SIZE SELECTED mRNA INDUCES EXPRESSION OF P-AMINOHIPPURATE TRANSPORT IN XENOPUS OOCYTES

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Abstract. **Xenopus oocytes were injected with size-fractionated mRNA isolated from the renal cortex of rabbit kidney and after 4 days incubation, PAH uptake in oocytes injected with mRNA (0.7 - 1.3 kb) was 8 to 45 fold that of the water injected controls. The oocyte to medium ratio of accumulated PAH was 1.95. The Km and Vmax for transport were 333 μM and 66.6 nmoles·oocyte^{-1}· min^{-1}, respectively. This Km is similar to that reported for PAH transport in intact kidneys and slices. The uptake of PAH was unaffected by the absence of Na^+ or the presence of probenecid. Expression of the transport represents the first step in an effort to clone and identify the gene for PAH transport.**

Introduction

The renal proximal tubule actively secretes many organic anions, including anionic metabolites, toxins and drugs. From kinetic measurements of transport in isolated tubules, membrane vesicles and tissue slices, there is general consensus that mediated transport occurs in both the apical and basolateral membranes of the proximal tubule epithelium, but that the active step is at the basolateral surface (1). Nonetheless, despite over 30 years of extensive research, the physicochemical nature of the transporter(s) remains unknown. The principal impediment to identification of the components of this system has been the lack of specific inhibitors or other ligands with high affinity which could serve as a probe. We have previously attempted to label components of the system using a photoaffinity analogue of the prototypical anion, i.e., para-aminohippuric acid (PAH), which is transported by this system; however, four proteins were labelled making interpretation of the data equivocal (2).

An alternative approach to identification of membrane transport proteins, specifically the intestinal Na dependent glucose transporter, has been reported recently by Hediger et al. (3). These investigators utilized Xenopus oocytes to express transport activity following injection of the appropriate purified mRNA. Following their lead, we demonstrate here that a PAH transport protein is expressed by oocytes injected with size fractionated mRNA isolated from rabbit renal cortex. Such a demonstration is a prerequisite for isolation and identification of the gene for PAH transport.

Materials and Methods

Total RNA and poly(A)+ RNA were isolated by procedures previously described (4-6). Briefly, RNA was extracted from rabbit renal cortex using guanidium thiocyanate extraction followed by cesium chloride gradient centrifugation. Poly(A)+ RNA was selected by oligo (dT) cellulose chromatography (Sigma, St. Louis, MO) and fractionated by sucrose density gradient velocity centrifugation. The total mRNA was applied to a 5-25% (W/W) sucrose gradient, centrifuged for 4 hr at 20°C at 45,000 rpm in a SW 50.1 rotor, and subsequently divided into 14 fractions. Formaldehyde gel electrophoresis with ethidium bromide staining was used to examine the quality and the size range of each RNA fraction.

Oocytes were hand-dissected from ovarian fragments of Xenopus laevis, treated with collagenase to remove follicular cells and incubated in modified Barth's medium at 19°C (7). After 15-20 hr, the healthy oocytes were injected with about 100 nl of mRNA (about 1 ng/nl) or the same volume of water and incubated in modified Barth's solution with gentamicin at 19°C for another 3-4 days with daily changes of medium. Subsequently, PAH uptake into oocytes was measured by a radiotracer technique in the presence and absence of probenecid (1 mM). In brief, oocytes were incubated for 30 min in Na free buffer (100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes-tris, pH 7.5) and then placed in a Na (100 mM NaCl) or Na free (100 mM choline chloride) buffer containing 5 μCi of PAH (P-[glycyl-2-^{3}H], 4.4 Ci/mmol, Dupont). After incubation for 60 min at 25°C, the uptake was terminated by washing the oocytes with four 9 ml aliquots of ice cold choline chloride buffer. Each oocyte was dissolved in 0.5 ml of 10% $NaDodSO_4$ and the ^{3}H-PAH was assayed by liquid scintillation spectrometry.

