Biochemical Pharmacology, Vol. 30, No. 11, pp. 1175–1183, 1981.
Printed in Great Britain.

0006-2952/81/111175-09 $02.00/0

BENZAMIL BINDING TO KIDNEY CELL MEMBRANES

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(*Received* 27 *August* 1980; *accepted* 14 *November* 1980)

Abstract—The binding of [^{3}H]benzamil (*N*-benzylamidino-3,5-diamino-6-chloropyrazine carboxamide), a blocker of passive sodium channels, was investigated in tissues from several species (rat, guinea-pig, rabbit, chicken, frog and two species of fish). Tissues used were kidney, colon epithelium, cardiac and skeletal muscle, brain, liver, epididymis and red cell membranes. Displaceable binding was detected principally in target tissues, and particularly in the kidney. In rat and rabbit kidney considerably more binding activity was present in the cortex than in the medulla. In rat kidney cortex, a saturable binding component (2 nmoles g^{-1} protein) with high affinity (approx. 5×10^7 M^{-1}) was detected. Other agents which are known to interact with sodium channels competed with [^{3}H]benzamil for the binding site. The affinity of amiloride detected in this way was 10^5 M^{-1}. Neither adrenalectomy nor pretreatment with aldosterone affected the amount of benzamil binding activity. Sodium had no effect on either the affinity or the capacity of binding. The binding activity appeared not to be associated with the base carrier known to be present in the proximal convoluted tubule. Binding activity was increased in both crude plasma membrane fractions and in fractions enriched in brush border membranes. The possible physiological role of the diuretic receptor is discussed.

Benzamil, and its better known analogue amiloride, are both pyrazine carboxamides with natriuretic activity [1]. In addition, both drugs are known to inhibit sodium transport in a variety of epithelia, such as amphibian skin [2] and the large intestine [3]. Using [^{14}C]amiloride or [^{3}H]benzamil saturable, low capacity binding sites have been detected in amphibian epithelia with affinities similar to those for transport inhibition [4–6]. Although there is considerable interest in the nature of the sodium entry sites located in the apical membranes of sodium transporting cells, they are so sparsely distributed in amphibian epithelia that isolation from such tissues has so far been impossible. Thus we have turned our attention to the mammalian kidney as a possible alternative source. In this paper we have set out to answer three preliminary questions. (1) Are high affinity, low capacity binding sites for benzamil demonstrable in target tissues such as kidney and colon? (2) Do other drugs which are known to interact with the amiloride site affect benzamil binding in a meaningful way? (3) Can the binding sites be unequivocally identified? It will be seen that the first two questions can be answered in the affirmative, while the third will require alternative experimental approaches.

MATERIALS AND METHODS

Preparation of homogenates. Homogenates were prepared from kidney, liver, cerebral cortex, cardiac muscle, skeletal muscle, epididymis, colonic epithelium and red cell membranes. After dissection the solid tissues were finely chopped with a scalpel. The colonic mucosal epithelium was scraped off with a microscope slide. Washed red cells were haemolysed in distilled water and the membranes separated by centrifugation at 80,000 g_{av} for 30 min.

All tissues, except skeletal muscle, were homogenised in 5 volumes (w/v) of potassium phosphate buffer, 50 mM, pH 7.4 in a Teflon–glass homogeniser (10 strokes at 500 rpm). Skeletal muscle required more vigorous treatment with a Polytron blender (setting 5 for 10 sec). The tissue homogenates were used either immediately or stored at −10° for up to four days.

Fractionation of kidney homogenates. Rat kidney homogenates prepared as above were used to make crude plasma membrane fractions. Homogenates were centrifuged at 500 g_{av} for 5 min and the pellet discarded. The supernatants were recentrifuged at 10,000 g_{av} for 20 min and the pellet again discarded. The pellet produced by centrifuging the supernatants at 40,000 g_{av} for 1 hr consisted of membrane fragments. In general the yield was small, about 2–3 per cent of the total tissue protein. For binding studies the pellet was gently homogenised (10 strokes by hand in a Teflon–glass homogeniser) to give a final protein concentration of around 4 mg ml^{-1}.

Pelleted fractions were prepared for electron microscopy by fixation overnight in a sodium cacodylate buffer (100 mM, pH 7.0–7.2) containing $CaCl_2$, 3 mM and glutaraldehyde, 2.5% v/v. The pellet was treated with osmic acid before embedding and sectioning.

Fractions enriched in brush border membranes were prepared from rat kidney homogenates by a modification of the method of Malathi *et al.* [7]. Fresh kidney cortices were homogenised in 5 volumes (w/v) of 50 mM mannitol/2 mM Tris–HCl buffer, pH 7.0 in a Teflon–glass homogeniser (10 strokes at 500 rpm). 1 M $CaCl_2$ solution was added to a final concentration of 10 mM and the mixture stirred in an ice bath for 10 min. The homogenate was centrifuged at 3000 g_{av} for 15 min and the pellet discarded. The supernatant was then centrifuged at

43,000 g_{av} for 20 min. The pellet, representing brush border membranes, was washed superficially with buffer and then resuspended in the same buffer by gentle homogenisation. The final protein concentration of the suspension was around 3 mg ml^{-1}.

