Biochemical Identification of Two Types of Phenamil Binding Sites Associated with Amiloride-Sensitive Na^+ Channels†

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ABSTRACT: The existence of distinct forms of the epithelium Na^+ channel that differ in their sensitivity to amiloride has been repeatedly suggested by physiological data. The biochemical basis for these differences was analyzed by using phenamil, the most potent inhibitor known so far for the epithelium Na^+ channel. [^{3}H]Phenamil of high radioactive specific activity (30 Ci/mmol) was prepared and used to titrate [^{3}H]-phenamil binding sites in pig kidney membranes. Kinetic experiments, equilibrium binding studies, and competition experiments indicated the presence in crude membrane preparations of two classes of independent binding sites. A first binding site was characterized by a high affinity for phenamil (K_{d1} = 0.4 nM) and for amiloride (K_{d1} = 0.1 μM). A second binding site recognized phenamil and amiloride with lower affinities [K_{d2}(phenamil) = 28 nM, K_{d2}(amiloride) = 4 μM]. The ratio of the respective amounts of low- and high-affinity binding sites was 14 ± 2 in different membrane preparations (range: 6–22). The two types of binding sites for [^{3}H]phenamil copurified and were still observed after purification of the epithelium Na^+ channel to homogeneity. These results indicate that at least two types of pharmacologically distinguishable Na^+ channels exist in the kidney. They correspond either to two isoforms of the apical Na^+ channel or to one single type of channel under two different states of covalent regulation.

Amiloride is a K^+-sparing diuretic that exerts its action by blocking Na^+ channels in the apical membrane of renal distal and collecting tubules (O'Neil & Boulpaep, 1979). Amiloride also inhibits Na^+ transport across a number of tight epithelia such as the frog skin, toad urinary bladder, and the colon in higher vertebrates (Bentley, 1968; Benos, 1982). Apical Na^+ channels have been extensively studied by using electrophysiological techniques (Lindemann, 1984; Sariban-Sohraby & Benos, 1986a). It is only recently that a biochemical approach has provided information on their molecular structure (Barbry et al., 1987; Benos et al., 1987).

In amphibian epithelia, EC_{50} values for amiloride blockade of the short circuit current are comprised between 50 and 500 nM (Benos, 1982). In some species of frog skin, the Hill coefficient describing the interaction of amiloride with the Na^+ channel is markedly lower than 1 (Benos et al., 1979), suggesting that there may be different types of Na^+ channel with different affinities for amiloride or that negative cooperative interactions between amiloride binding sites exist within a single class of apical Na^+ channels. It has also been shown recently that the amiloride-sensitive electrogenic $^{22}Na^+$ flux across vesicles from toad bladder (Garty, 1984; Asher et al., 1987) and from mammalian kidney (Kleyman et al., 1986; Barbry et al., 1986) can be separated into two components that differ in their sensitivity to amiloride and its derivatives. Finally, Na^+ channels that have a low affinity for amiloride (EC_{50} > 1 μM) have recently been observed in lamb lung (Olver et al., 1986), in late proximal tubule of the rabbit (Gogelein & Greger, 1986), and in toad urinary bladder (Garty et al., 1987).

The radiolabeled amiloride derivatives that have been used to identify Na^+ channels had a relatively low specific radioactivity (1–3 Ci/mmol) (Cuthbert & Edwardson, 1981; Kleyman et al., 1986; Barbry et al., 1986; Lazorick et al., 1985; Sariban-Sohraby et al., 1986b). These ligands have not revealed the presence of multiple amiloride binding sites. We describe in this paper the synthesis of [*phenyl*-4-^{3}H]phenamil of high specific radioactivity (30 Ci/mmol) and its use to titrate phenamil binding sites in pig kidney membranes. Phenamil is the most potent amiloride derivative known to inhibit the epithelium Na^+ channel (Garvin et al., 1985; Barbry et al., 1986; Asher et al., 1987; Ehrenfeld et al., 1987). The results indicate the presence in pig kidney membranes of both high- and low-affinity binding sites for amiloride and phenamil. The two types of binding sites copurify and are likely to be associated with identical or very similar protein structures.

Experimental Procedures

Materials. Amiloride [3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazinecarboxamide] and phenamil [3,5-diamino-6-chloro-*N*-[amino(phenylamino)methylene]pyrazinecarboxamide] were synthesized as previously described (Cragoe et al., 1967). QAE Sephadex A-25 and Sephadex G-50 were purchased from Pharmacia. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)[1] was from Boehringer Mannheim. *Bandeiraea simplicifolia* lectin coupled to Sephadex A-25 was generously provided by Prof. F. Delmotte (CNRS, Orléans, France).

