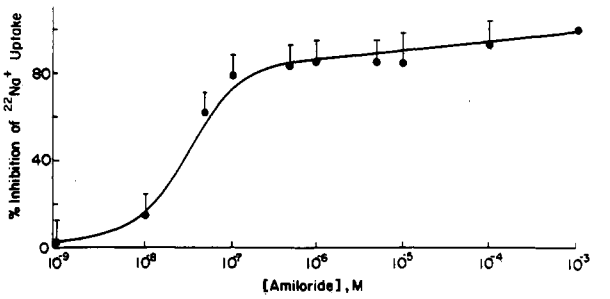

FIGURE 2: Dose–response curve of $^{22}Na^+$ uptake inhibition by amiloride in bovine papilla V_1. The incubation time with tracer was 30 s at all amiloride concentrations. NaCl concentration in the uptake buffer was 0.93 μm. For each amiloride concentration, the amount of flux inhibition is expressed as percent of the amiloride-sensitive flux measured at the highest amiloride concentration (1 mM). Means ± SD of four independent experiments performed in triplicate are shown.

$^{22}Na^+$ Influxes into Apical Plasma Membrane Vesicles. Detailed characterization of $^{22}Na^+$ uptake by A6 vesicles was described previously (Sariban-Sohraby et al., 1984a). Na^+ transport has been documented in the papilla of mammalian kidney (Rau & Fromter, 1974; Rocha & Kudo, 192; Burg, 1986). Results of $^{22}Na^+$ influx in bovine papilla vesicles are shown as Figures 1 and 2. Unidirectional $^{22}Na^+$ influx into the vesicles was linear up to 30 s, and about 98% of the initial influx was inhibited by 0.1 mM amiloride (Figure 1). The

Table I: Effect of Different Detergents on Protein Solubilization and Recovery of Specific [^{3}H]CH_3BrA Binding Sites[a]

condition	sp binding act. (pmol/mg of protein)	solubilization of total V_1 protein (%)	recovery of initial V_1 sp binding sites (soluble/insoluble) (%)
beef V_1	0.223		100
10 mM CHAPS	0.465	46.4	95.0/0
1% Triton X-100	0.123	60.0	32.7/0
30 mM digitonin	0.392	19.2	33.5/0
2% octyl glucoside	0.139	60.3	36.9/28.3
30 mM sodium cholate		14.0	0/0

[a] Extraction was allowed to proceed for 1 h at 4 °C. Total protein was adjusted to be 2–3 mg/mL during detergent extraction. Each number represents the average of duplicate determinations. The solubilization medium consisted of PBS containing 1 mM EDTA, 175 μg/mL PMSF, 5 mg/mL egg phosphatidylcholine, and the appropriate detergent in the concentration indicated. Displaceable [^{3}H]methylbromoamiloride binding was not observed in any detergent-insoluble fractions, except for octyl glucoside. In this case, the specific binding activity was 0.16 pmol/mg of protein.

maximal extent of inhibition by amiloride varied from preparation to preparation, ranging from 42% to 98% (n = 12). Amiloride (0.1 mM) inhibited the initial rate of $^{22}Na^+$ uptake by greater than 95% in two-thirds of the preparations. The amiloride-sensitive component of $^{22}Na^+$ uptake (i.e., total influx minus amiloride-insensitive influx) was shown to occur in an osmotically active space; i.e., when the osmolarity of the external medium was raised progressively from 100 to 400 mosM, $^{22}Na^+$ influx was decreased proportionately in a linear fashion (data not shown). The amount of amiloride-insensitive, osmotically inactive "influx" varied from 0% to 44% of the total influx in different preparations.

Figure 2 shows that amiloride inhibition of $^{22}Na^+$ influx in bovine papilla vesicles was dose-dependent and essentially complete at 0.1 mM. In the presence of 1 μM NaCl in the outer vesicular solution, the half-maximal inhibition constant ($K_{1/2}$) of amiloride was 36 nM, a result in good agreement with the values of $K_{1/2}$ calculated in intact A6 cells and A6 vesicles at the same external NaCl concentrations (Sariban-Sohraby et al., 1983, 1984a). Although the maximal extent of amiloride inhibition of $^{22}Na^+$ uptake varied, the $K_{1/2}$ for amiloride inhibition ranged from 22 to 70 nM between the various preparations.

