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Modulation of Insulin Transport in Rat Brain Microvessel Endothelial Cells by an Ecto-Phosphatase Activity

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Abstract The physiological function of alkaline phosphatase (ALP) remains controversial. It was recently suggested that this membrane-bound enzyme has a role in the modulation of transmembranar transport systems into hepatocytes and Caco-2 cells. ALP activity expressed on the apical surface of blood-brain barrier cells, and its relationship with ^{125}I-insulin internalization were investigated under physiological conditions using *p*-nitrophenylphosphate (*p*-NPP) as substrate. For this, an immortalized cell line of rat capillary cerebral endothelial cells (RBE4 cells) was used. ALP activity and ^{125}I-insulin internalization were evaluated in these cells. The results showed that RBE4 cells expressed ALP, characterized by an ecto-oriented active site which was functional at physiological pH. Orthovanadate (100 μM), an inhibitor of phosphatase activities, decreased both RBE4-ALP activity and ^{125}I-insulin internalization. In the presence of L-arginine (1 mM) or adenosine (100 μM) RBE4-ALP activity and ^{125}I-insulin, internalization were significantly reduced. However, D-arginine (1 mM) had no significant effect. Additionally, RBE4-ALP activity and ^{125}I-insulin internalization significantly increased in the presence of the bioflavonoid kaempferol (100 μM), of the phorbol ester PMA (80 nM), IBMX (1 mM), progesterone (200 and 100 μM), β-estradiol (100 μM), iron (100 μM), or in the presence of all-trans retinoic acid (RA) (10 μM). The ALP inhibitor levamisole (500 μM) was able to reduce ^{125}I-insulin internalization to 69.1 ± 7.1% of control. Our data showed a positive correlation between ecto-ALP activity and ^{125}I-insulin incorporation ($r = 0.82$; $P < 0.0001$) in cultured rat brain endothelial cells, suggesting that insulin entry into the blood-brain barrier may be modulated through ALP. J. Cell. Biochem. 84: 389–400, 2002.

Key words: alkaline phosphatase; RBE4 cells; insulin; receptor mediated endocytosis; PI-PLC; blood brain barrier

Insulin receptors and insulin signaling proteins are widely distributed throughout the central nervous system (CNS). Previous experiments have suggested a role for insulin signaling in the regulation of food intake [Schwartz et al., 1992; Baskin et al., 1999], and, by affecting neuronal growth and differentiation, in learning and memory [Heidenreich, 1993; Robinson et al., 1994]. Moreover, insulin inhibits the formation of brain lesions (neurofibrillary tangles) that characterize Alzheimer's disease [Hoyer, 1997]. Insulin was recently shown to be transported across the blood-brain barrier (BBB) [Miller et al., 1994; Porte et al., 1998].

The BBB separates brain interstitial space from blood, and it is mainly formed by brain capillary endothelial cells fused together by epithelial-like tight junctions [see review by Cserr and Bundgaard, 1984]. The cerebral endothelial cells contain high levels of enzymes such as gamma-glutamyl transpeptidase (GTP) and alkaline phosphatase (ALP), which contribute to the enzymatic barrier of the BBB [Meyer et al., 1990]. In relation to insulin transport, the primary pathway for its cellular internalization is receptor-mediated endocytosis. Moreover, it is well established that transcytosis has an important role in the delivery of insulin to its target cells in tissues, such as the CNS.

ALP acts as an enzyme to regulate extracellular concentrations of some phosphate compounds such as pyridoxal-5′-phosphate (the cofactor form of vitamin B_6), phosphoethanola-

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mine [Whyte et al., 1988] and some phosphoproteins [Chan and Stinson, 1986].