Synthesis of [phenyl-4-^{3}H]Phenamil. The synthesis started with (iodophenyl)amiloride. (4-Iodophenyl)guanidinium was prepared according to McKee (1901) and methyl 3,5-diamino-6-chloropyrazinecarboxylate according to Cragoe et al.

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TEA, triethanolamine.

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(1967). The two compounds were condensed into 4-iodophenamil [3,5-diamino-6-chloro-*N*-[amino[(4-iodophenyl)amino]methylene]pyrazinecarboxamide] according to the procedure described by Cragoe et al. (1967) for the preparation of phenamil. The product was taken out of the reaction mixture by using chloroform. The organic phase was extracted with 10% aqueous HCl and the product precipitated by addition of NaOH. Recrystallization was conducted in dimethylformamide/water. The structure of synthesized compound was checked by IR, ^1H NMR, and mass spectrometry.

[*phenyl*-4-^3H]Phenamil was prepared from (iodophenyl)amiloride. (Iodophenyl)amiloride (15 nmol) was reacted for 30 min with 25 Ci (0.92 TBq) of tritium gas in the presence of 13 mg of 5% palladium catalyst. The reacting solution was filtered on Millex SR (0.5-μm) filters, and labile ^3H atoms were removed by dilution into methanol to yield 310 mCi (11.47 GBq) of product. The final product was purified by thin-layer chromatography using acetonitrile/ethanol (70/30) as solvent. After the extraction, 57 mCi (2.1 GBq) of [*phenyl*-4-^3H]phenamil was obtained. Specific radioactivity was 30 Ci/mmol (1.11 TBq/mmol). Radiochemical purity was checked after HPLC on C-18 inverse phase using acetonitrile/water/trifluoroacetic acid (20/80/0.1) as eluent. It was 98.4% pure. In some experiments, [*phenyl*-4-^3H]phenamil was diluted with unlabeled phenamil to obtain specific radioactivities ranging from 2.8 to 30 Ci/mmol.

Binding Assay. Pig kidney membranes were prepared as previously described (Barbry et al., 1986). For binding assays, kidney membranes were incubated at 4 °C in a solution consisting of 1 mM EDTA and 15 mM TEA-HCl (pH 8.0). Association kinetics were initiated by the addition of [^3H]-phenamil (2.8–30 Ci/mmol). After different times of incubation, specifically bound radioactivity was determined by filtration under reduced pressure using Whatmann GF/B glass fiber filters that had been treated with 0.3% poly(ethylenimine) (Bruns et al., 1983). After 30 min of incubation, once specific binding had reached a plateau value, dissociation kinetics were followed by measuring the decrease in bound [^3H]phenamil following a 10-fold dilution with the incubation buffer, or a displacement of labeled phenamil by 10 μM unlabeled phenamil. For equilibrium binding experiments, membranes were incubated with increasing concentrations of [^3H]phenamil for 60 min at 4 °C. Nonspecific binding was determined in parallel incubations in the presence of 10 μM unlabeled phenamil. Inhibition of specific [^3H]phenamil binding by unlabeled phenamil, benzamil, or amiloride was measured under the equilibrium binding conditions described above. In all experiments, the free ligand concentration was calculated by subtracting the bound ligand concentration from the total ligand concentration. Experimental data were fitted to the equation for one ligand and two different receptor sites (Munson & Rodbard, 1980).

Purification of the Epithelium Na^+ Channel. A 1100-fold purification of the pig kidney epithelium Na^+ channel was achieved by a two-step procedure as described previously (Barbry et al., 1987). The first step involves an ion-exchange column using QAE Sephadex. The second step was an affinity chromatography on a *B. simplicifolia* lectin column. [^3H]-Phenamil binding assays to the purified Na^+ channel were performed as described (Barbry et al., 1987). Protein concentrations were measured according to Peterson (1977). Iodination of the purified protein was carried out by using Iodo-Gen as supplied by Pierce (Fraker & Speck, 1978). SDS–PAGE was carried out according to Laemmli (1970), using 6% polyacrylamide gels under denaturing and reducing conditions. Gels were fixed, stained with Coomassie blue and exposed to Kodak XAR5 films for 30 h. Means ± SD are indicated.