Results

Fig. 1 is a representative formaldehyde gel electrophoregram of fractionated mRNA obtained by sucrose density gradient centrifugation. The size of the mRNA fractions was highly reproducible in four experiments, however, the amount of RNA in each fraction varied as determined by visual inspection of ethidium staining. mRNA from each of 14 fractions (Fig. 2) from a sucrose gradient was injected into oocytes and PAH uptake evaluated. As shown in Fig. 2, fractions 3 and 4, corresponding to mRNA of length 0.7 - 1.3 kb, produced an 8 to 45 fold ($\bar{x}$ = 18.6) increase in PAH uptake in four separate trials compared to control. Fig. 3 depicts a typical distribution of mRNA from a sucrose

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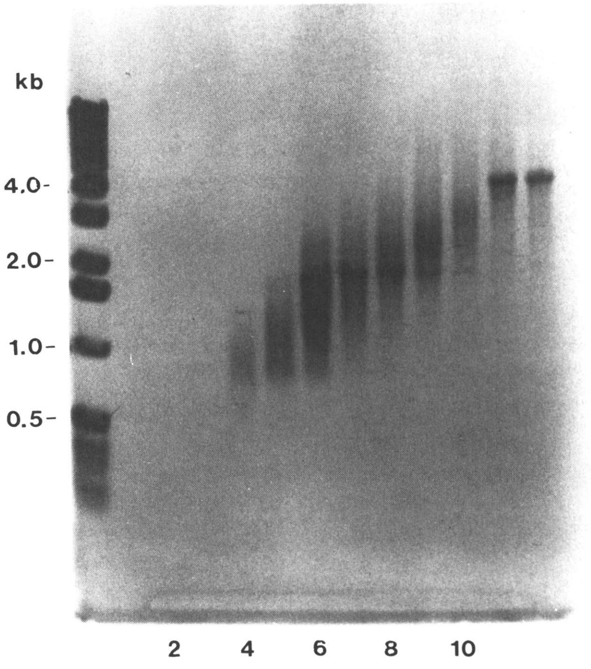

Figure 1. Representative formaldehyde gel electrophoregram of 12 mRNA fractions following sucrose gradient density centrifugation. The numbers on the abscissa refer to the fraction number. Gels were stained with ethidium bromide. The left lane is a 1-kb DNA fragment ladder (Bethesda Research Laboratories).

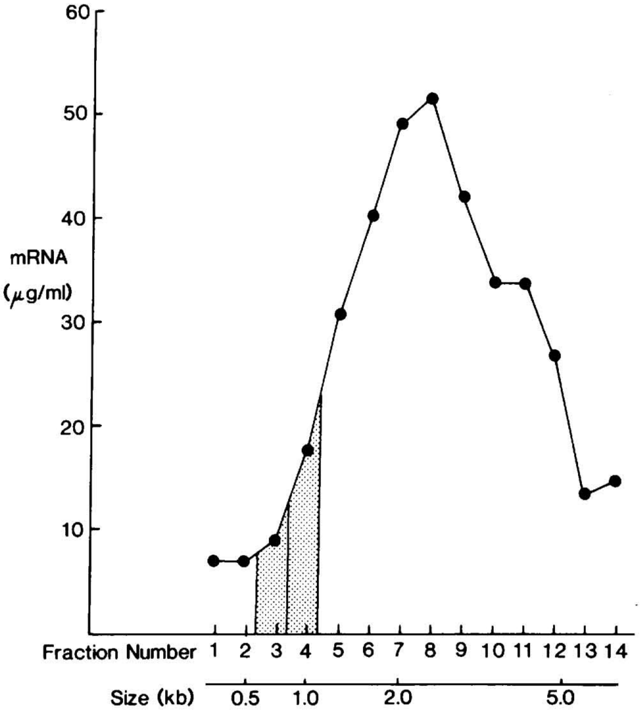

Figure 3. Poly(A)$^+$ mRNA distribution in the sucrose gradients. The shaded panel represents mRNA in Fractions 3 and 4. Size was computed from DNA marker fragments. Concentration was determined by absorbance at 260 nm.