Solubilization of Plasma Membrane Vesicles. A6 and bovine papilla plasma membrane vesicles (V_1) were optimally solubilized by the zwitterionic detergent CHAPS in the presence of protease inhibitors at 4 °C. A6 homogenate was used in the initial solubilization trials (Figure 3A). Maximal solubilization of protein was achieved after a 1-h incubation with 10 mM CHAPS. Once the time of incubation and detergent concentrations were determined, V_1 was assayed for extent of solubilization following a 1-h exposure to different

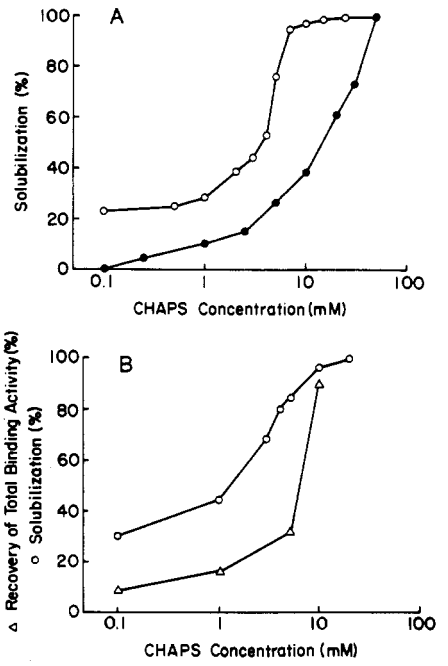

FIGURE 3: (A) Detergent solubilization of A6 cell homogenate. Samples of homogenate were incubated on ice for 30 (filled circles) or 60 min (open circles) with solubilization buffer containing 10 mM CHAPS, 150 mM NaH_2PO_4, 1 mM EDTA, 175 μg/mL PMSF, and 0.2% (w/v) phosphatidylcholine. Solubilization at any CHAPS concentration was expressed as a percentage of maximal solubilization (i.e., after a 2-h exposure to 50 mM CHAPS). Values are means of duplicate determinations. (B) Detergent solubilization of A6 V_1. Samples of V_1 (~5 mg of protein/mL) were incubated on ice for 60 min (open circles) in solubilization buffer (with 10 mM CHAPS). The amount of solubilization is expressed as the percent of that achieved after a 1-h exposure of homogenate to 50 mM CHAPS. The open triangles indicate the percentage of recovery of total specific [^{3}H]CH_3BrA binding sites in the soluble fraction compared to V_1. Values are means of duplicate determinations.

concentrations of CHAPS as shown in Figure 3B (open circles). The starting protein concentration was 5 mg/mL. Optimal solubilization of V_1 protein from both A6 cells and bovine papilla was likewise achieved with 10 mM CHAPS. A6 V_1 was also exposed for 2 h with 10 mM CHAPS without a further increase in solubilization. Protein recovery in the soluble fraction was 44 ± 4% of V_1 protein (n = 13). The ability of sodium cholate, digitonin, Triton X-100, and octyl glucoside to solubilize bovine papilla V_1 was also evaluated. The protein recoveries were 14%, 19%, 60%, and 60%, respectively, in the presence of 5 mM PC (Table I). However, the recovery of total specific binding activity of [^{3}H]CH_3BrA was markedly decreased in Triton X-100, digitonin, and octyl glucoside soluble fractions compared to CHAPS (Table I). No binding activity was present when sodium cholate was used. The recovery of total binding activity was maximal (90 ± 2.8%) at a CHAPS concentration of 10 mM and rapidly decreased at lower concentrations as shown for the A6 soluble

Table II: Effect of Different Phospholipid/Detergent Ratios on Recovery of Specific [^{3}H]CH_3BrA Binding Sites in Solubilized A6 V_1[a]

