[^{3}H]Phenamil Binding Protein of the Renal Epithelium Na^+ Channel. Purification, Affinity Labeling, and Functional Reconstitution†

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ABSTRACT: This paper describes a large-scale purification procedure of the amiloride binding component of the epithelium Na^+ channel. [^{3}H]Phenamil was used as a labeled ligand to follow the purification. The first two steps are identical with those previously described [Barbry, P., Chassande, O., Vigne, P., Frelin, C., Ellory, C., Cragoe, E. J., Jr., & Lazdunski, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4836–4840]. A third step was a hydroxyapatite column. The purified material consisted of a homodimer of two 88-kDa proteins that migrated anomalously in SDS–PAGE to give an apparent M_r of 105 000. Deglycosylation by treatment with neuraminidase and endoglycosidase F or with neuraminidase and glycopeptidase F indicated that less than 5% of the mass of the native receptor was carbohydrate. Sedimentation analysis of the purified Na^+ channel in H_2O and D_2O sucrose gradients and gel filtration experiments led to an estimated molecular weight of the [^{3}H]phenamil receptor protein–detergent–phospholipid complex of 288 000 and of the native [^{3}H]phenamil receptor protein of 158 000. [^{3}H]Br-benzamil is another labeled derivative of amiloride that recognized binding sites that had the same pharmacological properties as [^{3}H]phenamil binding sites and that copurified with them. Upon irradiation of kidney membranes, [^{3}H]Br-benzamil incorporated specifically into a 185-kDa polypeptide chain under nonreducing electrophoretic conditions and a 105-kDa protein under reducing conditions. The same labeling pattern was observed at the different steps of the purification. Reconstitution of the purified phenamil receptor into large unilamellar vesicles was carried out. A low but significant phenamil- and amiloride-sensitive electrogenic Na^+ transport was observed.

Sodium reabsorption across tight epithelia is a two-step process, where Na^+ is first reabsorbed by the epithelial cells at the apical side via a Na^+-selective channel and is then excreted at the basolateral side by the (Na^+,K^+)ATPase. In the kidney, this coupled transport system is responsible for the final adaptation of Na^+ and water excretion by the distal and collecting tubules. It is under hormonal control by aldosterone and by vasopressin (Garty & Benos, 1988).

The diuretic drug amiloride is a potent blocker of epithelial Na^+ channels (Benos, 1982). Phenamil is a derivative of amiloride that is more potent than amiloride for inhibiting epithelium Na^+ channels (Asher et al., 1987; Barbry et al., 1986; Garvin et al., 1985). It has been radiolabeled and used to titrate epithelium Na^+ channels in pig kidney membranes (Barbry et al., 1987), in membranes of endothelial cells from brain microvessels (Vigne et al., 1989), in membranes of thyroid cells (Verrier et al., 1989), and in the toad urinary bladder (Garvin et al., 1986). The [^{3}H]phenamil receptor has been solubilized from pig kidney membranes by using the zwitterionic detergent CHAPS[1] and purified by a two-step procedure. The purified material consisted of a dimer of two identical subunits of apparent M_r = 105 000 (Barbry et al., 1987). On the other hand, Benos et al. (1986, 1987) reported a procedure for the purification of the amiloride receptor from toad bladder cells and from bovine kidney using [^{3}H]-methylbromoamiloride. Their preparation consisted of a 700-kDa complex composed of 300–315-, 149–180-, 95–110-, 71–85-, and 55–66-kDa polypeptide chains. Finally, Kleyman et al. (1986) affinity labeled the amiloride receptor with [^{3}H]Br-benzamil. They covalently labeled a series of proteins in bovine kidney with apparent M_r of 176 000, 77 000, and 47 000.

The experiments reported in this paper were specifically designed (i) to determine whether the [^{3}H]phenamil receptor is part of a large molecular weight complex, (ii) to identify the amiloride binding protein in photoaffinity labeling experiments using [^{3}H]Br-benzamil, and (iii) to show by using reconstitution experiments that the channel activity was retained in the purified material.

Experimental Procedures

Materials. 3,5-Diamino-6-chloro-*N*-[amino(phenylamino)methylene]pyrazinecarboxamide (phenamil), 3,5-diamino-6-chloro-*N*-[amino(benzylamino)methylene]pyrazinecarboxamide (benzamil), 3,5-diamino-6-bromo-*N*-[amino(benzylamino)methylene]pyrazinecarboxamide (Br-benzamil), amiloride, and [*phenyl*-3-^{3}H]Br-benzamil (36.6 mCi/mg) were kindly provided by Dr. E. J. Cragoe, Jr. (Merck, Sharp & Dohme). [*phenyl*-4-^{3}H]Phenamil (30 Ci/mmol) was synthesized as previously described (Barbry et al., 1989). When necessary, [^{3}H]phenamil and [^{3}H]Br-benzamil were diluted with unlabeled phenamil and Br-benzamil to obtain specific activities down to 2 Ci/mmol. *Bandeiraea simplicifolia* lectin, as a mixture of AB_3 and A_3B isoforms, coupled to Sephadex A-25 and QAE Sephadex A-25 were from Pharmacia. Hydroxyapatite was from IBF (France). Soybean phosphati-

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[1] Abbreviations: SBPC, soybean phosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TEA, triethanolamine; BS1, *Bandeiraea simplicifolia* lectin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IAA, iodoacetamide; MS, methanesulfonate; β-ME, β-mercaptoethanol.

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dylcholine (type II-S), dioleoyl-L-α-phosphatidylcholine, gramicidin, valinomycin, and D_2O were from Sigma. CHAPS was from Boehringer. Frozen pig kidneys were purchased from Collectorgane (Lyon, France).