In both cases marker enzymes were measured as an indication of membrane purification. These were alkaline phosphatase (EC.3.1.3.1) as a (brush border) plasma membrane marker, monoamine oxidase (EC.1.4.3.4) as an outer mitochondrial membrane marker and succinate dehydrogenase (EC.1.3.9.9.1) as an inner mitochondrial membrane marker. Alkaline phosphatase was estimated by the liberation of *p*-nitrophenol from its phosphate ester [8], monoamine oxidase was estimated using [^{3}H]tyramine as substrate [9] and succinate dehydrogenase by the formation of formazan from *p*-iodonitrotetrazolium violet [10].

Binding of [^{3}H]*benzamil to homogenates and membrane preparations.* Centrifugation methods were used, as described below. The samples were incubated with the label, plus where necessary other substances, in potassium phosphate buffer (50 mM, pH 7.4) at 30° and then centrifuged. The supernatant was aspirated and the surface of the pellet washed twice with ice cold buffer. It was then suitably dispersed and the contained radioactivity measured. Two methods of centrifugation were used:

Method A: Incubation samples (10 ml containing 3 to 5 mg protein ml^{-1}) were centrifuged at 40,000 g_{av} for 1 hr or 80,000 g_{av} for 30 min. The resulting pellet was dispersed in buffer and aliquots taken for scintillation counting. This method was used for all experiments with membrane preparations and for some with homogenates.

Method B: Limitation of the number of samples together with time required for centrifugation made it necessary to find an alternative rapid method for use with tissue homogenates, particularly in experiments where many samples were required or when the time course of radiolabel uptake was studied. Aliquots (250 μl containing around 0.5 mg protein) of incubation mixtures were centrifuged at 8,700 g_{av} for 1 min in a Beckman 152 microfuge. The resulting pellets were washed, the tips of the plastic microfuge tubes were clipped off into scintillation vials and the pellet dispersed in scintillant by shaking.

Measurement of sodium transport in a model system. Sodium transport was measured as short circuit current (SCC) in isolated frog skin (*Rana temporaria*) by methods described elsewhere [2].

Miscellaneous. The scintillant used for radioactivity measurements was butyl BPD, ethoxyethanol, toluene, 0.6%:33%:67%, w/v/v. Throughout protein concentrations were measured by the method of Lowry *et al.* [11]. [^{3}H]Benzamil was synthesised from [^{3}H]benzylamine as described elsewhere [12]. Its initial specific activity was 21.0 Ci $mmol^{-1}$ and correction for radioactive decay was made periodically.

Calculation of results. Non-specific binding is defined as the radioactivity retained by the pellets in the presence of 1 μM unlabelled benzamil. When other ligands were used to compete with [^{3}H]benzamil non-specific binding was defined as the level at which a plot of the percentage of uninhibited binding against inhibitor concentration flattened out. Where the foot of the curve could not be sufficiently well defined, usually because of solubility problems, non-specific binding was taken as the binding insensitive to benzamil, 1 μM.

The volume of solution entrapped within the microfuge pellets was determined with [^{3}H]mannitol (sp. act. 2.65 Ci $mmol^{-1}$) at a concentration of 3.77×10^{-8} M. The volume of [^{3}H]mannitol solution entrapped was 3.4 ± 0.1 μl ($n = 16$) in pellets of a typical homogenate. The volume equivalent of the non-specific binding of [^{3}H]benzamil in the same homogenate was 32 μl. Thus 90 per cent of the non-specific binding component is accounted for by bound material.

The affinity constant, K_a, for an inhibitor was calculated from the concentration of the compound, IC_{50}, required for 50 per cent inhibition of the specific binding of [^{3}H]benzamil, using the relationship,

$$K_a = \frac{K_{benz} \cdot [B] + 1}{IC_{50}} \quad (1)$$

where B is the radioligand concentration and K_{benz} is its affinity constant. In the special case of unlabelled benzamil this expression simplifies to

$$K_{benz} = \frac{1}{IC_{50} - [B]} \quad (2)$$

assuming that substitution of one atom of tritium for hydrogen has no effect on the binding properties of the ligand.

These calculations suppose that the interactions between the ligands and the specific binding sites obey simple mass action kinetics with a single set of sites. This was tested for benzamil by linear regression analysis of conventional unweighted Hill plots of

$$\log \frac{100 - \%\text{ uninhibited binding}}{\%\text{ uninhibited binding} - \%\text{ non-specific binding}}$$

against log[benzamil]. A Hill coefficient of unity indicates a simple mass action situation.

