BINDING OF [^{3}H]PHLORIZIN TO RAT KIDNEY PLASMA MEMBRANES

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1. Introduction

Phlorizin, a known inhibitor of D-glucose transport, has been used as a tool to study transport and binding characteristics of sugars in the kidney tubule. The binding of phlorizin to kidney brush border membranes was first characterized by Kinne et al. [1,2]. They and others [3] have demonstrated the competitive inhibition of phlorizin binding by D-glucose and used this technique to study the characteristics of D-glucose binding. On the basis of physiological studies in dogs [4], it has been proposed that the phlorizin inhibition of D-glucose transport at the luminal surface is competitive while that at the antiluminal surface of the tubular cell is not. We have demonstrated the binding of [^{3}H]phlorizin to a rat kidney membrane preparation enriched for antiluminal membranes. This binding is not affected by D-glucose and must be termed 'non-specific'. The binding observed is reversible and membrane bound [^{3}H]phlorizin may be dissociated by high concentrations of phlorizin and resorcinol.

2. Materials and methods

Adult male Sprague–Dawley rats fed ad libitum on Purina rat chow and water were sacrificed and the kidneys removed. All operations were performed at 4°C. Plasma membranes were isolated by differential centrifugation using the method of Fitzpatrick et al. [5] in a slightly modified form. Whole decapsulated kidneys were sliced with a Stadie–Riggs microtome and the slices homogenized in 3 X the volume of SEA buffer (0.25 M sucrose, 0.005 M EDTA, 726 units/ml penicillin G, 64 μg/ml streptomycin sulfate, and 140 mM NaCl, pH 7.4). The final pellet was suspended in SEA buffer.

The binding assay was patterned after that of Bode et al. [1]. It consisted of 0.5 ml of freshly prepared membranes (0.2–2.0 mg/ml) in SEA buffer at 22°C to which 0.2 μCi [^{3}H]phlorizin and 0.2 μCi D-[^{14}C]mannose were added. Unlabeled phlorizin was added to the concentration desired. For competition experiments, the membranes were suspended in SEA containing 100 mM D-glucose or 100 mM resorcinol for use in the binding assay. The mixture was stirred on a Vortex genie for 1/2 min and then centrifuged at 20 000 *g* for 20 min at 0°C. The supernatants were removed and excess liquid wiped from the walls of the centrifuge tube using Kimwipes. The membrane pellets were solubilized in 1 ml Soluene 100 (Packard) and counted in 10 ml dilute Liquifluor (Packard) in a Packard Tri-Carb scintillation counter. Counting efficiency and crossover values were determined using the automatic external standard.

The amount of phlorizin bound (b) was calculated after using mannose to correct for diffusion. 'Free' (f) phlorizin was calculated from the expression $f = T - b$ where T is the total amount of phlorizin in the incubation mixture. Bound phlorizin was then

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converted to pmoles/mg of protein to compare different preparations. The expressions b/f and b are given per mg of protein throughout. Protein concentration was determined by the method of Lowry et al. [6].

To determine if the phlorizin bound could be displaced, standard binding assays were performed after which the membrane pellets were resuspended in SEA buffer alone or in SEA buffer containing either 1 mM unlabeled phlorizin, 100 mM D-glucose, or 100 mM resorcinol. After thorough resuspension, the pellets were allowed to incubate for 5 min at 22°C before re-centrifugation at 20 000 g for 20 min at 0°C. Pellets were then treated as described above.

3. Results and discussion

The binding of [^{3}H]phlorizin to rat renal plasma membranes was studied over a concentration range of 0.04 μM–10.4 μM. As can be seen in fig. 1, phlorizin binding was not saturable over this range. Addition of 100 mM D-glucose or 100 mM resorcinol in the binding assay produced no significant inhibition of phlorizin binding. Resorcinol resembles the phenolic portion of the phlorizin molecule and was used to see if the phlorizin binding observed might involve the phenolic site. In fig. 2, a Scatchard plot of the data shows a biphasic curve which indicates the presence of two different binding sites. Neither binding site was affected by the presence of 100 mM D-glucose or 100 mM resorcinol.

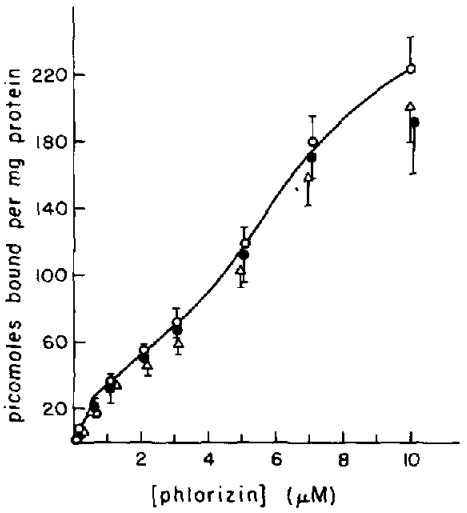

Fig. 1. Effect of phlorizin concentration on phlorizin binding to rat renal plasma membranes. Plasma membranes were incubated under standard binding assay conditions in SEA buffer containing 0.2 μCi [^{3}H]phlorizin with the addition of unlabeled phlorizin to the desired concentration. The final concentrations of phlorizin varied from 0.04 μM–10.04 μM. Assays were performed in the absence (○) and presence of 100 mM D-glucose (△) or 100 mM resorcinol (●). Each point represents the mean of 21–35 determinations from 7–12 different preparations. One standard error from the mean is indicated.