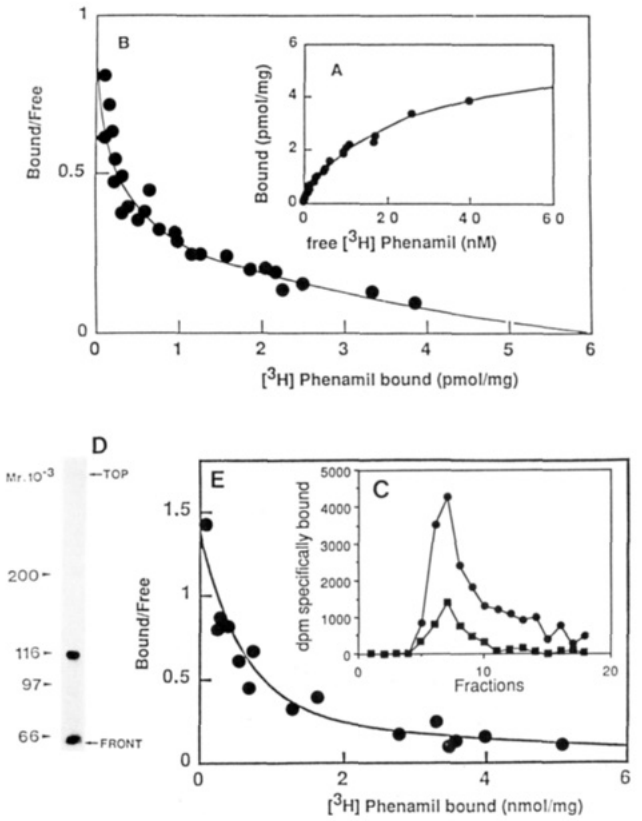

FIGURE 1: Equilibrium binding of [^3H]phenamil to pig kidney membranes and to the purified Na^+ channel. Panel A: Specific [^3H]phenamil binding to pig kidney membranes plotted as a function of the [^3H]phenamil concentration used. Panel B: Scatchard plot for the specific [^3H]phenamil binding. Kidney membranes were incubated with increasing concentrations of [^3H]phenamil of variable specific radioactivity (2.8–30 Ci/mmol). The Scatchard plot was resolved into two linear components with the following parameters: K_{d1} = 0.4 nM, K_{d2} = 26 nM, B_{max1} = 0.36 pmol/mg of protein, and B_{max2} = 5.9 pmol/mg of protein. Panel C: Copurification of the low- and high-affinity binding sites for [^3H]phenamil from a QAE Sephadex column. Experimental conditions were as previously described (Barbry et al., 1987). Each fraction eluted from the column was assayed for [^3H]phenamil binding by using 0.7 nM (30 Ci/mmol, ■) or 20 nM (3 Ci/mmol, ●) [^3H]phenamil. The specific binding component is shown. Panel D: Autoradiogram of SDS–PAGE of the purified Na^+ channel used in the binding assays. Electrophoresis has been carried out under reducing conditions. Molecular weights were determined from Coomassie blue stained gels. Panel E: Scatchard plot for the specific [^3H]phenamil binding component to the purified Na^+ channel. It was resolved into two linear components with parameters K_{d1} = 0.73 nM, K_{d2} = 45 nM, B_{max1} = 1.08 nmol/mg of protein, and B_{max2} = 7.1 nmol/mg of protein.

Results

Equilibrium Binding of [^3H]Phenamil to Kidney Membranes and to the Purified Na^+ Channel. Figure 1A shows the concentration dependence of the specific [^3H]phenamil binding component to pig kidney membranes. The Scatchard plot for the specific binding shown in Figure 1B is curvilinear with an upward concavity, suggesting that [^3H]phenamil binds to two populations of binding sites. In 10 different membrane preparations the dissociation constants K_{d1} and K_{d2} for the high- and low-affinity binding sites have mean values of 0.42 ± 0.08 nM and 27.9 ± 3.4 nM, respectively. The ratio of the respective amounts of low- and high-affinity binding sites (B_{max2}/B_{max1}) was 14.1 ± 2.0. The reason why the high-affinity binding sites were not detected in our previous experi-

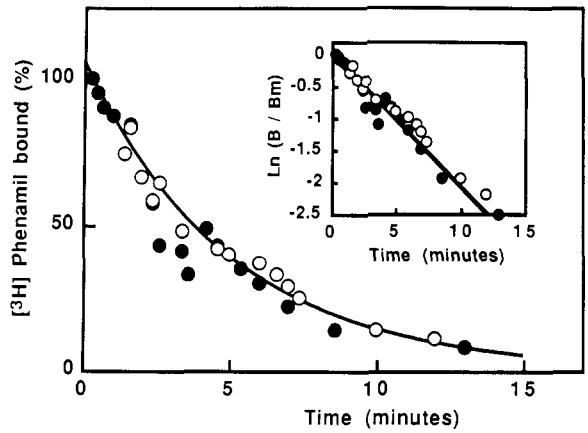

FIGURE 2: Kinetics of dissociation of [^{3}H]phenamil bound to pig kidney membranes. Main panel: Time courses of dissociation. Inset: First-order kinetics representation of the data. After equilibration of kidney membranes with 0.05 nM phenamil (30 Ci/mmol, ●) or with 90 nM phenamil (3 Ci/mmol, ○), dissociation of bound [^{3}H]-phenamil was initiated by the addition of 10 μM unlabeled phenamil (●) or by a 10-fold dilution of the incubation medium (○). B and B_m are the bound ligand concentrations at a given time and at the start, respectively. Identical symbols were used for the main panel and the inset.

ments (Barbry et al., 1986, 1987) is due to the fact that the radiolabeled ligand that we used had a specific activity which was too low to reveal the relatively small number of high-affinity sites.