	A	B	C	D	E	F	G
[CHAPS] (mM)	0	10	10	10	50	50	50
[PC] (mM)	ND	ND	2	2	1.4	5	10
[PC]/[CHAPS]			0.2	0.2	0.028	0.1	0.2
recovery of total binding sites (%)	100	25.5 ± 6 (n = 3)	90 ± 5 (n = 3)	90 ± 2 (n = 5)	2.5 ± 2.5 (n = 3)	79 (n = 2)	95 ± 2 (n = 3)

[a] Solubilization was carried out for 1 h on ice. Column A: control V_1 fraction (i.e., not solubilized). Column B: solubilization of V_1 with 10 mM CHAPS with no addition of exogenous PC. Column C: same as column B except that 2 mg/mL PC was added to the solubilization buffer. Column D: same as column C except that binding was assayed between 24 h and 3 weeks after initial solubilization. Columns E–G: same as column C except that phosphatidylcholine was added 30 min prior to the binding assay. ND = not determined.

Table III: Specific [^{3}H]CH_3BrA Binding and Protein Recovery during CHAPS Solubilization

fraction	protein (mg)	recovery (%)	sp act. (pmol/mg)	total binding sites pmol	%
A6 homogenate (40 filters)	292 ± 11[a]		0.45 ± 0.13[c]	131 ± 88	
V_1	8.0 ± 1.5[a]	100	4.00 ± 1.36[c]	32 ± 7.0	100
solubilized V_1	3.5 ± 0.3[b]	43.7 ± 4.4	8.24 ± 0.14[d]	28.8 ± 1.41	90
WGA-specific material	0.22 ± 0.04[c]	2.7 ± 0.21	66.6 ± 7.7[c]	14.6 ± 1.4	46
bovine renal papilla homogenate (4 kidneys)	944 ± 109[c]		0.12 ± 0.05[c]	113 ± 66	
V_1	24.2 ± 3.4[c]	100	0.39 ± 0.14[c]	9.4 ± 5.2	100
solubilized V_1	10.8 ± 1.5[c]	44.2 ± 6.2	0.83 ± 0.22[c]	9.0 ± 5.1	96
WGA-specific material	0.74 ± 0.30[c]	3.9 ± 1.7	13.9 ± 5.7[c]	10.2 ± 5.8	108

[a] $n = 15$. [b] $n = 13$. [c] $n = 5$. [d] $n = 4$.

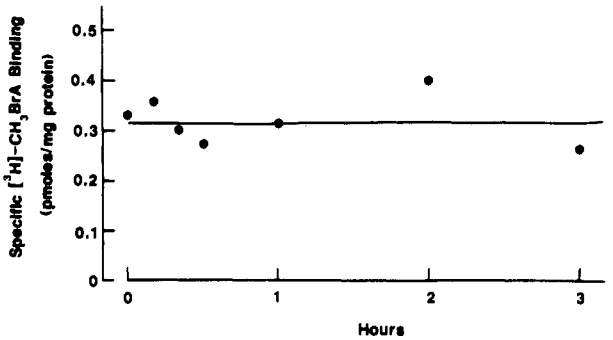

FIGURE 4: Specific [^{3}H]CH_3BrA bound to solubilized bovine papilla V_1 at 21 °C vs. time. Binding at experimental time = 0 was measured at 0 °C. Thereafter, the solubilized V_1 protein was warmed to 21 °C for various times, and aliquots were removed for binding. All binding measurements were performed at 0 °C as described under Materials and Methods. Values are means of duplicate determinations.

V_1 fraction in Figure 3B (open triangles).

Table II demonstrates the effect of various lipid to detergent ratios on the recovery of specific [^{3}H]CH_3BrA binding sites. CHAPS solubilization of V_1 without subsequent addition of exogenous PC resulted in the loss of 75% of the binding sites (column B), while 90% of the binding sites were recovered after the addition of 2 mM PC (column C) This soluble specific binding activity was stable for at least 3 weeks at 4 °C (column D). When the CHAPS concentration was increased (column E), the binding activity was almost completely inhibited within 1 h but could be restored by a proportionate increase in PC concentration (columns F and G). Therefore, we concluded that CHAPS was the detergent of choice to solubilize both A6 and papilla V_1 provided that the PC to CHAPS concentration ratio was maintained close to the optimal value of 0.2.

