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EXTRACTION OF A [^{3}H]BENZAMIL BINDING COMPONENT FROM KIDNEY CELL MEMBRANES

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Abstract—The [^{3}H]benzamil binding component in rat kidney cortex cell membranes was extracted with digitonin. Incomplete extraction was achieved, but binding activity was not destroyed by the treatment. The binding of [^{3}H]benzamil to the digitonin extracts was assayed on Sephadex G-25C columns. Characteristic peak and trough elution profiles were obtained which enabled the binding affinities of benzamil and amiloride to be estimated. Half-saturating concentrations were 6 nM and 6 μM, respectively. Using gel filtration, the apparent molecular weight of the binding component was found to be large (1–2 million), which suggests that the extract may consist of small membrane fragments. Failure to improve the ratio of specific to non-specific benzamil binding in the digitonin extract supported this view. Radiation inactivation analysis gave a molecular weight of 650,000 for the benzamil binding site *in situ*.

A saturable binding component for benzamil, with high affinity and low capacity, has been demonstrated in membranes prepared from rat kidney cortex (1). This component has a number of properties which suggest that it is associated with sodium ion translocation in the nephron, although the evidence is not unequivocal. Unfortunately there is no simple test for receptor *function* which might be applied to kidney homogenates and we are unaware of any biochemically-linked process associated with passive transmembrane sodium movement. Ideally, the ability of purified receptor material to transport sodium ions across a bilayer structure would provide excellent evidence of function, provided the transport was sensitive to benzamil. An essential first step towards these ends is solubilisation and purification of the receptor. This paper shows that the benzamil receptor can be extracted using digitonin and some properties of the extracted material are described.

MATERIALS AND METHODS

Experiments were performed on homogenates or cell membrane preparations prepared from cortices of rat kidneys as described in the preceding paper [1].

Extraction of [^{3}H]*benzamil binding material.* Rat kidney cortex homogenates in potassium phosphate buffer (pH 7.4, 50mM) were centrifuged at 1000 g_{av} for 10 min. The resulting pellet was resuspended in 5 vol. (w/v) of potassium phosphate buffer containing the potential solubilising agent and incubated at 0° for 1 hr. Afterwards the incubate was centrifuged at 1000 g_{av} for 10 min and the pellet retained. The supernatant was respun at 100,000 g_{av} for 1 hr and the supernatant retained. [^{3}H]Benzamil binding was estimated in the resuspended pellet by the microcentrifuge method (Method B of the previous paper [1]). [^{3}H]Benzamil binding in the supernatant was assayed by gel filtration (see below).

Membrane fractions prepared on sucrose gradients were solubilised in a similar way, but only the supernatants from the 100,000 g_{av} spins were examined further.

Binding measurements in extracts. Sephadex G-25C gel, swollen in potassium phosphate buffer (pH 7.4, 50 mM) containing 0.02% sodium azide, was used to prepare columns with a bed volume of approximately 5 ml. Columns were equilibrated with the buffer containing azide, the appropriate solubilising agent and [^{3}H]benzamil, 0.5 nM, in the presence or absence of benzamil, 1 μM.

Samples of extracted material (200 μl containing approximately 1.5 mg protein) were applied to the column and eluted with buffer containing the appropriate additions. Samples (400 μl approximately) were collected directly into scintillation vials and 10 ml scintillation fluid (butyl PBD, Triton X-100, toluene, 0.6%:30%:70% w/v/v) added. Initially the samples were collected into tared vials to check that the drop size remained constant after the samples were added. Early experiments were carried out at 4° but it was shown later that identical results could be obtained at room temperature (22°).

Solubilising agents. Eight potential solubilising agents or techniques were examined. Solubilisation was attempted with Triton X-100 (1%); Triton X-100 (1%) with dithiothreitol, 2 mM; Nonidet P-40 (1%); Tween 85 (1%); Lubrol WX (1%); Digitonin (1%) and potassium iodide (1M). Kidney homogenate was also treated with maleic anhydride as follows. The 1000 g_{av} pellet was homogenised in 2 volumes of phosphate buffer and diluted 15 times with distilled water. The pH was adjusted to 8.0 and

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Table 1. Extraction of ^{3}H-benzamil binding activity from homogenates of rat kidney cortex.

