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Regulation of phosphate transport by second messengers in capillaries of the blood-brain barrier

Lise Dallaire, Sylvie Giroux and Richard Béliveau

Laboratoire de membranologie, Groupe de recherche en biothérapeutique moléculaire, Université du Québec à Montréal, Montréal (Québec) (Canada)

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Regulation of phosphate uptake by the blood-brain barrier was studied in isolated bovine capillaries. Dibutyryl cAMP, in the presence of 3-isobutylmethylxanthine, resulted in a dose-dependent inhibition of phosphate uptake. Phosphate influx, with or without 3-isobutylmethylxanthine, was not different. Inhibition of phosphate uptake was also observed when capillaries were preincubated with isoproterenol, parathyroid hormone, insulin and acidic or basic fibroblast growth factors. Treatment of capillaries with vasoactive intestinal peptide, prostaglandin E_1, angiotensin II, epidermal growth factor and phorbol esters did not affect phosphate transport. Endothelin I increased phosphate uptake by 15%. Preincubation with cholera toxin also resulted in a dose-dependent decrease in phosphate uptake. In addition, pertussis toxin inhibited phosphate transport by 29%, but only in the presence of 3-isobutylmethylxanthine. These results demonstrate that generation of second messengers, following receptor stimulation, can induce physiological effects on capillary phosphate influx and suggest that G proteins may modulate this transport.

Introduction

The capillaries of the brain are formed by a specialized endothelium whose function is to regulate the movement of solutes between blood and brain [1]. A number of laboratories have reported the presence of receptors for various hormones and neurotransmitters on brain capillary plasma membranes [2]. Light- and electron-microscopic observations indicate a rich innervation of cerebral blood vessels [3]. One of the functions of the blood-brain barrier receptors may be to alter its permeability to circulating nutrients, water and ions [1]. Precise control of brain volume, through adjustment of cell water and electrolyte content, is important for the normal function of the brain. There is some indication that brain vasculature is under neuronal and hormonal influence; especially notable is the change in blood flow and water permeability of the brain vasculature in response to adrenergic stimulation or administration of vasopressin [4,5].

We have previously developed a method to isolate bovine brain capillaries [6] and demonstrated the presence of a facilitated phosphate uptake system [7]. However, the mechanisms involved in the regulation of this transport are still unknown. We therefore examined whether blood-brain barrier phosphate transport was influenced by hormonal and non-hormonal stimuli.

Three distinct pathways involving various receptors and second messengers were investigated. These include (i) stimulation of adenylate cyclase by isoproterenol, parathyroid hormone, prostaglandin E_1, vasoactive intestinal peptide, cholera and pertussis toxins, (ii) activation of phospholipase C by angiotensin II and endothelin I and (iii) stimulation of tyrosine kinases by insulin, epidermal and fibroblast growth factors. Phosphate transport of the isolated capillaries was increased by stimulation of phospholipase C while activation of adenylate cyclase and tyrosine kinases decreased it.

Materials and Methods

Materials

Dibutyryl cAMP (db cAMP), 3-isobutylmethylxanthine (IBMX), cholera toxin, pertussis toxin, isoproterenol, parathytroid hormone (PTH, fragment 1–

Correspondence to: R. Béliveau, Laboratoire de membranologie, Groupe de recherche en biothérapeutique moléculaire, Université du Québec à Montréal, C.P. 8888, Succ. A, Montréal, (Québec), Canada H3C 3P8.

34), propanolol, insulin, prostaglandin E_1 (PGE_1), endothelin I, angiotensin II, epidermal growth factor (EGF, fragment 20–31), acidic and basic fibroblast growth factors (aFGF, bFGF), phorbol ester 12-myristate 13-acetate (PMA), 4α-phorbol ester 12,13-didecanoate (4α-PD) were purchased from Sigma (St. Louis, MO). Vasoactive intestinal peptide (VIP) was from Peninsula Laboratories (Belmont, CA). IBMX solutions were prepared daily and dissolved in the physiological buffer described below. Isoproterenol and propanolol solutions were prepared fresh and dissolved in distilled water, before being diluted in the incubation medium. Phorbol esters were dissolved in DMSO 100%, PGE_1 in EtOH 95% and the other drugs in 5 mM Hepes-Tris buffer (pH 7.5), and diluted in the incubation medium before use. [^{32}P]Orthophosphate (carrier-free) was obtained from Dupont-New England Nuclear (Mississauga, Ontario). [^{32}P]Nicotinamide adenine dinucleotide (NAD) was from ICN (Costa Mesa, CA). Other chemicals were of the highest purity commercially available.

Capillary isolation

Capillaries were isolated by mechanical homogenization of cortex obtained from fresh bovine brain, as described previously [6]. The capillaries were resuspended in a cryoprotective solution (147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes-Tris buffer (pH 7.5), 14% glycerol and 1.4% sorbitol) and stored in liquid nitrogen. Enrichment for the marker enzyme γ-glutamyl transpeptidase was routinely 20-fold [6]. Before use, capillaries were washed (1 : 30, v/v) with a solution containing 147 mM NaCl, 4 mM KCl, 5 mM glucose and 15 mM Hepes-Tris buffer (pH 7.5) and collected by centrifugation at 25000 $\times g$ for 10 min at 4°C. The pellet was resuspended in the same medium to a concentration of 7–10 μg of protein/μl.

