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MOLECULAR SIZE OF THE Na^+-H^+ ANTIPORT IN RENAL BRUSH BORDER MEMBRANES, AS ESTIMATED BY RADIATION INACTIVATION

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The radiation inactivation method was applied to brush border membrane vesicles from rat kidney, in order to estimate the molecular size of the Na^+-H^+ antiporter. Sodium influx (1mM) driven by an acid intravesicular pH was unaffected by the high osmolarity of the cryoprotective solution. Initial rate of influx was estimated by linear regression performed on the first 10 seconds of transport: 0.512 pmol/µg protein/s. There was no binding component involved. Incubation performed in the presence of 1 mM amiloride, an inhibitor of the Na^+-H^+ antiport gave an initial rate of only 0.071 pmol/µg/s, an 82% inhibition. Membrane vesicles were irradiated at -78°C in a Gammacel Model 220. Sodium influx was reduced, as the dose of radiation increased, but the influx remained linear for the period of time (10s) during which the initial rate was estimated, indicating no alteration of the proton driving force during this time period. Amiloride-insensitive flux remained totally unaffected by the radiation dose, indicating that the passive permeability of the membrane towards sodium was unaffected. The amiloride-sensitive pathway presented a monoexponential profile of inactivation, allowing the molecular size to be estimated at 321 kDa. Based on DCCD-binding studies suggesting the molecular size of the monomer to be around 65 kDa for rat kidney, our results suggest that the functional transporter in the membrane to be a multimer.

The Na^+-H^+ antiport is a plasma membrane-associated transporter which mediates electroneutral Na^+-H^+ exchange. This antiport is involved in many different aspects of cell function (1,2). The luminal membrane of the renal proximal tubule presents a Na^+-H^+ exchange activity which is used as an experimental model for this antiport system (3). Despite extensive

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characterization of the kinetics, very little is known about the nature of the membrane protein responsible for this antiport activity. The reported molecular weights of the protein obtained by chemical labelling vary from 50 000 to 120 000 (5,6,7). Unprotonated carboxyl groups have been suggested as likely candidates for Na^+ binding sites and inhibition of the antiport by N-ethoxycarbonyl-2-ethoxy-1,2 dihydroquinoline, a carboxyl group modifier, has been reported (4). Affinity labelling with dicyclohexyl carbodiimide (DCCD) has suggested that a protein of 65 kDa is the Na^+-H^+ exchanger in rat renal brush border membranes (6). In the rabbit, the DCCD-binding protein had a molecular weight of 100 000 (8), suggesting species differences with respect to the renal Na^+-H^+ exchanger (9).

Radiation inactivation is a powerful analytical tool that can be applied to estimate the molecular size of membrane proteins in situ (10,11) without the need to purify the enzyme. We have recently reported that the radiation inactivation method is an analytical tool which can be applied to transporters in renal brush border membrane vesicles (12). We showed that the structural integrity of the vesicles, passive diffusion of substrates and intravesicular volumes were unaffected up to 8 Mrad. In this study, we analyze the effect of irradiation on the Na^+-H^+ antiport activity and we report the estimated molecular size to be 321 kDa.

Material and Methods

Membrane vesicles were prepared from rat kidney cortex by the magnesium chloride precipitation method (13). The mean enrichment for alkaline phosphatase, as measured by hydrolysis of p-nitrophenyl phosphate was 12.3-fold. The final pellet containing brush border membranes were resuspended in a cryoprotective medium consisting of 150 mM KCl, 40 mM Tris/Mes, pH 5.5, 14% glycerol and 1.4% sorbitol. The frozen samples (-78°C) were irradiated in a Gammacell Model 220 at a dose rate of about 2 Mrad/h (1). The following empirical equation (14) was used to relate molecular size (RIS) to $D_{37,t}$, the radiation dose in Mrad necessary to inactivate an enzyme or a transporter to 37% of its initial value, and to t, the temperature (in °C):

$$\text{Log RIS} = 5.89 - \log D_{37,t} - 0.0028t \quad \text{(eq. 1)}$$

$D_{37,t}$ values were obtained from a semi-logarithmic plot of uptake versus dose using a least-square fit.

A rapid-filtration technique was used for uptake studies (15). Incubation media contained 40 mM Tris/Hepes, pH 7.5, 14% glycerol, 1.4% sorbitol, 150 mM KCl, 1 mM $^{22}NaCl$ in the presence or absence of 1 mM amiloride. Uptake studies

performed in triplicate at 25°C were initiated by the addition of 80-120 µg of membrane protein. After incubation, the reactions were stopped by the addition of 1 ml of ice-cold stop solution. The stop solutions used for the sodium transport experiments contained 20 mM Tris/Hepes buffer, pH 7.5, 150 mM KCl, 14% glycerol and 1.4% sorbitol. The vesicle suspension was applied to 0.45 µm pore-size filters under vacuum. Filters were washed with 8 ml of ice-cold stop solution and processed for liquid scintillation counting.