RESULTS

[^{3}H]*Benzamil binding*

Specific benzamil binding, defined as the uptake of [^{3}H]benzamil at 1 nM which is inhibited by unlabelled drug at 1 μM, was measured in six or seven tissues from four species by method A. Tissues chosen were kidney cortex and colon epithelium, both know to be target tissues for the inhibitory actions of pyrazine carboxamides [1, 3] and cardiac muscle, skeletal muscle, epididymis, liver, cerebral cortex and red cells. Effects of amiloride on cardiac muscle, skeletal muscle, red cells and fluid absorption in the epididymis have been described [13–15] but in general the effects appear only at high concentration and it is not established that the effects are due to blockade of passive sodium channels.

The profiles of binding activity for tissues of the rat, guinea-pig, rabbit and chicken are shown in Fig. 1. In the rat there is appreciable binding to the two

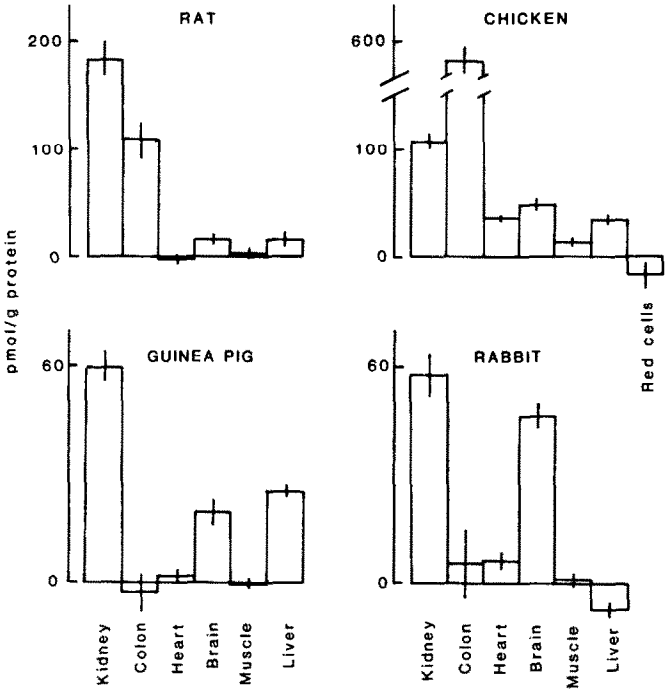


Fig. 1. Specific binding of [^{3}H]benzamil (displaced by benzamil, 1 μM) in pmoles g^{-1} protein in tissues from four species. Homogenates of various tissues were incubated in potassium phosphate buffer (pH 7.4, 50 mM) at 30° for 10 min with [^{3}H]benzamil (1 nM) in the presence and absence of unlabelled benzamil (1 μM). Subsequently the samples were centrifuged at 80,000 g_{av} for 30 min and the radioactivity bound to the pellets measured. Values were obtained for five aliquots for each homogenate and means ± S.E. are shown.

target organs and only small amounts elsewhere. In the guinea-pig and rabbit the kidney contains substantial amounts of binding material while appreciable amounts are present in rabbit and guinea-pig brain and guinea-pig liver. In the chicken the colon contains more binding activity than any other tissue studied while the kidney contains an amount similar to that found in mammalian kidneys. It cannot, of course, be assumed that the binding material is the same in all, or even any two, of these tissues but it is striking that, apart from the extraordinary value for the chicken colon, the kidney of all four species is a major source of benzamil binding activity. No particular pattern of non-specific binding was apparent in these tissues.

Out of convenience, the rat was chosen for a more detailed investigation. The profile described for the rat was confirmed using the microfuge technique (method B) and it was shown that kidney tissue from six other species also contained significant amounts of specific binding material using this method (Table 1).

Table 1. [^{3}H]Benzamil binding in tissue homogenates from various species measured by method B

Species	Tissue	[^{3}H]Benzamil bound (pmoles g^{-1} protein) Total	Non-specific	Displaceable
Rat	kidney cortex	114.6 ± 5.0	52.9 ± 1.9	62.0 ± 3.7 (68)*
Rat	cerebral cortex	120.0 ± 2.7	116 ± 3.8	4.1 ± 4.6 (1)
Rat	cardiac muscle	35.0 ± 3.1	29.3 ± 1.0	5.7 ± 3.2 (1)
Rat	skeletal muscle	113.0 ± 7.7	105 ± 5.8	7.0 ± 9.7 (1)
Rat	liver	104.0 ± 5.1	98.2 ± 2.0	5.5 ± 5.5 (1)
Rat	epididymis	53.4 ± 4.0	51.1 ± 3.1	2.3 ± 1.6 (3)
Guinea-pig	kidney cortex	164.0 ± 4.6	68.5 ± 1.8	92.8 ± 4.9 (5)*
Rabbit	kidney cortex	141.0 ± 4.6	64.9 ± 3.2	77.5 ± 5.0 (3)*
Chicken	kidney	93.6 ± 3.1	49.5 ± 2.5	47.1 ± 2.4 (3)*
Frog	kidney	153.0 ± 5.9	80.4 ± 3.0	69.8 ± 4.3 (6)*
Cottus bubalis	kidney	21.2 ± 0.7	13.8 ± 0.6	7.4 ± 1.0 (1)*
Cottus bubalis	heart	38.4 ± 1.4	37.1 ± 0.7	1.3 ± 1.6(1)
Pleuronectes platessa	kidney	39.1 ± 2.0	29.0 ± 1.5	10.1 ± 2.5 (1)*