The [^{3}H]phenamil receptor was solubilized from the pig kidney membranes by using CHAPS as a detergent and glycerol as a stabilizing agent and then purified by a two-step procedure as described previously (Barbry et al., 1987). The two [^{3}H]phenamil binding sites coeluted from the QAE Sephadex column (Figure 1C) and were present at all stages of the purification. The purified preparation consisted of a single 105-kDa band in SDS–PAGE performed under reducing conditions (Figure 1D). The Scatchard plot for the specific [^{3}H]phenamil binding to the purified Na^+ channel was curvilinear (Figure 1E). It was resolved into two linear components with the following parameters: K_{d1} = 0.73 nM, B_{max1} = 0.8 nmol/mg of protein, K_{d2} = 45 nM, and B_{max2} = 7.1 nmol/mg of protein.

Association and Dissociation Kinetics Relative to the Interaction of [^{3}H]Phenamil with Kidney Membranes. Dissociation kinetics obtained after equilibration of the membranes with 0.05 and 90 nM [^{3}H]phenamil are shown in Figure 2. Semilogarithmic representations of the data were linear and identical for the two conditions (Figure 2, inset), indicating (i) that the dissociation was a first-order process and (ii) that the dissociation kinetics were independent of the [^{3}H]phenamil concentration used. No difference was observed when dissociations were initiated by dilution or by addition of unlabeled phenamil. In six independent experiments performed by using [^{3}H]phenamil concentrations between 0.05 and 90 nM, the mean first-order rate constant of dissociation (k_d) was 2.45 $\times$ 10^{-3} $\pm$ 0.12 $\times$ 10^{-3} s^{-1}.

Association kinetics were performed at different [^{3}H]-phenamil concentrations. In all cases, a semilogarithmic plot of the data was linear. Figure 3A, B shows the results obtained for the two extreme concentrations (0.05 and 90 nM). Figure 3C shows that the apparent rate constant of association, measured at [[^{3}H]phenamil] > 20 nM, was linearly related to the [^{3}H]phenamil concentration used. Since under these conditions, more than 90% of the bound radioactivity was associated with the low-affinity binding sites, the slope of the relationship is a measure of the second-order rate constant of

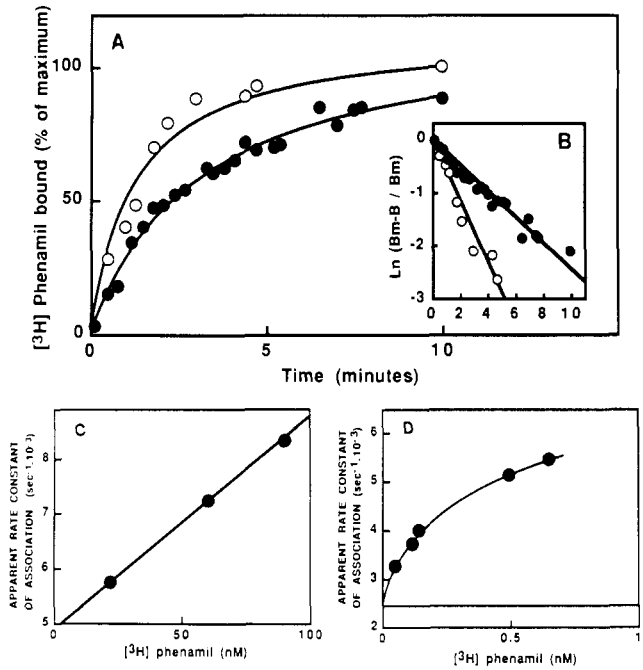

FIGURE 3: Kinetics of association of [^{3}H]phenamil to pig kidney membranes. Panel A: Kinetics of association performed at 0.05 nM [^{3}H]phenamil (●) and at 90 nM [^{3}H]phenamil (○). Panel B: Semilogarithmic plot of the data presented in panel A. Symbols are identical with those of panel A. B and B_m are the bound ligand concentrations at a given time and at the plateau value, respectively. Panel C: Relationship between the apparent rate constant of association and [[^{3}H]phenamil] for [[^{3}H]phenamil] > 20 nM. Panel D: Relationship between the apparent rate constant of association and [[^{3}H]phenamil] for [[^{3}H]phenamil] < 1 nM. The horizontal line indicates the value of k_d (2.45 $\times$ 10^{-3} s^{-1}). In all experiments, conditions were chosen to ensure that the free [^{3}H]phenamil concentration did not vary by more than 10% during the association processes.