[^{3}H]Phenamil and [^{3}H]Br-benzamil binding experiments to kidney membranes or to the solubilized receptor were carried out as described previously (Barbry et al., 1987). Nonspecific binding was determined in the presence of 10 μM unlabeled phenamil or unlabeled Br-benzamil. Proteins were determined according to Peterson (1977) or Bradford with reagents supplied by Bio-Rad. Bovine serum albumin was used as standard.

Purification of the Phenamil Receptor. All buffers used during the different steps were prepared with fresh Milli-Q water and filtered on 0.2-μm Sartorius filters before use. Protease inhibitors (1 mM IAA, 1 mM *o*-phenanthroline, 0.1 mM phenylmethanesulfonyl fluoride, and 1 μM pepstatin A) were added to all buffers during the purification.

Kidney membranes were prepared as described previously (Barbry et al., 1987) and solubilized in 0.5% CHAPS, 10% glycerol, 0.1% SBPC, and 20 mM TEA-HCl at pH 7.0. After a 1-h incubation at 4 °C, the mixture was spun at 32000 rpm on a 35TI Beckman rotor for 45 min. The supernatant was recovered and used immediately for the purification. The solubilized material (1.5 g of protein, 500 mL) was loaded onto a 500-mL QAE Sephadex column equilibrated in a low ionic strength buffer (0.05% CHAPS, 0.01% SBPC, 15 mM TEA-HCl at pH 7.0). The column was washed with 3 volumes of the equilibration buffer. Once absorbance at 280 nm had stabilized at the lowest value, the elution was initiated with a linear 0–200 mM NaCl gradient, and 10-mL fractions collected. Phenamil binding activity eluted as a single peak at conductivities of 4–8 mS. The active fractions (about 200 mL) were pooled and adjusted to 1 mM $CaCl_2$.

A 2-mL BS1 column equilibrated in 0.05% CHAPS, 0.01% SBPC, 1 mM $CaCl_2$, 50 mM NaCl, and 20 mM TEA-HCl buffer at pH 7.0 was loaded with the active material from the QAE Sephadex column. After washing with 100 mL of equilibration buffer, activity was eluted in a single step with 0.05% CHAPS, 0.01% SBPC, 150 mM NaCl, and 100 mM melibiose buffered at pH 7.0 with 20 mM TEA-HCl.

A suspension of hydroxyapatite was equilibrated in a blue tip with a 0.05% CHAPS, 0.01% SBPC, and 20 mM potassium phosphate buffer at pH 6.8. The active fractions from the BS1 column were applied to the column. After washing with 10 mL of equilibration buffer, the activity was eluted either in a single step with the same buffer supplemented with 250 mM potassium phosphate or with a linear 20–500 mM potassium phosphate gradient. The active fractions were dialyzed overnight at 4 °C against a phosphate-free solution (0.05% CHAPS, 0.01% SBPC, 50 mM NaCl, 20 mM TEA-HCl at pH 7.0) to eliminate phosphate. Phosphate was found to inhibit [^{3}H]phenamil binding to its receptor with a $K_{0.5}$ value of 10 mM. The purified material could be frozen at −70 °C and stored for several weeks. Upon thawing, no significant loss in binding activity and in properties was observed.

Iodination of the Purified Receptor. About 5 pmol of purified receptor in 200 μL was treated with 5 μCi of $Na^{125}I$ (Amersham, 2000 Ci/mmol), in an Eppendorf tube along the wall of which 0.1 μg of Iodogen (Pierce) had been dried. After 15 min of reaction at 4 °C, the mixture was loaded onto a 5-mL Sephadex G-50 column. The first peak of radioactivity eluting from the column was recovered.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoreses. SDS–PAGE was carried out by using 8% polyacrylamide gel slabs and an acrylamide to bis(acrylamide) ratio of 37.5. Samples were denatured in 75 mM Tris, 2% SDS, and 7.5% glycerol at pH 6.8 in the presence (reducing conditions) or the absence of 50 mM β-mercaptoethanol (nonreducing conditions). Electrophoresis was carried out at a constant current of 70 mA/1.5-mm slab, under permanent refrigeration. Electrophoretic mobility was calibrated by using standards of known molecular weight (Bio-Rad). Gels were stained with Coomassie blue or with Silver (Merril et al., 1981). Fergusson plot analyses were performed by using 4–15% polyacrylamide gradients poured perpendicularly to the axis of migration and overlayered with a stacking gel of 3% polyacrylamide. The purified receptor (200 pmol) and standard proteins were layered on the gel. Gels were stained with Coomassie blue. Quantification of the polyacrylamide percentage was evaluated by weighting constant areas of the dried gel. Data were analyzed according to Frank and Rodbard (1975).

Sucrose Gradient Sedimentation Analysis. Linear 20-mL sucrose gradients (5–15% w/v) were formed in H_2O or D_2O. The gradients contained 0.1% CHAPS, 0.02% SBPB, 50 mM NaCl, and 20 mM TEA-HCl at pH 7.0. Gradients were overlayered with 20 pmol of purified receptor or with a mixture of protein standards of known sedimentation behavior: cytochrome *c*, alkaline phosphatase (both from Boehringer), and catalase (Pharmacia). Gradients were centrifuged at 50000 rpm in a Beckman VTI50 rotor for 3 h. The gradients were fractionated from the bottom and fractions assayed for the specific [^{3}H]phenamil binding, catalase (A406), alkaline phosphatase (Cathala et al., 1975), and cytochrome *c* (A450).