Incubation of capillaries with drugs

Drugs to be tested were added to capillaries in a small volume (2 μl) of diluent, and the same volume of diluent were added to the control. When IBMX was to be assayed with another drug, each was added in a volume of 1 μl. Capillaries were preincubated 20 min at 25°C with drugs, except with cholera and pertussis toxins where preincubations of 30 min at 25°C were needed.

Phosphate transport

A rapid filtration technique was used to measure transport. Phosphate uptake, performed in quadruplicate at 25°C, was initiated by the addition of the incubation medium to 70–100 μg of protein/10 μl, preincubated 60 s at 25°C. The incubation medium contained, in a volume of 50 μl, 200 μM [^{32}P]phosphate (2 μCi), 147 mM NaCl, 4 mM KCl, 5 mM glucose and 15 mM Hepes-Tris buffer (pH 7.5). After incubation, the reaction was stopped by addition of 1 ml of ice-cold stop solution (147 mM NaCl, 4 mM KCl and 15 mM Hepes-Tris buffer (pH 7.5)). The suspension was filtered under vacuum through a 0.45-μm pore-size Millipore filter. The filter was rinsed with 8 ml of stop solution and the radioactivity was counted. Non-specific binding to the filters was determined by filtering [^{32}P]phosphate solutions without capillaries. Non-specifically bound radioactivity was subtracted from measured radioactivity. Variations in phosphate uptake were observed between different preparations of purified capillaries. These could reflect uncontrollable parameters such as the age, sex and diet of the animals. An appropriate control was thus performed with each experiment. Non-specific binding to the capillaries was determined by adding the capillary suspension to the ice-cold stop solution, before the addition of the [^{32}P]phosphate. These values were subtracted from transport values. Statistical analyses were made using non-paired Student's t-test. All values are expressed as means ± S.E. of at least three experiments done in quadruplicate.

Results

Db cAMP (0.2 mM), a permeant analog of cAMP, inhibited significantly phosphate uptake by 19% (Table I). Db cAMP together with 10 μM IBMX, an inhibitor of the breakdown of cAMP by phosphodiesterases, diminished phosphate uptake by 40%. Phosphate influx, in presence and absence of IBMX was not changed. The decrease of phosphate transport into capillaries was significantly different in the presence of IBMX and db cAMP than with db cAMP alone. On the basis of this experiment, all subsequent preincubations of the capillaries with db cAMP were done in the presence of 10 μM IBMX. Capillary cells preincubated with different concentrations of db cAMP resulted in a

TABLE I

Effect of db cAMP on phosphate uptake in brain capillaries

Capillaries were preincubated for 30 min at 25°C with 200 μM db cAMP alone or in the presence of 10 μM IBMX. Phosphate transport was measured as described in Materials and Methods. The values are expressed as percent of the mean control value (0.128 ± 0.02 nmol phosphate/mg protein per 30 s). Data are means + S.E. of five experiments in quadruplicate. * $P < 0.1$ vs. control. ** $P < 0.05$ vs. control. † $P < 0.1$ vs. db cAMP alone.

Effectors	Phosphate transport (% of control)
Control	100
Db cAMP	81.4 ± 8.1 *
Db cAMP + IBMX	59.7 ± 10.2 **,†

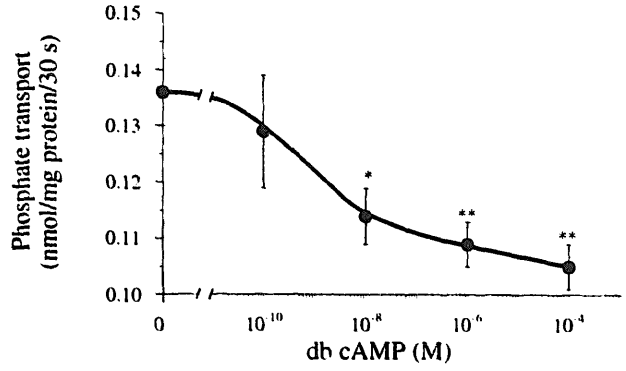

Fig. 1. Dose-response of db cAMP on phosphate uptake in brain capillaries. Experiments were performed after a 20 min preincubation at 25°C, in the presence of various concentrations of db cAMP and 10 μM IBMX. Data are means ± S.E. of five experiments in quadruplicate. * $P < 0.01$ vs. control. ** $P < 0.005$ vs. control.