ALP anchored to the plasma membrane may function as an ecto-phosphatase, dephosphorylating extracellular substrates, or cell-surface proteins. It was recently demonstrated that membrane-bound ALP concentration in different tissues is positively correlated with the extent of exchange surface per unit volume of the tissue, suggesting an association between ALP and transport systems [Calhau et al., 1999]. Additionally, evidence was obtained for an involvement of ALP in the modulation of organic cation transport in hepatocytes [Martel et al., 1996, 1998a,b; Calhau et al., 2000], as well as in Hek293 [Martel et al., in press] and Caco-2 cells [unpublished results]. Thus, we considered the hypothesis of an influence of ecto-ALP on insulin transport at the BBB. The experiments were performed by using an immortalized cell line of rat capillary cerebral endothelial cells (RBE4 cells). This cell line was obtained by transfection of rat brain microvessel endothelial cells with a plasmid containing the E1A adenovirus gene [Roux et al., 1994]. These cells display a non-transformed endothelial phenotype expressing the brain microvessel-associated enzymes GTP, inducible nitric oxide synthase, ALP, as well as P-glycoprotein [Roux et al., 1994; Naassila et al., 1996; El Hafny et al., 1997; Nobles and Abbott, 1998] and GLUT1 [Regina et al., 1997]. Thus, RBE4 cells provide the opportunity to investigate ecto-enzyme functions on BBB, without interference from other tissue elements.

MATERIALS AND METHODS

Materials

^{125}I-insulin (specific activity 629 Ci mmol^{-1}) (New England Nuclear Chemicals, Dreieich, Germany); adenosine, ALP substrate (*p*-nitrophenyl phosphate), ALP standard (*p*-nitrophenol), ALP type XXXII (from bovine kidney), D-arginine hydrochloride, L-arginine hydrochloride, corticosterone, cytochalasin D (from *Zygosporium mansonni*), beta-estradiol, ferrous sulfate, HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), insulin (from bovine pancreas), 3-isobutyl-1-methylxanthine (IBMX), kaempferol, levamisole hydrochloride, penicillin/streptomycin solution, phenylarsine oxide, phorbol 12-myristate 13-acetate (PMA), phospholipase C phosphatidylinositol-specific (PI-PLC; from *Bacillus cereus*), progesterone, all-trans retinoic acid (RA), sodium dodecyl sulfate (SDS), sodium orthovanadate, testosterone, Tris (tris-(hydroxymethyl)-aminomethane hydrochloride), trypsin-EDTA solution, (Sigma, St. Louis, MO, USA); DMSO (dimethylsulphoxide) (Merck, Darmstadt, Germany). Corticosterone, β-estradiol, progesterone, and testosterone were dissolved in ethanol (1%) and cytochalasin D, kaempferol, PAO, and RA were dissolved in DMSO (1%). Controls for these drugs were run in the presence of the respective solvent.

Cell and Culture Conditions

The RBE4 cell line was kindly supplied by Dr. Françoise Roux (INSERM U. 26, Hôpital Fernand Widal, Paris, France). RBE4 clone was maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. These cells (passages 30–60) were grown in Minimum Essential Medium/Ham's F10 (1:1) supplemented with 300 ng ml^{-1} neomycine, 10% fetal bovine serum, 1 ng ml^{-1} basic fibroblast growth factor, 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B, 100 μg ml^{-1} streptomycin, and 25 mM HEPES (all from Sigma). The cell medium was changed every 48 h, and the cells reached confluence after 3–4 days culture. For subculturing, the cells were dissociated with 0.25% trypsin-EDTA (Sigma), diluted 1:6 and subcultured in Petri dishes with a 21-cm^2 growth area (Corning Costar, Badhoevedorp, The Netherlands). For the experiments, the RBE4 cells were seeded in 24-well plastic cell culture clusters (2-cm^2; ⌀ 16 mm; Corning Costar). For 24 h prior each experiment, the cell medium was made free of fetal bovine serum. All experiments were performed 7–10 days after initial seeding and each cm^2 contained about 200 μg of cell protein. In chronic experiments, compounds to be tested ($FeSO_4$, ALP, levamisole or all-trans RA) were dissolved in culture media (0.1%).

Assay of Phosphohydrolase Activity of Ecto-ALP in Intact RBE4 Cells

ALP phosphohydrolase (ecto-*p*-NPPase) activity was assessed by measuring *p*-nitrophenol release from *p*-nitrophenylphosphate (*p*-NPP) at physiological pH by absorbance spectrophotometry at 410 nm. Ecto-*p*-NPPase assays were realized on intact RBE4 cells.

