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EFFECT OF PHOSPHOLIPASE AND TRYPSIN ON HISTIDINE UPTAKE BY MOUSE BRAIN SLICES

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SUMMARY

1. Mouse brain slices accumulated histidine from a wide range of external histidine concentrations. On 60 min incubation in oxygenated Krebs–Ringer bicarbonate medium supplemented with glucose, saturation was reached with 20 mM histidine, the uptake being 32 μmoles/g tissue.

2. Phospholipases A and C inhibited histidine uptake by brain slices from varying histidine concentrations. Initial influx of histidine into brain slices pretreated with phospholipase A was inhibited to an extent that depended on enzyme concentration.

3. Trypsin did not interfere with histidine uptake. Preincubation of brain slices with trypsin enhanced the inhibiting effect of phospholipase A on uptake of histidine.

4. Treatment of brain slices with phospholipase A under conditions required to completely stop histidine accumulation resulted in slight hydrolysis of their phosphatidylcholine and phosphatidylethanolamine.

INTRODUCTION

Transport of small molecules through membranes and their subsequent accumulation within cells are processes depending on membrane integrity and maintenance of structure. It is therefore of interest to study how changes in membrane components affect influx and efflux of small molecules.

In recent years enzymes are being used for specifically removing membrane constituents, thus making possible study of the relation between membrane structure and function[1–5]. In the experiments reported here the action of phospholipases and trypsin on mouse brain slices has been studied in terms of their ability to affect uptake and efflux of histidine. This amino acid was chosen in view of its known high uptake by this tissue[6].

MATERIALS AND METHODS

Brain slices

Male white mice of a local strain, weighing 20–22 g were used throughout. The mice were killed by decapitation. The brains were rinsed in saline and after removal of

the brain stem cut transversely into thin slices with a razor blade. Routinely, 12 slices were obtained from each brain, having a total weight of 0.375 ± 0.003 g (mean ± S.E.; 15 determinations).

Histidine uptake

In the standard experiment the slices obtained from one brain were suspended in 10 ml of Krebs–Ringer bicarbonate buffer containing 0.3 % glucose and [^{14}C]histidine at a concentration of 0.5 μmole/ml (specific activity 8 μC/mmole, unless otherwise stated). The suspension was gassed with O_2–CO_2 (95:5, v/v) and incubated at 37° for 60 min. The suspension was filtered and the slices homogenized in 2 ml of cold 5 % trichloroacetic acid. Preliminary experiments showed that rinsing of the slices with Krebs–Ringer buffer did not remove any radioactivity from them. After centrifugation, 1 ml of clear supernatant was mixed with 5 ml solution according to BRAY[7] and counted in a Packard liquid scintillation spectrometer with an efficiency of 52 %. In control experiments radioactivity in the medium at the end of the incubation period was also determined. Under the standard conditions described, the ratio of radioactivity in the trichloroacetic acid-soluble material obtained from 1 g brain (calculated) to radioactivity in 1 ml medium at 60 min was 7.82 ± 0.36 (mean ± S.E.; 8 determinations). Other control experiments showed that 96 % of the radioactivity in the slices was in the trichloroacetic acid-soluble material and that 95 % of the radioactivity in this fraction chromatographed as histidine.

Histidine efflux

Slices preincubated for histidine uptake under the standard conditions described above were transferred into 10 ml of a glucose-containing Krebs–Ringer medium. After varying periods of incubation the trichloroacetic acid-soluble material in the slices was separated and counted and the radioactivity related to that of a control sample prepared immediately after uptake (0 min efflux). All determinations were done on triplicates.

Lipid extraction and analysis

Brain lipid was extracted by chloroform–methanol (2:1, by vol.). Phospholipids were separated on Silica gel G plates by two dimensional chromatography, using chloroform–methanol–water (65:25:4, by vol.) as the first solvent system and 1-butanol–acetic acid– water (60:20:20, by vol.) as the second. Phosphorus in phospholipid spots was determined as described by ROUSER AND FLEISCHER[8].

