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THE EFFECT OF COLCHICINE ON CHOLESTEROL BIOSYNTHESIS IN CONCANAVALIN A-STIMULATED LYMPHOCYTES

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Summary: Attempting to test the hypothesis that colchicine blocks lymphocyte activation at the commitment point, we determined whether or not colchicine inhibited the ConA-induced increase in the synthesis and content of cholesterol in lymphocyte cultures. Colchicine decreased the incorporation of (^{14}C) acetate into cholesterol by about 50%. However, the decrease affected unstimulated and stimulated lymphocytes equally. The presence of colchicine for as long as 48 hours did not prevent stimulated lymphocytes from accumulating cholesterol. The results suggest that an intact microtubular system is not required for the initiation of blastogenesis and the activation of cholesterol synthesis.

Introduction: Colchicine and other antimicrotubular drugs inhibit DNA synthesis in lectin-stimulated lymphocytes (1,2). Where in the cell cycle these antimicrotubular drugs act to block lymphocyte activation is presently unknown. Kinetic studies by Edelman and co-workers (1,2) showed that colchicine blocks lymphocyte activation at or near the point at which lymphocytes become committed to undergo DNA replication. These authors raised the possibility that microtubules play a crucial role in transmitting the signal from the cell surface to the cell interior. However, a controversy exists in the literature about this hypothesis. Rudd, et. al. (3) recently questioned the validity of Edelman's conclusion by showing the time course of colchicine effect. Other investigators have reported that some reactions considered to be early manifestation of blastogenesis were not inhibited by antimicrotubular drugs. Thus, colchicine does not inhibit the increased incorporation of (^{14}C)oleate into phosphatidylcholine (4), the

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Methods and Materials

Rabbit renal brush border membrane vesicles were prepared as described by Malathi et al. (10). The purity of the membranes was routinely evaluated by specific marker enzymes (11) and occasionally by electron microscopy. Transport was assayed by the Millipore filtration technique as described previously (11).

Recovery and identification of intravesicular contents: Membrane vesicles were incubated with 0.32 mM [1-^{14}C]glycyl-L-proline in NaCl medium as described in the transport assay (11) for different time intervals. The membrane vesicles were collected on Millipore filters. For each time point, 24 filters were used. After washing, the filters were transferred to 20 ml of boiling water. This step was necessary to inactivate the glycyl-L-proline hydrolase activity of the membrane vesicles. The filters were cut into small pieces and the extraction was continued at 25°C in a shaker for 30 min. The extract was filtered and freeze-dried. The residue was dissolved in a minimum volume of 95% ethyl alcohol and subjected to paper chromatography, using n-butyl alcohol/acetic acid/water (4:1:1, v/v) as the solvent. The spots were identified by a Packard Radiochromatogram Scanner. Recovery of the intravesicular content by this method was 94 $\pm$ 8%. No recovery corrections were used in subsequent calculations.

Kinetics of hydrolysis: The hydrolysis of [1-^{14}C]glycyl-L-proline by the brush border vesicles was studied in the incubation system used in the transport assay. Hydrolysis was stopped by heat inactivation of the enzyme by placing the tube containing the reaction mixture in boiling water for 1.5 min. The hydrolytic products were separated by paper chromatography using n-butyl alcohol/acetic acid/water (4:1:1, v/v) as the solvent. With the guidance of the reference standards, the glycine spots were cut out and quantitated by liquid scintillation counting. Ninhydrin was used to visualize the standard spots.

Intravesicular volume: Intravesicular volume was calculated by subtracting extravesicular volume, measured with [methoxy-^{14}C]inulin from the total water content. Total water content was measured by drying as well as by using [^{3}H]water.

Materials:

[1-^{14}C]Glycyl-L-proline (specific activity, 7 mCi/mmol) was purchased from the Radiochemical Center, Amersham, England. [Methoxy-^{14}C]inulin and [^{3}H]water were from New England Nuclear Corporation, Boston, MA. All other chemicals were of analytical grade.

Results and Discussion

Microperfusion techniques designed to study the handling of peptides by the proximal tubule have employed small linear peptides such as bradykinin and angiotensin II. Using these hormonal peptides, Carone and coworkers (4-6) have demonstrated that small linear peptides are hydrolyzed by the brush border membrane of the tubular cells, followed by the reabsorption of most of the hydrolytic products. Silbernagl also has shown (7,8) with a similar technique that dipeptides are split by the brush border enzymes and are reabsorbed as free amino acids. Welch and Campbell (9) have recently employed purified renal brush border membrane vesicles to study the handling of L-alanyl-glycine by

these vesicles. They showed that a dipeptidase, highly active towards this dipeptide, is enriched in the renal brush border vesicles. It was also found that hydrolysis of this dipeptide by the purified vesicles was instantaneous, and the characteristics of the uptake of radiolabels from L-alanyl-[^{3}H]glycine and [^{14}C]glycine were similar. They suggested that renal dipeptidase could hydrolyze the dipeptides in the glomerular filtrate with the subsequent reabsorption of the resultant free amino acids, thus making separate dipeptide transport systems unnecessary.