On the day of experiment, the culture medium was removed and cell monolayers were

washed once with Hanks' medium at 37°C. Thereafter, the cell monolayers were preincubated for 20 min in Hanks' medium at 37°C. Hanks' medium had the following composition (mM): NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $CaCl_2$ 0.25, $MgCl_2$ 1.0, Tris-HCl 0.15, and sodium butyrate 1.0, pH 7.4. The reaction was carried out in 300 μl of Hanks' medium containing 2.86 mM *p*-NPP (buffer assay). After the reaction, the supernatant solution was transferred into hemolyse tubes containing 0.02 M NaOH at 4°C (in order to stop the reaction), and the amount of *p*-nitrophenol released was measured. In experiments performed in order to evaluate the effect of drugs on ecto-ALP activity, drugs were present during both the preincubation and incubation periods.

The results are expressed as milliunits (mU) of ecto-*p*-NPPase activity per milligram of cell protein. One unit (U) of enzyme activity corresponds to the enzyme activity which hydrolyzes 1 μmol *p*-NPP per min at 37°C, at pH 7.4.

^{125}I-Insulin Internalization Into RBE4 Cell Monolayers

For measurement of ^{125}I-insulin internalization into RBE4 cells, confluent monolayers were incubated with ^{125}I-insulin (100 pM) in 300 μl of incubation medium (KRH) with the following composition (mM): NaCl 119, KCl 4.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, HEPES 16, and glycose 5, supplemented with 1% fetal bovine serum, pH 7.4. After a 60 min incubation at 37°C, monolayers were washed twice with ice-cold KRH medium, pH 7.4, to remove free ^{125}I-insulin. Separation of membrane-bound from internalized radioactivity was achieved by acid washing. Cells were incubated for 10 min at 4°C with KRH medium adjusted to pH 2.8 with HCl; at the end of this period, the medium was collected, and the acid-sensitive radioactivity was measured. The cells were then solubilized in 0.1% SDS for 60 min at room temperature, and the acid-resistant radioactivity was measured [Yagil et al., 1988]. To distinguish ^{125}I-insulin nonspecifically bound from internalized ^{125}I-insulin, some cell monolayers were incubated with a high concentration of insulin (10 μM) plus the same concentration (100 pM) of ^{125}I-insulin.

PI-PLC Cell Treatment: Soluble-ALP Assay

PI-PLC of bacterial origin is known to release glycosylphosphatidylinositol-anchored proteins from cells [Low, 1987]. So, this enzyme was used to release ecto-ALP into the incubation medium. On the day of experiment, the culture medium was removed, and cell monolayers were washed once with NaCl/$MgCl_2$-free Hanks' medium (solution A) at 37°C. After a 20 min preincubation in solution A (pH 7.4, 37°C), cells were incubated with or without 0.1 U ml^{-1} PI-PLC (whose activity is inhibited by NaCl [Sundler et al., 1978]) for 30 min. At the end of the incubation, the medium was collected and supplemented with 137 mM NaCl, and 1 mM $MgCl_2$, and the soluble-ALP activity of the incubation buffer was measured (in the presence of 2.86 mM *p*-NPP).

Protein Determination

The protein content of cells was determined by the method of Bradford [1976], with human serum albumin as standard.

Calculations and Statistics

For the calculation of IC_{50}'s, the parameters of the Hill equation for multisite inhibition [Segel, 1975] were fitted to the experimental data by a nonlinear regression analysis, using a computer assisted method. The IC_{50}'s are given as geometric means [Cheng and Prusoff, 1973].

Values are expressed as the arithmetic mean ± SEM and geometric means are given with 95% confidence limits. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance (ANOVA test) followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. Differences were considered to be significant when $P < 0.05$.