The solubilized methylbromoamiloride receptor was stable at 21 °C for at least 3 h (Figure 4). The stability of binding activity at other temperatures has not yet been evaluated.

[^{3}H]Methylbromoamiloride Binding. Binding of [^{3}H]-CH_3BrA to both A6 cell homogenate (H) and plasma membrane vesicles (V_1) reached equilibrium in 10–15 min at 0 °C at 0.14 μM [^{3}H]CH_3BrA concentration (Figure 5). The half-time of the association reaction was 180 s. Binding to V_1 (as well as to solubilized V_1) was reversible upon addition of a 1000-fold molar excess of unlabeled amiloride (Figure 5A). The half-time of the displacement step was 15 s. Figure 5 also displays the semilogarithmic linearization of the data from which the values of k_d (first-order rate constant of dissociation) and k_a (the rate constant of association) were calculated. These values were 49.5×10^{-3} s^{-1} and 3.6×10^5 M^{-1} s^{-1}, respectively. The resulting equilibrium dissociation constant K_D (k_d/k_a) was 138 nM.

The specific binding of [^{3}H]CH_3BrA to V_1 saturated with increasing drug concentration (Figure 6A). Scatchard analysis of the data is shown as Figure 6B. The K_D of [^{3}H]CH_3BrA binding to V_1 was 130 nM. This value is in good

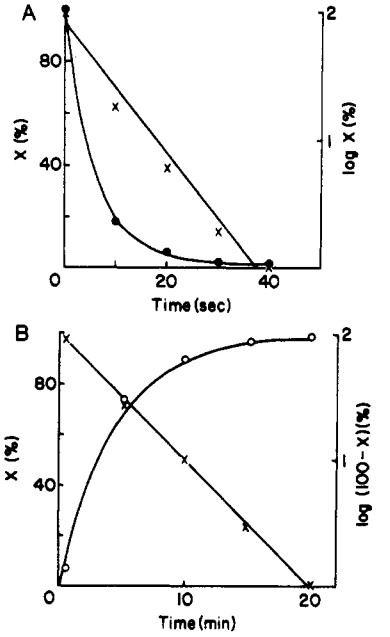

FIGURE 5: (A) Time course of displacement of [^{3}H]CH_3BrA bound to V_1 by unlabeled amiloride. Binding is expressed as the percent of binding in the absence of amiloride (filled circles). Nonspecific binding was subtracted from each point. Samples were incubated with 0.14 μM [^{3}H]CH_3BrA for 30 min on ice, at which point 0.1 mM amiloride was added, and bound [^{3}H]CH_3BrA was measured at the indicated times. The logarithm of the binding values (X) plotted as a function of time yields a straight line with slope = $-k_d$ (the dissociation rate constant, 49.5×10^{-3} s^{-1}). Once $-d[AS]/dt$ and k_d are known, k_a (the association rate constant) can be calculated to be 3.6×10^5 M^{-1} s^{-1} at a [^{3}H]CH_3BrA concentration of 1.4×10^{-7} M. The results are representative of three independent experiments. (B) Semilogarithmic plot of [^{3}H]CH_3BrA specific binding (X) to V_1 as a function of time. Data are taken from panel A; the concentration of [^{3}H]CH_3BrA was 0.14 μM. Binding is also expressed as percent of maximum (open circles). The slope of the line, $-d[AS]/dt$ (where [AS] represents the complex [^{3}H]CH_3BrA–substrate), is 99.5×10^{-3} s^{-1} (determined by linear regression analysis, $r = 0.99$).

agreement with the half-maximal $^{22}Na^+$ influx inhibition constants of amiloride in intact A6 cells (100 nM), plasma membrane vesicles (126 nM), and single A6 Na^+ channels incorporated into planar lipid bilayers (100 nM) (Sariban-Sohraby et al., 1983, 1984a,c).