In the present study, we used glycyl-L-proline to investigate this problem. We have recently shown that purified renal brush border vesicles possess glycyl-L-proline hydrolytic activity (11). However, the activity was low, and about 80% of glycyl-L-proline in the medium remained intact even after 30 min incubation with the vesicles in a regular transport assay using 0.5-0.6 mg of the membrane protein.

Kinetics of hydrolysis: The hydrolysis of glycyl-L-proline by the renal brush border vesicles was studied over a substrate concentration range of 0.32 mM-6.4 mM. The results are shown in Fig. 1 as a Lineweaver-Burk plot. The kinetic constants calculated by least squares fit analysis of the data showed that the Km for glycyl-L-proline hydrolysis was 0.69 $\pm$ 0.06 mM and Vmax was 773 $\pm$ 12 pmol/min/mg membrane protein. According to Welch and Campbell (9), the Km for hydrolysis of L-alanyl-glycine was 2.73 $\pm$ 0.68 mM and Vmax was 47.6 $\pm$ 19.6 µmol/min/mg membrane protein. While the Km values for the hydrolysis of the two dipeptides were comparable, Vmax values differed by several orders of magnitude. Glycyl-L-proline was about 60,000 times less susceptible to hydrolysis by the renal brush border vesicles than L-alanyl-glycine.

Identification of intravesicular contents: The intravesicular contents were chromatographed following incubation with 0.32 mM [1-^{14}C]glycyl-L-proline for 1 min and 30 min in NaCl medium. Fig. 2 shows that transport occured predominantly as intact glycyl-L-proline with 1 min incubation, even though there

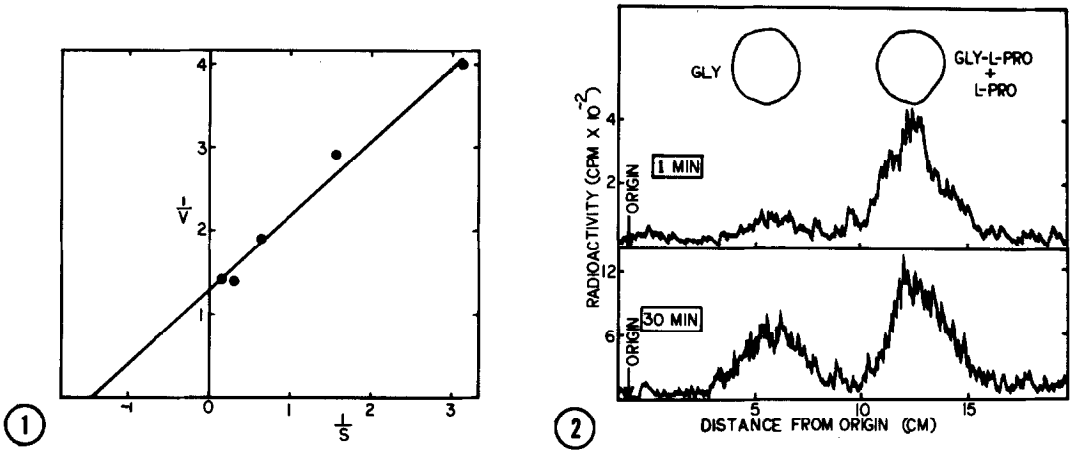

Figure 1: Lineweaver-Burk plot of glycyl-L-proline hydrolysis by rabbit renal brush border membrane vesicles. The membrane vesicles were present in 1 mM Hepes-Tris buffer, pH 7.5 containing 300 mM mannitol. Hydrolysis was initiated by adding 50 µl of membrane suspension (0.75 mg protein) to 100 µl of the medium containing 1 mM Hepes-Tris buffer, pH 7.5, 100 mM mannitol, 100 mM NaCl and varying concentrations of glycyl-L-proline (including traces of [1-^{14}C]glycyl-L-proline). After 30 min incubation at 25°C, the hydrolysis was stopped by heat treatment.

V, nmol of glycyl-L-proline hydrolyzed x min^{-1} x mg $protein^{-1}$.

S, concentration of glycyl-L-proline in mM.

Figure 2: Radiochromatogram scans of the intravesicular contents of membrane vesicles incubated with 0.32 mM [1-^{14}C]glycyl-L-proline in the presence of a Na^+-gradient for 1 min and 30 min. With n-butyl alcohol/acetic acid/water (4:1:1, v/v) as the solvent system, the R_f values of glycyl-L-proline and L-proline were identical. However, this did not complicate the interpretation of the scans because L-proline moiety of [1-^{14}C]glycyl-L-proline was not labelled.

was a trace amount of free glycine. Glycyl-L-proline represented more than 80% of the total radiolabel present inside the vesicle. With 30 min incubation, although the absolute amounts of both glycyl-L-proline and free glycine inside the vesicles increased, only about 65% of the total radiolabel was glycyl-L-proline.

Fig. 3 shows the time course of the accumulation of intact glycyl-L-proline and free glycine inside the vesicles from NaCl medium containing 0.32 mM glycyl-L-proline.