RESULTS

Ecto-*p*-NPPase Activity

RBE4 cells have a high level of ALP expression [El Hafny et al., 1997]. In order to investigate the phosphohydrolase activity of cell surface ALP, substrate hydrolysis was measured directly on RBE4 cells under physiological conditions (i.e., pH 7.4 at 37°C). A commonly used in vitro substrate for ALP, *p*-NPP, was chosen because it does not penetrate the cellular membrane [DePierre and Karnovsky, 1974]. In preliminary experiments, the time course of *p*-nitrophenol formation was determined, using a saturating concentration (2.86 mM) of *p*-NPP (data not shown). The

formation of *p*-nitrophenol increased linearly with time for at least 80 min. Thus, in all subsequent experiments in which ALP activity was determined, cells were incubated for 60 min with substrate.

Orthovanadate decreased ALP activity in a concentration-dependent way, with an IC_{50} of 0.2 μM (0.012–2.3 μM; $n = 3$) (Fig. 1). It produced a maximal decrease of 92% in ALP activity (Table I). In the presence of L-arginine (1 mM) or adenosine (100 μM), ALP activity was significantly reduced. However, D-arginine (1 mM) had no significant effect (Table I).

On the other hand, progesterone and beta-estradiol produced an increase in ALP activity. In the presence of 200 μM progesterone and 100 μM beta-estradiol, ALP activity was increased to 489 and 126% of control, respectively (Table I). The other steroids tested (testosterone 100 μM and corticosterone 100 μM) had no effect on ALP activity (Table I). The increase in ALP activity produced by progesterone (10–200 μM) was found to be concentration-dependent (Fig. 2a), and dependent on the preincubation time. As shown in Figure 3, ALP activity after a preincubation of the cells with progesterone (200 μM) for 20 or 60 min was increased to 489 and 757% of control, respectively.

The ecto-ALP inhibitor orthovanadate (100 μM), the endocytosis inhibitors cytochalasin D (10 μg ml^{-1}) and PAO (100 μM) significantly reduced the effect of progesterone (200 μM) on ALP activity (Fig. 4a). However, cytochalasin D (10 μg ml^{-1}) had no effect on ALP activity and PAO (100 μM) increased the activity of this enzyme (Table I). These results suggest that the progesterone-induced increase in ALP activity may occur through a recruitment of new enzyme molecules from an intracellular pool.

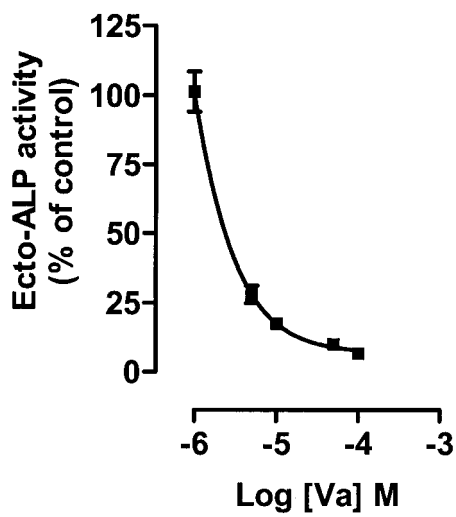

Fig. 1. Effect of orthovanadate on ecto-ALP activity in RBE4 cells. Cells were incubated at 37°C with *p*-NPP (2.86 mM) for 60 min, and the enzyme activity was measured in the absence or presence of increasing concentrations (1–100 μM) of orthovanadate. Symbols are means of four experiments in triplicate and vertical lines show SEM.

Additionally, ALP activity in these cells significantly increased in the presence of the bioflavonoid kaempferol (100 μM), of the phorbol ester PMA (80 nM), IBMX (1 mM) (Table I) or in the presence of RA (10 μM) (Table I).

Chronic treatment of RBE4 cells. Treatment of RBE4 cells for 48 h with RA (10 μM) increased ecto-ALP activity (Table I). Treatment of RBE4 cells for 24 h with $FeSO_4$ (100 μM) or with purified bovine kidney ALP (4 U ml^{-1}) produced a significant increase in ecto-ALP activity. This ecto-ALP activity was insensitive to levamisole, because a 24 h treatment with levamisole (500 μM) did not modify the effect of purified bovine kidney ALP (4 U ml^{-1}) (Table I).