association for the low-affinity binding sites (k_{a2}). This value was 38 800 $M^{-1}\cdot s^{-1}$. Figure 3D shows that, at [[^{3}H]phenamil] < 1 nM, the relationship between the apparent rate constant of association and the concentration of [^{3}H]phenamil was no longer linear. A theoretical treatment of the system for low concentrations of phenamil (see Appendix) shows that, even at low [^{3}H]phenamil concentrations, the apparent rate constant of association is a complex function of [[^{3}H]phenamil] and of the second-order rate constants of association for the high-(k_{a1}) and low-affinity (k_{a2}) binding sites. Analysis of the data allows computation of the ratio k_{a1}/k_{a2}, which is 165. Knowing k_{a2} (k_{a2} = 38 800 $M^{-1}\cdot s^{-1}$), we estimated k_{a1} to be 6.4 $\times$ 10^6 $M^{-1}\cdot s^{-1}$. The equilibrium dissociation constants ($K_d = k_d/k_a$) are therefore K_{d1} = 0.38 nM and K_{d2} = 63 nM.

Competition Experiments. The ability of unlabeled phenamil and amiloride to inhibit [^{3}H]phenamil binding to kidney membranes was tested in competition experiments involving constant concentrations of membranes and of [^{3}H]phenamil and variable concentrations of amiloride or unlabeled phenamil. Experiments were first performed at a low concentration of [^{3}H]phenamil (0.07 nM) to favor the occupancy of the high-affinity binding sites. Figure 4A shows that the concentration of unlabeled phenamil that reduced specific [^{3}H]phenamil binding by 50% ($K_{0.5}$) was 0.5 nM. Since the concentration of [^{3}H]phenamil is far below the dissociation constant of the [^{3}H]phenamil–high-affinity site complex (K_{d1} = 0.4 nM), the true K_d value for unlabeled phenamil is of course very close to the $K_{0.5}$ value that has been determined; hence K_{d1}(phenamil) = 0.5 nM. Similarly, Figure 4A shows that the $K_{0.5}$ value for amiloride inhibition of the specific [^{3}H]phenamil binding to its high-affinity binding sites in

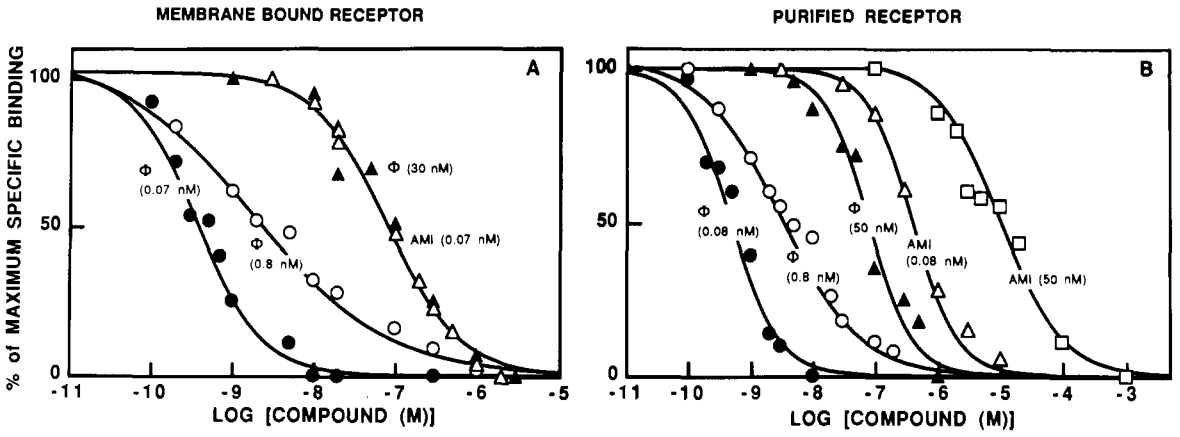

FIGURE 4: Inhibition by unlabeled phenamil and amiloride of [^{3}H]phenamil binding to pig kidney membranes and to the purified Na^+ channel. Panel A: Pig kidney membranes. Panel B: Purified Na^+ channel. Competing ligands are unlabeled phenamil (Φ: ●, ○, ▲) and amiloride (AMI: △, □). The concentrations of [^{3}H]phenamil used are indicated in parentheses.

kidney membranes was 100 nM. Therefore, K_{d1}(amiloride) = 100 nM.