The figures relate to the amount of [^{3}H]benzamil bound when incubated with the homogenates at a concentration of 1 nM in the absence and presence of unlabelled benzamil (1 μM). The figures in parentheses indicate the number of separate experiments. The values for the total and non-specific binding were significantly different as indicated by the asterisks (*$P < 0.001$). Homogenates were incubated in potassium phosphate buffer (pH 7.4, 50 mM) at 30° for 1 hr before centrifugation.

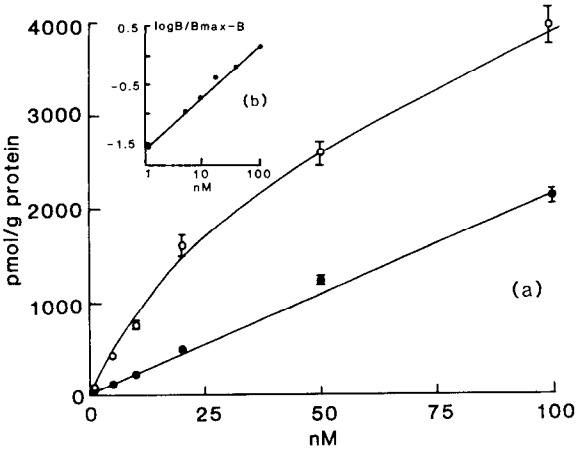


Fig. 2. Binding of [^{3}H]benzamil by rat kidney cortex homogenate at different concentrations (method B). In (a) binding in the absence (open circles) or presence (closed circles) of unlabelled benzamil, 1 μM. Incubation was carried out in potassium phosphate buffer (50 mM, pH 7.4) for 1 hr at 30°. Points represent means ± S.E. of eight determinations. Standard error bars are not shown where they lie within the dimensions of the points. Fitting the difference curve gave values of 2.4 ± 0.2 nmoles g^{-1} protein for maximal specific binding and 29 ± 6 nM for the half-saturating concentration. In (b) a Hill plot for the difference curve obtained from data given in (a) is shown. The regression line has a gradient of 1.07 ± 0.05 ($r = 0.995$, $P < 0.001$).

The binding of [^{3}H]benzamil to rat kidney cortex homogenate was measured at different times. Equilibrium was established within 5 min and binding remained constant for periods of up to 2 hr. Incubation times in excess of those required to achieve equilibrium have been employed throughout.

The relation between specific [^{3}H]benzamil binding and concentration was investigated on three occasions, one of which is illustrated in Fig. 2. Binding was curvilinear with concentration but in the presence of excess unlabelled benzamil binding was simply linear with concentration. The resulting difference curve was hyperbolic and binding appeared to show saturation. Analysis of the difference curve by the Wilkinson method [16] gave a value of 2.4 ± 0.2 nmoles g^{-1} protein for the maximal binding capacity and a value of 29 ± 6 nM for the half-saturating concentration. The mean values from three experiments for maximal specific binding and half-saturating concentration were 1.6 ± 0.5 nmoles g^{-1} protein and 17 ± 6 nM respectively.

A Hill plot derived from the specific binding component (Fig. 2) had a slope of 1.07 ± 0.05 with a regression coefficient of 0.99, suggesting simple mass action kinetics. Analyses of the type described above are valid only when the concentration of the sites is lower than the dissociation constant of binding and when the non-specific component of binding is small. When this is not so depletion of the ligand will affect the values obtained. Depletion can be minimised by lowering the protein concentration. However at very low protein concentrations the pellets become so small that they are difficult to handle, and standard errors become very large. We have not been able to avoid depletion and it remains for us to estimate the consequences of this for our measurements.

The total ligand concentration $[D_t]$ is given by

$$[D_t] = [D_f] + [DR] + [D_f] \cdot c$$

where $[D_f]$ is the concentration of free ligand, $[DR]$ is the concentration bound specifically to receptors and c is the concentration of non-specific sites per unit drug concentration. It can be shown that

$$[D_t] = K_m \left(\frac{1}{1-f}\right)(1 + c) + f \cdot [R_t]$$

where K_m is the half-saturating concentration, f the fractional receptor occupancy and $[R_t]$ the total receptor concentration. Thus when $[R_t]$ is small compared to K_m, and c is also small the drug concentration for 50 per cent occupancy approximates to K_m. Allocating entirely reasonable values for $[D_t(\frac{1}{2})]$, c and $[R_t]$ of 20 nM, 0.15 and 5.0 nM respectively, K_m has a value of 15 nM. Thus the K_m values are overestimated by about 30 per cent of their true value by this method. Incidentally, the Hill slope would have a value of 1.06 rather than 1.00 using the same parameters. An additional drawback of method B is that not all of the protein in the homogenate is spun down. Around 50–60 per cent of the total protein remains suspended with the microfuge method. A large proportion of this is soluble protein which showed no binding activity when measured on Sephadex G-25C columns (see following paper). However, because small membrane fragments are not spun down by the microfuge the binding capacity will be underestimated. This will not, of itself, affect the values for the affinities of various ligands.

