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AMINO ACID TRANSPORT BY ISOLATED MAMMALIAN RENAL TUBULES

III. BINDING OF L-PROLINE BY PROXIMAL TUBULE MEMBRANES

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SUMMARY

A particulate, brush border-rich membrane fraction was prepared from isolated rabbit renal tubules by agitation in 5 mM disodium EDTA and differential centrifugation. This membrane fraction bound L-proline. Binding was maximal during the first minute of incubation and reached equilibrium within 5 min. Saturation occurred at medium L-proline concentrations of 75 mM. Binding was inhibited completely by *p*-chloromercuribenzoate and by thermal denaturation. Deletion of Na^+ from the incubation medium reduced binding significantly as did NaCN. Ouabain and 2,4-dinitrophenol, however, had no inhibitory effect on binding. Glycine and L-alanine, known to share renal transport sites with proline, inhibited binding, but L-valine and L-phenylalanine did not. These results suggest that the observed binding process represents the initial step in the transtubular transport of L-proline.

INTRODUCTION

In a previous paper[1] in this series[1,2], we studied the transport of L-proline by isolated renal tubule segments prepared by collagenase digestion of rabbit kidney cortex. This imino acid was transported by energy-dependent, substrate-specific mechanisms which were separated into three kinetically distinct transport systems by the use of competitive inhibitors. Although this tissue preparation has many advantages over kidney cortex slices for studying transport[2], even in the isolated tubule the transport process remains a "black-box" where only the end result, accumulation of the substrate by the cell, can be measured.

In 1961, MILLER AND CRANE[3] prepared brush border membranes from hamster small intestine. This preparation was subsequently shown to preferentially bind D-glucose[4,5]. We reasoned that a similar membrane fraction prepared from the isolated renal tubule might be of great assistance in differentiating specific membrane binding from the other complex processes involved in transtubular transport of organic solutes.

The present paper describes the preparation of a brush border-rich particulate membrane fraction from isolated rabbit renal tubule segments and presents evidence that L-proline is specifically bound by such membrane fragments.

METHODS

Preparation of brush border membranes

Isolated renal tubule segments were prepared by collagenase digestion of minced rabbit kidney cortex as described previously[2,6]. The tubule suspension, after the second washing with Krebs–Ringer–bicarbonate–saline buffer (pH 7.4) supplemented with acetate (NaCl, 115 mM; KCl, 5 mM; sodium acetate, 10 mM; NaH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 1.7 mM; $NaHCO_3$, 15 mM; gassed with O_2–CO_2 (95:5, v/v)), was filtered through four layers of surgical gauze (Johnson and Johnson No. 7604). The filtrate was centrifuged at 250 × *g* in an International Model UV centrifuge at room temperature for 2 min. Tubule segments were then suspended in 40 ml of ice-cold 5 mM disodium EDTA at pH 7.4 and agitated by magnetic stirring at 0° for 10 min in a trypsinizing flask. During this hypotonic treatment the tubule segments underwent several morphologic changes, as seen by phase microscopy. First, the tubules swelled. Then individual epithelial cells with distinct brush borders separated from the tubules (Fig. 1). Upon further agitation, many of these brush border forms separated from the cells as discrete identifiable units. Following the agitation, the suspension was centrifuged at 75 × *g* in a Sorvall RC-3 centrifuge at 0° for 5 min yielding three variably separated tissue layers. The top layer (Fig. 2) contained nuclei, small membrane vesicles, and the separated brush borders. The middle layer con-