Results and Discussion

Sodium uptake in the cryoprotective medium was linear for the first 10 seconds of incubation (Fig. 1). The initial rate of uptake, calculated by linear regression, was 0.512 pmol/µg protein/s. When amiloride was added to the incubation medium, the uptake was inhibited by 82%, the initial rate being only 0.071 pmol/µg protein/s. These values are in agreement with results obtained in non-cryoprotective medium of much lower osmolarity (300 mosm) where the inhibition caused by amiloride was 89% (16). This indicates that the high osmolarity conditions used in the experiments reported here do not affect the behavior of the antiport.

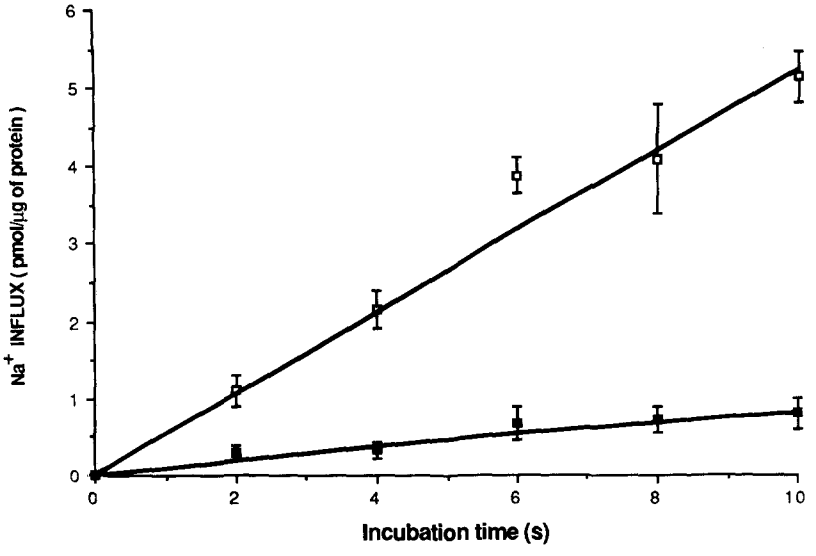

Fig. 1. Effect of amiloride on Na^+ uptake in the presence of a proton gradient. Membrane vesicles were equilibrated in 150 mM KCl, 14% glycerol, 1.4% sorbitol, 40 mM Tris/Mes, pH 5.5 and then incubated with 1 mM $^{22}NaCl$ (1µCi) in 150 mM KCl, 14% glycerol, 1.4% sorbitol and 40 mM Tris/Hepes, pH 7.5 in the presence (■) or absence (□) of 1 mM amiloride. One representative experiment is shown.

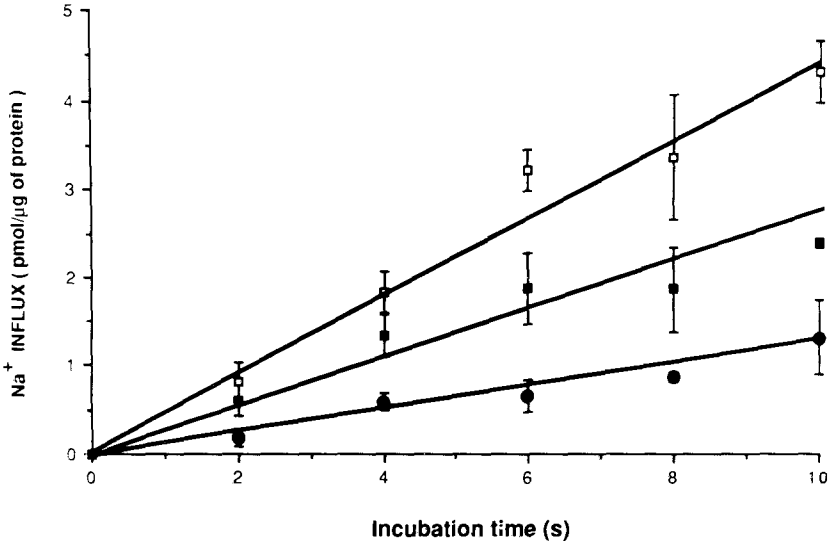

Fig. 2. Effect of irradiation on the initial rates of Na^+ uptake by brush border membrane vesicles. Vesicles were irradiated as described under "Materials and Methods". The membranes were exposed to different doses (□) 0 Mrad, (■) 1.9 Mrad, (●) 3.8 Mrad. Na^+ uptake was measured as described in the legend of Fig. 1. Na^+-H^+ exchange was calculated as the difference between the uptake from the medium free of amiloride and the medium containing this inhibitor (n=4).

