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Effect of the cationic polypeptide polylysine on neutral amino acid transport in isolated brain microvessels

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Summary. The functionality of isolated brain microvessels – used as an *in vitro* model of the blood-brain barrier – can be influenced by interaction with cationic proteins. The various polylysines (M_r ranging from 0.9 to 180 kDa) tested affected the activity of both the Na^+-dependent ("A") and the Na^+-independent ("L") systems for neutral amino acid transport. Exposure to the 180 kDa polylysine caused a conspicuous inhibition of both transport systems, associated to an increased passive permeability. There was a constant, M_r-dependent, inhibition of the the L-system-mediated uptake of hydrophobic neutral amino acids. The activity of the A-system was enhanced, upon exposure to polymers larger than 22 kDa reaching its peak at 68 kDa and and declining at higher M_r values. The effect which was Na^+-ions dependent and abolished by phloretine, could be essentially ascribed to an increased affinity of the MeAIB for its carrier (K_m value decreasing from 265 to 169 μM in presence of 68 kDa polylysine).

Keywords: Amino acids – Cationic polypeptide – Blood brain barrier – Neutral amino acids transport

Introduction

The concept of blood-brain barrier (BBB) derives from the observation that the brain remained colorless after intravenous injection of trypan blue (and/or of other acidic dyes), while most body tissues were permeated by the dye. The anatomical counterpart of the barrier was subsequently identified with the endothelial cells of brain microvessels, which possess the ability of regulating the restricted movements of nutrients between the blood and the brain compartments (Bradbury, 1979; 1985; Cardelli-Cangiano et al., 1981; 1984; Cangiano et al., 1983a,b; Pardridge, 1983). The most relevant morphologic characteristics

of the blood-brain barrier are the presence of tight junctions between the endothelial cells, and the poor formation of pinocytotic vesicles or of transendothelial channels crossing the endothelium from the luminal side to the abluminal one (Reese and Karnovsky, 1967). In the microvessels of other tissues, the endothelial cell membranes possess areas with negative surface charge (Seilor et al., 1975; Charonis et al., 1983), whose function has not yet been fully clarified but which generally predominate in the membrane regions where only very few vesicles and/or transendothelial channels are formed (De Bruyn et al., 1978; Simionescu, 1983). In the brain microcirculation, such negatively-charged areas may be of importance in regulating and/or maintaining the barrier function. In vivo experience has shown that a transient opening of the blood-brain barrier can be obtained using cationic or "cationized" proteins (Hardebo and Johanson, 1980). The activity of the blood brain barrier can thus be influenced by modifying the distribution and the movements of the negatively charged areas on the cell surface (Hardebo and Kahrstrom, 1985). The present study evaluates the influence of cationic polylysine of different size in the 0.9 to 180 kDa range, on the neutral amino acid transport systems (Cangiano et al., 1983a) and passive diffusion.

Materials and methods

Chemicals

α1-(^{14}C)-methylaminoisobutyrate (MeAIB) 4.95 mCi/mmol, L-(U^{14}C)-leucine 335 mCi/mmol, D-(U^{14}C)-labeled sucrose and Aquasol-2^{R} were obtained from New England Nuclear Corp. (Boston, MA, U.S.A). Poly(L-lysine) hydrobromide (M_r ranging from 0.9 to 180 kDa), HEPES and γ-glutamyl-paranitroanilide were from Sigma Chemical Corp. (St. Louis, MO, U.S.A.). Luciferine and luciferase were from LKB (Bromma, Sweden), carboxyfluorescein diacetate from Molecular Probes Inc. (Junction City, OR, U.S.A.). All other chemicals were from Merck (Darmstadt, Germany) or from Fluka (Buchs, Switzerland).

Isolation of microvessels

Fresh bovine brains, obtained from the local slaughterhouse, are transported on ice to the laboratory. Meninges were removed, and only the cortex was used. The capillaries were isolated as previously described by Cangiano et al. (1983a). The gray matter was homogenized by hand (1 : 1, w/v), using a loose teflon pestle homogenizer, in a buffer containing 10 mM glucose, 122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4 and 10 mM HEPES, pH 7.4 and equilibrated with 95% O_2 + 5% CO_2. The homogenate was passed through a 86 μm pore nylon sieve and the retained material was washed with a spray of cold buffer, subjected to a second homogenization step, filtered again through the nylon sieve and repeatedly washed with a spray of cold buffer to remove the residual non-vascular tissue. The isolated brain microvessels were then collected in a plastic tube, resuspended in the same buffer and placed at 0°C under a 95% O_2 + 5% CO_2 atmosphere until use. For some of the experiments, after the isolation step, microvessels were resuspended in Na^+-free buffer in which NaCl and $NaHCO_3$ had been replaced, respectively, by choline chloride and $KHCO_3$.