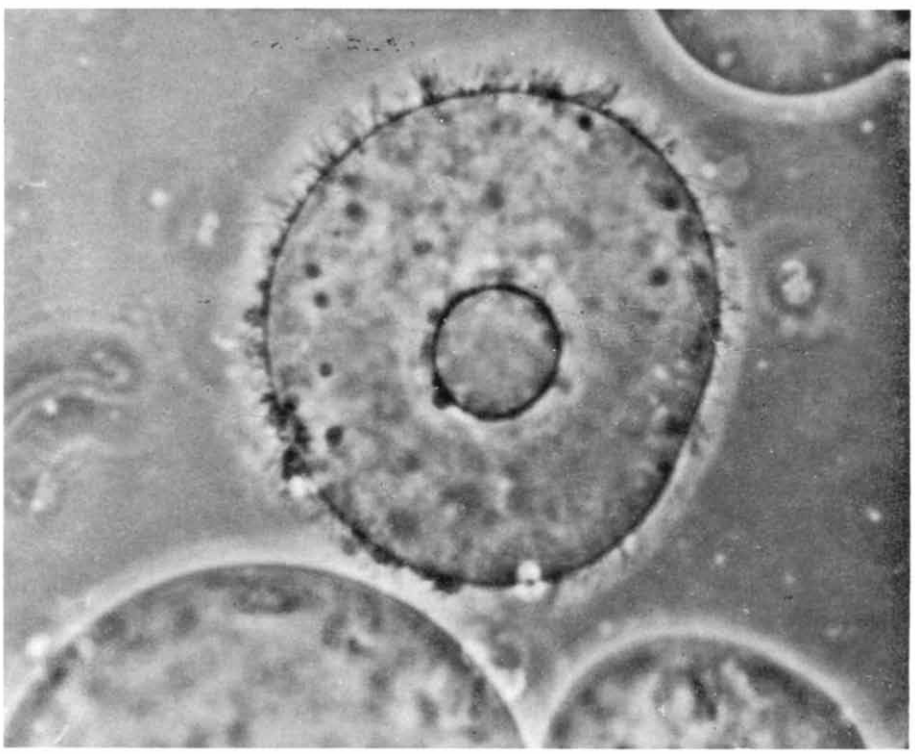

Fig. 1. Phase-contrast micrograph of isolated renal tubular cell demonstrating intact brush border. Such cells appeared during agitation of intact proximal tubules with disodium EDTA (5 mM) as described in detail under METHODS. (× 500.)

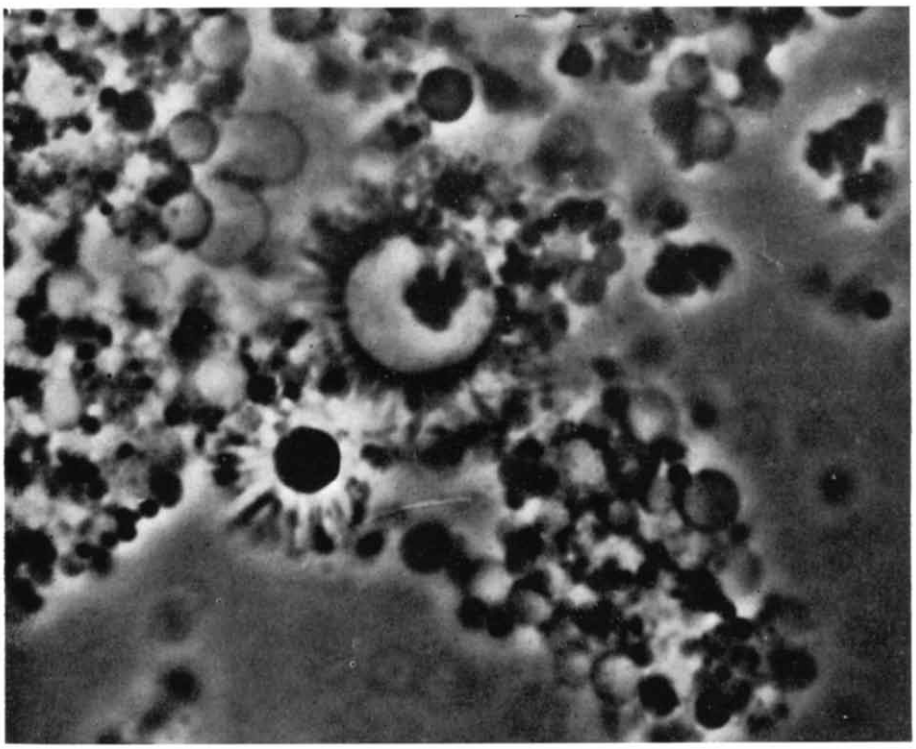

Fig. 2. Phase contrast micrograph of brush border-rich membrane fraction derived from isolated proximal tubules. Note the brush border forms at the center of the field and the membrane vesicles of varying size at the periphery. (× 500.)

tained individual tubule cells with partially separated brush borders. The bottom layer consisted of tubule cells with intact membranes and a few swollen tubule segments. The top and most of the middle layer were removed with a 10-ml serologic pipet and centrifuged again at 75 × *g* in a Sorvall RC-3 centrifuge at 0° for 5 min. This additional centrifugation allowed easy separation of the preparation into two distinct tissue layers. If glass centrifuge tubes were used, no identifiable nuclei were seen in the top layer after this centrifugation. If plastic tubes were used, the remaining nuclei could be removed by filtration through glass wool. The top layer was removed and then centrifuged at 3000 × *g* in a Sorvall RC-2B centrifuge at 0° for 5 min. The supernatant was discarded and the pellet consisting of large membrane fragments rich in microscopically identifiable brush borders was utilized for the binding studies to be described subsequently. When this pellet was suspended in the bicarbonate–saline buffer described above, the brush borders gradually lost their characteristic appearance over a 30–60-min period and formed intact vesicles similar to those seen in the brush border preparations from hamster intestine[3]. The final yield of membrane fragments utilizing both kidneys from a 4–5-lb white New Zealand rabbit averaged 24 mg dry wt. of membrane. The yield could be increased by further agitation of the second and third layers of the membrane suspension, but no morphologically identifiable brush borders were seen in these particulate fractions.