Enzymes and chemicals

Phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4) was isolated from the venom of *Hemachatus haemachates* by paper electrophoresis and purified by gel filtration of a 4 % trichloroacetic acid precipitate, as described by ALOOF-HIRSCH[9]. The material electrophoresed as a single band on a polyacrylamide gel. Phospholipase C Type I (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Clostridium welchii* was purchased from Sigma Chemical Co. and trypsin, 2 times crystallized, from Worthington Biochemical Corp. Lysolecithin was prepared from rat liver lecithin and purified according to SAUNDERS[10]. Bovine albumin, fatty acid poor, was purchased from Gallard Schlesinger Chemical Mfg. Corp.; L-[ring-2-^{14}C]histidine from the Radio-

chemical Centre Amersham and Silica gel G from Merck AG. All chemicals used were analytically pure.

RESULTS

Histidine uptake

Phospholipases. The effect of phospholipase A on histidine uptake from various concentrations of the amino acid is shown in Fig. 1. At 10 μg/ml the enzyme inhibited uptake from a wide range of histidine concentrations (0.25–20 mM) to about the same extent, causing a decrease of 50–60 % in the amount accumulated in brain slices on 60 min incubation. Time dependent accumulation of histidine from two different initial concentrations of the amino acid (0.5 and 5.0 mM) both in the absence and presence of phospholipase A is described by the curves in Fig. 2. In agreement with findings by NEAME[6], the rate of histidine accumulation in brain slices decreased with time, but saturation was not reached within 60 min. In the presence of phospholipase

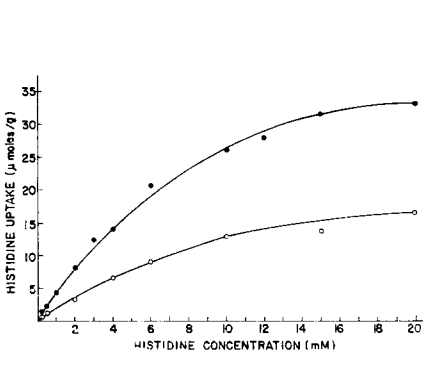


Fig. 1. Effect of phospholipase A on histidine uptake from varying initial concentrations of histidine. Incubation time 60 min, specific activity of histidine 1.6 μC/mmole. ●—●, without enzyme; ○—○, with phospholipase A, 10 μg/ml.

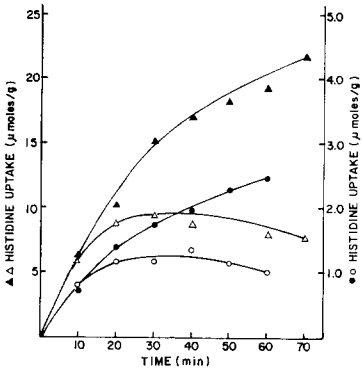


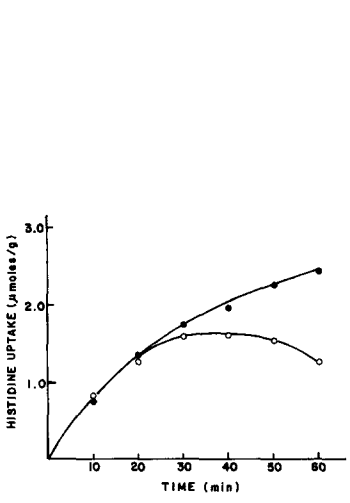
Fig. 2. Kinetics of histidine uptake in presence of phospholipase A. Specific activity of histidine at 5 mM, 1.6 μC/mmole and at 0.5 mM, 8 μC/mmole. ▲—▲, 5 mM histidine, without enzyme; △—△, 5 mM histidine, with phospholipase A, 10 μg/ml. ●—●, 0.5 mM histidine, without enzyme; ○—○, 0.5 mM histidine, with phospholipase A, 10 μg/ml.

A histidine uptake from both concentrations was unaffected during 10 min, slight inhibition being observed first at about 20 min. Little more histidine was taken up between 20 and 40 min. Beyond 40 min a gradual decrease in the amount of radioactivity in the slices was found at both histidine concentrations.

To test the possibility that inhibition of histidine uptake by phospholipase A is due to the action of free fatty acids or lysocompounds formed, uptake experiments were performed in the presence of various fatty acids and lysolecithin. Palmitic, lauric, linoleic and oleic acids, each tested separately at concentrations up to 1 mM, and lysolecithin at concentrations up to 400 μg/ml did not affect uptake of histidine under standard conditions. Albumin at 20 mg/ml did not diminish the inhibitory effect of phospholipase A on histidine uptake by brain slices. In contrast, phospholipase C did interfere with histidine uptake (Fig. 3). At a concentration of 100 μg/

ml inhibition started at 30 min; otherwise the kinetics were similar to those found with phospholipase A. Thus it appears that impairment of histidine uptake by phospholipase A is a result of degradation of membrane phospholipids *per se*.