Gel Filtration Analysis. An HPLC column (AP714, S-5, 300 Å, DIOL) (YMC Inc.) was equilibrated in 0.1% CHAPS, 1% glycerol, 200 mM NaCl, and 100 mM potassium phosphate at pH 6.8. The purified receptor (40 pmol) was injected and the column was eluted at a flow rate of 0.75 mL/min. Fractions (250 μL) were collected and assayed for the specific [^{3}H]phenamil binding. Calibration was achieved with standards of known Stokes radius (thyroglobulin, aldolase, catalase, bovine serum albumin, chymotrypsinogen A, ribonuclease A from Pharmacia, alkaline phosphatase, lactoperoxidase and cytochrome *c* from Boehringer). Data were analyzed as described by Ackers (1967) using partial specific volumes of 0.984 and 0.86 cm^3/g for phosphatidylcholine and CHAPS, respectively. The latter value was calculated from the partial specific volume of taurocholate according to Steele et al. (1978).

Enzymatic Deglycosylation. A purified and iodinated preparation of the [^{3}H]phenamil receptor protein was treated for 18 h at 37 °C with 2 IU/mL neuraminidase (Boehringer) in a 0.1 M sodium phosphate buffer at pH 5.5 supplemented with 1% CHAPS, 0.1% SDS, 20 mM ethylenediaminetetraacetic acid, and protease inhibitors. Half of the reaction mixture was then treated with 2.4 IU/mL endoglycosidase F (Boehringer) for an additional 18 h at 37 °C. The other half of the reaction mixture was adjusted to pH 7.4 and treated with 5 IU/mL glycopeptidase F (Boehringer) for 3 h at 37 °C. The reaction mixtures were analyzed by SDS–PAGE under reducing or nonreducing conditions. Gels were dried and exposed with Kodak X-Omat AR films at −70 °C and revealed after 3 days of exposition.

Photolabeling with [^{3}H]Br-benzamil. Pig kidney membranes (10–15 pmol of phenamil receptor) or purified preparations of the [^{3}H]phenamil receptor (10–15 pmol) were equilibrated with 100 nM [^{3}H]Br-benzamil for 1 h in dim light. Samples were then irradiated for 20 s by a high-intensity

UV light with a 2000-W mercury lamp (Philips HP2000) at a distance of 20 cm. Wavelengths of less than 300 nm were cut off with a protective quartz filter. The yield of covalent binding was increased by reducing the optic way through the sample to 1 mm. Samples were analyzed by SDS–PAGE under reducing or nonreducing conditions. Gels were treated for 30 min with 1 M sodium salicylate, dried, and exposed to Kodak X-Omat AR films with a Du Pont Cronex intensifying screen for 30 days at −70 °C. In other experiments, gels were sliced and slices digested with 10 μg/mL proteinase K (Boehringer) in a 25 mM $(NH_4)HCO_3$ buffer at pH 8.2. After a 24-h incubation at 37 °C, 4 mL of Aquassure (NEN) was added and the radioactivity counted.

Reconstitution into Lipid Vesicles. Large unilamellar vesicles were prepared according to Szoka and Papahadjopoulos (1978) using dioleoyl-L-α-phosphatidylcholine. The purified receptor was inserted into liposomes as previously described (Rigaud et al., 1983). The aqueous phase was 0.2 M mannitol and 50 mM Na^+-MS (or 50 mM K^+-MS) buffered at pH 8.0 with 20 mM Tris–MS. Vesicles were dialyzed overnight at 4 °C or diluted into a large volume of aqueous phase, centrifuged at 100000*g* for 1 h, and then resuspended into 0.5 mL of aqueous phase. [^{3}H]Phenamil binding experiments indicated that 20–25% of the receptors were reinserted in the correct orientation.

Flux experiments were performed following the protocol described by Garty et al. (1983). Briefly, $^{22}Na^+$ uptake was measured against a large Na^+ gradient ($[Na^+]_i$ = 50 mM, $[Na^+]_o$ < 1 mM) or against a large K^+ gradient ($[K^+]_i$ = 50 mM, $[K^+]_o$ < 1 mM, in the presence of 1 μM valinomycin). The accumulation of tracer amounts of $^{22}Na^+$ is, under both conditions, a measure of the electrical diffusion potential that has been imposed across the membrane (Garty et al., 1983). High pH values were chosen to favor an open state of the Na^+ channel (Harvey et al., 1988; Palmer & Frindt, 1987).

To remove extravesicular Na^+ (or K^+), 30-μL aliquots of the vesicle suspension were diluted into 1.2 mL of external buffer (0.3 M mannitol buffered at pH 8.0 with 20 mM Tris–MS) and applied to a Dowex AG50W-X8 column equilibrated in 0.3 M mannitol and 20 mM Tris–MS at pH 8.0. The column was centrifuged at 1000*g* for 10 s. No change in extravesicular pH was observed after passage on the Dowex column. The eluted material was recovered, and 10 μCi of carrier-free $^{22}NaCl$ was added to initiate the uptake. When phenamil was used, it was added 1 min before $^{22}Na^+$. At the end of the incubation period at 37 °C, 200-μL aliquots were filtered on the Dowex resin as described above to remove extravesicular $^{22}Na^+$, and the eluate was counted. The accumulation ratio of $^{22}Na^+$ was defined by comparing the amount of $^{22}Na^+$ accumulated inside the vesicle to that accumulated by vesicles incubated for 10 min in a 50 mM Na^+ medium in the presence of 1 μM gramicidin. Means ± SDM are indicated.

