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Radiation target sizes of the Na,K-ATPase and *p*-aminohippurate transport system in the basolateral membrane of renal proximal tubule

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Basolateral membrane vesicles made from rabbit kidney proximal tubules were frozen and irradiated with a high energy electron beam and the effects of irradiation on Na,K-ATPase activity, *p*-aminohippurate (PAH) transport, the membrane diffusion barrier and vesicle volume were measured. The vesicle volume and diffusion barrier were not significantly changed by radiation exposure. Na,K-ATPase activity was inactivated as a simple exponential function of radiation dose. Target size analysis of the data yielded a molecular size of 267 ± 17 kDa, consistent with its existence as a $(\alpha\beta)_2$ dimer. The carrier-mediated PAH uptake by basolateral membrane vesicles was also inactivated as a function of radiation dose. A target molecular size of 74 ± 16 kDa was calculated for the PAH transport system. This study is the first measurement of the functional size of the organic acid transport system based directly on flux measurements.

Introduction

Renal proximal tubules avidly secret many organic anions, e.g., *p*-aminohippurate (PAH), against high transepithelial concentration gradients. The active step in secretion is uptake of PAH across the basolateral membrane against an electrochemical gradient and intracellular PAH accumulation above the plasma concentrations [1]. Efforts have been made for the molecular identification of the transporter responsible for the active transport of PAH across the basolateral membrane. In the first experiments the transporters were labeled using a photoaffinity analog of PAH. Four polypeptides were specifically labelled making interpretation of the data equivocal [2]. More recently, PAH transport was expressed in *Xenopus* oocytes injected with size-selected mRNA from kidney cortex [3]. Expression of PAH transport was found in mRNA fractions with size range of 700–1300 bases, indicating that the expressed protein probably has a molecular mass under 47 kDa. While these approaches provide information on components of the transport system, they do not indicate the molecular assembly of the system in the membrane.

In this communication, the native size of the PAH transport system was determined using radiation target size analysis. This approach has been used to determine quaternary structure of a number of membrane bound transport and enzyme systems including the Na,K-ATPase [4–7], the inorganic anion exchanger [8], the glucose transporter [9,10], Na/glucose co-transporter [11], and amino acid transporters [12]. Since the molecular size of transport systems vary from tens to hundreds of thousands of daltons, the information obtained here can substantially narrow the size range to be investigated in subsequent purification.

Materials and Methods

Basolateral membrane vesicles (BLM) were prepared from rabbit kidney cortex employing differential and density gradient centrifugation as described previously [2]. Na,K-ATPase, a marker enzyme for the basolateral membrane was enriched 9–14-fold over the homogenate, whereas the marker enzymes for brush-border (alkaline phosphatase) and endoplasmic reticulum (glucose-6-phosphatase) were enriched under 2-fold. BLM were suspended in 100 mM mannitol, 100 mM KCl and 20 mM Hepes-Tris, pH 7.4 (Hepes, 4-(2-hydroxyethyl)-

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1-piperazineethanesulfonic acid, titrated to pH 7.4 with Tris, 2-amino-2-hydroxymethylpropane-1,3-diol). The membrane vesicles (10 mg protein/ml) were spreaded as a thin film in open aluminium trays, frozen at −70 °C and stored in liquid nitrogen.

Radiation inactivation was performed using a Van de Graf generator producing a beam of 1.5 MeV electrons as previously described [13]. During irradiation, the chamber was cooled by a stream of liquid nitrogen and the sample temperature was maintained between −45 °C and −55 °C. Dosimetry was performed with the bleaching of Blue Cellophane (DuPont MSC-300) at the sample irradiation temperature. After irradiation, trays were kept at −70 °C until use. Vesicular uptake of PAH and Na,K-ATPase actvity were measured immediately after thawing.

Decay of enzyme activity and PAH transport was fitted to a single exponential function

$$Y = A\,e^{-KD} \qquad (1)$$

where Y is fractional activity (activity or uptake at a given radiation dose/activity or uptake without radiation) and D is radiation dose. A and K were computed using a procedure of nonlinear least-square fit [14]. The target size molecular weight (M_r) was derived from the equation $M_r = 6.4 \cdot 10^5 \cdot K$ [13].

Na,K-ATPase activity was assayed according to Jørgensen and Skou [15]. Samples containing 0.25 mg of membrane protein were preincubated in 1.0 ml total volume containing 25 mM imidazole (pH 7.0), 2 mM EDTA, and 0.6 mg/ml deoxycholate. After 30 min of preincubation, 100 μl aliquots were transferred to assay tubes containing 130 mM NaCl, 20 mM KCl, 5 mM ATP, 5 mM $MgCl_2$ and 30 mM histidine (pH 7.5). Ouabain (1 mM) was added to determine the fraction of activity attributable to Na,K-ATPase. After 10 min at 37 °C the reaction was stopped by adding 200 μl of ice-cold 6% perchloric acid. Inorganic phosphate released was determined as described by Fiske and SubbaRow [16].