In order to test the possibility of radiation artifacts caused, for example, by an increase in the permeability of the membrane, sodium uptake by membranes irradiated at various doses was also studied in initial rate conditions. As shown in Fig. 2, sodium uptake, driven by intravesicular acid pH, by membranes irradiated at 1.9 and 3.8 Mrad remained linear during the first 10 seconds of incubation, indicating that the driving force of the proton gradient remained unaltered during this period. Initial rates of sodium influx at increasing doses of radiation was estimated by linear regression performed on the data from the first 3 s period. As shown in Fig. 3, the antiport activity was lost progressively as a function of the radiation dose. Sodium uptake measured in the presence of amiloride remained totally unaffected by the irradiation. As this amiloride-insensitive influx has already been shown to be diffusional (16), reflecting the permeability of the membrane towards sodium, the lack of effect on this flux indicates that the physical integrity of the membrane is preserved, at these low radiation doses. These results are in agreement with studies where we showed that the low doses of irradiation did not alter the physical properties

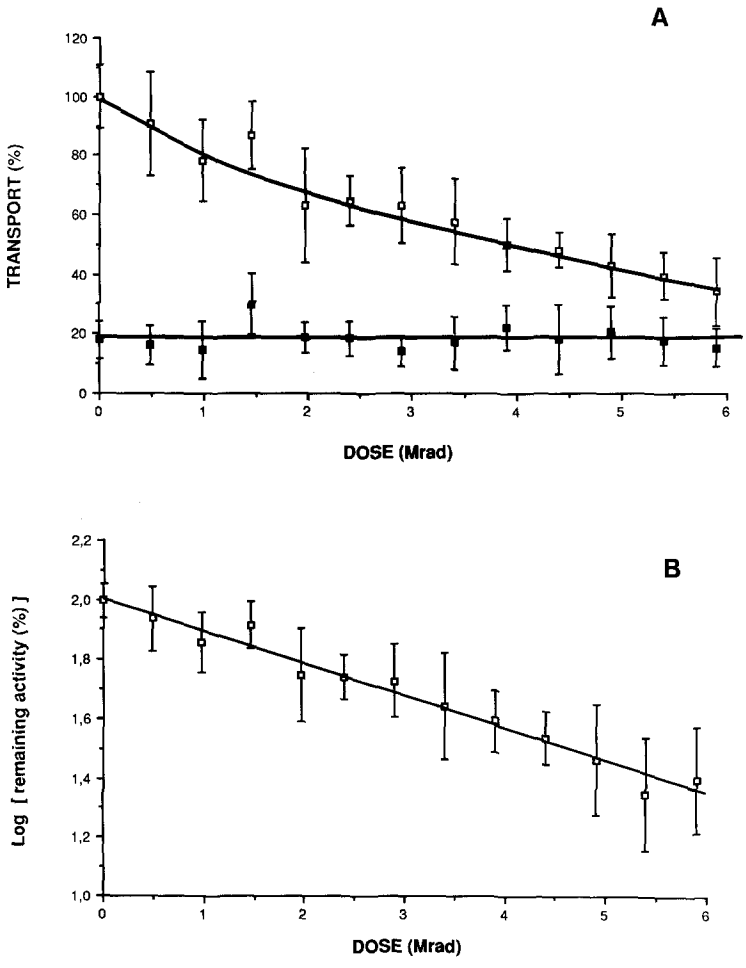

Fig. 3. Determination of the molecular size of the Na^+-H^+ exchanger. A) Uptake was measured in the presence (■) of absence (□) of amiloride as described in the legend of Fig. 1. B) Na^+-H^+ exchange was calculated as the difference between the uptake in presence of absence of amiloride in the incubation media and expressed as the log of the percentage of the remaining activity (n=4).

of the membrane (12): intravesicular volumes, sodium-independent fluxes of phosphate and glucose and protein composition were reported to remain unaltered by these low doses. When the amiloride-insensitive flux is subtracted from the total flux, the flux mediated by the antiport is obtained. The results, expressed as the logarithm of the percentage of the remaining activity, compared to unirradiated vesicles, allow the molecular size of the antiport to be estimated. The molecular size is obtained from the radiation dose necessary to inactivate an enzyme to 37% of its initial value, as indicated earlier (eq. 1). The size obtained is 321 kDa.

Knowledge of the molecular size of the Na^+-H^+ antiporter has important implications in understanding the structure-function relationship in this carrier. There are very few data available concerning the size of the Na^+-H^+ antiport. Chemical modification of carboxyl residues has indicated a wide variety of proteins modified by DCCD. Radiolabeled dicyclohexylcarbodiimide was reported to bind covalently, in an amiloride-sensitive fashion, to a 100 kDa protein in rabbit membranes (8) and to a 65 kDa protein in rat membranes (16). It was suggested that these proteins could represent the Na^+-H^+ exchanger or a subunit. The results presented here suggest that the carrier is formed of an association of several submits.

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