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[^{3}H]PHENAMIL, A RADIOLABELLED DIURETIC FOR THE ANALYSIS OF THE AMILORIDE-SENSITIVE Na^+ CHANNELS IN KIDNEY MEMBRANES

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SUMMARY. The interaction of amiloride and amiloride derivatives with the Na^+ channels of pig kidney membranes was studied from $^{22}Na^+$ uptake experiments. The order of potency of the different molecules tested is : phenamil > benzamil > amiloride, ethylisopropylamiloride. [^{3}H]labelled phenamil was prepared and used to titrate Na^+ channels in pig kidney membranes. Kinetics experiments, equilibrium binding studies and competition experiments between [^{3}H]phenamil and unlabelled phenamil indicate that phenamil recognizes a single family of binding sites with a K_d value of 20 nM and a maximum binding capacity of 11.5 pmol/mg of protein. The order of potency of different amiloride analogs tested in [^{3}H]phenamil competition experiments is identical to that found for the inhibition of $^{22}Na^+$ uptake by apical Na^+ channels.

INTRODUCTION. High resistance epithelia actively transport Na^+ from the luminal side to the blood (1). The first step in transepithelial Na^+ transport is the diffusion of Na^+ across the apical membrane via Na^+ channels that are inhibited by amiloride (2-4) and that are regulated by hormones like vasopressin and aldosterone (5-7). Apical Na^+ channels have been extensively studied using electrophysiological techniques (8-10). Previous attempts to characterize biochemically the protein structure responsible for apical Na^+ transport have used [^{3}H]benzamil, as a radiolabelled ligand (11). Benzamil is a derivative of amiloride that is about 10 times more potent than amiloride for inhibiting the Na^+ channel in the frog skin (11-13). This paper describes the properties of interaction of another amiloride derivative, phenamil, with the Na^+ channel of pig kidney membranes. Phenamil which has been reported to be more potent than benzamil for inhibiting the short circuit current in the frog skin (13, 14), appears to be a very good tritiated ligand for the biochemical identification of the epithelial Na^+ channel.

MATERIALS AND METHODS. [4-^{3}H-Phenyl]phenamil (2.8 Ci/mmol) was synthesized as previously described (15, 16). Carrier-free ^{22}NaCl (0.5 Ci/mg) and ^{86}RbCl (4.5 mCi/mg) were purchased from Amersham.

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Membrane preparations. Membranes kidney outer medulla and cortex were prepared according to ref. 17. The final pellet was suspended in the homogenization buffer at a protein concentration of 60 mg/ml and stored frozen. For ion flux experiments microsomes were prepared as described above except that 50 mM NaCl, 50 mM KCl or 50 mM LiCl were added to all buffers in order to load membranes with adequate monovalent cations. Membrane preparations used in ion flux studies were used within 4 hr after their isolation.

Ion flux experiments. Na^+-loaded membranes (20-30 µl at 60 mg of protein/ml) were mixed with Dowex 50WX8 (200-400 mesh, Tris form), poured onto Biorad, Econo columns, and centrifuged for 1 min at 700 rpm in a bench centrifuge. The eluate was supplemented with the desired concentration of inhibitor and carrier-free $^{22}NaCl$ (5 µCi/ml) and incubated at 4°C. After various times of incubation 180 µl aliquots (0.2-0.3 mg of protein) were rapidly filtered through 0.22 µm Sartorius filters. Filters were washed twice with 5 ml of prefiltered ice-cold 0.1 M $MgCl_2$. Filters were counted in a gamma counter.

Binding of [^{3}H]phenamil to kidney membranes. Membranes (routinely 0.5 mg/ml) were assayed at 4°C in 1 ml of a solution containing 1 mM EDTA, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid pH 8.0 and the required concentration of [^{3}H]phenamil (2.8 Ci/mmol). After 90 min of incubation, the reaction was stopped by rapid filtration through Whatman GF/B glass fiber filters that have been pretreated with 0.3% polyethyleneimine (18). Fifty microliters of the reaction mixture were also counted for measurement of the total [^{3}H]phenamil present. Non-specific binding was determined in parallel incubations in the presence of an excess (10 µM) of unlabelled phenamil.