Results

Purification of the Phenamil Receptor. The first two steps of the purification of the [^{3}H]phenamil receptor were performed essentially as previously described (Barbry et al., 1987) except that all solutions used were supplemented with 0.1% soybean phosphatidylcholine. The scaling up of the purification procedure while keeping high yields was achieved at the expense of the final purity. SDS–PAGE analysis under reducing conditions of the material recovered after the first two steps of purification (Figure 1) showed that the 105-kDa protein which is the putative [^{3}H]phenamil receptor protein (Barbry et al., 1987) represented about 50% of the total

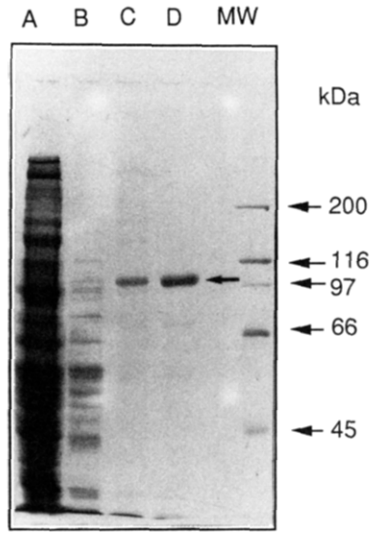

FIGURE 1: SDS–PAGE patterns of the [^{3}H]phenamil receptor at different stages of the purification. Fractions resulting from the different steps of the same purification were analyzed under reducing conditions. The different lanes are as follows: solubilized material (lane A), QAE Sephadex purified material (lane B), BS1-purified material (lane C), and hydroxyapatite-purified material (lane D). Molecular weight markers (MW) are myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). The gel was stained with Coomassie blue.

proteins. Other bands with apparent M_r of 170000, 140000 (diffuse band), 70000, 80000, and 30000–40000 were the most commonly observed contaminants in our preparations. They may be analogous to the polypeptide chains described by Benos et al. (1987) in their preparation of the epithelium Na^+ channel from bovine kidney. We found however that these different proteins appeared in variable amounts in different preparations and that the specific [^{3}H]phenamil binding activity correlated best with the presence of the major 105-kDa protein. For instance, the gel pattern presented in lane C of Figure 1 mainly shows the presence of high molecular weight proteins, whereas the gel pattern obtained in another preparation and presented in Figure 2 shows that the major contaminating protein was a smaller 40-kDa band.

Final purification of the [^{3}H]phenamil receptor was achieved by using a third chromatographic step which consisted of a hydroxyapatite column. The lower panel of Figure 2 shows SDS–PAGE patterns of the different fractions that have been eluted from an hydroxyapatite column using a linear phosphate gradient. It shows that both high and low molecular weight contaminants eluted off the column before the 105-kDa protein. The only faint bands that copurified with the 105-kDa band had apparent M_r of 70000 and 80000. Fingerprint analyses of tryptic digests of electroeluted 70- and 80-kDa bands indicated however these two bands were likely degradation products of the 105-kDa band (not shown). In agreement with this finding, we observed that the relative importance of the 70- and 80-kDa proteins increased upon storage of the purified preparations. The upper panel of Figure 2 compares the elution profiles of the 105-kDa protein and of the [^{3}H]phenamil binding activity. It shows that the two activities coeluted from the hydroxyapatite column.

Deglycosylation Experiments. Deglycosylation of the purified [^{3}H]phenamil receptor was achieved with neuraminidase and glycopeptidase F, or with neuraminidase and endoglycosidase F. Figure 3 shows that the two treatments decreased the apparent molecular weight of the major protein from 182000 to 176000 when SDS–PAGE was carried out

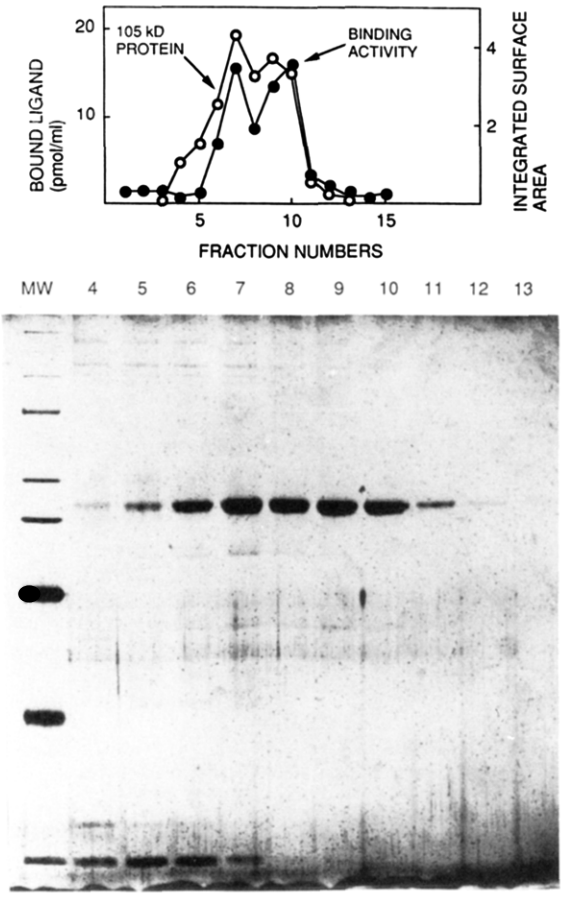

FIGURE 2: Copurification of the 105-kDa protein and of the [^{3}H]-phenamil receptor during hydroxyapatite chromatography. BS1-purified material was loaded on a hydroxyapatite column, and the column was eluted with a linear 20–500 mM phosphate gradient. Fractions were assayed for [^{3}H]phenamil binding and analyzed by SDS–PAGE after reduction with β-mercaptoethanol. The lower panel shows SDS–PAGE patterns obtained after silver staining. Lanes shown are molecular weight standards (MW) and fractions 4–13 eluted from the hydroxyapatite column. The upper panel compares the elution profiles of the [^{3}H]phenamil specific binding activity (●) and of the 105-kDa protein (○). The 105-kDa protein in each lane was quantitated by scanning the gel with a LKB densitometer. [[^{3}H]-Phenamil] was 10 nM.