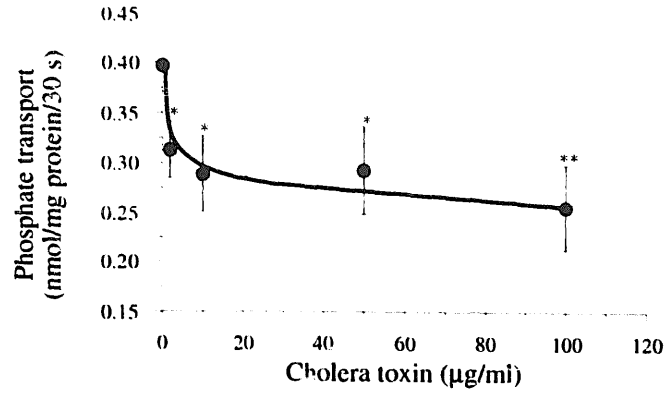

Fig. 3. Effect of cholera toxin on phosphate uptake by blood brain barrier capillaries. Experiments were performed after a 30 min preincubation at 25°C. Data are means ± S.E. of four experiments in quadruplicate. * $P < 0.025$ vs. control. ** $P < 0.01$ vs. control.

dose-dependent inhibition of phosphate uptake (Fig. 1). Half-maximal inhibition of phosphate influx was observed at 10^{-9} M db cAMP.

Preincubation of capillary cells with two stimulators of adenylate cyclase, PTH (10^{-5} M) and isoproterenol (10^{-4} M), inhibited phosphate uptake significantly by 22% and 26%, respectively (Fig. 2). Addition of IBMX with PTH did not modify the response to PTH. However, addition of IBMX with isoproterenol increased phosphate uptake by 31%, over isoproterenol alone. Propanolol (10^{-4} M) reversed the inhibition caused by isoproterenol, but had no effect when added alone.

Cholera toxin is a protein produced by *Vibrio cholerae* that activates adenylate cyclase by ADP-ribosylation [8]. As shown in Fig. 3, preincubation of capillary cells with cholera toxin, at concentrations of 2 to 100 μg/ml, led to a significant dose-dependent decrease in phosphate uptake. The inhibition was up to 36% of the control at 100 μg/ml of cholera toxin. Capillaries incubated without cholera toxin transported 0.397 ± 0.026 nmol phosphate/mg protein per 30 s while those incubated with 100 μg/ml of cholera toxin transported only 0.256 ± 0.043 nmol/mg protein per 30 s (Fig. 3). Half-maximal inhibition was observed at 2 μg/ml. Unexpectedly, addition of IBMX increased phosphate transport by 25%, over preincubation with cholera toxin alone (Table II). Pertussis toxin at doses as high as 10 μg/ml had no effect on phosphate uptake by brain capillaries (Table II). However, pertussis toxin in the presence of IBMX inhibited phosphate uptake by 29% (Table II).

Two other effectors which are thought to activate adenylate cyclase (PGE_1 and VIP) [8] were preincubated with brain capillaries, but did not alter significantly phosphate uptake (Table III). Known stimulators of protein kinase C, angiotensin II, PMA and endothelin I, were also studied. Only endothelin I

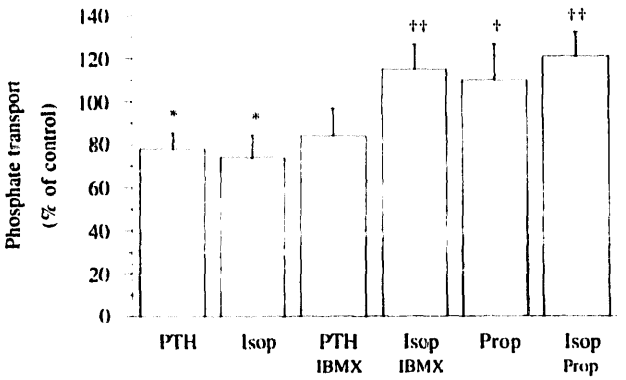

Fig. 2. Effect of activators of adenylate cyclase on phosphate transport in isolated capillaries. Phosphate uptake was measured after a preincubation of 20 min at 25°C with the various agents. Isoproterenol and propanolol were used at a final concentration of 10^{-4} M, and PTH and IBMX, at 10^{-5} M. The values are expressed as percent of the mean control value (0.276 ± 0.027 nmol phosphate/mg protein per 30 s). Data are means ± S.E. of five experiments in quadruplicate. * $P < 0.05$ vs. control. † $P < 0.05$ vs. isoproterenol. †† $P < 0.01$ vs. isoproterenol.

TABLE II

Effect of toxins on phosphate transport in capillaries of the blood brain barrier

Capillaries were preincubated 30 min at 25°C with cholera toxin (100 μg/ml) or pertussis toxin (10 μg/ml), in the presence or absence of 10 μM IBMX. Phosphate transport was measured after 30 s as described in Materials and Methods. The values are expressed as percent of the mean control value obtained in absence of toxins and inhibitors (0.342 ± 0.022 nmol phosphate/mg protein per 30 s). Data are means ± S.E. of four experiments in quadruplicate. * $P < 0.05$ vs. control. † $P < 0.1$ vs. cholera toxin.

Toxin	Phosphate transport (% of control)
Control	100
Cholera	73 ± 8.4 *
Cholera + IBMX	91 ± 6.5 †
Pertussis	102 ± 7.4
Pertussis + IBMX	71 ± 13 *