Binding of L-[^{14}C]*proline*

The brush border-rich pellet, suspended in the bicarbonate–saline buffer described above, was pipetted into 12-ml Sorvall glass centrifuge tubes and placed in a constant temperature water bath for 5 min to assure temperature equilibration. Metabolic or competitive inhibitors were added prior to this equilibration period. Following this equilibration, L-[^{14}C]proline and unlabeled L-proline were added to the tube and the suspension was agitated for 1–2 sec using a Vortex mixer. The membrane suspension was then incubated at constant temperature for intervals ranging from 0.3 to 10 min. The reaction was terminated by pipetting duplicate 2-ml samples of the suspension on to tared 1.2-μ Millipore filters attached to a vacuum assembly. It should be emphasized that these filters are not inert and will of themselves bind significant quantities of unlabeled and radioactive substrates unless saturated with buffer solution. The saturation of the filters was assured by putting 10 ml of buffer in the filter holder and by not turning on the suction until 0.3 min prior to pipetting the sample. The samples were then washed with four 10-ml aliquots of ice-cold buffer and dried in an air flow hood. After the filters were reweighed, they were immersed in 10 ml of Liquiflor and counted in a β liquid scintillation spectrometer. Background radioactivity and weight were determined with simultaneous tissue-free blanks of the same specific activity as the test samples incubated and filtered in the same way as tubes containing the membrane suspension. Separate aliquots of the tissue suspension were spun at 50000 $\times$ *g* in a Sorvall RC-2B at 0° for 10 min. Duplicate 25-μl samples of the supernatant medium were then dried on Millipore filters and counted in the same manner as the tissue samples. Binding was expressed as net counts per min of L-[^{14}C]proline retained on the filter corrected for dry tissue weight and adjusted to constant medium radioactivity of 200000 counts/min per ml. All data presented are the mean of 8–16 observations except where stated otherwise.

Activity of (Na^+-K^+)-ATPase in membrane fragments was determined by the method of KATZ AND EPSTEIN[7].

Uniformly labeled L-[^{14}C]proline was purchased from New England Nuclear Corp. L-Proline, L-alanine and glycine were obtained from Mann Laboratories. The collagenase, obtained from Nutritional Biochemicals, was prepared from *Clostridium histolyticum* and had a specific activity of 125–350 μmoles of amino acids digested per mg, expressed as L-leucine. *p*-Chloromercuribenzoate and 2,4-dinitrophenol were purchased from Eastman Chemicals. Disodium EDTA was obtained from Fisher Chemicals.

RESULTS

Morphologic studies

The relatively gentle methods used in the preparation of the brush border suspension allowed the identification of brush borders throughout the procedure by phase microscopy (Figs. 1 and 2). The final preparation contained membrane vesicles of fairly uniform size and was particularly rich in the brush border forms.

(Na^+-K^+)-ATPase activity

The final membrane preparation liberated 21.4 μmoles of phosphate per mg of membrane protein per h. This value compares favorably with that reported for cell

membranes from rat whole kidney homogenates (14.4 ± 2.85) (ref. 8), from rat kidney cortex (9.6 ± 0.8) (ref. 9), and from hamster kidney cortex (19.8 ± 1.2) (ref. 9).

Specificity of binding

L-Proline, glycine, and D-glucose, all known to be actively transported by the renal tubule were bound by the brush border preparation. (Only proline binding is further discussed in this paper.) Inulin, which is not transported, did not demonstrate any binding to this preparation. Preboiling of the tissue preparation prevented any proline binding, as did the addition of strong acids or bases to the membrane suspension.

Weight dependence of proline binding

When membranes were incubated for 5 min at an initial medium proline concentration of 0.05 mM, proline binding was directly proportional to the dry weight of membranes used (Fig. 3). Binding was linear from 0.19 to 1.2 mg dry wt. Dry weights greater than 1.5 mg exceeded the filtering capacity of the system. Most experiments utilized between 0.6 and 0.9 mg dry wt. per filter. Under these conditions 213 ± 38 counts/min were bound per mg dry wt.