Agent	Binding activity Non-extracted	Extracted
Triton X-100	–	–
Triton X-100 with DTT	–	–
Nonidet P-40	+	–
Tween 85	+	–
Lubrol WX	+	–
Digitonin	+	+
Potassium iodide	+	–
Maleic anhydride	+	–

A plus indicates that binding activity was present, while a minus indicates absence of detectable binding.

Note that complete extraction is not achieved with digitonin.

held constant with a pH stat as maleic anhydride (30 mg) was added slowly.

Table 1 shows the effects of the eight treatments on [^{3}H]benzamil binding activity. Seven of the procedures failed to extract [^{3}H]benzamil binding material and in six instances activity was still detectable in the undissolved pellet. When Triton X-100 was used no activity was recoverable in either the solubilised or insoluble material. [^{3}H]Benzamil binding activity was apparently extracted by digitonin.

In an experiment to examine the efficiency of extraction with digitonin it was found that 70 per cent of the total activity was present in the digitonin extract and 30 per cent remained in the residue. The total amount of activity in extract plus residue was the same as that in an equivalent amount of untreated homogenate, indicating that no activity was lost on treatment with digitonin.

Molecular weight estimations in digitonin extracts. Attempts were made to measure the molecular weight of the digitonin solubilised [^{3}H]benzamil binding material by gel filtration, using Sephadex G-150C (exclusion limit 0.4 million), Sepharose 6B (exclusion limit 5.0 million), Bio-Gel A-1.5m (exclusion limit 1.5 million) and Bio-Gel A-15m (exclusion limit 15 million).

Dextran blue (molecular weight 2 million) was used to measure the void volume with Sephadex G-150C and Bio-Gel A-1.5m. The void volumes determined with dextran blue on Sepharose 6B and Bio-Gel A-15m gels were only apparent values as it is not completely excluded. These apparent void volumes were compared with the elution volumes for [^{3}H]benzamil binding activity (volume of eluate which precedes the peak of binding activity). Protein concentrations of the eluates were monitored by UV absorption at 280 nm using an LKB 8300 Uvicord II UV analyser.

Molecular weight estimations in crude homogenates. The technique of radiation inactivation analysis was used. Samples of crude rat kidney cortex homogenate in potassium phosphate buffer (pH 7.4, 50 mM) were frozen and lyophilised in thin pyrex tubes. Afterwards the tubes were gassed with nitrogen, stoppered and cooled on dry ice before irradiation using the 20 MeV linear accelerator at Addenbrooke's Hospital, Cambridge. This procedure minimises non-specific inactivation by free radicals [2]. Doses of 0.1 to 5.0 Mrad were given at a rate of 1 Mrad min^{-1}. After irradiation samples were rehydrated with distilled water and [^{3}H]benzamil binding estimated by method B [1]. A single ligand concentration (1nM) was used in the presence and absence of benzamil, 1 μM.

RESULTS

Binding in digitonin extracts. Elution profiles for digitonin extracts applied to Sephadex G-25C columns showed a peak of radioactivity in the void volume where the high molecular weight material in the extract emerged. A well-defined trough followed the peak after which the radioactivity in each sample increased back to the control value. If the extract was pre-equilibrated with [^{3}H]benzamil at the same concentration as in the column (0.5 nM) then a peak of the same size, but no trough, characterised the elution profile. This indicates that within the period of assay (about 3 min) binding of [^{3}H]benzamil with the high molecular weight material in the extract achieves equilibrium.

The radioactive peaks were taken to represent bound [^{3}H]benzamil and were integrated, after subtraction of the background level of [^{3}H]benzamil, to give the amount bound. Inclusion of unlabelled benzamil (1μM) in the eluting fluid consistently reduced the size of the peaks, and the difference in the amount of radioactivity bound in the presence and absence of unlabelled benzamil was taken as the specifically bound material.