Competition experiments

Known transport inhibitors. The affinity of a variety of substances for the binding sites in rat kidney cortex homogenates was investigated by examining their competition with [^{3}H]benzamil. As [^{3}H]benzamil was used at a concentration of only 1 nM in these experiments the occupancy was low (< 5%). Thus the concentration of inhibitor which displaces 50 per cent of the specifically bound benzamil (IC_{50}) approximates to the half-saturating concentration of inhibitor.

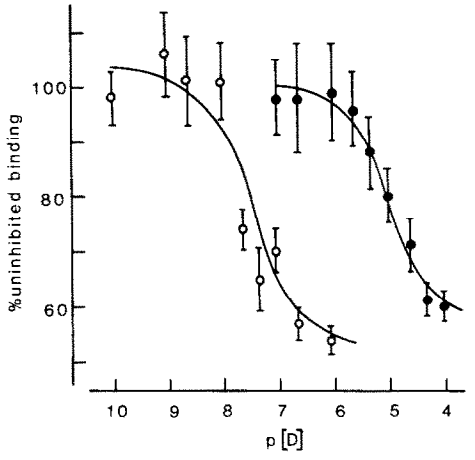


Fig. 3 Concentration dependence of the displacement of bound [^{3}H]benzamil by unlabelled benzamil (open circles) and by amiloride (closed circles) (method B). Homogenates were incubated in potassium phosphate buffer (50 mM, pH 7.4) at 30° for 1 hr with the ligands before microcentrifugation. Rat kidney cortex was used throughout. Points are the means ± S.E. for eight determinations and the [^{3}H]benzamil concentration was 1 nM. Curves were drawn by eye. IC_{50} values were estimated as 25 nM and 10 μM respectively for benzamil and amiloride.

Figure 3 shows the displacement of [^{3}H]benzamil from rat kidney cortex homogenate by unlabelled benzamil and amiloride. With benzamil the IC_{50} was 25 nM (corresponding to an affinity of $4 \times 10^7 M^{-1}$). When the results from seven similar experiments were pooled the IC_{50} from the composite curve was 40 nM. A Hill plot of the pooled data gave a Hill slope of 0.99 ± 0.11 ($r = 0.98$; $P < 0.001$) supporting the contention that the interaction can be described by simple mass action kinetics. The mean value of the specific benzamil binding at a concentration of 1 nM was 62.0 ± 3.7 pmoles g^{-1} protein ($n = 68$).

The displacement of bound [^{3}H]benzamil by unlabelled benzamil and amiloride was also measured for kidney homogenates from other species. Table 2 shows the IC_{50} values obtained in the different species. The values given for benzamil, amiloride and their ratio vary by little more than one order of magnitude between the species.

Several other pyrazine carboxamides and furanterene, an aminopteridine diuretic [17], were compared with benzamil in their ability to displace [^{3}H]benzamil from rat kidney cortex homogenate. The detailed results are given in Table 3. For comparison the IC_{50} values for these agents as inhibitors of sodium transport in frog skin are given. The percentage of non-specific binding, that is the amount of [^{3}H]benzamil remaining bound in the presence of a large excess of competing ligand, is also included in the table. The relative constancy of these percentage values for non-specific binding suggests that different competing ligands displace [^{3}H]benzamil from the same population of binding sites. Benzimidazole guanidine which both stimulates and, at higher concentration, inhibits transport in frog skin [18], was a relatively weak displacer of [^{3}H]benzamil. The loop diuretic frusemide [19] affected neither SCC nor [^{3}H]benzamil binding at a concentration of 100 μM.

Inhibitors of weak organic base secretion. As benzamil and amiloride are weak organic bases (pK_a 8.2 and 8.7, respectively) it is possible that binding was to the base carrier of proximal tubular cells. *N*-Methylnicotinamide is known to be a substrate for the carrier system, with a half-saturating concentration of 2 mM [20]. In two experiments and at a concentration of 10 mM *N*-methylnicotinamide reduced benzamil binding to only 96 ± 5 per cent and 89 ± 3 per cent of the control values, suggesting that benzamil was not bound to the base carrier.