PAH uptake was measured using a rapid filtration method as described previously [2]. Briefly, the experiments were started by adding vesicles (5 mg/ml) in an aliquot equal to 12.5% of the volume of an incubation medium containing 100 mM NaCl, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.4), 0.1 mM PAH, 10 μCi/ml of [*glycyl*-1-^{14}C]PAH (New England Nuclear), and when required, 2 mM probenecid. At appropriate times, 100 μl of the reaction mixture was quickly filtered and washed with 5 ml of ice-cold solution containing 100 mM NaCl, 100 mM mannitol and 20 mM Hepes-Tris (pH 7.4). Radioactivity retained in the filters was quantified by liquid scintillation counting. The average values of triplicate determinations were taken from each preparation. The inhibition of PAH uptake by probenecid at 30 s of incubation was taken as a measure of facilitated PAH transport. Equilibrium uptake of PAH (60 min incubation) was determined to assess vesicle volume.

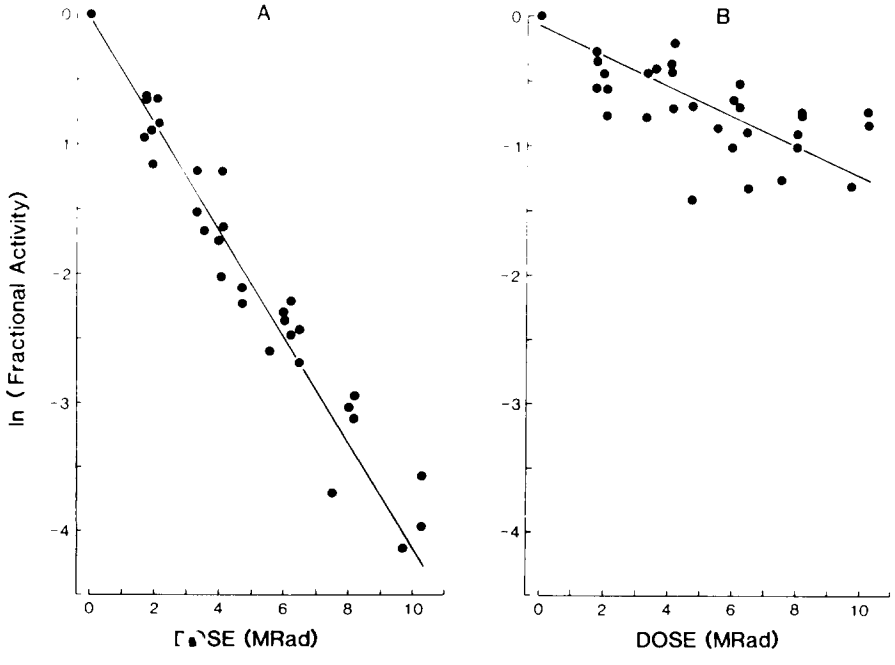

Fig. 1. Inactivation of Na,K-ATPase activity (A) and facilitated PAH uptake (B). Data are plotted on the same scale for comparison. The lines are from $y = 0.9975\ e^{-0.4174x}$ (A) and $y = 0.9282\ e^{-0.1159x}$ (B) which are obtained from a nonlinear least-square fit [13] using Eqn. 1. The 95% confidence ranges of the target sizes were 267000 ± 17000 daltons for the Na,K-ATPase and 74200 ± 16400 daltons for the PAH transporter. See text for other details.

Results

The Na,K-ATPase activity was inactivated as a simple exponential function of radiation dose (Fig. 1A). A target size of 267 kDa for the ATPase was computed as described above. From analysis of the slope (K of Eqn. 1), the 95% confidence interval of the target size was calculated to be from 250 to 284 kDa.

To determine the effects of radiation on vesicle configuration and lipid components of the membrane, equilibrium uptake of PAH and diffusional flux of PAH were measured. The equilibrium uptake of PAH was taken as an indicator of intravesicular volume and was not significantly changed by irradiation (Table I). Also, probenecid-insensitive PAH uptake at 30 s of incubation in the presence of 2 mM probenecid was not significantly altered by radiation (Table I) suggesting that the diffusion of PAH was not affected by radiation exposure.