Similar types of experiments were performed at a higher [^{3}H]phenamil concentration (30 nM) to favor the occupancy of low-affinity binding sites. Figure 4A shows that, under these conditions, the $K_{0.5}$ value for phenamil inhibition of specific [^{3}H]phenamil binding was 100 nM. The true K_d value for unlabeled phenamil is given by

$$K_{0.5} = K_d\{1 + [[^3H]\text{phenamil}]/K_d([^3H]\text{phenamil})\}$$

where [[^{3}H]phenamil] is the concentration of [^{3}H]phenamil used in the experiment (30 nM) and K_d([^{3}H]phenamil) is the dissociation constant of the receptor complex found between [^{3}H]phenamil and the low-affinity site (28 nM). By use of these values K_{d2}(phenamil) was estimated to be 50 nM.

Competition experiments using kidney membranes were also performed at intermediate [^{3}H]phenamil concentrations (0.8 nM) so that both high- and low-affinity binding sites contributed to the specific binding component. Figure 4A shows that, under these conditions, the inhibition curve for unlabeled phenamil covered more than 3 orders of magnitude of concentrations between 10^{-10} and 10^{-6} M with a $K_{0.5}$ value at 4 nM.

Figure 4B shows the results of competition experiments performed with the purified receptor preparation. At a low concentration of [^{3}H]phenamil (0.08 nM), $K_{0.5}$ values for inhibitions by phenamil and amiloride of the specific [^{3}H]-phenamil binding were 0.7 nM and 0.5 μM. Hence K_{d1}-(phenamil) = 0.7 nM and K_{d1}(amiloride) = 0.5 μM. At a higher concentration of the tritiated ligand (50 nM), $K_{0.5}$ values for phenamil and amiloride inhibitions were 100 nM and 8 μM, respectively, corresponding to K_{d2} values of 52 nM and 4.2 μM [using a K_{d2}([^{3}H]phenamil) value of 45 nM]. At an intermediate concentration of the ligand (0.8 nM), the dose–response curve for phenamil inhibition of the specific [^{3}H]-phenamil binding extended over more than 3 orders of magnitude of concentrations. The $K_{0.5}$ value for phenamil inhibition was, in that case, observed at 5 nM (Figure 4B).

Table I summarizes kinetics and thermodynamic parameters for the binding of [^{3}H]phenamil and various amiloride derivatives to pig kidney membranes and to the purified Na^+ channel.

Table I: Kinetic and Thermodynamic Parameters for the Binding of [^{3}H]Phenamil and Amiloride Derivatives to Pig Kidney Membranes and to the Purified Na^+ Channel[a]

parameter	high-affinity binding sites	low-affinity binding sites
	Membrane-Bound Receptor	
k_a	6.4×10^6 $M^{-1}\cdot s^{-1}$	3.88×10^4 $M^{-1}\cdot s^{-1}$
$K_d = k_d/k_a$	0.38 nM	63 nM
K_d(equilibrium)	0.42 ± 0.08 nM (n = 10)	27.9 ± 3.4 nM (n = 10)
B_{max}	0.36 ± 0.070 pmol/mg (n = 10)	5.9 ± 1.1 pmol/mg (n = 10)
K_d(phenamil)	1.1 ± 0.5 nM (n = 5)	60 ± 11 nM (n = 5)
K_d(amiloride)	0.1 ± 0.05 μM (n = 3)	4 ± 2 μM (n = 5)
K_d(benzamil)	50 ± 10 nM (n = 2)	400 ± 160 nM (n = 3)
	Purified Na^+ Channel	
K_d(equilibrium)	0.73 ± 0.20 nM (n = 3)	45 ± 10 nM (n = 3)
B_{max}	0.8 ± 0.3 nmol/mg (n = 3)	7.1 ± 0.4 nmol/mg (n = 3)
K_d(phenamil)	0.8 ± 0.2 nM (n = 3)	53 ± 13 nM (n = 4)
K_d(amiloride)	0.5 ± 0.1 μM (n = 2)	4.2 ± 1.5 μM (n = 2)
K_d(benzamil)	40 ± 10 nM (n = 2)	500 ± 100 nM (n = 2)

[a] Values given are means ± SEM from the number of observations indicated in parentheses.