The specific [^{3}H]CH_3BrA binding activity in A6 and papillary H, V_1, and solubilized V_1 is shown in Table III. There was no measurable displaceable [^{3}H]CH_3BrA binding in the CHAPS-insoluble fraction. Over 90% of the binding sites present in V_1 were recovered in the soluble fraction. V_1 represents a preparation enriched 10 times in apical membranes compared to A6 cell homogenate (Sariban-Sohraby et al., 1984a). Bovine V_1 was enriched 5.0 ± 2.2, 4.2 ± 0.9, and 0.4 ± 0.2 ($n = 5$) times relative to homogenate in alkaline phosphatase, γ-glutamyltransferase, and succinic dehydrogenase activities, respectively. The enrichment in specific

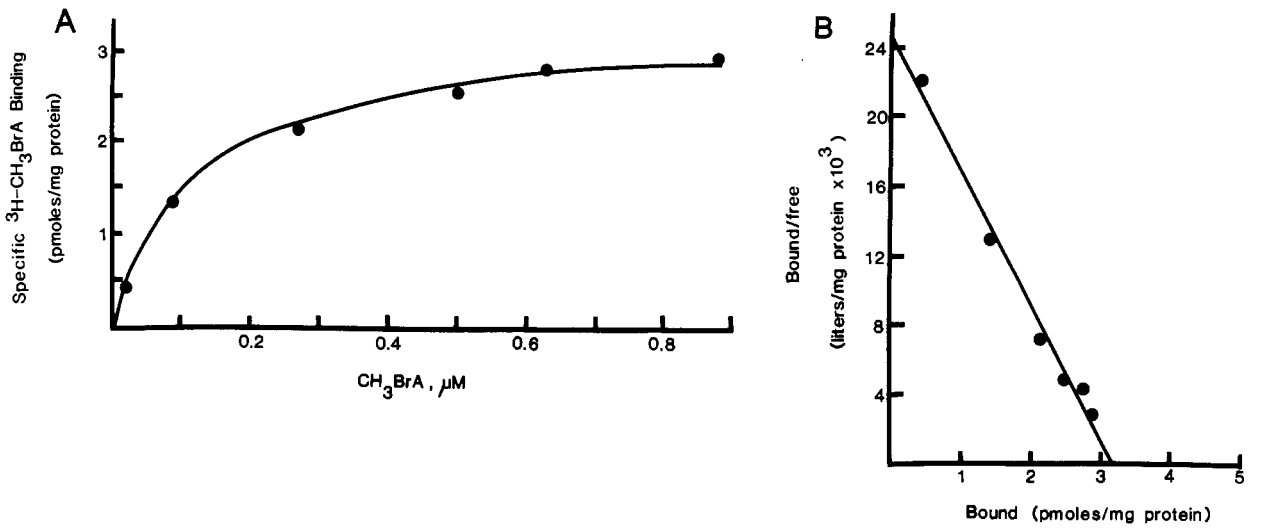

FIGURE 6: (A) Specific binding of [^{3}H]CH_3BrA to V_1 as a function of [^{3}H]CH_3BrA concentration. The incubation time was 30 min on ice. This experiment is representative of five individual experiments. (B) Scatchard plot of specific [^{3}H]CH_3BrA binding to V_1. V_1 samples were incubated for 30 min on ice with 0.02–0.9 μM [^{3}H]CH_3BrA in the absence and presence of 0.1 mM amiloride. The K_D, given by the inverse of the slope, was 130 nM, and the total number of binding sites (x intercept) was 3.2 pmol/mg of protein. The data for this plot were taken from panel A.