Association kinetics were started by the addition of 32 nM [^{3}H]phenamil to a solution consisting of 1 mM EDTA, 10 mM Hepes NaOH pH 8.0 and containing 0.55 mg of protein/ml. After different times of incubation at 4°C, bound radioactivity was separated from the free radioactivity as described above. Non-specific binding was determined in parallel incubations which contained 10 µM unlabelled phenamil.

After 70 min of association, at which time the amount of specifically bound [^{3}H]phenamil had reached a plateau value, the incubation mixture was made 10 µM with unlabelled phenamil. Dissociation kinetics were followed by measuring the decrease in bound [^{3}H]phenamil.

Inhibitions of [^{3}H]phenamil binding by unlabelled phenamil, benzamil, amiloride or EIPA were measured under equilibrium binding conditions as described above. Time equilibration was 90 min.

Proteins were determined according to Hartree (19) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION.

Pharmacological properties of $^{22}Na^+$ transport through Na^+ channels of pig kidney membranes. A procedure to assay the amiloride-sensitive $^{22}Na^+$ fluxes in toad bladder membranes has recently been devised (20). In this assay, Na^+-loaded membranes are incubated in a low Na^+ medium resulting in a transient electrical diffusion potential for Na^+ in vesicles that are highly permeable to Na^+, i.e., across vesicles that contain electrogenic Na^+ channels. The labelled isotope then tends to equilibrate across the membrane according to potential that has been artificially created (20).

The main panel of Fig. 1 presents a typical time-course of $^{22}Na^+$ uptake by pig kidney membranes in the presence of an outward Na^+ gradient ($[Na^+]_i$ = 50 mM, $[Na^+]_o$ < 10 µM). $^{22}Na^+$ accumulation reached a maximum at >10 min and then stabilizes. Phenamil (0.1 mM) inhibits 50 to 60% of $^{22}Na^+$ uptake (Fig. 1 main panel).

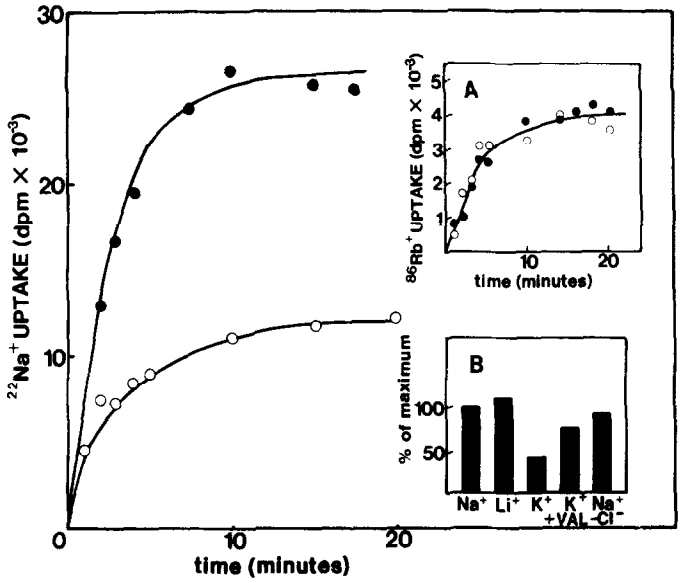


Fig. 1 : Evidence for electrogenic Na^+ channels in pig kidney membranes :
Main panel : Time-course of $^{22}Na^+$ uptake by Na^+-loaded kidney membranes. Experiments were performed in the absence (●) or the presence (○) of 100 µM phenamil.
Inset A : Time-course of $^{86}Rb^+$ uptake by Na^+-loaded membranes incubated in the absence (●) or the presence (○) of 10 µM phenamil.
Inset B : Comparison of the rates of phenamil-sensitive $^{22}Na^+$ uptake by kidney membranes loaded with NaCl, LiCl, KCl in the absence or in the presence of 10 µM valinomycin and Na methanesulfonate. External medium contains Tris-Cl or Tris-methanesulfonate.