Discussion

[^{3}H]Phenamil, the labeled derivative of amiloride used in this work, has a higher specific radioactivity than other labeled derivatives of amiloride previously used to characterize the epithelium Na^+ channel (Cuthbert & Edwardson, 1981; Barbry et al., 1986, 1987; Kleyman et al., 1986; Lazorick et al., 1985; Sariban-Soraby & Benos, 1986b; Benos et al., 1986, 1987). Using this ligand, we showed that the Scatchard plot for the specific [^{3}H]phenamil binding, measured under equilibrium conditions, was curvilinear (Figure 1B), suggesting the presence of more than one class of binding sites for [^{3}H]phenamil. Data from detailed kinetic analysis of the binding of [^{3}H]phenamil to kidney membranes are also consistent with the existence of two classes of binding sites that differ in their rate constant of association for [^{3}H]phenamil (Figure 3), but not in their rate constant of dissociation (Figure 2). Equilibrium dissociation constants for the two classes of binding sites for [^{3}H]phenamil differ by a factor of about 100. The K_d value of [^{3}H]phenamil for the high-affinity binding site is 0.38–0.50 nM. The K_d value of [^{3}H]phenamil for the low-affinity binding site is 26–63 nM. Finally, competition experiments also show the presence of two affinity states for amiloride. K_d values for amiloride were 0.1 μM and 4 μM for the high and low [^{3}H]phenamil binding sites respectively.

Therefore, high-affinity binding sites for phenamil correspond to high-affinity binding sites for amiloride, and low-affinity binding sites for phenamil correspond to low-affinity binding sites for amiloride.

Other Na^+-transporting systems also have different affinity states for their specific ligands. A typical example is the interaction of the voltage-sensitive Na^+ channel with its specific toxins: tetrodotoxin and sea anemone toxins. In brain synaptic terminals, innervated mammalian muscles, and avian heart cells, voltage-dependent Na^+ channels have a high sensitivity to tetrodotoxin (K_d = 1–5 nM) and a low sensitivity to sea anemone toxins (K_d = 1 μM) (Lombet et al., 1982; Frelin et al., 1984a). Conversely, in other tissues the affinity to tetrodotoxin is reduced by a factor of 100–1000 while the affinity to sea anemone toxins increases in parallel by a factor of 100 (Lombet et al., 1982; Frelin et al., 1984b). In mammalian heart cells and in noninnervated muscle cells, high- and low-affinity binding sites for tetrodotoxin are simultaneously present (Frelin et al., 1983; Renaud et al., 1983). Similarly, (Na^+,K^+)ATPases in different tissues have different affinities for ouabain (Sweadner, 1979). In cardiac cells high- (K_d = 30 nM) and low- (K_d = 3 μM) affinity states for ouabain coexist (Kazazoglou et al., 1983).

One hypothesis could be that the two affinity states of the epithelium Na^+ channel for phenamil and amiloride correspond to two distinct protein structures. A procedure for the purification of the epithelium Na^+ channel has recently been described (Barbry et al., 1987). The purified material consists of a 180-kDa protein that migrates in SDS–PAGE as a 105-kDa protein under reducing conditions (Figure 1D). Another purification (Benos et al., 1987; Garty & Benos, 1988) has led to the identification of multiple polypeptides with molecular masses 300–315, 149–180, 95–110, 71–85, and 55–65 kDa. [^{3}H]Phenamil binding experiments were performed at all stages of the purification and indicated that the high- and low-affinity phenamil binding sites copurified. The properties of interaction of the highly purified Na^+ channel with phenamil, benzamil, and amiloride are very similar to those of the membrane-bound structure (Table I). High-affinity phenamil binding sites represent 10–20% of the level of low-affinity binding sites in the highly purified Na^+ channel preparations. Since our experiments failed to show clear and reproducible heterogeneities in the electrophoretic protein patterns of the purified material, a likely conclusion is that the two different receptors with two different affinity states for phenamil and amiloride are located on the same or on very similar protein structures that cannot be resolved by gel electrophoresis.

Another hypothesis could be that a covalent modification, possibly associated to a regulatory process, could change the affinity of a single type of apical Na^+ channel protein for its inhibitors. An example of this sort of situation has been described for the membrane receptor for epidermal growth factor (EGF). In many cell types, two distinct affinity states of EGF receptor are present (King & Cuatrecasas, 1982). They are due to a kinase C mediated phosphorylation of the receptor that converts the high-affinity state of the receptor into a low-affinity state (Shoyab et al., 1979). The epithelium Na^+ channel may be the target of transmethylation and/or phosphorylation reactions (Sariban-Sohraby & Benos, 1986a).

In the same pig kidney microsomes as those used in binding experiments, a component of the electrogenic $^{22}Na^+$ uptake is inhibited by amiloride (EC_{50} = 6 μM) and by phenamil (EC_{50} = 70 nM) (Barbry et al., 1986). These properties are similar to those of the low-affinity phenamil binding sites [K_{d2}(phenamil) = 50 nM, K_{d2}(amiloride) = 4 μM]. This would suggest that the low-affinity amiloride binding sites identified in this work are associated to functional Na^+ channels in pig kidney membranes. High-affinity phenamil binding sites might also be associated to functional Na^+ channels. However, due to their low density as compared to the low-affinity binding sites, their activity would not contribute to a significant and measurable extent to the total electrogenic $^{22}Na^+$ flux. The same problem has previously been found for high- and low-affinity binding sites for tetrodotoxin associated with the mammalian muscle Na^+ channel (Frelin et al., 1984b; Sherman et al., 1983). The properties of the high-affinity binding site for phenamil and amiloride reported here for pig kidney membranes are similar to the properties of the Na^+ channel in renal cortical collecting tubules (O'Neil & Boulpaep, 1979; Palmer & Frindt, 1986).