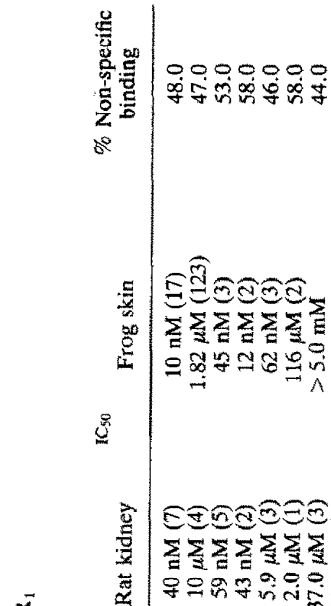
Darstine and cyanine 863 are both inhibitors of the base carrier system with half-saturating concentrations of 1.7 μM and 25 μM respectively [20]. Both of these compounds inhibited [^{3}H]benzamil binding, but to a greater extent than unlabelled benzamil itself. For example, with darstine the inhibition curve had not levelled out even at 10 mM at which concentration [^{3}H]benzamil binding had fallen to 36 per cent, while in the same experiment non-specific binding accounted for 55 per cent of the total uptake. With the cyanine dye a similar result was obtained. At a concentration of 10 μM, binding was reduced to 40 per cent of the control value and yet maximal inhibition had not been achieved. Eosin, a dye which is not an inhibitor of the base transport mechanism reduced binding to 20 per cent of the control value at a concentration of 100 μM, far in excess of the inhibition that could be achieved with unlabelled benzamil. With both the cyanine dye and eosin the

Table 2. Displacement of [^{3}H]benzamil (1 nM) by benzamil and amiloride in different species (method B)

	IC_{50}		
	Benzamil	Amiloride	Ratio
Rat, cortex	40 nM (7)	10 μM (4)	250
Guinea-pig, cortex	71 nM (3)	4 μM (3)	56
Rabbit, cortex	6.5 nM (1)	2.8 μM (2)	430
Chicken, whole kidney	77 nM (1)	7.7 μM (1)	100
Frog, whole kidney	141 nm (3)	20 μM (2)	142

IC_{50}'s were calculated from the pooled results from inhibition curves. Figures in parentheses refer to the number of experiments. Tissues were incubated for 1 hr at 30° in potassium phosphate buffer, pH 7.4, 50 mM.

Table 3. Displacement of ^{3}H-benzamil (1 nM) by known transport inhibitors in rat kidney cortex homogenates (Method B)



Compound	R_1	R_2	IC_{50} Rat kidney	IC_{50} Frog skin	% Non-specific binding
Benzamil	$-CH_2-C_6H_5$	$-NH_2$	40 nM (7)	10 nM (17)	48.0
Amiloride	$-H$	$-NH_2$	10 μM (4)	1.82 μM (123)	47.0
Naphthylamiloride	$-CH_2-C_{10}H_7$	$-NH_2$	59 nM (5)	45 nM (3)	53.0
o-Chlorobenzamil	$-CH_2-C_6H_4Cl$	$-NH_2$	43 nM (2)	12 nM (2)	58.0
Hydroxyethylamiloride	$-CH_2-CH_2-OH$	$-NH_2$	5.9 μM (3)	62 nM (3)	46.0
Cyclopropylamiloride	$-H$	$-NH-CH_2-C_3H_5$	2.0 μM (1)	116 μM (2)	58.0
Benzimidazole guanidine			37.0 μM (3)	> 5.0 mM	44.0
Furanterene			31.3 μM (3)	1.3 μM (1)	46.0
Furosemide			No inhibition	—	—

Rat kidney homogenates were incubated in potassium phosphate buffer (pH 7.4, 50 mM) for 1 hr at 30°. The numbers in parentheses indicate the number of separate experiments. IC_{50} values were calculated from the pooled results of inhibition curves. Values for inhibition of SCC in frog skin were made in *Rana temporaria*, bathed on both sides with Ringer solution. The data for frog skin was taken from references [2] and [18].

pellet became heavily stained and, furthermore, both dyes could displace [^{3}H]benzamil even in the presence of unlabelled benzamil (1 μM). These agents, therefore, seem to displace both specific and non-specific binding, which makes it impossible to interpret the results obtained.

The alkylating agent dibenamine has also been reported to inhibit base transport [21] although this is clearly not its only effect. Incubation of homogenates for 3 hr at 20° with dibenamine (1 μM) followed by inactivation of the remaining alkylating agent with thiosulphate reduced specific binding of benzamil by 80 per cent.