Garty (21) has previously defined some of the important properties of electrogenic $^{22}Na^+$ transport in toad bladder membranes. The same strategy has been followed with pig kidney membranes and similar results have been obtained : **(i)** There is no phenamil-sensitive $^{86}Rb^+$ uptake component in Na^+-loaded membranes (Fig. 1 inset A). **(ii)** The rate of the phenamil-sensitive $^{22}Na^+$ uptake is higher in Li^+-loaded vesicles than in Na^+-loaded vesicles. It is much lower in K^+-loaded vesicles (Fig. 1B). High rates of phenamil-sensitive $^{22}Na^+$ are restored in K^+-loaded vesicles by the addition of 10^{-5} M of valinomycin (Fig. 1B). **(iii)** The rate of phenamil-sensitive $^{22}Na^+$ uptake in Na^+-loaded vesicles is independent of the presence of Cl^- (Fig. 1B).

All these experiments taken together indicate that the phenamil-sensitive $^{22}Na^+$ uptake component by pig kidney membranes is an electrogenic process that occurs in vesicles that have the following permeability profile for monovalent cations : $Li^+ > Na^+ > K^+ > Rb^+$. This sequence is identical to that described for apical Na^+ channels (22, 23).

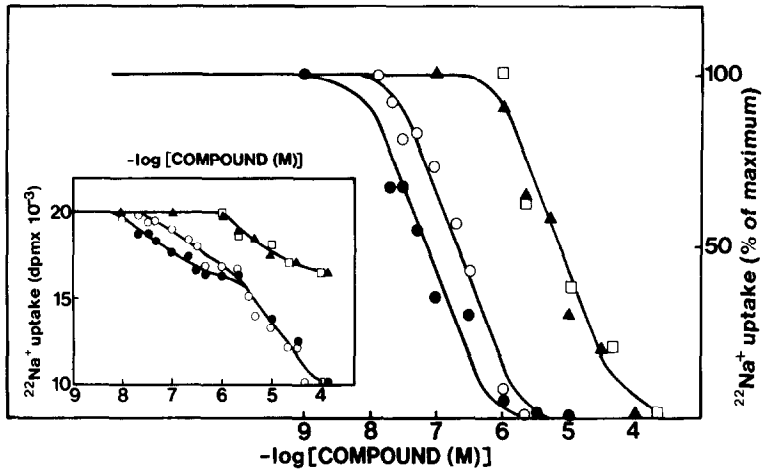


Fig. 2 : Pharmacological properties of the phenamil-sensitive $^{22}Na^+$ uptake component :
Inset : Dose-response curves for phenamil (●), benzamil (○), amiloride (▲) and EIPA (□) inhibition of $^{22}Na^+$ uptake by NaCl-loaded vesicles.
Main panel : Dose-response curves for amiloride derivatives inhibition of $^{22}Na^+$ uptake. The ordinate scale represents percent inhibition of the high affinity phenamil-sensitive component. Symbols are the same as for the inset.

The inset of Fig. 2 shows the dose-response curves for the inhibition of $^{22}Na^+$ uptake by phenamil, benzamil, amiloride and EIPA. The inhibitory action of phenamil and benzamil is observed over a 10 000-fold range of concentration between 10 nM and 0.1 mM. The inhibitory action of amiloride and EIPA is observed between 1 µM and 0.1 mM. The inhibition produced by 0.1 mM amiloride or EIPA is only 40% that produced by 0.1 mM phenamil or benzamil. Dose-response curves for phenamil and benzamil inhibition of the rate of $^{22}Na^+$ uptake are biphasic with a marked plateau near 1 µM. The inhibition produced by 1 µM phenamil or benzamil is identical to that produced by 0.1 mM amiloride or EIPA. The data suggest that there are two components of phenamil-sensitive $^{22}Na^+$ uptake. One of the components, representing about 40% of the total phenamil-sensitive $^{22}Na^+$ uptake is inhibited by the four amiloride derivatives with the following order of potency : phenamil > benzamil > amiloride, EIPA.