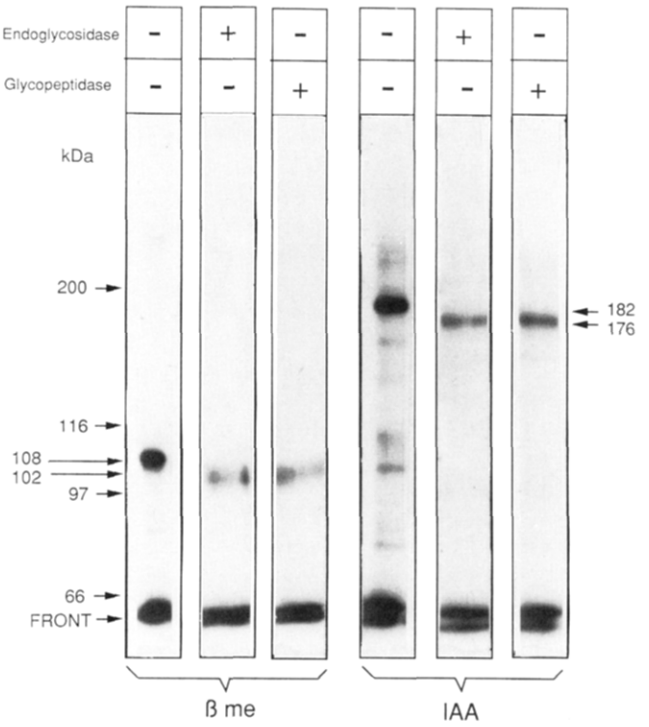

FIGURE 3: SDS–PAGE patterns of the [^{3}H]phenamil receptor after enzymatic deglycosylation. The purified and iodinated [^{3}H]phenamil receptor was treated with neuraminidase plus glycopeptidase F or with neuraminidase plus endoglycosidase F as indicated on the top of the lanes. Left lanes: Reducing conditions (β-ME). Right lanes: Nonreducing conditions (IAA).

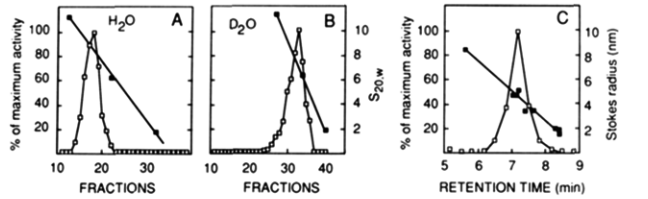

FIGURE 4: Sedimentation profiles of the purified [^{3}H]phenamil receptor on 5–15% sucrose gradients. Distribution of the [^{3}H]phenamil binding activity on 5–15% sucrose gradients in H_2O (panel A) and D_2O (panel B). The symbol (■) shows the sedimentation behavior of standard proteins: catalase, alkaline phosphatase, and cytochrome *c*. Panel C: Elution profile of the [^{3}H]phenamil binding activity from a DIOL HPLC column. The retention times for the standard proteins are indicated.

under nonreducing conditions and from 105 000 to 102 000 when it was performed under reducing conditions. These results show (i) that both the 185- and 105-kDa proteins were glycosylated and (ii) that sugars represented at least 5% of their mass.

Fergusson Analysis. Fergusson analysis was used to obtain a precise estimate of the molecular weight of the [^{3}H]phenamil receptor. Fergusson plots for the 105-kDa protein extrapolated to a relative migration at 0% acrylamide that was intermediate between that of myosin and that of other standard proteins. A plot of the retardation coefficient of each of the standard proteins as a function of their molecular weights indicated a molecular weight of the phenamil receptor of 88 000 (not shown).

Sedimentation and Gel Filtration Analysis. The size of the nondenatured phospholipid–detergent–receptor complex was estimated from sedimentation experiments in H_2O and in D_2O sucrose gradients. Figure 4A,B shows that the [^{3}H]phenamil binding activity sedimented as a single peak in both conditions. The true partial specific volume of the [^{3}H]phenamil receptor protein–detergent micelle was calculated according to Clarke (1975) and found to be 0.806 ± 0.016 cm^3/g. Using this value, the corrected $s_{20,w}$ was 8.72 ± 0.07 S (N = 2).

Gel filtration analysis of the phospholipid–detergent–[^{3}H]phenamil receptor protein complex was performed on a DIOL HPLC column. When a hydroxyapatite-purified preparation of the [^{3}H]phenamil receptor was injected, the binding activity eluted as a single peak with the major peak of proteins (Figure 4C). The effective Stokes radius of the phospholipid–detergent–protein complex was 5.7 ± 0.2 nm (N = 2). Combining this value to the data from centrifugation studies yields an estimate of the molecular weight of the complex of 288 000. The partial specific volume of micelles composed of 5:1 mixture of CHAPS and SBPC (Hartshorne et al., 1980) being 0.88 cm^3/g and that of most proteins being 0.73 ± 0.02 cm^3/g, we calculated that the complex was composed of 55% protein and 45% CHAPS + phospholipids. Accordingly, the molecular weight of the native [^{3}H]phenamil receptor was 158 000 ± 17 000.

Properties of the [^{3}H]Br-benzamil Binding Site. Br-benzamil is another derivative of amiloride that has previously been used to label the epithelium Na^+ channel in toad bladder and bovine kidney membranes (Kleyman et al., 1986). Figure

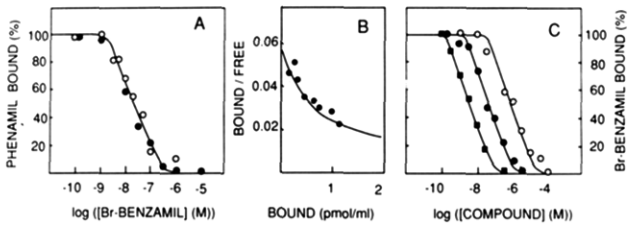

FIGURE 5: Properties of interaction of Br-benzamil with the epithelium Na^+ channel. Panel A: Inhibition by unlabeled Br-benzamil of the specific [^{3}H]phenamil binding to pig kidney membranes (○) and to a purified preparation of the epithelial Na^+ channel (●). [[^{3}H]-Phenamil] = 0.07 nM. Panel B: Scatchard plot for the specific [^{3}H]Br-benzamil binding to a purified preparation of the epithelium Na^+ channel. The line shows the relationship that would have been obtained if [^{3}H]Br-benzamil labeled two families of independent binding sites with the following parameters: K_{d1} = 10 nM, B_{max1} = 0.4 nmol/mg, K_{d2} = 400 nM, and B_{max2} = 7.1 nmol/mg. B_{max} values were calculated from [^{3}H]phenamil binding experiments performed on the same preparation. Panel C: Inhibition by amiloride derivatives of the specific [^{3}H]Br-benzamil binding to a purified preparation of the epithelium Na^+ channel. Compounds used are phenamil (■), benzamil (●), and amiloride (○). [[^{3}H]Br-benzamil] was 14 nM.