Initial influx of histidine into brain slices preincubated with phospholipase A is presented by the curves in Fig. 4. The slices were subjected to the action of this enzyme at varying concentrations for 60 min, filtered, rinsed with saline and transferred into a [^{14}C]histidine-containing medium for uptake. It is seen that influx of the amino acid, determined at 2.5 and 5 min, was inhibited in all pretreated brain samples to an extent that depended on enzyme concentration. The curves also indicate that even following fairly drastic treatment with phospholipase A (50 μg/ml; 60 min) brain slices retain some of their ability to take up histidine.



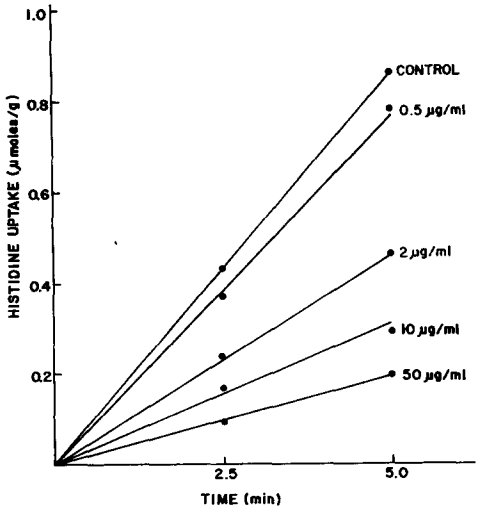


Fig. 3. Effect of phospholipase C on histidine uptake. 2.5 mM $CaCl_2$ was added to all the flasks. ●—●, without enzyme; ○—○, with phospholipase C, 100 μg/ml.

Fig. 4. Initial influx of histidine into phospholipase-treated brain slices. Slices were incubated for 60 min in Krebs–Ringer medium containing varying concentrations of phospholipase A. After being rinsed with saline the slices were introduced into the standard medium for uptake. Specific activity of histidine 16 μC/mmole.

Trypsin. In preliminary experiments the trypsin preparation was found to have in the standard Krebs–Ringer medium a specific activity of 2.9 units/mg on denatured casein, this being 92 % of its activity in 0.1 M phosphate buffer, pH 7.6. In contrast to the marked inhibitory effect of phospholipase A, trypsin at 100–500 μg/ml decreased the amount of histidine accumulated in brain slices on 60 min incubation by 5–15 % only; inhibition was unrelated to enzyme concentration. An action of trypsin on brain slices could be demonstrated when used together with phospholipase A (Fig. 5). Slices preincubated with trypsin for 20 min and then subjected to phospholipase, accumulated histidine normally for 10 min, but did not take up any more of the amino acid beyond this period. In control slices treated with phospholipase alone, histidine uptake stopped at 20 min incubation. Thus pretreatment of brain slices with trypsin enhanced the inhibitory action of phospholipase on

histidine uptake. This effect was observed only when treatment of the slices with this enzyme preceded the addition of phospholipase.

Histidine efflux

The effect of phospholipase A and trypsin on histidine efflux is shown in Fig. 6. With the technique employed, enzyme-treated and untreated slices lost about 15 % of their radioactivity within the first min of efflux. In the absence of enzyme a gradual loss of histidine occurred thereafter, leaving about 55 % of the amino acid in the slices on 45 min incubation. Presence of phospholipase A in the efflux medium at 10 μg/ml resulted in the loss of an additional 15 % of radioactivity on 45 min incubation. With trypsin at 100 μg/ml, increase in the rate of histidine efflux was about half that observed with phospholipase.

The effect of ouabain on histidine efflux was studied for comparison. As the enzyme required 20 min for expression of its inhibiting activity in standard uptake experiments whereas ouabain caused pronounced inhibition at 2.5 min (C. KIRSCHMANN, unpublished results), histidine efflux was determined with ouabain added to the medium before or 20 min after addition of the slices. The curves in Fig. 7 demonstrate a remarkable acceleration of histidine efflux caused by ouabain; they also show that when added at 20 min its effect was much smaller and similar to that caused by phospholipase A, shown in Fig. 6.