For the assay of enzymatic activities, the microvessels were homogenized in a Potter-Elvehjem motor-driven apparatus in the appropriate buffer. Alkaline phosphatase, γ-glutamyl transpeptidase, were assayed as previously described (Cardelli-Cangiano et al., 1987). Passive permeability of the brain microvessels was tested by measuring the carboxyfluorescein efflux from microvessels which had been preloaded with carboxyfluorescein

diacetate (Strom et al., 1973). The intracellular ATP content was assayed as previously described (Cardelli-Cangiano et al., 1984).

Uptake experiments

The ability the isolated brain microvessels to take up a labeled amino acid was measured either, at different time intervals (up to 30 min), or as initial velocity (within the first two minutes) in relation to amino acid concentration. After the appropriate incubation interval, 0.6 ml aliquots of the microvessel suspensions were poured on a 44 μm pore nylon sieve on a vacuum manifold and washed three times with 5 ml of cold (4°C) buffer. The sieves with the retained microvessels were then placed in disposable tubes containing 1.8 ml of 1 N NaOH, left overnight at room temperature, and then sonicated for 1 min. Portions were withdrawn for protein determinations (Lowry et al., 1951), while 0.5 ml aliquots were transferred to liquid scintillation vials containing an equal volume of 1 N HCl. After addition of 10 ml of Aquasol 2^R, the vials were counted in a Beckman 9800 liquid scintillation spectrometer. Non-specific radioactivity, due to the binding of the labeled amino acid to the nylon sieve, was also tested in triplicate by omitting the microvessels; the radioactivity remaining on the sieve never exceeded 100 dpm. The initial rate of uptake measured within the first two minutes showed, when plotted as a function of amino acid concentration, the presence of a saturable component superimposed on a non-saturable one. Non-linear regression analysis was used to obtain the optimal estimate of the kinetic parameters and to evaluate the standard error of the two transport systems. The values of the kinetic parameters were plotted in the v/S *vs* v ("Eadie-Hofstee") plot which allows a fairly simple statistical analysis as described by Wilkinson (1961).

Results

Integrity and viability of the microvessel preparations

The isolated brain microvessel preparations appeared to consist essentially of branching capillary segments with some arterioles and venules. As previously reported (Cangiano et al., 1983a; Cardelli-Cangiano et al., 1984) neither phase-contrast light microscopy nor scanning electron microscopy showed any contamination by nervous or glial cells. Accurate measurements of passive

Table 1. Carboxyfluorescein efflux from isolated brain microvessels

Incubation medium	Carboxyfluorescein efflux (%)	
	after 5 min	after 30 min
Buffer alone	3.10 ± 0.03	13.01 ± 0.10
Polylysine 68 kDa, 0.5 mg/ml	4.61 ± 0.06	18.05 ± 0.07
Polylysine 180 kDa, 0.5 mg/ml	6.21 ± 0.09	31.02 ± 0.04

The microvessels were preloaded with carboxyfluorescein by incubation for 20 min with carboxyfluorescein diacetate (50 μg/ml) – the diffusion of the unesterified dye liberated within the cells by hydrolytic enzyme(s) being more restricted than that of its non-polar precursor. After having been extensively washed by filtration on a nylon sieve, they were resuspended in warm (37°C) buffer with the appropriate polylysine. The dye efflux, expressed as percent of the total amount of entrapped carboxyfluorescein, was measured after 5 and 30 min at 37°C. Data are the mean values ± S.D. of three different experiments.

permeability of cell membranes, was performed by measuring the efflux of the dye using microvessels preloaded with a non-polar, hydrolysable precursor of carboxyfluorescein, (Strom et al. 1973). There was a moderate increase of the outward diffusion of carboxyfluorescein when the microvessels were exposed to polylysine with $M_r > 68$ kDa; this effect became considerably more consistent, over 30% of the entrapped dye being released after 30 min at 37°C, when the 180 kDa polypeptide was used (Table 1). The microvessel suspensions were found to be enriched with respect to the gray matter of some enzymatic activities such as γ-glutamyl transpeptidase (200 ± 20 *vs* 6 ± 0.1 nmol/mg protein/min) and alkaline phosphatase (215 ± 18 *vs* 20 ± 5 nmol/mg protein/min).