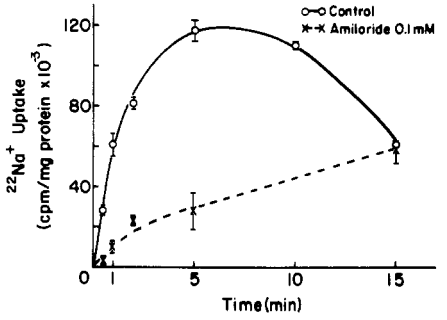

FIGURE 7: Time course of $^{22}Na^+$ uptake into liposomes containing solubilized A6 V_1 proteins in the absence and presence of 0.1 mM amiloride.

[^{3}H]CH_3BrA binding sites in V_1 vs. homogenate was also less in the papillary preparation as compared to A6 (3.2 vs. 8.8). Because we do not have any information about the existence or functionality of intracellular or basolateral amiloride binding sites, we feel it is appropriate to consider the V_1 binding sites as the reference point for recovery calculations.

$^{22}Na^+$ Fluxes into Solubilized V_1 Reconstituted Liposomes. A crucial step in the solubilization of any membrane transport protein is to test whether or not transport function is maintained subsequent to detergent treatment. To this end, we measured $^{22}Na^+$ uptakes into PC liposomes following reconstitution of the detergent-soluble A6 V_1 proteins into them. Na^+ uptake occurred against a large electrochemical Na^+ gradient, as described above for the bovine papillary apical membrane vesicles. Figure 7 shows the results of these experiments. Unidirectional $^{22}Na^+$ influx was linear for 60 s, peaked at about 5 min, and then slowly declined from its maximum value over the next 10 min. Amiloride (0.1 mM) added to the external medium inhibited both the initial rate and maximal extent of $^{22}Na^+$ uptake by 96% and 79%, respectively. In the absence of protein in the PC liposomes, the absolute magnitude of $^{22}Na^+$ uptake was greatly reduced and there was no effect of 0.1 mM amiloride on $^{22}Na^+$ uptake. Essentially identical results were obtained with the bovine V_1 preparation, solubilized and reconstituted into PC liposomes (n = 3, data not shown).

Wheat Germ Affinity Chromatography. The solubilized V_1 material from both A6 cells and bovine papilla was absorbed onto WGA–agarose, a lectin with sugar specificity for *N*-acetylglucosamine and sialic acid (Hedo, 1984). Both specific and nonspecific fractions were tested for [^{3}H]CH_3BrA binding. Approximately 6% and 7% of the applied solubilized V_1 protein from A6 and bovine papilla, respectively, were retained (Table III). Specific elution resulted in over 90% recovery of the retained protein as well as between 46% and 100% of the specific [^{3}H]CH_3BrA binding sites present in the solubilized V_1 material. The specific binding activities of [^{3}H]CH_3BrA to WGA protein from A6 cells and bovine papilla averaged 66.6 and 13.9 pmol/mg of protein, respectively (Table III), yielding 148- and 116-fold enrichments in binding. The nonspecific protein samples did not show any specific binding of [^{3}H]CH_3BrA.

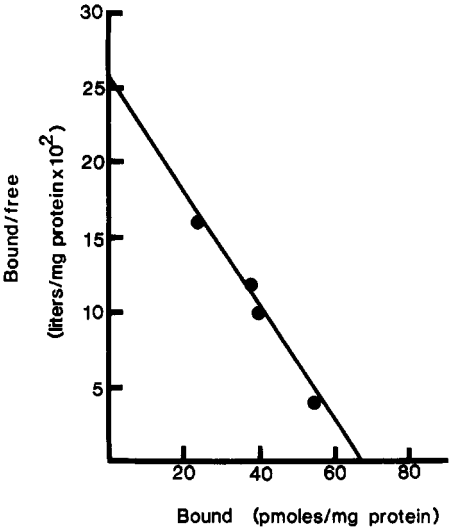

FIGURE 8: Scatchard plot of specific [^{3}H]CH_3BrA binding to WGA samples. Samples were incubated for 30 min with 0.075–0.7 μM of [^{3}H]CH_3BrA in the absence and presence of 0.1 mM amiloride. The K_D for binding was 128 nM, and the total number of binding sites was 66.6 pmol/mg of protein. This experiment is representative of five independent measurements.