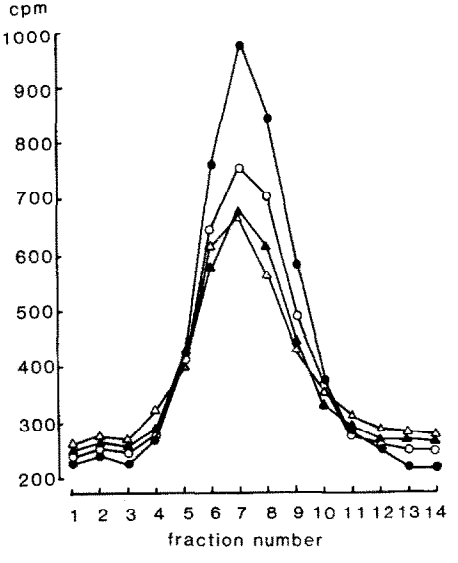

Fig. 1. Binding of [^{3}H]benzamil in a digitonin extract of a crude homogenate of the rat kidney cortex. 200 μl samples of the extract were added to a Sephadex G-25C column and eluted with buffer. The column and buffer contained [^{3}H]benzamil, 0.5 nM plus various concentrations of unlabelled benzamil: 0 (closed circles), 10 nM (open circles), 100nM (closed triangles) and 1μM (open triangles). Fraction size was 400 μl.

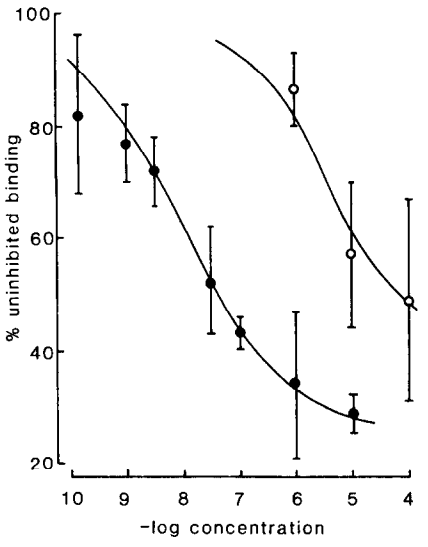

Fig. 2. Inhibition of [^{3}H]benzamil (0.5 nM) binding in a digitonin extract of a crude homogenate of rat kidney cortex by benzamil (closed circles) and amiloride (open circles). Points are means ± S.E. (3 determinations). Curves are drawn by eye.

Intermediate concentrations of benzamil produced a graded reduction of peak size, the dependence of peak size upon concentration being used to obtain an estimate of affinity.

Using 0.5 nM [^{3}H]benzamil the specific binding was found to be 200 ± 41 pmoles g^{-1} protein (n = 19) in the digitonin extracts, which represents 39 ± 3 per cent of the uninhibited binding. Figure 1 shows data from an experiment using several concentrations of benzamil to displace the radioligand bound to aliquots of a single extract. Benzamil, 0.1 and 1.0 μM, produced approximately equal displacement of [^{3}H]benzamil (around 33%), while benzamil, 0.01μM gave an intermediate value (around 24%).

Figure 2 shows results using another digitonin extract in which displacement of [^{3}H]benzamil by both benzamil and amiloride was investigated. In this experiment the percentage of binding which was displaceable by benzamil (70%) was greater than in other experiments. Amiloride was less active than benzamil in displacing the radioligand. The concentrations causing 50 per cent displacement, and approximating to the half saturating concentration at low [^{3}H]benzamil concentration, were 6 nM and 6 μM for benzamil and amiloride respectively. No interaction between [^{3}H]benzamil and sodium ions in the digitonin extract could be detected.

In several experiments crude homogenates of rat kidney cortex were fractionated on discontinuous sucrose gradients (1.0/1.25/1.50/1.75/2.0 M spun at 100,000 g_{av} for 1 hr) and the fractions were treated with digitonin in the normal way. Although some fractions were modestly enriched in their [^{3}H]benzamil binding activity compared to the crude homogenate there was no substantial enhancement in the percentage of specific (displaceable) binding. No binding was detected in the soluble fraction which did not penetrate the gradient.

Molecular weight estimation of [^{3}H]*benzamil binding material.* [^{3}H]Benzamil binding material in digitonin extracts was excluded from Sephadex G-150C. With Sepharose 6B the elution volume for [^{3}H]benzamil binding activity was greater than the apparent void volume for dextran blue in three out of four preparations, suggesting a MW of less than 2 million. Using Bio-Gel A-1.5m the binding activity