5A shows that unlabeled Br-benzamil prevented [^{3}H]phenamil binding to pig kidney membranes and to the BS1-purified material with a $K_{0.5}$ value at 25 nM. Since experiments were performed at a [^{3}H]phenamil concentration (0.07 nM) that was lower than the value of the dissociation constant of the high-affinity phenamil–receptor complex (0.8 nM; Barbry et al., 1989), the K_d value of the Br-benzamil–receptor complex is close to the $K_{0.5}$ value, i.e., 25 nM. As a comparison, the K_d value for benzamil interaction with the high-affinity phenamil binding site of pig kidney membranes is 40 nM (Barbry et al., 1989).

[^{3}H]Br-benzamil was found to bind specifically to kidney membranes with a maximum binding capacity (2–4 pmol/mg of protein) comparable to that of [^{3}H]phenamil. No evidence for a number of binding sites as large as 130 pmol/mg of protein (Kleyman et al., 1986) could be obtained. The [^{3}H]Br-benzamil specific binding activity was retained at all steps of the purification procedure that led to a highly enriched preparation of the [^{3}H]phenamil receptor. Figure 5B shows a Scatchard plot for the specific [^{3}H]Br-benzamil binding to the purified material. A linear regression of the data, which assumed the presence of a single class of independent binding sites, gave apparent K_d and B_{max} values of 38 nM and 1.7 pmol/mL, respectively. Binding isotherms may however be more complex. We showed previously that [^{3}H]phenamil, benzamil, and amiloride recognized two families of binding sites in pig kidney membranes and that the two binding sites are associated to the same protein structure in purified Na^+ channel preparations (Barbry et al., 1989). In the same purified material as the one used for [^{3}H]Br-benzamil binding experiments, the Scatchard plot for the specific [^{3}H]phenamil binding was curvilinear. It was resolved into two linear components with parameters K_{d1} = 0.8 nM, B_{max1} = 0.4 nmol/mg, K_{d2} = 18 nM, and B_{max2} = 7.1 nmol/mg (not shown). Figure 5B presents the curvilinear Scatchard plot obtained by using the values K_{d1} = 10 nM, K_{d2} = 400 nM, B_{max1} = 0.4 nmol/mg, and B_{max2} = 7.1 nmol/mg, i.e., by assuming that [^{3}H]Br-benzamil recognized the two phenamil binding sites with affinities of 10 nM and 400 nM. It shows that our data on [^{3}H]Br-benzamil binding are also consistent with a two-site model. Defining whether a one-site model (linear Scatchard plot) or a two-site model (curvilinear Scatchard plot) is more appropriate to fit the [^{3}H]Br-benzamil binding data would require a series of detailed experiments such as those that have been performed with [^{3}H]phenamil (Barbry et al., 1989).

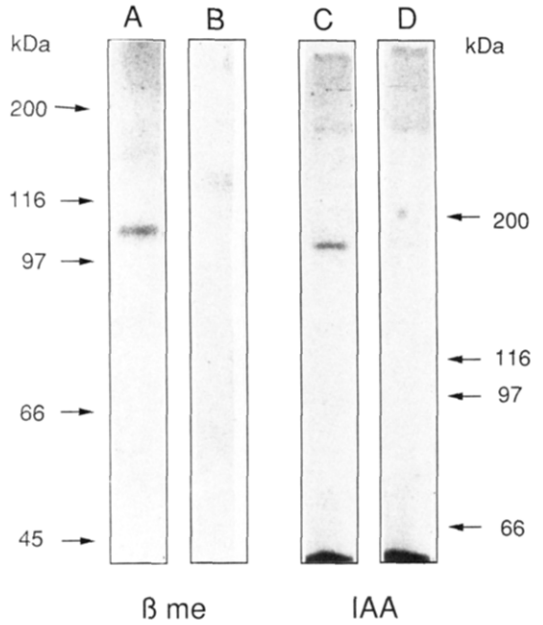

FIGURE 6: Photolabeling of the amiloride binding site with [^{3}H]-Br-benzamil in kidney membranes. Nonspecific labeling (lanes B and D) was obtained by using 10 μM unlabeled phenamil. The migration of molecular weight standards is indicated. Lanes A and B: Reducing conditions (β-ME). Lanes C and D: Nonreducing conditions (IAA).

Such experiments were not carried out with [^{3}H]Br-benzamil for very high concentrations of the ligand (up to 1 μM) would have to be used in order to saturate a large fraction of the low-affinity binding sites. At such high concentrations, reliability in the data is limited by the presence of a high nonspecific binding component.