Binding of [^{3}H]CH_3BrA to WGA-specific protein from both A6 and bovine papilla likewise reached equilibrium in 10–15 min at 0 °C. The specific binding of [^{3}H]CH_3BrA to WGA material from A6 cells also saturated with increasing drug concentration, and the K_D for binding, as determined by

Scatchard analysis, was 128 nM (Figure 8), a value in excellent agreement with that measured in V_1 (cf. Figure 6B). Comparable results were obtained using the WGA material originating from bovine papilla.

Discussion

The amiloride-binding and sodium-transporting components of the epithelial Na^+ channel from the A6 cultured toad kidney cell line have been solubilized. In addition, we have begun the characterization of the bovine kidney papilla as an alternative source of Na^+ channels to be used in the eventual purification of this protein.

The main limiting factor in the isolation of the epithelial Na^+ channel is the source material. For example, native amphibian tissues such as frog skin are multilayered epithelia comprised of heterogeneous cell types and are therefore unattractive for purification purposes. Dissection of individual renal tubules to obtain appropriate amounts of starting material is impractical. The culture of amphibian cells is advantageous because of both relative cell homogeneity and control of cell environment allowing for uniform starting preparations. The main problems with this technique are relatively low protein yield, high cost, and extensive laboratory space requirements.

In order to show amiloride-sensitive transport, A6 cells have to be grown on porous supports mounted as filter cups (Sariban-Sohraby et al., 1983). Because of the space and time constraints imposed by the manufacture of the filter cups and the handling of the cells, we could carry up to 40 cups plus the stock cultures in our laboratory. A6 cells grown on filter-bottomed cups (102 cm^2 per cup) yield 0.072 mg of total protein/cm^2 of confluent epithelium. By comparison, this starting material from 40 filters represents 0.033% of the amount of protein provided by one eel electric organ (Agnew et al., 1978) or 0.34% of brain homogenate from 30 rats (Hartshorne & Catterall, 1984) for isolation of the voltage-sensitive Na^+ channel. The contribution of the epithelial Na^+ channel protein to the apical plasma membrane total protein is unknown. Also, there is a complete lack of information concerning the existence of intracellular amiloride binding sites and/or Na^+ channels. Studies using either blocker molecule binding or current fluctuation analysis (noise) have attempted to quantify the density of the Na^+ channel in the apical membrane. Reversible (Cuthbert & Shum, 1975a,b; Aceves et al., 1979) as well as irreversible (Garvin et al., 1986) blockers used in binding studies in various intact amphibian tissues have yielded values for binding sites between 130 and 455 sites/μm^2 (see Table IV). Noise analysis estimation of the Na^+ channel density varied between 1 and 38 sites/μm^2 of tissue (Van Driessche & Lindemann, 1979; Christensen & Bindslev, 1982; Li et al., 1982; Zeiske et al., 1982; Loo et al., 1983). In intact A6 epithelia, the specific binding of [^{3}H]-CH_3BrA, an irreversible blocker molecule, upon ultraviolet light irradiation, was 0.22 pmol/mg of whole cell protein (unpublished observations). This value corresponds to 70 sites/μm^2 of epithelium, assuming a 1:1 molar binding ratio for the blocker molecule. However, this assumption may not be appropriate. Kinetic studies of the amiloride–sodium entry site interaction in frog skin epithelia have suggested multiple binding sites for amiloride on the basis of negative Hill coefficients (Benos et al., 1979). The discrepancy in channel density between binding and noise data could be explained by the fact that the electrical measurement reflects only the number of conducting channels, while binding may occur independent of the channel's functional state (i.e., conducting or nonconducting). However, it is not known whether amiloride can bind to both open and closed channels. Also in binding studies, especially when a reversible blocker is used, the number of sites could be overestimated because of incomplete correction for nonspecific binding.