The main panel of Fig. 2 presents comparative dose-response curves for the inhibition of this component of $^{22}Na^+$ uptake by these four compounds using an expanded scale. With this representation complete inhibition is assumed for 1 µM phenamil or benzamil or for 0.1 mM amiloride or EIPA. Half-maximum inhibitions are observed at 70 nM, 250 nM, 6 µM and 6 µM for phenamil, benzamil, amiloride and EIPA

respectively. This pharmacological profile is typical of apical Na^+ channels, such as those that have been studied in amphibia epithelium (12, 13, 22). It differs from the pharmacological profiles of other Na^+ transporting systems such as the Na^+/H^+ exchange system and of the Na^+/Ca^{2+} exchange system that are also inhibited by amiloride and its derivatives (24-29).

The pharmacological properties of the Na^+/H^+ exchange system of renal, as well as in non-renal cells, has been analyzed both from $^{22}Na^+$ uptake experiments and from [^{3}H]EPA (Ethyl-[^{3}H]propyl-amiloride) binding studies. For the Na^+/H^+ exchange system, 5-N disubstituted derivatives of amiloride, like EIPA, are the most potent inhibitors, with a K_d value 10 to 100 times lower than the K_d value for amiloride. In contrast benzamil and other derivatives of amiloride that are substituted on the guanidino moiety are > 10 times less active than amiloride for the inhibition of the Na^+/H^+ exchange system (24, 25). The pharmacological properties of the Na^+/Ca^{2+} exchange system are quite similar to those of apical Na^+ channels, i.e. benzamil is more potent than amiloride or EIPA (26, 27). However, the IC_{50} values for the inhibition of the Na^+/Ca^{2+} exchange system by these amiloride derivatives are higher than 100 µM instead of the less than micromolar values found in this work.

The second component of phenamil-sensitive $^{22}Na^+$ uptake (Fig. 2) is inhibited by phenamil and benzamil with similar potenties (IC_{50} = 10 µM). It is not altered by amiloride or EIPA at concentrations up to 10^{-4} M. This component could represent the operation of another family of apical Na^+ channels having a low affinity for phenamil or benzamil. The existence of channels subtypes with a different pharmacology has been well established for voltage-dependent Na^+ channels where subtypes with high and low affinity sites for tetrodotoxin and sea anemone toxins have been found (30, 31).

Titration of [^{3}H]phenamil binding sites in pig kidney membranes. Typical kinetics of association of [^{3}H]phenamil to pig kidney membranes are shown in Fig. 3A. The semi-logarithmic plot of the association kinetics is linear, as expected for a pseudo-first order reaction. The observed rate constant is

$$k = k_a[[^3H]\text{phenamil}] + k_d \qquad (1)$$

where k_a and k_d are the second order constant of association and the first order rate constant of dissociation respectively. The value of k was calculated to be 6.2 10^{-3} s^{-1}.

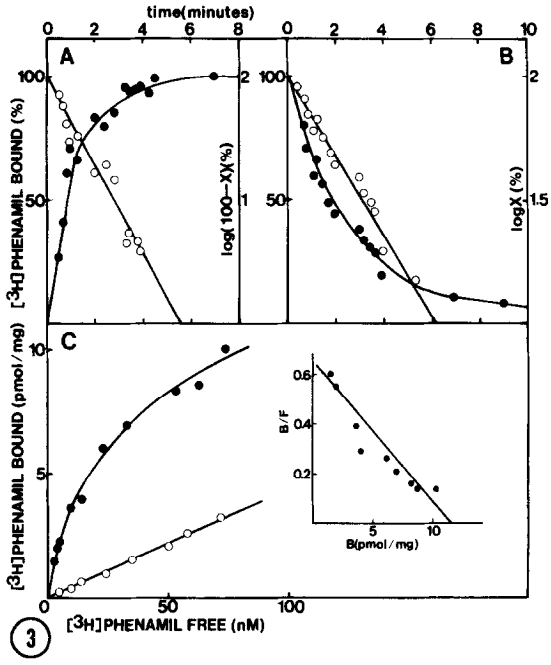
Fig. 3B shows that [^{3}H]phenamil bound to kidney membranes can be displaced by unlabelled phenamil. The rate constant of the dissociation process, k_d, was calculated from the linear semi-logarithmic plot of the data shown in Fig. 3B. The value of k_d is 2.6 10^{-3} s^{-1}. Incorporation into equation (1) of the values of k and k_d leads to a k_a value of 1.1 10^5 M^{-1} s^{-1}. K_d, the dissociation constant of the [^{3}H]phenamil-receptor complex obtained from kinetic data is : $K_d = k_d/k_a$ = 24 nM.