Effect of polylysine on the transport of neutral amino acids

Addition of polylysine modified both the A- and the L-systems of neutral amino acid transport of isolated brain microvessels. The presence of 68 kDa polylysine accelerated the transport of methylaminoisobutyrate, which is A-system specific (Fig. 1), while the activity of the L-system was inhibited (Fig. 2). These effects were dependent on the polylysine size: the stimulatory action of the cationic polypeptide on the A-system of transport could be evidenced in the M_r range from 22 to 68 kDa (Fig. 3); a further increase in molecular weight caused instead a progressive inhibition of MeAIB uptake (Fig. 3). In terms of kinetic parameters, the most prominent effect of the 68 kDa polylysine was on the affinity of the MeAIB carrier (the K_m value decreasing from 265 to 169 μM), with minor alterations of the V_{max} of transport (from 201 to 189 pmol/mg protein/min) and

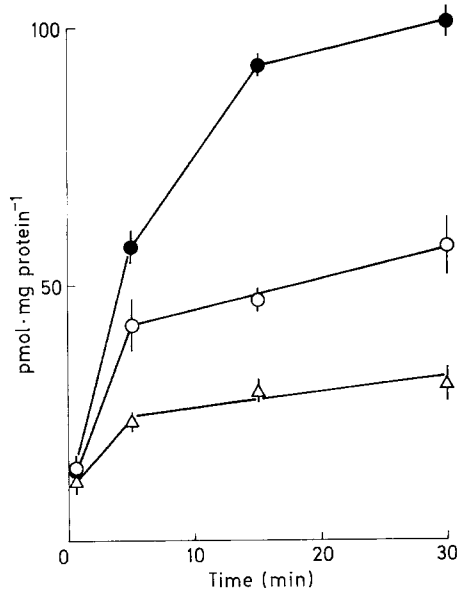

Fig. 1. Time course uptake of labeled MeAIB measured in the presence of different molecular weight polylysine (●) 68 kDa polylysine (0.5 mg/ml), (△) 180 kDa polylysine (0.5 mg/ml), (○) in the presence of buffer alone. Data shown are the means of three different experiments ± S.D.

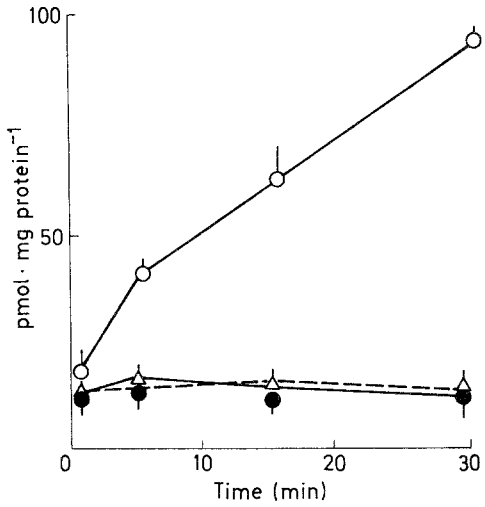

Fig. 2. Uptake over a 30 min period of labeled leucine measured in the presence of different molecular weight polylysine: (●) 68 kDa polylysine (0.5 mg/ml), (△) 180 kDa polylysine (0.5 mg/ml), (○) in the presence of buffer alone. Data shown are the mean of three different experiments ± S.D.

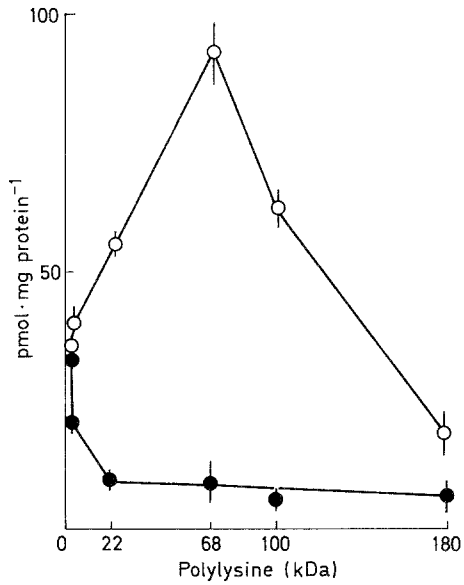

Fig. 3. Effect of the degree of polymerization on the uptake either of MeAIB (○) or of leucine (●). In the presence of the appropriate polylysine used at a final concentration of 0.5 mg/ml. The internal radioactivity of the isolated microvessels was measured after 15 min of incubation with the labeled amino acid. Data shown are the mean of two different experiments ± S.D.

of the non-saturable diffusional component K_d (from 0.60 to 0.70 min^{-1}). The intracellular ATP content, which could be estimated to be around 500 pmol/mg protein, was not modified. Addition of phloretine (Fig. 4), as well as the absence of Na^+ ions (data not shown) from the incubation medium, abolished the