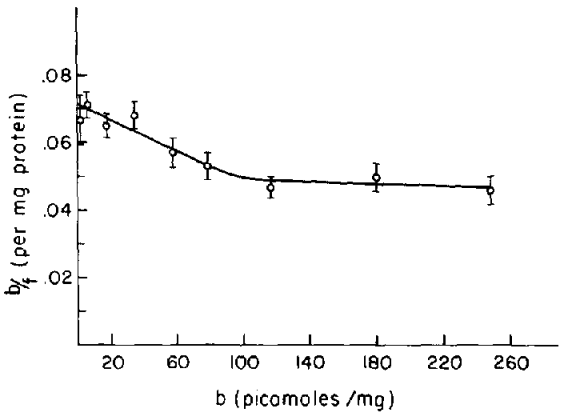

Fig. 2. A Scatchard plot of phlorizin binding to rat renal plasma membranes. Data from fig. 1.derived from the standard assay with no additions of glucose or resorcinol are shown. Each point represents the mean of 21–35 determinations from 7–12 different membrane preparations.

Results from studies in which pellets with bound [^{3}H]phlorizin were resuspended in buffer or buffer containing 1 mM phlorizin, 100 mM D-glucose, or 100 mM resorcinol are shown in fig. 3. The amount of [^{3}H]phlorizin bound per milligram of protein to plasma membrane preparations was not decreased by 'washing' (resuspension and re-centrifugation) of the pellet in SEA buffer or in SEA buffer containing 100 mM D-glucose. However, pellets from the binding assay which were resuspended in SEA containing 1 mM unlabeled phlorizin or 100 mM resorcinol did exhibit displacement of the bound material to different extents. Approximately 30% of the [^{3}H]phlorizin bound was displaced by resuspension of the pellets in 1 mM unlabeled phlorizin while about 40% of the membrane bound phlorizin was removed by subsequent exposure to 100 mM resorcinol.

It would seem that, although the phlorizin binding

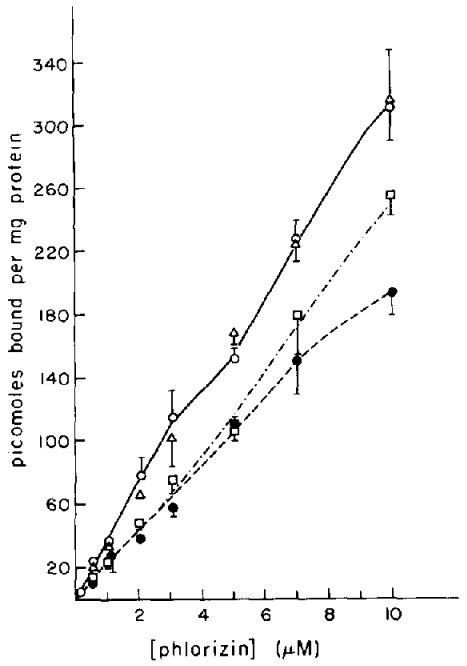

Fig. 3. Displacement of membrane-bound phlorizin. Standard binding assays were performed after which membrane pellets were resuspended in SEA buffer alone (○) or in SEA containing 1 mM unlabeled phlorizin (□), 100 mM D-glucose (△), or 100 mM resorcinol (●). The resuspended pellets were then re-centrifuged and [^{3}H]phlorizin bound per mg protein determined. Each point represents the mean of triplicate determinations from a single plasma membrane preparation. The abscissa shows the original concentration of phlorizin in the standard binding assay.

observed is reversible to some extent, dissociation of the bound material from the membrane is relatively difficult. Bound [^{3}H]phlorizin can, however, be dissociated by high concentrations of phlorizin or resorcinol. No significant effect of D-glucose can be observed on the binding of [^{3}H]phlorizin to or dissociation of bound [^{3}H]phlorizin from plasma membranes. This supports the proposal of Silverman et al. [4], derived from in vivo studies in dog kidney, that phlorizin binding to antiluminal membranes does not involve competitive inhibition with D-glucose.

These results reflect what has been called 'non-specific' binding of phlorizin to the membrane. Glossman and Neville [3] have proposed the existence of three separate sites to explain their data for phlorizin binding to rat renal brush border membranes. Only one of these sites, designated R_1, was considered to exhibit 'specific' binding which was inhibited by D-glucose, similar to the R_1 site described by Frasch et al. [2]. The relative purification factor for the unspecific site (R_3) was seen to decrease during brush border isolation. They do not elaborate on their R_2 site. The membrane preparations used in the present study showed a 6-fold to 8-fold purification of Na^+K^+-ATPase and a 2-fold purification of alkaline phosphatase over that in the homogenate, indicating enrichment for basal or antiluminal over microvillar membranes. We have shown that 'non-specific' phlorizin binding sites (perhaps R_2 and R_3) occur in rat renal plasma membranes and that the 'specific' R_1 site of Frasch et al. and of Glossman and Neville is not present in a plasma membrane preparation enriched for antiluminal membranes.

The physiological significance of phlorizin binding is not yet fully understood. Investigators working with kidney brush border membranes have inferred the presence of three binding sites, however, the specificity of these sites is far from clear. Others have shown phlorizin–glucose interaction at one site (R_1) on the luminal membrane, but we have demonstrated no such interaction using preparations enriched for antiluminal membranes. While phlorizin binds to these membranes, the binding must be termed 'non-specific' for the present since it may eventually be found to relate to effects of phlorizin on processes other than sugar transport.

Acknowledgements

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