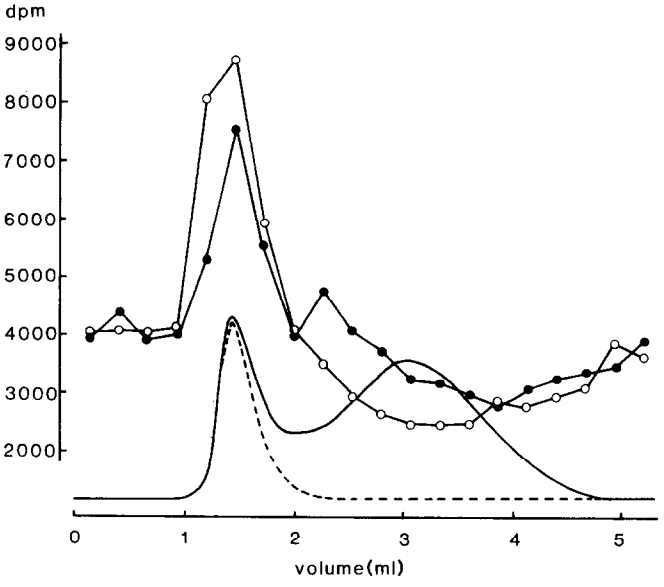

Fig. 3. Radioactive elution profiles for a digitonin extract of a crude homogenate of rat kidney cortex on Bio-Gel A-1.5m. The [^{3}H]benzamil concentration was 0.5 nM and elution was carried out in the absence (open circles) and presence (closed circles) of unlabelled benzamil (1μM). Samples added to the column were 100 μl and fractions were each of 250 μl. U.V. absorption traces for protein (solid line) and for dextran blue (dotted line).

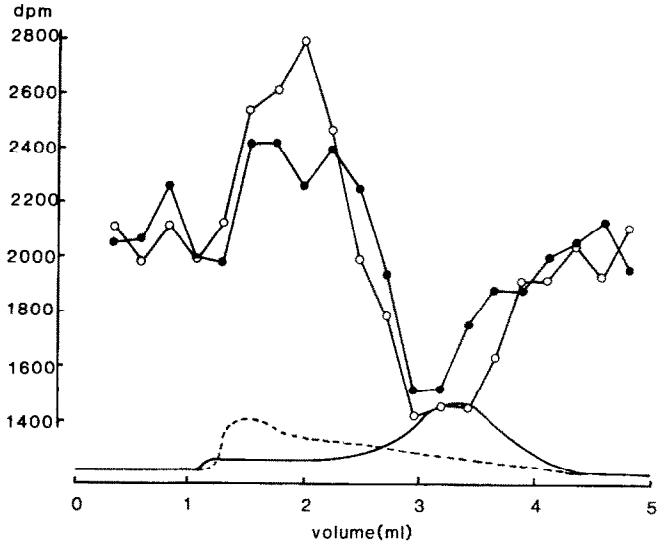

Fig. 4. Radioactive elution profiles for a digitonin extract of a crude homogenate of rat kidney cortex on Bio-Gel A-15m. The [^{3}H]benzamil concentration was 0.5 nM and elution was carried out in the absence (open circles) and presence (closed circles) of unlabelled benzamil (1μM). 100 μl samples were added to the column and 250 μl samples were collected. U.V. absorption traces for protein (solid line) and dextran blue (dotted line) are also shown. Note that the elution volumes for the radioactive peaks are greater than the void volume.

was totally excluded with three out of four preparations. The elution profiles for a typical experiment with Bio-Gel A-1.5m is shown in Fig. 3, along with u.v. absorption traces for dextran blue and protein in the same extract and measured in the same column. Elution was carried out in the absence and presence of unlabelled benzamil, 1μM. The peaks are coincident in the presence and absence of unlabelled ligand and are equal to the void volume, indicating a molecular weight of greater than 1.5 million for the [^{3}H]benzamil binding material. There appears to be no [^{3}H]benzamil bound, either specifically or non-specifically, corresponding with the second protein peak delayed by the gel.

Using Bio-Gel A-15m the elution volume for the [^{3}H]benzamil peak was greater than the apparent void volume, given by dextran blue, on five separate occasions indicating that the [^{3}H]benzamil binding material is not totally excluded from the column (Fig. 4).

It might be thought that elution from Bio-Gel A-1.5m or 15m gels provides a way to purify the [^{3}H]benzamil binding activity. However, active fractions re-run a second time on columns were found to have lost much binding activity.