The carrier-mediated (probenecid-sensitive) flux of PAH was found to decrease upon irradiation in a dose-dependent manner (Fig. 1B). A radiation sensitive target size of 74.2 ± 16.4 kDa (95% confidence interval) was calculated for the PAH transport system.

Discussion

The primary aim of this study was to measure the target size of the organic acid transport system. The validity of this measurement depends on the fulfillment of two criteria. The first is that radiation must not alter vesicle volume, which would of course alter the rate of uptake. Secondly, the nonmediated transport of PAH (diffusional barrier) must be less sensitive to radiation than the mediated transport. As shown in Table I, neither the equilibrium uptake of PAH, which reflects vesicular volume, nor the probenecid insensitive transport, a measure of the integrity of the diffusion barrier, was altered by the radiation doses employed in these experiments.

The target size of the Na,K-ATPase was simultaneously determined in this study because the enzyme is known to be resident in basolateral membranes, the subunits of this enzyme are well characterized, and radiation inactivation of this enzyme has been measured in other laboratories. It was felt, therefore, that this enzyme would serve as an internal standard against which to compare the size of the organic anion transport system. Incidentally, this is the first measurement of the Na,K-ATPase in proximal tubule membranes.

There have been a number of studies of inactivation of Na,K-ATPase in different membrane preparations. While radiation target sizes of the partial reactions of the enzyme such as K-phosphatase, and nucleotide, ouabain and vanadate bindings were the size of the α-subunit [4,5,7], the target sizes of Na and K dependent ATP hydrolysis and Na-pump activity were consistently larger than the α-subunit [4–7]. Various in situ organizations of the enzyme have been proposed; $(\alpha\beta)_2$ dimer, $(\alpha\beta)$ monomer, and α_2 dimer. The data in Fig. 1A appear to be most consistent with $(\alpha\beta)_2$ dimeric form of the enzyme in the kidney cortex, although we cannot rigorously rule out other possibilities, i.e., α_2 dimer.

It is clear from Fig. 1B that the organic acid transporter is considerably smaller than the Na,K-ATPase. Relatively large variation of PAH uptake data compared to that of Na,K-ATPase activity is inherent in the method of vesicle uptake measurement. This is to be expected when comparing a transport process to a measurement of enzyme activity [10–12]. However, a statistically reliable measurement of the inactivation curve can be made and a 95% confidence in molecular size established at between 58 and 90 kDa for the PAH transporter.

Target size provides information about the in situ size of membrane proteins. This is particularly valuable if the subunits of the protein are known, in which case the molecular assembly can be obtained. The subunits of the PAH transporter in the proximal tubule have not been determined. Photoaffinity labelling, however, revealed four candidate peptides with molecular weights of 108, 65, 52 and 26 kDa [2]. It is speculated that the 26 kDa protein may be a subunit of the PAH transporter because of the following reasons; (1) it is the protein predominantly labelled by the photoaffinity probe, (2) it seems to be an integral membrane protein

TABLE I

Effect of radiation on equilibrium and probenecid-insensitive PAH uptake

The equilibrium (60 min) uptake of PAH provides a measure of intravesicular space. The 30 s uptake in the presence of 2 mM probenecid provides a measure of the rate of non-mediated transport. Values are average from two preparations. Nonlinear regression fit [13] of the above data to Eqn. 1 showed that the slopes (K values) were not significantly different from 0. The 95% confidence ranges of the slopes for the equilibrium uptake and probenecid-insensitive uptake were -0.0224 to 0.0407 and -0.0443 to 0.0452, respectively. In a separate experiment, the equilibrium and probenecid-insensitive uptake were measured on vesicles irradiated at 0 and 10.24 Mrad. The values at 10.24 Mrad were 95 to 105% of those at 0 Mrad, indicating that the diffusion barrier and intravesicular space were not changed at the highest radiation dose shown in Fig. 1.

Radiation dose (Mrad)	PAH uptake (pmol/mg protein)	
	at equilibrium (60 min)	at 30 s in presence of probenecid
0	167	60.4
1.72	155	70.9
3.29	152	67.7
4.71	147	63.6
6.43	160	63.9

since it remains associated with membrane after alkali treatment [2], and (3) the size of mRNA that induced PAH transport in *Xenopus* oocytes [3] indicates that the expressed protein must be smaller than 47 kDa. From the target size of approx. 75 kDa obtained here, it could be envisioned that the PAH transporter may be a heterodimer of the 26 kDa protein and another, e.g., the 52 kDa protein labeled by the probe, although other possibilities cannot be ruled out at present. Determination of precise assembly requires purification of the transport protein. Cloning of the cDNA that induces expression of PAH transport in oocytes [3] could prove to be a valuable start for the purification.

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