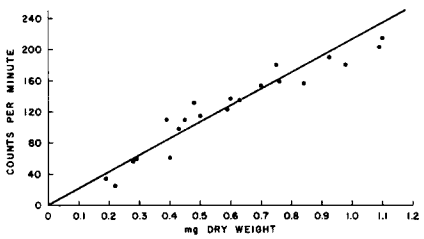

Fig. 3. Linear relationship between proline binding (ordinate) and membrane dry wt. (abscissa). These data were obtained from experiments in which L-proline (0.05 mM) was incubated with the particulate membrane suspension for 5 min. Each point represents a single observation.

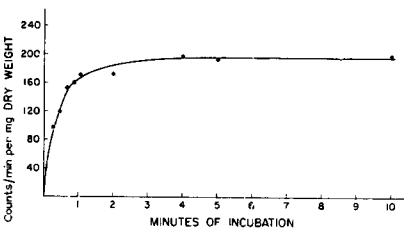

Fig. 4. Time-course of binding of L-proline (0.05 mM) by brush border-rich membrane fraction from isolated renal tubules. The data represent single observations from one of five representative time curves. Incubation was conducted at 37°.

Time studies

Proline (0.05 mM) binding by the brush border preparation was maximal in the first minute of incubation (Fig. 4). There was an additional small increment of binding between 1 and 4 min of incubation. After 5 min, the binding remained constant.

Temperature dependence

At equilibrium (5 min), binding by the membrane preparation was constant at temperatures between 10 and 38°. At 0° binding was reduced by about 30 %. At temperatures greater than 45°, binding decreased. At 56° binding was 20 % less than that observed at 37°, and at temperatures greater than 56°, the membranes became gelatinous and only small amounts of proline were bound (1–10 % of the binding at 37°). Repeated studies utilizing preboiled membrane preparations failed to show any bound radioactivity.

Saturation

At equilibrium (5 min), binding was a saturable process (Fig. 5). Maximal binding occurred at initial medium concentrations of proline of 75 mM suggesting that the substrate concentration required to achieve half maximum binding (K_m) is in the range of 30–35 mM. Maximal binding averaged 40 μg proline per mg dry wt. of membrane preparation per 5 min.

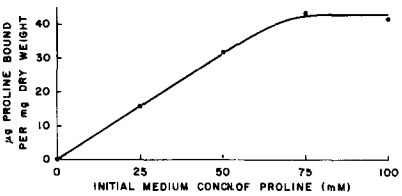

Fig. 5. Evidence for saturability of L-proline binding by brush border-rich membrane fraction. Incubation was carried out for 5 min at 37°. Each point represents the mean of at least eight observations. See text for estimation of K_m derived from these data.

TABLE I

INFLUENCE OF METABOLIC INHIBITORS AND Na^+ CONCENTRATION ON PROLINE BINDING BY BRUSH BORDER MEMBRANES

Membranes were incubated in Na^+-free buffer or with the compounds listed for 5 min at 37° prior to 5 min incubation with L-proline (0.05 mM). Na^+-free buffer was prepared by equimolar substitution with Tris or choline. Values represent the mean ± S.E.

Experimental conditions	*Inhibition (−) or stimulation (+) (%)*	*Number of observations*
p-Chloromercuribenzoate (1 mM)	−89.8 ± 8.3	8
NaCN (0.01 M)	−64.4 ± 12.8	8
Ouabain (0.5 mM)	+18.7 ± 13.3	6
2,4-Dinitrophenol (0.25 mM)	− 0.5 ± 20.5	8
ATP (5.4 mM)	+ 5.3 ± 7.5	4
Na^+-free medium	−43.2 ± 1.9	8

Inhibitor studies

As shown in Table I, the addition of 1 mM *p*-chloromercuribenzoate to the incubation medium inhibited proline binding by more than 90 %. NaCN (0.01 M) inhibited binding by 60 %. Each of these compounds changed the physical characteristics of the membrane preparation and made it more gelatinous. Ouabain (0.5 mM) failed to inhibit proline binding and rather appeared to increase binding by 10–30 % in these experiments. Removal of Na^+ from the incubation medium reduced binding by 43 %. 2,4-Dinitrophenol (0.1 and 0.25 mM) and ATP (2.7 and 5.4 mM) had no effect on binding.

Competitive inhibition

L-Alanine and glycine, amino acids known to share transport systems with proline[1] in the intact tubule, inhibited proline binding maximally by 34 and 38 %, respectively (Table II). L-Valine and L-phenylalanine, which have not been demonstrated to share transport systems with proline, had no affect on proline binding.