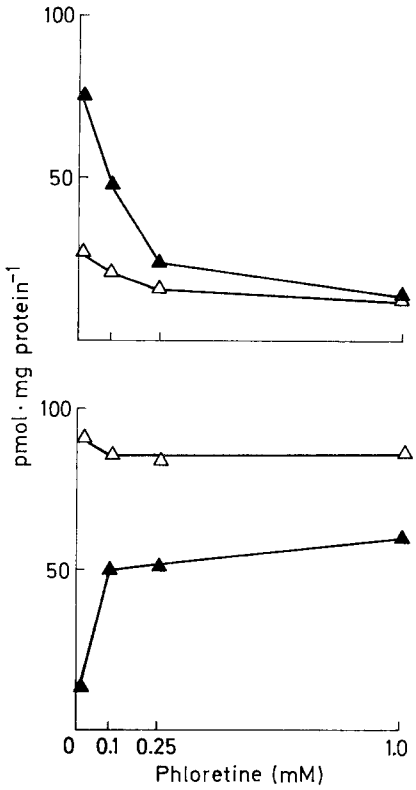

Fig. 4. Effect of different concentration of phloretine on the time course uptake either of MeAIB (upper panel) or of leucine (lower panel) in the presence of 68 kDa polylysine (0.5 mg/ml) (▲) or in its absence (△). The microvessels were left 15 min in the presence of the labeled amino acid and the internal radioactivity was then measured. Data shown are the mean of three different experiment ± S.D.

stimulatory effect of 68 kDa polylysine. The L-system, which is specific for the large neutral amino acids, was inhibited by all the polylysine homopolymers tested, the extent of inhibition increasing throughout the whole range from 5 to 180 kDa. Addition of phloretine partially reverted this inhibition.

Discussion

The endothelial cells of the cerebral blood vessels are joined by tight junctions and contain few pinocytotic vesicles. This structure, which is impermeable to large molecular weight substances differently than capillaries of other body tissues, regulates the restrict movements of solute through the blood brain barrier (Bradbury, 1985; Cangiano and Cardelli-Cangiano, 1989; Cardelli-Cangiano and Cangiano, 1990). Thus the possibility for different compounds to cross the BBB is related either to their lipid solubility (Crone et al., 1965) or to a specific affinity for a carrier-mediated transport system.

Brain microvessels possess, similarly to the capillaries of other tissues, areas with a relatively high density of anionic residues, the major contribution to the negative surface charge coming from carboxyl groups of sialic acid residues (De

Bruyn et al., 1978). In most microvessels, these negatively charged areas are reportedly characterized by the absence of endocytotic vesicles and of trans-endothelial channels (Simionescu, 1983; De Bruyn et al., 1978). These areas, which are present on both the luminal and abluminal sides of brain microvessels, contribute to the preservation of the barrier function, probably with a role similar to that described for some epithelial cells (Nag and Arsenau, 1983; Hart et al., 1985). In our study we used the cationic polypeptide polylysine to clarify some functional roles of the anionic sites present at the isolated brain microvessels. Addition of the largest polylysine homopolymer induced an increased permeability of the endothelial cell membranes, presumably through its specific binding to negatively charged areas (Hardebo and Kahrstrom, 1985). If the polylysine size was below 100 kDa, there was however only a moderate increase in passive permeability, the presence of polylysine affecting predominantly the saturable permeation pathways, as shown by the selective modification of the kinetic parameters of the two aminoacid transport systems. Why larger molecules of polylysine act differentely from the smaller ones was not clear (the final concentration in the microvessel suspension was throughout the experiments 0.5 mg/ml, independently from the M_r of the polylysine used) but it can be suggested that the different effect could be ascribed to the degree of polymerization of the polylysine tested. An inhibition of the L-system was observed throughout the whole size range of polylysine molecules, while treatment with homoplymers ranging from 22 to 68 kDa resulted in an increased activity of the A-system-mediated uptake of MeAIB. This change was mainly due to an increase in the affinity ($1/K_m$) of the saturable component, whereas there were only negligible variations in the V_{max} values. In order to clarify some aspect of the polylysines interaction with the A- and L-systems of neutral amino acid transport the effect of 68 kDa polylysine was tested either in the presence of phloretine or in a Na^+ ions depleted medium. Phloretine inhibited the polylysine (68 kDa) effect of the energy requiring A-system, by abolishing the endocellular glucose consumption – glucose was the only energetic supply of the isolated brain microvessels during the experiments –, as consequence the isolated microvessels became rapidly energy depleted, otherwise in these experimental conditions the energy independent L-system activity was partially restored. This effect was associated to the possibility of the microvessels to use glucogenic neutral amino acid to requilibrate the energetic balance. As reported in the results, the 68 kDa polylysine effect was linked to the Na^+ ions concentration. In fact in their presence the (68 kDa) polylysine increased the MeAIB transport by the A-system as a consequence of a partial membrane hyperpolarization leading to an enhanced outside/inside flux of Na^+ ions. In preliminary experiments, however, polylysine could not be effectively substituted by other cationic polypeptides such as polyarginine (unpublished results).

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