Figure 5C defines the pharmacological profile of the [^{3}H]Br-benzamil binding site. In the purified material, [^{3}H]Br-benzamil binding was prevented by different amiloride derivatives with the following order of potency: phenamil ($K_{0.5}$ = 2 nM) > benzamil ($K_{0.5}$ = 30 nM) > amiloride ($K_{0.5}$ = 1 μM). Since the K_d value for the [^{3}H]Br-benzamil–receptor complex is 10–25 nM (Figure 5A,B) and since a low concentration of the ligand (14 nM) was used in these experiments, $K_{0.5}$ values are close (within a factor of 2) to the true K_d values. As a comparison the K_d values for phenamil, benzamil, and amiloride binding to the high-affinity [^{3}H]-phenamil binding site are 0.8 nM, 40 nM, and 0.5 μM (Barbry et al., 1989). The good agreement between the two series of K_d values indicates that [^{3}H]phenamil and [^{3}H]Br-benzamil share a common site on the epithelium Na^+ channel.

Photolabeling Experiments Using [^{3}H]Br-benzamil. The results of photolabeling experiments using [^{3}H]Br-benzamil are shown in Figures 6 and 7. SDS–PAGE autoradiograms of irradiated membranes show that [^{3}H]Br-benzamil specifically labeled only one 105-kDa band under reducing conditions (Figure 6, lanes A and B) which migrated as a 185-kDa band under nonreducing conditions (Figure 6, lanes C and D). The radioactivity profiles of sliced gels presented in Figure 7 show that an identical result was obtained with QAE Sephadex purified material (Figure 7A) and with BS1-purified material (Figure 7B).

Reconstitution of the Na^+ Channel Activity into Lipid Vesicles. The purified amiloride binding site was reconstituted into large unilamellar vesicles, and its activity was assayed by the $^{22}Na^+$ flux technique developed by Garty et al. (1983). Figure 8A shows that when a large outward Na^+ gradient was imposed to vesicles that had incorporated the purified Na^+

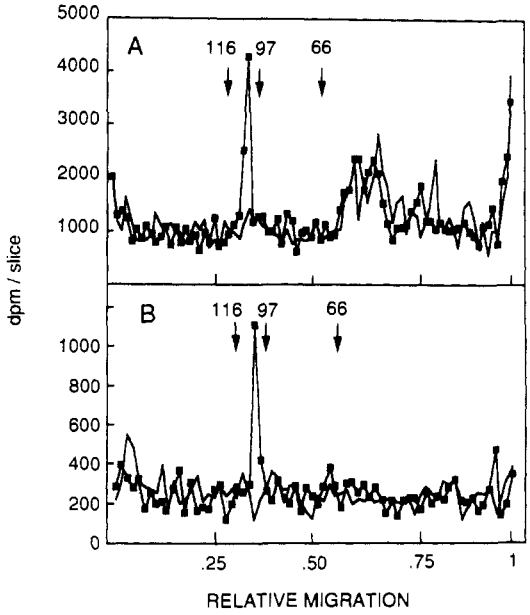

FIGURE 7: Photolabeling of the amiloride binding site with [^{3}H]-Br-benzamil at different stages of the purification of the receptor. Panel A: QAE Sephadex purified material. Panel B: BS1-purified material. The symbol (■) represents the total labeling. Nonspecific labeling, obtained with 10 μM unlabeled phenamil, is indicated by the continuous line. For reasons of clarity, experimental points corresponding to the nonspecific labeling have been omitted. Electrophoreses were performed under reducing conditions.

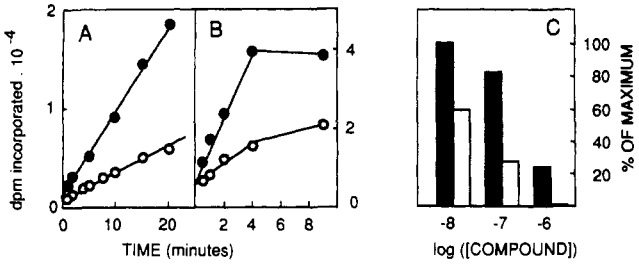

FIGURE 8: Reconstitution of Na^+ transport activity in large unilamellar vesicles. Panel A: Time course of $^{22}Na^+$ uptake in vesicles containing the reconstituted Na^+ channel in the presence of a Na^+ diffusion potential. $[Na^+]_i$ = 50 mM; $[Na^+]_o$ < 1 mM. Experiments were performed in the absence (●) or the presence (○) of 10 μM phenamil. Each point is the mean of two or three determinations performed on the same preparation. Panel B: Time course for $^{22}Na^+$ uptake in vesicles containing the reconstituted Na^+ channel in the presence of a K^+ diffusion potential ($[K^+]_i$ = 50 mM, $[K^+]_o$ < 1 mM) imposed by 1 μM valinomycin. Experiments were performed in the absence (●) or the presence (○) of 10 μM phenamil. Each point is the mean of two determinations performed on the same preparation. Panel C: Dose–response curves for phenamil (open bars) and amiloride (filled bars) inhibition of the $^{22}Na^+$ uptake by vesicles containing the reconstituted Na^+ channel. Experiments were performed in the presence of an outward Na^+ gradient. Activity was defined from the linear portion of the uptake and by using at least four data points.

channel, $^{22}Na^+$ uptake proceeded linearly for at least 20 min. Phenamil or amiloride (10 μM) reduced the $^{22}Na^+$ flux component by about 50% (Figure 8A). In typical experiments, a net accumulation ratio of $^{22}Na^+$ of up to 3 was observed. A similar accumulation ratio was obtained with 0.1 nM gramicidin. Larger concentrations of gramicidin (1 μM) produced much larger $^{22}Na^+$ fluxes with an accumulation ratio of up to 60. The $^{22}Na^+$ flux component induced by gramicidin was insensitive to 10 μM phenamil.

Experiments were also performed in the presence of a K^+ diffusion potential induced by 1 μM valinomycin to show that $^{22}Na^+$ accumulated in response to the membrane potential that had been imposed. Figure 8B shows that, under these conditions, a phenamil-sensitive $^{22}Na^+$ flux component was also observed. This phenamil-sensitive $^{22}Na^+$ flux component was completely abolished when the extravesicular K^+ concentration was raised to 50 mM, i.e., under conditions in which no electrical potential developed across the membrane. Finally, Figure 8C shows that phenamil was more potent than amiloride for inhibiting $^{22}Na^+$ uptake. $K_{0.5}$ values were observed at 20 nM and 0.3 μM for phenamil and amiloride, respectively. These values are in good agreement with the K_d values determined in [^{3}H]phenamil binding experiments.