The shape of displacement (inhibition of binding) curves using competing ligands will be affected by ligand depletion. Assuming simple competition between the radioligand, D, and the competing ligand, I, it can be shown that

$$[D_t] = [D_f]\cdot[1 + c] + \frac{[R_t]\cdot[D_f]\cdot K_D}{1 + [D_f]\cdot K_D + [I]\cdot K_I}$$

where K_D and K_I are the affinity constants of D and I respectively. In the case where the depleting ligand was benzamil ($K_D = K_I$) the equation given above was solved for various values of I using values of 5×10^7 M^{-1}, 0.15, 5 nM and 1 nM for K_D (K_I), c, $[R_t]$ and $[D_t]$ respectively. The amounts bound at each inhibitor concentration were then calculated from

$$\frac{[R_t]\cdot[D_f]\cdot K_D}{1 + [D_f]\cdot K_D + [I]\cdot K_I} + [D_f]\cdot c$$

Thus two displacement curves were constructed with and without depletion and using reasonable experimental parameters. IC_{50} values were 2.0×10^{-8} M for the non-depleted condition and 2.5×10^{-8} M when depletion was allowed for, indicating that our values for IC_{50} are overestimated by about 20 per cent.

Interestingly even if much larger values of c and $[R_t]$ are used the effect of depletion on IC_{50} values is still small partly because this has the effect of reducing the total amount of diplacement which can be achieved with I.

Further properties of binding

The regional distribution of binding activity was measured in the kidney of rat and rabbit (Table 4). In each instance there was significantly more binding in the cortex compared to the medulla. The papillary tissue was devoid of binding activity.

No statistically significant difference in the amount of [^{3}H]benzamil bound specifically at a concentration of 1 nM was found in rat kidney cortex tissue from adrenalectomised animals (12 and 34 days) compared with sham operated controls. Similarly injections of aldosterone (1 μg 100g^{-1} body weight given twice at 90 min intervals and animals killed 4 hr after the first injection) caused no statistically significant change in benzamil binding activity of kidney homogenate when compared with saline injected controls.

No effect of sodium on either the affinity or capacity of binding of [^{3}H]benzamil was detected.

Table 4. Regional distribution of [^{3}H]benzamil binding in the kidney (method B)

		[^{3}H]Benzamil bound (pmole g^{-1} protein) Uninhibited	Non-specific	Specific
Rat	cortex	121 ± 6.2	57.8 ± 2.3	63.8 ± 6.2*
	medulla	70.7 ± 6.4	55.7 ± 2.6	14.8 ± 5.4*
	papilla	61.1 ± 6.0	70.9 ± 4.4	−9.8 ± 7.4
Rabbit	cortex	167 ± 12.2	76.3 ± 4.1	90.3 ± 12.9*
	medulla	107 ± 22.9	69.7 ± 5.9	37.0 ± 23.7
	papilla	57.4 ± 7.5	57.0 ± 8.2	0.4 ± 11.3

Throughout the [^{3}H]benzamil concentrations was 1 nM. Unlabelled benzamil (1 μM) was used to determine non-specific binding. Tissues were incubated for 1 hr at 30° in potassium phosphate buffer (pH 7.4, 50 mM) before microcentrifuging. Values are means ± S.E. for multiple determinations (16 in the rat tissue and 8 for the rabbit). Asterisks indicate statistically significant ($P < 0.001$) amounts of specific binding.

Binding to crude plasma membrane fractions

Specific binding of [^{3}H]benzamil in plasma membrane fractions was consistently found to be enriched, compared with the crude homogenate (method A). In one particular experiment, for example, the specific binding component was enriched 5.2-fold, while the specific activity of the membrane marker alkaline phosphatase was enriched 3.6-fold. Enrichments for the mitochondrial markers monoamine oxidase (outer membrane) and succinate dehydrogenase (inner membrane) were 1.2-fold and 1.0-fold, respectively.

The concentration dependence of the inhibition of [^{3}H]benzamil binding by unlabelled benzamil in this fraction is shown in Fig. 4. In this instance the IC_{50} was 100 nM, somewhat larger than the value of 40 nM obtained for the crude homogenate. A Hill plot of the data of Fig. 4 gave a value of 1.15 ± 0.11 ($r = 0.99$; $P < 0.001$) for the Hill coefficient.

The 40,000 g_{av} fraction was found to consist of membrane fragments, many of which appeared to form closed vesicles of diameter approximately 0.1–0.2 μm.

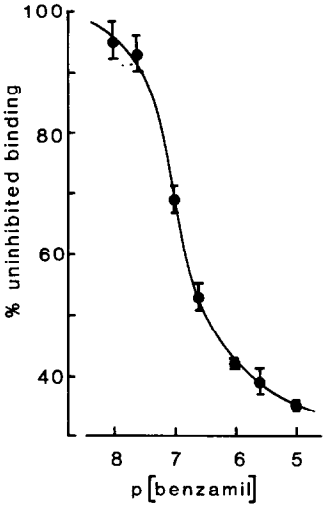


Fig. 4. Inhibition of [^{3}H]benzamil binding by unlabelled benzamil in a membrane preparation from rat kidney cortex (method A). Points are means ± S.E. for five determinations. The radioligand was used at a concentration of 1 nM. The curve was fitted by eye.