Radiation inactivation analysis. When rat kidney cortex homogenates in phosphate buffer were lyophilised and then reconstituted by addition of distilled water the specific binding activity fell by approximately 20 per cent. If lyophilised homogenates were heated at 50° for ten minutes before reconstitution there was no further loss of binding activity. The stability of the binding material to these conditions allowed us to use radiation inactivation analysis as an estimate of molecular weight.

Initial experiments showed that irradiation with high energy electrons at doses of around 2.0 Mrad reduced specific binding to around 10 per cent of the value for lyophilised, but non-irradiated samples. Subsequently three separate experiments were made using different homogenates and a series of doses between 0.1 and 2.0 Mrad. The percentage specific binding in relation to control is plotted against dose in Fig. 5. Essentially a linear inactivation plot is obtained consistent with the inactivation of a target of uniform size by a single hit process [3]. The apparent mol. wt was obtained from the empirical relation mol. wt = 650,000/D_{37} where D_{37} is the dose in Mrad which reduces the binding fraction to 1/*e* of its original value. The experiments indicate that the [^{3}H]benzamil may bind to a component with a mol. wt of 0.65 million. Acetylcholinesterase (using washed red cells) was used as a calibrating marker in these experiments.

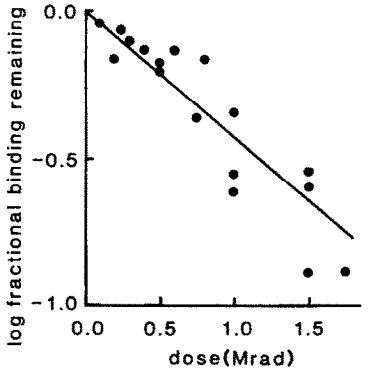

Fig. 5. Inactivation of specific [^{3}H]benzamil binding in a rat kidney cortex homogenate by high energy electron irradiation. The [^{3}H]benzamil concentration used was 1nM. Data represents results from three experiments. The dose causing 37% loss of activity was approximately 1 Mrad.

DISCUSSION

A [^{3}H]benzamil binding component extracted by digitonin from membranes of rat kidney cortex has been detected on columns equilibrated with the radioligand. The fractional occupancy was kept low (approx. 0.08 at 0.5 nM [^{3}H]benzamil) in order to achieve a compromise between peak height and the background against which the peaks were measured.

Although the extracted material did not sediment at 100,000 g_{av}, it is not clear whether digitonin actually solubilised the macromolecule, presumably a protein, or whether the material consisted of small membrane fragments associated with an unknown number of digitonin molecules. Gel filtration gave variable molecular weights in the region of 1–2 million, which suggests that the latter is the case. The failure to improve significantly the ratio of specific to non-specific binding in the digitonin extracts and the co-migration of these two components of binding on gels represents further evidence that complete solubilisation has not been achieved.

Harsher detergents than digitonin, such as Triton X-100, destroyed all binding activity, indicating that the binding component may require a membrane environment in order to retain a high affinity for benzamil. It is not altogether uncommon, in fact, for solubilised membrane components to exhibit changed ligand binding properties. An interesting example is the voltage sensitive sodium channel in excitable tissues which binds, at different sites, both saxitoxin and scorpion toxin. Membranes solubilised with Triton X-100 retain their binding activity to saxitoxin while that to scorpion toxin is completely lost [4]. In addition the non-specific component of tetrodotoxin binding is entirely lost in Triton X-100 extracts suggesting that the conformation of the recognition site for TTX and STX is independent of membrane integrity [5]. In this study extraction of kidney cell membranes produced a modest increase in affinity for both amiloride and benzamil when compared to the values for untreated membranes.

The technique of radiation inactivation analysis is the only method available for estimating molecular weight *in situ*. The application of classical target theory to radiation inactivation data has allowed molecular weights to be estimated for a number of important biological molecules [6, 7]. Kepner and Macey [2] combined empirically determined values for the energy dispersion of sparsely ionising radiation with assumed values for the average density of protein. They arrived at the empirical relationship used here. Our value for the benzamil receptor (0.65 million) is three times that for the tetrodotoxin receptor (0.23 million) estimated by the same method [8].

Assuming the benzamil receptor is a globular protein with a density of 0.81 daltons Å^{-3} its radius is approximately 60 Å. If it is concerned with ion translocation it appears to be of sufficient size to span the cell membrane.

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