Discussion

This paper describes a large-scale procedure for the purification of the epithelium Na^+ channel using [^{3}H]phenamil as a labeled ligand and defines some of the properties of the purified protein. In pig kidney, the binding site for amiloride and its derivatives is a glycosylated, 88-kDa protein that migrates in SDS–PAGE with an apparent M_r of 105 000. Under nonreducing conditions, it exists as an 180–185-kDa homodimer. An important point is that, in our experiments, the [^{3}H]phenamil receptor protein did not appear to be part of a large molecular weight complex composed of several polypeptide chains cross-linked by disulfide bridges such as the one described by Benos et al. (1986, 1987). We do find, in partially purified preparations, the presence of a number of protein bands that have apparent molecular weights similar to those of Benos et al. (1987). We observed however that these polypeptide chains were not all present in the different preparations that have been performed. This means that the stoichiometry of these different proteins in a putative large molecular weight complex was not constant. We also found that hydroxyapatite chromatography removed all these additional proteins except for two 70- and 80-kDa bands that were probable degradation products of the major 105-kDa protein. Since the hydroxyapatite chromatography was performed without reduction with β-mercaptoethanol, the most obvious conclusion is that the contaminating polypeptide chains that were still present at the end of the BS1 purification step were not covalently linked to the major 185/105-kDa protein. In addition, our previous sedimentation experiments (Barbry et al., 1987) and the more detailed analyses using H_2O and D_2O sucrose gradients as well as the gel filtration experiments reported in this paper are fully consistent with the idea that the amiloride binding site is not part of a large (>700 000) molecular weight complex. In fact, all the new experiments reported here support our previous conclusion that the nonreduced phenamil binding protein is a homodimer of two 105-kDa proteins (Barbry et al., 1987). It could be that the large molecular weight complexes observed by Benos et al. (1986, 1987) are a consequence of the storage of the solubilized membranes or of partially purified preparations. In all our experiments we took care to avoid delays between the solubilization of the membranes and the different steps of the purification.

Three independent pieces of evidence indicated that the 105-kDa monomer that had been purified was the binding site for diuretics of the amiloride family. First, the maximum specific [^{3}H]phenamil binding activity obtained was 8 nmol/mg of protein (Barbry et al., 1989). The maximum specific [^{3}H]phenamil binding activity expected for one [^{3}H]phenamil binding site per 88-kDa protein would be 11.4 nmol/mg of protein. This suggests that the purified material was >70% pure. On SDS–PAGE of the purified protein, the 105-kDa protein represented >90% of the total integrated stain intensity. Considering that the first estimate depends upon the assumption that the proteins were accurately measured

by the protein determination used, these results are consistent with the hypothesis that the 105-kDa protein is the receptor site for [^{3}H]phenamil. A second and more direct evidence is that the 105-kDa protein and the [^{3}H]phenamil binding activity coeluted from the hydroxyapatite column when a linear phosphate gradient was applied (Figure 2).

A final evidence has been obtained by direct photoaffinity labeling of the binding protein with [^{3}H]Br-benzamil. We first show (i) that the [^{3}H]Br-benzamil binding protein can be purified by using the same procedure as the one used for the purification of the [^{3}H]phenamil binding protein and (ii) that the different amiloride derivatives recognize the benzamil binding site of the Na^+ channel with properties identical with their properties of interaction with the phenamil binding site (Figure 5). Affinity labeling experiments presented in Figures 6 and 7 clearly show that [^{3}H]Br-benzamil specifically labeled a 185-kDa protein that migrated as a 105-kDa protein after reduction of disulfide bridges. The same result was obtained with kidney membranes (Figure 6) and preparations of the binding protein at the different stages of the purification (Figure 7). All together, these results indicate that the 105-kDa protein is indeed the protein that binds diuretics of the amiloride family.

Demonstrating the functional integrity of the purified epithelium Na^+ channel is difficult for two reasons. First there is no activator of epithelium Na^+ channels that would stabilize an open conformation of the channel such as batrachotoxin or veratridine, which have been used for the voltage-dependent Na^+ channel of excitable tissues (Hartshorne et al., 1985; Tanaka et al., 1983), or BayK8644 for the L-type Ca^{2+} channels (Curtis & Catterall, 1986). The second reason is that the activity of the epithelium Na^+ channel is regulated by a variety of as yet unknown mechanisms (Garty & Benos, 1988). For instance, electrophysiological experiments revealed the presence of epithelium Na^+ channels in renal collecting tubules only if animals had been maintained on a low-Na^+ diet (Palmer & Frindt, 1986a,b). In contrast, high aldosterone levels are not necessary to show the presence of a specific [^{3}H]phenamil binding component in kidney membranes. This suggests that [^{3}H]phenamil probably titrates both physiologically active and inactive forms of the channel.

This work shows that when purified phenamil receptor preparations were reincorporated into large unilamellar vesicles, an electrogenic Na^+ transport could be demonstrated (Figure 8). This Na^+ transport was inhibited by both amiloride and phenamil, phenamil being more potent than amiloride (Figure 8C). The relatively low transport capacity of the purified and reconstituted material could mean that, as suggested above, only a few of the reconstituted 105-kDa proteins were in an active form. It could also mean that the transport activity necessitates another protein than the 185/105-kDa protein which would be present in minor quantities in the preparation.

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Registry No. Na, 7440-23-5; phenamil, 2038-35-9; amiloride, 2609-46-3; Br-benzamil, 98057-31-9.

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