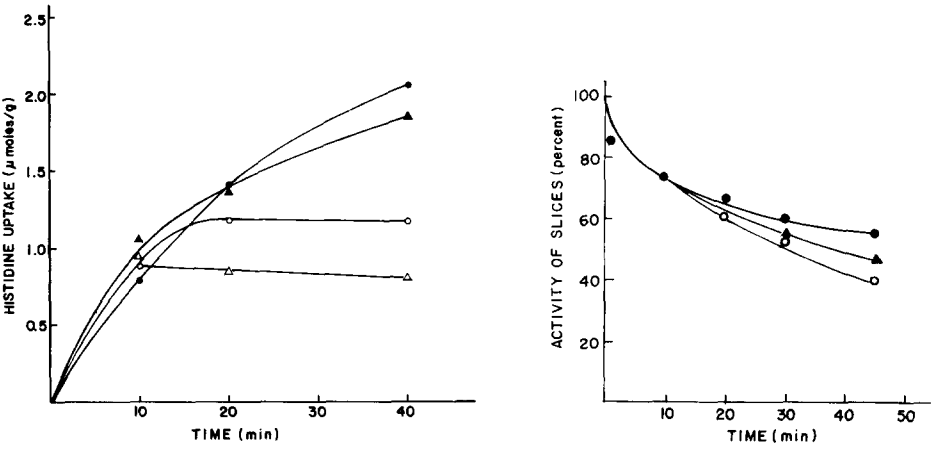


Fig. 5. Effect of phospholipase A and trypsin on histidine uptake. Two samples of brain slices were preincubated in glucose-containing Krebs–Ringer medium with trypsin at 37° for 20 min, two samples without trypsin. At 0 min [^{14}C]histidine was added to all samples and phospholipase as indicated. Enzyme concentrations: trypsin, 50 μg/ml; phospholipase A, 10 μg/ml. ●—●, without enzyme; ○—○, with phospholipase A; ▲—▲, with trypsin; △—△, with trypsin and phospholipase A.

Fig. 6. Effect of phospholipase A and trypsin on histidine efflux from brain slices. Procedure described under MATERIALS AND METHODS. ●—●, without enzyme; ▲—▲, with trypsin 100 μg/ml; ○—○, with phospholipase A, 10 μg/ml.

Effect of phospholipase A treatment on brain phospholipids

Brain slices, incubated for varying periods in phospholipase A containing Krebs–Ringer media, were extracted and analyzed for phospholipids as described in MATERIALS

AND METHODS. The results given in Table I indicate that at a concentration of 10 μg/ml phospholipase A caused on 20 min incubation only slight hydrolysis (10–12 %) of both phosphatidylethanolamine and phosphatidylcholine. Longer incubation or treatment with the enzyme at a higher concentration resulted in increased hydrolysis of these phospholipids.

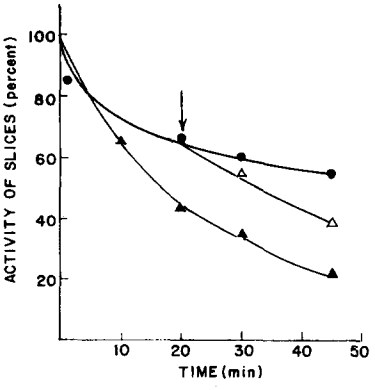


Fig. 7. Effect of ouabain on histidine efflux from brain slices. Procedure described under MATERIALS AND METHODS. ●—●, control; △—△, 1 mM ouabain at 20 min; ▲—▲, 1 mM ouabain at 0 min.

TABLE I

HYDROLYSIS OF BRAIN PHOSPHOLIPIDS BY PHOSPHOLIPASE A

Brain slices were incubated for varying periods in the standard medium for uptake supplemented with unlabeled histidine (0.5 μmole/ml) and phospholipase A. Slices from 3 brains, incubated under identical conditions, were pooled, thoroughly rinsed with saline and homogenized in a chloroform–methanol mixture (2:1, by vol.). Phospholipids in the extract were determined as described in MATERIALS AND METHODS.