Table IV: Na^+ Channel and Amiloride Binding Site Densities in Epithelia[a]

tissue	site density (sites/μm^2)	method of estimation	ref
hen coprodeum	38	noise analysis	Christensen and Bindslev, 1982
frog skin	15	noise analysis	Van Driessche and Lindemann, 1979
rabbit colon	6	noise analysis	Zeiske et al., 1982
toad urinary bladder	1	noise analysis	Li et al., 1982
rabbit urinary bladder	0.02	noise analysis	Loo et al., 1983
toad bladder	455	[^{3}H]phenamil binding	Garvin et al., 1986
frog skin	400	[^{14}C]amiloride binding	Cuthbert and Shum, 1975a
toad bladder cells	312	[^{14}C]amiloride binding	Cuthbert and Shum, 1975b
frog skin cells	130	[^{3}H]benzamil binding	Aceves et al., 1979
A6 cells	70	[^{3}H]CH_3BrA binding	this study

[a] For the noise results, site densities have been extrapolated to zero external [Na^+]. [^{3}H]Phenamil and [^{3}H]CH_3BrA were used as irreversible blockers while [^{14}C]amiloride and [^{3}H]benzamil were reversible.

In the detergent-soluble fraction, both [^{3}H]CH_3BrA binding and Na^+ transport activities are present. Our preliminary results indicate that the same is true for WGA material. We have shown that specific [^{3}H]CH_3BrA binding to the solubilized WGA material displays the same equilibrium dissociation constant ($\sim$130 nM) as that of V_1 material (Figure 8). We have not yet demonstrated that the soluble forms of the amiloride-binding protein and the sodium ion conducting protein are the same. However, amiloride inhibits the sodium conductance of intact A6 epithelia (Sariban-Sohraby et al., 1983) and of single Na^+ channels (derived from fraction V_1) incorporated into planar lipid membrane (Sariban-Sohraby et al., 1984c) with the same inhibitory constant as that determined by our equilibrium binding studies at the same Na^+ concentration. This result indicates that the transfer of the conducting unit into an artificial membrane is accompanied by the coupled transfer of the inhibitor binding site and strongly suggests that both binding and transport functions are intertwined, possibly because each subunit comprises part of a larger, single protein molecule. The similar values of K_D's for the soluble and membrane-bound receptor indicates that the detergent solubilization did not alter the conformation at the amiloride binding site.

Several authors have reported the requirement for exogenous phospholipids to stabilize detergent-solubilized channel proteins in a conformation capable of binding specific agonists or inhibitors, as well as to maintain functionality (Agnew & Raftery, 1979; Catterall et al., 1979; Barchi et al., 1980). Our results confirm the observation that phosphatidylcholine stabilizes the soluble receptor and also show that the loss of binding activity with high detergent to lipid concentration ratios is restored upon addition of phosphatidylcholine. This result indicates that the receptor has not been irreversibly denatured by the detergent but rather inactivated. The deleterious effect of high detergent concentrations on receptor activity has also been shown for CHAPS-solubilized opiate

receptors form cultured hybrid neuroblastoma–glioma cells (Simonds et al., 1980). We have also shown that the specific binding capacity of the CHAPS-solubilized protein was stable for at least 3 weeks at 4 °C (Table II).

An essential step toward purification of a membrane transport protein is solubilization with retention of biological activity. Detergent-solubilized V_1 proteins, upon reconstitution into phospholipid liposomes, still display amiloride-sensitive Na^+ transport activity. We have thus presented a procedure that allows the solubilization of the amiloride receptor in good yield and with maintenance of two major functions of epithelial sodium channels, namely, the amiloride-binding function and the sensitivity of sodium transport to amiloride inhibition. Further, affinity chromatography of this detergent-solubilized material onto agarose-immobilized wheat germ agglutinin resulted in retention of 46–100% of the total specific binding activity and produced a 116–148-fold enrichment of specific binding activity with respect to homogenate.

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Registry No. CHAPS, 75621-03-3; CH_3BrA, 98299-53-7; Na, 7440-23-5; Triton X-100, 9002-93-1; digitonin, 11024-24-1; octyl glucoside, 41444-50-2; sodium cholate, 361-09-1.

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