Acknowledgments

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Appendix

Equations that describe the kinetics of equilibration of one ligand (L) to two affinity states of the receptor (R_1 and R_2) are

	L	+	R_1	$\underset{k_d}{\overset{k_{a1}}{\rightleftharpoons}}$	LR_1
time = 0	l		a		0
time = t	$l - x - y$		$a - x$		x
	L	+	R_2	$\underset{k_d}{\overset{k_{a2}}{\rightleftharpoons}}$	LR_2
time = 0	l		b		0
time = t	$l - x - y$		$b - x$		y

Association rates are k_{a1} for the high-affinity receptor state and k_{a2} for the low-affinity receptor state. We define $\beta = k_{a1}/k_{a2}$ ($\beta \gg 1$). From the experimental data presented in Figure 2, k_d, the first-order rate constant of dissociation, is the same for the high- and low-affinity states of the receptor. The different concentrations at the start of the association process appear as l for [^{3}H]phenamil, a for high-affinity binding sites, and b for low-affinity binding sites. We define α as b/a. From the results of equilibrium binding experiments performed on the same membrane preparation we know that a = 0.080 nM and α = 22.

Differential equations that describe the system are

$$dx/dt = \beta k_{a2}(l - x - y)(a - x) - k_d x$$

$$dy/dt = k_{a2}(l - x - y)(\alpha a - y) - k_d y$$

For low concentrations of x and y (i.e., if $1 \leq K_{d1}$), the system can be linearized under a vectorial form:

$$d\mathbf{X}'/dt = -\mathbf{AX} + \mathbf{B}$$

where

$$\mathbf{X} = \begin{pmatrix} x \\ y \end{pmatrix} \qquad \mathbf{X}' = \begin{pmatrix} dx/dt \\ dy/dt \end{pmatrix}$$

$$\mathbf{A} = \begin{pmatrix} \beta k_{a2}(l + a) + k_d & \beta k_{a2} a \\ \alpha k_{a2} a & k_{a2}(l + \alpha a) + k_d \end{pmatrix}$$

$$\mathbf{B} = \begin{pmatrix} \beta l a k_{a2} \\ \alpha l a k_{a2} \end{pmatrix}$$

This differential system is completely solvable, and it is then

possible to calculate the total binding on both low- and high-affinity states of the receptor:

$$x + y = C \exp(-\lambda_1 t) + D \exp(-\lambda_2 t)$$

where λ_1 and λ_2 ($\lambda_1 > \lambda_2$), which are positive, are the two eigenvalues of matrix **A**. C and D are constants determined by initial conditions. Under the experimental conditions used, D is always less than 12% of C (from 6% at [[^{3}H]phenamil] = 0.05 nM to 12% at [[^{3}H]phenamil] = 0.14 nM). As a consequence, association data can be correctly fitted by a single exponential of time constant $1/\lambda_1$. λ_1 represents the apparent rate constant of association. It can be written under the form:

$$\lambda_1 = k_{a2}F(\alpha,\beta,l,a) + k_d$$

where

$$F(\alpha,\beta,l,a) = \{(\beta + 1)l + (\alpha + \beta)a + [(\beta - 1)^2l^2 + 2al(\beta - 1)(\beta - \alpha) + (\alpha + \beta)^2a^2]^{1/2}\}/2$$

Considering λ_1 as a function of F, λ_1 is linearly related to F with a slope of k_{a2}. For given values of α, β, and l, it is possible to determine F numerically and then to calculate the slope of $\lambda_1(F)$. F is not significantly altered when α is varied between the extreme values obtained in equilibrium binding experiments (6–30). Calculations were made by using $\alpha = 22$. This value was determined in equilibrium binding experiments performed on the same membrane preparation. β was then varied so that the slope of $\lambda_1(F)$ was equal to the k_{a2} value determined from the kinetics of association performed at high phenamil concentrations. This procedure yields a β value of 165.

Registry No. Na, 7440-23-5; amiloride, 2609-46-3; phenamil, 2038-35-9; [*phenyl*-4-^{3}H]phenamil, 119619-56-6; (4-iodophenyl)-guanidinium, 45964-99-6; methyl 3,5-diamino-6-chloropyrazine-carboxylate, 1458-01-1; 4-iodophenamil, 119619-57-7; benzamil, 2898-76-2.

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