TABLE II

INFLUENCE OF SECOND AMINO ACID ON PROLINE BINDING

Membranes were incubated with the listed amino acids for 5 min at 37° prior to 5-min binding studies with L-proline (0.05 mM). Fractional inhibition is expressed as the mean ± S.E. of at least four separate observations under each condition.

Amino acid added	*Inhibition* (%)	
	50 mM	*100 mM*
Glycine	−29.2 ± 6.4	−37.9 ± 5.0
L-Alanine	−15.5 ± 7.6	−33.6 ± 3.3
L-Valine	− 2.6 ± 2.4	0.0 ± 0.3
L-Phenylalanine	− 2.3 ± 5.2	Insoluble

Comparison of binding by brush borders and transport by the intact tubules

By utilizing previously published data[1], effects of metabolic and competitive inhibitors on binding of L-proline by the brush border suspension were compared to their effects on proline transport by renal tubule segments (Fig. 6). It is of particular interest that ouabain and 2,4-dinitrophenol, which decrease proline transport by the tubule markedly, exert no inhibitory effect on binding, whereas CN^- inhibits both transport and binding. Deletion of Na^+ likewise impairs both binding and transport, but the effect on transport is quantitatively much greater. The competitive inhibitory effects of alanine and glycine on proline binding and transport are about equal. It is also noteworthy that the estimated K_m for binding by the brush border membranes (30–35 mM) agrees very well with the estimated K_m of 30 mM for the low affinity proline transport system in intact tubules[1].

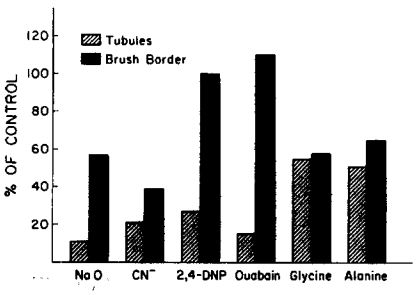

Fig. 6. Comparison of effects of Na^+ deletion and metabolic inhibitors on uptake of L-proline by intact tubules (hatched columns) versus binding of L-proline by brush border membrane fraction (closed columns). Medium concentration of L-proline was 0.05 mM throughout. See text for experimental details. 2,4-DNP = 2,4-dinitrophenol.

DISCUSSION

These results demonstrate that intact renal tubules, obtained by collagenase digestion of minced kidney cortex, provide a suitable tissue source from which to prepare a particulate membrane fraction rich in microscopically identifiable brush

borders. This fraction is almost surely admixed with other subcellular membranes, but the relatively low centrifugal force (3000 × *g*) used in preparing the final membrane suspension, the microscopic appearance, and the presence of cation-stimulated ATPase activity suggest that the preparation consists largely of tubular brush border membrane fragments. Initially, we were concerned that what we have called proline "binding" is merely nonspecific adsorption of proline to tissue fragments. However, the failure of inulin to be retained by the tissues, the inhibition of binding by boiling the tissue or by specific sulfhydryl reagents, and the selective inhibition of proline binding by amino acids known to compete with proline for transport *in vivo* and *in vitro* all argue against an interpretation of trivial nonspecific "sticking" or adsorption. The time-course and saturability of the proline binding process further support the thesis that we are studying the interaction between proline and its tubular "carrier" system(s) or "reactive site(s)".

If we assume that proline is being bound largely to brush border membranes and that such binding reflects the initial step in tubular reabsorption of this imino acid, it is interesting to compare the effects of various experimental conditions on binding with those observed on transport of proline by intact tubule segments. Glycine and L-alanine have similar maximal inhibitory effects on proline binding and on transport by intact tubules. This suggests that competition between these amino acids and proline occurs primarily during binding. Dinitrophenol, which uncouples oxidative phosphorylation, and ouabain, a specific inhibitor of (Na^+-K^+)-ATPase, inhibit transport markedly but do not impair binding. These data and the striking temperature independence of binding imply that the binding reaction is not dependent on oxidative metabolism or on ATPase activity which presumably affect the transport process at some step distal to the initial binding reaction. Deletion of Na^+ from the incubation medium impairs transport more than binding, suggesting that this ion may act at more than one site in the transport process. The striking inhibitory effect of *p*-chloromercuribenzoate on binding implies either that proline transport is initiated by a reaction in which the substrate interacts with a sulfhydryl group of a specific protein species or that lipid protein sulfhydryl interaction may be required for substrate binding to carrier.

If these initial promising results are confirmed by subsequent analyses with other substrates, hormones and drugs, it may be possible with further purification of the brush border-rich membrane fraction to advance one step closer to the ultimate goal of the study of renal transport mechanisms, namely, the isolation and characterization of the specific carrier proteins which catalyze and control transtubular transport.

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