Enzyme concn. (*μg/ml*)	*Time of incubation* (*min*)	*Amount hydrolyzed* (%)	
		Phosphatidyl-ethanolamine	*Phosphatidyl-choline*
0	60	0	0
10	20	10	12.5
10	60	28	22
20	60	—	32

DISCUSSION

The reduction of histidine uptake in brain slices caused by phospholipases A and C may be due to inhibition of influx or to enhancement of concomitant efflux, or both. A comparison of the kinetics of histidine uptake and efflux determined in the absence and presence of phospholipase A, allows to draw some conclusions regarding the process involved. Under standard conditions, with an external histidine concentration of 0.5 μmole/ml, untreated brain slices accumulated about 0.7 μmole histidine per g in the time interval between 20 and 40 min (Fig. 2). Phospholipase-treated slices did not take up amino acid during this period. Assuming uninhibited influx, the

enzyme-treated slices should have released into the medium an excess of 0.7 μmole histidine per g over the amount released by control slices. However, inspection of the histidine efflux curves (Fig. 6) shows that at 45 min the phospholipase-treated slices released only an additional 15 % of the amount preaccumulated during 60 min incubation (Figs. 1 and 2), *i.e.* about 0.36 μmole histidine per g. Apparently, the enzyme-treated slices did not take up histidine from the medium at the normal rate between 20 and 40 min; thus phospholipase A seems to have inhibited histidine influx. This conclusion is strongly supported by data on the initial influx of histidine into slices pretreated with the enzyme. Increasing damage to brain cells resulted in a corresponding inhibition of influx, determined shortly after introduction of the slices into the histidine containing medium, when occurrence of efflux should be negligible. In view of the inability of lysolecithin and fatty acids to affect histidine uptake by brain slices and the inhibiting effect of phospholipase C, inhibition seems to have resulted from loss of brain cell phospholipid. The portion of brain phosphatidylcholine and phosphatidylethanolamine hydrolyzed by phospholipase A under conditions which stop histidine uptake in slices was about 10–12 %. Although these two lipids together constitute about 75 % of mouse brain phospholipid (C. KIRSCHMANN, unpublished results), other phospholipids, not determined by us, may be more sensitive to the action of cobra phospholipase A. It is possible, however, that minor changes in total brain lipid bring about marked inhibition in uptake of the amino acid by the tissue.

Phospholipids could be directly involved in histidine transport into brain slices by ensuring functional structure of carrier molecules, presumed to mediate amino acid transport into cells[11–13]. Such relation between membrane lipid and protein has recently been suggested by WALLACH[14]. The curves given in Fig. 1 do indeed show partial loss of the saturable system for histidine uptake due to treatment with phospholipase A. This effect, however, is not specific, since uptake of other amino acids and of potassium ions in mouse brain slices have also been found inhibited following the same treatment (C. KIRSCHMANN, unpublished results). Alternatively, removal of phospholipid could indirectly affect amino acid uptake by changing another membranal activity possibly related to it, such as (Na^+,K^+)-dependent ATPase. We have assayed this activity in microsomes prepared from phospholipase A-treated brain slices and found it low and unstable compared to that in microsomes from control slices (C. KIRSCHMANN, unpublished results). However, the wet weight of microsomal pellets obtained from treated slices was consistently higher than that of pellets obtained from control slices by identical centrifugational fractionation, so that they may represent different subcellular particles. In contrast, the gross appearance and wet weight of whole brain slices did not change on 60 min treatment with phospholipase A. Thus, while our experiments do not establish damage of membrane ATPase as the mechanism underlying interference with histidine uptake caused by phospholipase A, an effect on some membrane constituent(s) functional in normal uptake is obvious.

Enhancement of the efflux of preaccumulated histidine molecules from brain slices is best ascribed to loss of membranes' ability to retain them. The effect observed is unlikely to represent inhibition of re-uptake of previously effluxed molecules, since the low specific activity of histidine used in these experiments makes detection of re-uptake impossible; it also seems too small to be leak through damaged membranes, though this effect cannot definitely be excluded. Enhanced efflux of previously

accumulated small molecules from tissue slices has been shown to occur under conditions which inhibit uptake and retention, such as treatment with ouabain[15,16]. The effect of phospholipase A on histidine efflux was found similar to that of ouabain added 20 min after start of efflux; both may therefore change histidine transport in the same way, namely by inhibiting uptake and retention.

Trypsin did not interfere with amino acid uptake, but preincubation with this enzyme enhanced inhibition of histidine uptake caused by phospholipase A. One could speculate that by breaking down protein components of brain cell membranes, this enzyme facilitates access of phospholipase A to its lipid substrate in the brain slices.

The non-interference by trypsin with histidine uptake in brain slices and the marked inhibition produced by phospholipases A and C lay stress on the role of membrane phospholipids in transport and retention of amino acids.

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