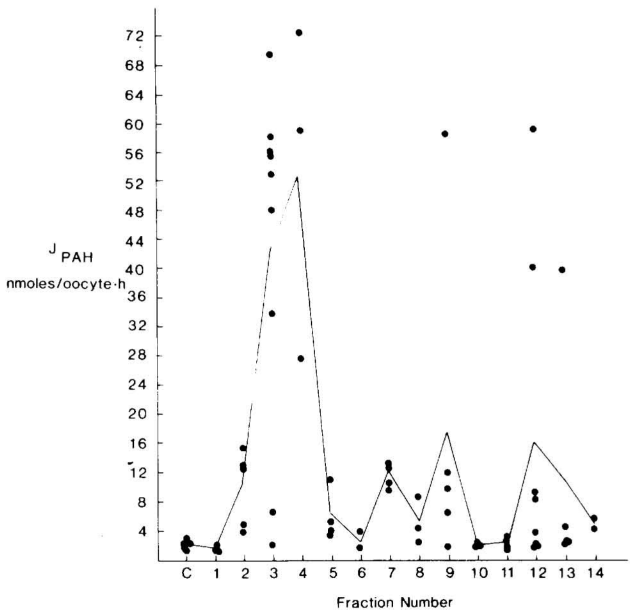

Figure 2. Uptake of PAH (J, nmol/oocyte·hr) into oocytes injected with mRNA fractions obtained following sucrose gradient density centrifugation (Fig. 4). (Note: These are not initial rates but rather a 1-hr integrated uptake.) Following injection with H_2O (C) or mRNA, oocytes were incubated for 4 days prior to measurement of PAH flux. The solid line is the mean uptake.

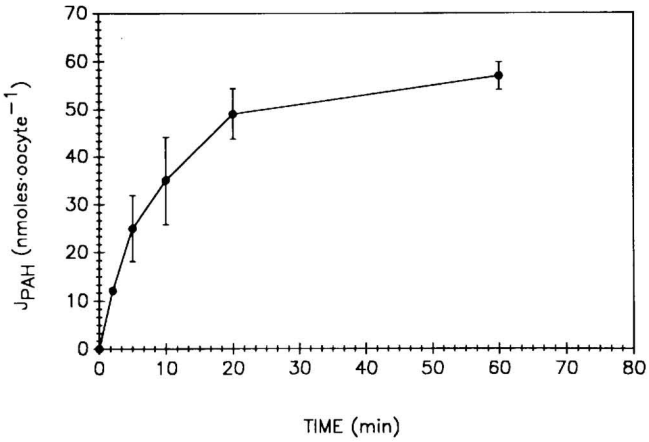

Figure 4. Time course of PAH uptake into oocytes injected with mRNA in Fraction 3 (Fig. 2) from the sucrose gradient. There was virtually no uptake in H_2O-injected controls (4 nmol PAH/hr).

density gradient and indicates the size and distribution of fractions 3 and 4. The time course for PAH uptake is shown in Fig. 4. The control uptake (not shown) was minimal, i.e., 4 nmol·oocyte^{-1} at 60 minutes. Kinetic analysis was performed using PAH concentrations from 20-2000 μM and a 2 min uptake as a measure of the apparent initial velocity (Table I). The Km for the

Table I. Dose Dependence of PAH Uptake in Oocytes Injected with Size Selected mRNA (~1 kb)

PAH (μM)	Uptake (nmol/oocyte·min)	*n*
20	2.5 ± 1.0	5
50	8.6 ± 2.0	5
100	14.8 ± 1.6	4
200	32.6 ± 3.9	6
1000	40.4 ± 19.0	3
2000	52.1	2

Note. Values are x̄ ± SEM. *n* = number of oocytes.