Transport in NaCl and KCl media: The intravesicular contents after incubation with 0.32 mM [1-^{14}C]glycyl-L-proline for 30 min in NaCl and KCl media were separated into glycyl-L-proline and glycine and quantitated. In KCl medium,

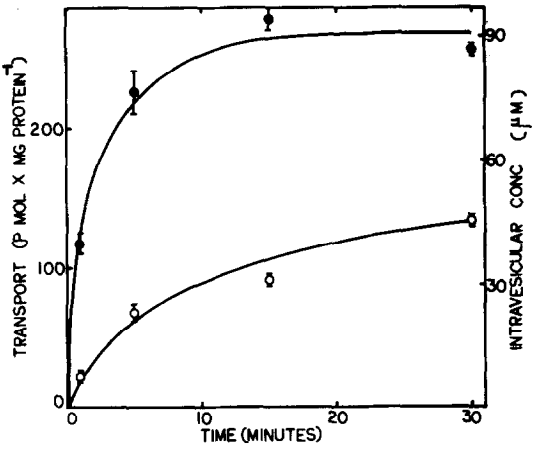

Figure 3: The time course of accumulation of glycine and glycyl-L-proline inside the brush border membrane vesicles. The membrane vesicles were incubated with 0.32 mM_+ [1-^{14}C]glycyl-L-proline for varying intervals of time in the presence of a Na^+-gradient.

●——● glycyl-L-proline

O——O glycine

the intravesicular content of glycyl-L-proline was 317.0 pmol/mg membrane protein and that of glycine was 217.6 pmol/mg membrane protein. In NaCl medium the intravesicular content of glycyl-L-proline was 326.9 pmol/mg membrane protein and that of glycine was 139.4 pmol/mg membrane protein. This shows that the amount of glycyl-L-proline inside the vesicles was the same in both KCl and NaCl media. But, the intravesicular glycine content in the KCl medium was greater than in the NaCl medium. We have previously shown (11) that the transport of radiolabel from [1-^{14}C]glycyl-L-proline was the same in both NaCl and KCl media during the initial periods of incubation (1-5 min) while the transport in the KCl medium was greater than in the NaCl medium during longer periods of incubation (20-40 min). Since free glycine transport is stimulated by the presence of a Na^+-gradient (12), the results suggest that the transport system for free amino acids was not responsible for at least a major portion of intravesicular glycine. The efflux of free glycine from the vesicles into the medium would be more in NaCl medium than in KCl medium and this would explain why there is more intravesicular glycine

in the presence of KCl as compared to NaCl. The efflux of glycyl-L-proline, if any, would be the same in both media.

These results also suggest that most of the intravesicular glycine should have resulted from the hydrolysis of glycyl-L-proline either during or after the transport of the dipeptide. Even though brush border membranes contain glycyl-L-proline hydrolase activity, the possibility that traces of soluble glycyl-L-proline hydrolase might have been trapped inside the vesicles which was undetectable by the paper chromatography method employed in the earlier study (11) could not be completely ruled out. The exact role of the membrane-bound glycyl-L-proline hydrolase in dipeptide transport or dipeptide hydrolysis or both remains to be elucidated.

The intravesicular volume was calculated by subtracting the extravesicular volume from the total water content. The conventional drying method always gave higher values for the total water content than the [^{3}H]water method. The total water content was 11.16 $\pm$ 0.18 µl/mg membrane protein by the drying method and 7.90 $\pm$ 0.10 µl/mg membrane protein by the [^{3}H]water method. It is possible that the weight loss in drying at 105-110°C, though commonly taken to be entirely made up of water, might include some components volatilized in organic decomposition of the cell constituents (13). On the contrary, the total water content determined by the [^{3}H]water method represented the volume of water exchangeable with [^{3}H]water (the so called 'functional' water). We believe that it would, therefore, be more appropriate to use the volume of the functional water to calculate the intravesicular concentration of the dipeptide rather than the total water content as determined by the drying method. The intravesicular volume was calculated by subtracting the extravesicular water content (4.95 $\pm$ 0.42 µl/mg membrane protein) from the 'functional' water. The intravesicular volume was 2.96 $\pm$ 0.43 µl/mg membrane protein.

The intravesicular concentration of glycyl-L-proline at 30 min incubation was calculated to be about 90 µM while the initial medium concentration was 320 µM. Though there was about 20-30% hydrolysis of the dipeptide in the medium

during 30 min incubation (11), the intravesicular concentration of glycyl-L-proline never seems to exceed the concentration in the incubation medium. Since many dipeptides have been shown to be potent inhibitors of glycyl-L-proline transport into renal brush border vesicles (11,14), transport of glycyl-L-proline appears to be a Na^+-independent, carrier-mediated process rather than simple diffusion. Evidence has been previously presented to show that the radiolabel from [1-^{14}C]glycyl-L-proline gets accumulated in an osmotically responsive intravesicular space (11). Renal tubular cells contain a very active cytosol glycyl-L-proline hydrolase (15). One of the functions of this enzyme may be to hydrolyze glycyl-L-proline entering the cell through this dipeptide transport system under normal physiological conditions. This would result in very low cytoplasmic dipeptide concentrations which would maintain a favorable peptide concentration gradient for further peptide transport.

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