Fig. 3C shows the results of equilibrium binding studies in which increasing concentrations of [^{3}H]phenamil were added to a fixed amount of pig kidney membranes. Specific [^{3}H]phenamil binding is defined as the difference between total binding and non-specific binding, determined in the presence of a large excess (10 µM) of unlabelled phenamil. The Scatchard plot for the specific [^{3}H]phenamil binding is linear (inset Fig. 3C), indicating the presence of a single family of high affinity binding sites. The dissociation constant of the [^{3}H]phenamil-receptor complex is 17 nM. The maximum binding capacity is 11.5 pmol/mg of protein.

The results of competition experiments between [^{3}H]phenamil and unlabelled amiloride derivatives for the binding of [^{3}H]phenamil to kidney microsomes are shown in the main panel of Fig. 4. The concentration of unlabelled phenamil that reduces the specific [^{3}H]phenamil binding by 50% ($K_{0.5}$) is 100 nM. The true K_d value for unlabelled phenamil is given by :

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

where [[^{3}H]phenamil] is the concentration of [^{3}H]phenamil used in this experiment (30 nM) and K_d([^{3}H]phenamil) is the dissociation constant of the [^{3}H]phenamil-receptor complex (20 nM). Accordingly the K_d for unlabelled phenamil is 44 nM. Benzamil, amiloride and EIPA also prevent [^{3}H]phenamil binding to kidney membranes (Fig. 4). $K_{0.5}$ values for benzamil, amiloride and EIPA inhibition of the specific [^{3}H]phenamil binding are observed at 0.7 µM, 10 µM and 3 µM, which correspond to K_d values of 300 nM, 4 µM and 1 µM respectively. A comparison of efficacies of the different compounds on $^{22}Na^+$ flux and in binding experiments is presented in the inset of Fig. 4.



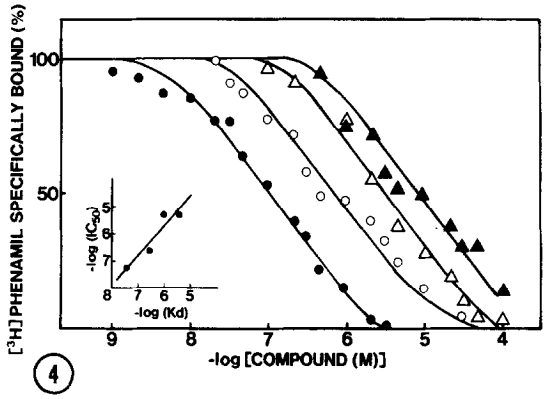


Fig. 3 : Titration of [^{3}H]phenamil binding sites in kidney membranes :
Panel A and B : Kinetics of association (A) and dissociation (B) of [^{3}H]phenamil to kidney membranes. Open symbols represent the same data after linearisation according to a pseudo-first order process (A) or a first order process (B). [^{3}H]phenamil concentration was 32 nM. The free concentration of [^{3}H]phenamil varied less than 10% during the course of association. The specific binding component is shown. The non-specific binding component did not vary during the course of association.
Panel C : Main panel : Equilibrium [^{3}H]phenamil binding to kidney membranes. Both the specific binding component (●) and the non-specific binding component (○) are shown. **Inset :** Scatchard plot for the specific [^{3}H]phenamil binding to kidney membranes.

Fig. 4 : Competition between [^{3}H]phenamil and compounds of the amiloride series :
Compounds used are unlabelled phenamil (●), benzamil (○), amiloride (▲) and EIPA (△).
Inset : Relationship between the IC_{50} for amiloride derivatives inhibition of $^{22}Na^+$ uptake and their K_d values, measured from [^{3}H]phenamil binding studies.

Taken together all these data indicate that the [^{3}H]phenamil binding sites that have been characterized in this study are likely to be associated with apical Na^+ channels. [^{3}H]Phenamil, because it has a reasonably high affinity (K_d = 20 nM) for its binding site and because of the high proportion of the specific versus non-specific binding will probably be useful in all biochemical studies of apical Na^+ channels, including purification.

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