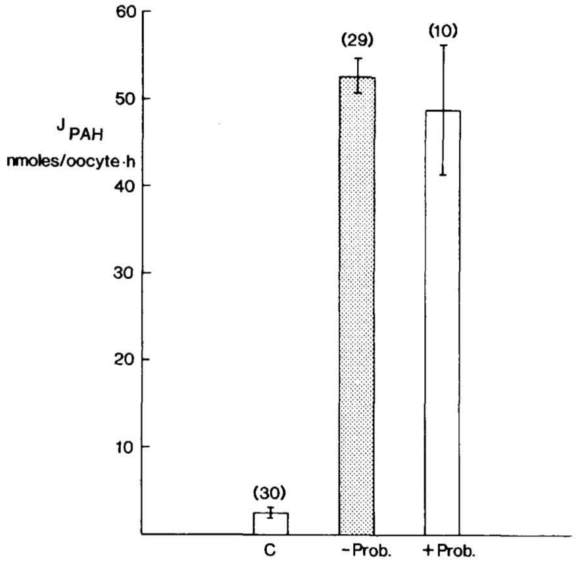

Figure 5. PAH uptake following mRNA injection (as in Fig. 2) in the presence and absence of probenecid, a classical inhibitor of PAH uptake. C is the uptake into water-injected control oocytes.

transport is 333 μM and Vmax is 66.6 nmole·oocyte^{-1}·min^{-1}. This affinity is similar to that obtained for PAH uptake in intact kidneys and slices (8,9). The average volume of each oocyte is 0.523 μl, assuming the egg is a sphere of 0.5 mm radius. If it is assumed the oocyte is 70% H_2O, then the oocyte to medium PAH ratio at 60 minutes is 1.95, indicating an apparent transport against an electrochemical gradient. The oocyte has a membrane potential of -70 to -80 mV (10). The uptake of PAH was not affected by the presence or absence of sodium in the medium nor was the uptake sensitive to probenecid (Fig. 5), a widely utilized inhibitor of active PAH secretion.

Discussion

Our results suggest that mRNA isolated from kidney cortex is indeed capable of expressing a Na independent probenecid-insensitive PAH transport in ovo. Alternatively, the isolated mRNA may have expressed an activator of a preexisting oocyte anion transport system or a PAH binding protein. Following expression, the oocyte appears to be able to concentrate PAH (oocyte/medium PAH ratio > 1); however, little is known about metabolism of PAH in this cell or intraoocyte PAH binding, either of which could vitiate the data. Clearly, however, no binding occurs extracellularly since H_2O injected controls show no significant uptake. Therefore, even if binding accounts for some or all of the uptake, it must be intracellular and thus, the binding would necessarily be preceded by an mRNA induced increase in transport. The size of the mRNA (0.7 to 1.3 kb) suggests the proteins synthesized are in the range of 17 to 58 kD. PAH secretion in the proximal tubule is both Na dependent and probenecid sensitive; however, recent evidence suggests the Na dependence may be due to an indirect coupling of PAH transport to a Na dependent cotransport process (11,12). If that is indeed the case the lack of Na dependence is not surprising. While probenecid is a potent inhibitor of PAH secretion in intact kidneys or slices, it is less effective in isolated membrane systems, e.g., vesicles. Therefore, the lack of a probenecid response in the oocyte may not be significant but rather may suggest that the inhibition of organic anion secretion by probenecid may either be nonspecific or more complicated than a simple interaction with the transporter. Alternatively, inhibitor sensitivity may have been lowered or lost during expression. It has been reported, for example, that the Na,K ATPase has reduced ouabain sensitivity following translation in oocytes (13).

It is not at all clear whether the expression represents a system normally residing in the basolateral membrane or the apical membrane. Recently, Pimplikar and Reithmeier (14) identified a single anion transport protein (130 kD) in the apical membrane of canine proximal tubule using a stilbene affinity column. If this protein component is the apical membrane PAH "carrier", then it would not be likely to be coded for by the mRNA in the size range we have isolated. It is tempting to suggest, on the basis of size, that the mRNA fraction which induces expression in oocytes is coding for basolateral membrane proteins which we have identified using photoaffinity labelling (14). Unequivocal evidence supporting this hypothesis will require cloning and identification of the gene.

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