Binding to brush border membrane fractions

Specific [^{3}H]benzamil binding was also enriched in brush border membrane fractions. The results from a single experiment are shown in Table 5. The

Table 5. [^{3}H]Benzamil binding (method A) and marker enzyme activities in a crude homogenate and a brush border membrane fraction of rat kidney cortex

	Crude homogenate	Brush border membrane fraction	Relative specific activity
Specific benzamil binding (1 nM) (pmoles g^{-1} protein)	151	849	5.6
Alkaline phosphatase specific activity (μmoles $min^{-1} \cdot mg^{-1}$ protein)	0.354	1.551	4.4
Monoamine oxidase specific activity (nmoles $min^{-1} \cdot mg^{-1}$ protein)	0.640	0.135	0.21
Succinate dehydrogenase specific activity (μmoles $min^{-1} \cdot mg^{-1}$ protein)	0.048	0.008	0.17

specific binding component was enriched 5.6-fold, while the specific activity of alkaline phosphatase was increased 4.4-fold. The specific activities of monoamine oxidase and succinate dehydrogenase were both reduced approximately 5.0-fold.

DISCUSSION

The binding profiles obtained for [^{3}H]benzamil are, in general, as would be predicted from the known spectrum of activity of the pyrazine diuretics. In all species except the chicken the kidney is the major site of binding. The extraordinary level of binding activity in the chicken colon (and coprodeum) was a constant finding, and its characteristics will be described in detail elsewhere. In other tissues usually only small amounts of specific binding are present, rabbit brain and guinea-pig liver being notable exceptions. It cannot be assumed, however, that the characteristics of binding are the same in these tissues as in the rat kidney, which was the most extensively studied tissue.

There are no reports of the pharmacological effects of benzamil when perfused through different sections of kidney tubules. However, a number of studies have been carried out with amiloride. As we have been able, indirectly, to measure the affinity of amiloride for binding sites in the kidney cell membranes it is important to compare our values with these direct measurements.

Undoubtedly amiloride inhibits sodium transport in the distal part of the nephron, causing a reduction or even a reversal of luminal negativity, reduced sodium flux and reduced transepithelial conductance [22–24]. In early studies Stoner and his colleagues found that 10 μM amiloride perfused through the lumen of the rabbit distal tubule reduced net sodium flux by 90 per cent [23]. More recently O'Neil and Boulpaep, using rabbit cortical collecting duct, found that 70 nM amiloride reduced the sensitive SCC by 50 per cent [25]. This concentration is some 35 times lower than our value (2.5 μM) for the rabbit. Although the order of potency for various amiloride analogues from our study was the same as that for frog skin (a supposed model for the distal tubule) the potency ratios for the two preparations were very varied. In themselves the individual potency ratios are unimportant, since amiloride affinity in frog skin differs by at least one order of magnitude in different species [26]. However, the variation in the different potency ratios makes it unlikely that the binding sites we have detected are unique to the distal tubule.

The observed enrichment of benzamil binding activity in crude plasma membrane fractions of rat kidney indicates that the binding site is likely to be located in the plasma membrane. Furthermore, the enrichment of binding activity in brush border membrane fractions suggests that at least some of the detectable binding may be to proximal tubule membranes. This would be consistent with our observation that binding is detected principally in the cortex of rat and rabbit kidney and would explain the failure of aldosterone to influence binding. Our results with *N*-methylnicotinamide indicate that the binding site is not identical to the base carrier. Although inhibitors of the base carrier mechanism interfered with benzamil binding, they did so in an anomalous manner, and were effective even in the presence of excess unlabelled benzamil. These compounds, therefore, did not yield any useful information.

Although the important antikaluretic action of amiloride is exerted in the distal tubule, there is evidence that this drug also affects sodium transport in the proximal tubule. For example, when amiloride (10^{-4} M) is perfused through the lumen of the rat proximal tubule there is a reduction in sodium reabsorption [27, 28]. In the proximal tubule, which is a typical 'leaky' epithelium, sodium moves across the tissue both through and between the cells. At high concentrations amiloride can affect junctional sodium conductance as well as the cellular route [29], thus providing two potential binding sites associated with the brush border of the proximal tubule.

Recently amiloride has been shown to inhibit competitively a sodium-proton exchange mechanism in brush border membrane vesicles prepared from rabbit kidney cortex [30]. The K_i for this effect (15.6 μM) is of the same order as the dissociation constant for amiloride (2.8 μM) obtained from our binding studies.

In conclusion, we have detected a high affinity, low capacity binding site for benzamil in the kidney of several species. We have been able to define only one category of binding site, although we recognise that other binding sites with even smaller capacities could be obscured. The available evidence suggests that the binding site is not identical to the sodium entry site of the distal tubule or to the base carrier protein. Some component of the mechanisms responsible for sodium handling in the proximal tubule is the most likely candidate for the binding component. Displacement of [^{3}H]benzamil from kidney homogenates may be a useful method for the preliminary screening of potential diuretic substances.

Acknowledgements—This work was supported by the Medical Research Council and the National Kidney Research Fund.

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