PI-PLC-Induced Release of *p*-NPPase Activity (Soluble-ALP)

Incubation of cultured cells with bacterial PI-PLC, known to selectively remove glycosylphospatidylinositol-membrane anchored proteins [Low, 1987], was used to determine the contribution of glycosylphosphatidylinositol-anchored ALP to ecto-*p*-NPPase activity. In the absence of PI-PLC, *p*-NPPase activity was not detected in the incubation medium (data not shown). PI-PLC (0.1 U ml^{-1}) addition to cells caused a release of soluble-ALP activity into the incubation medium (0.184 ± 0.048 nmol mg^{-1} protein min^{-1}, $n = 3$). This treatment released about 62% of the ecto-ALP activity into the medium. In the presence of orthovanadate (100 μM), soluble-ALP activity released into the incubation medium was totally inhibited (data not shown). Progesterone (200 μM) increased the PI-PLC-induced soluble-ALP activity of the incubation medium (0.916 ± 0.099 nmol mg^{-1} protein min^{-1}; $n = 3$). The increase of soluble-ALP activity of the incubation medium induced by progesterone (to 497% of control) was quantitatively similar to the increase in anchored ecto-ALP induced by this drug (Table I). When progesterone (200 μM) was present also during cell incubation with PI-PLC,

TABLE I. Effect of Several Compounds on Ecto-ALP Activity and ^{125}I-Insulin Internalization by RBE4 Cells*

Compound	% of control	
	Ecto-ALP activity	^{125}I-insulin internalization
Control	100.0 ± 4.0	100.0 ± 4.8
Orthovanadate, 100 μM	7.6 ± 1.5**	80.0 ± 5.5**
L-Arginine, 1 mM	51.2 ± 14.0**	76.9 ± 5.3**
D-Arginine, 1 mM	83.9 ± 2.5	95.0 ± 7.8
Adenosine, 100 μM	68.3 ± 6.5**	68.0 ± 7.7**
Control (ethanol)	100.0 ± 4.7	100.0 ± 4.6
Progesterone, 100 μM	159.3 ± 10.6**	236.6 ± 18.8**
Progesterone, 200 μM	489 ± 37.5**	286.0 ± 17.1**
Beta-estradiol, 100 μM	125.6 ± 10.4**	160.3 ± 14.1**
Corticosterone, 100 μM	124.6 ± 15.6	120.3 ± 12.3
Testosterone, 100 μM	115.4 ± 6.9	132.9 ± 12.7
Control (DMSO)	100.0 ± 9.8	100.0 ± 3.8
Cytochalasin D, 10 μg/ml	118.7 ± 3.9	109.1 ± 8.6
PAO, 100 μM	158.9 ± 8.2**	167.4 ± 7.2**
Kaempferol, 100 μM	193.7 ± 30.5**	134.8 ± 9.4**
PMA, 80 nM	189.8 ± 24.9**	128.2 ± 10.4**
IBMX, 1 Mm	134.6 ± 12.1**	133.2 ± 10.8**
RA, 10 μM	209.0 ± 24.0**	171.0 ± 12.6**
24 h		
Control (DMSO)	100.0 ± 3.8	100.0 ± 3.0
RA, 10 μM	109.4 ± 1.7	115.5 ± 2.8**
Control	100.0 ± 4.0	100.0 ± 3.6
$FeSO_4$, 100 μM	171.0 ± 10.0**	126.6 ± 5.0**
ALP, 4 U/ml	177.8 ± 11.6**	65.4 ± 5.6**
ALP, (4 U/ml) + Levamisole 500 μM	175.9 ± 3.3**	61.2 ± 5.9**
48 h		
Control (DMSO)	100.0 ± 0.8	100.0 ± 11.3
RA, 10 μM	159.1 ± 5.1**	179.5 ± 15.3**

*For ecto-ALP determination, cells were incubated at 37°C with *p*-NPP (2.86 mM) for 60 min. For assay of ^{125}I-insulin internalization, cells were incubated at 37°C with ^{125}I-insulin (100 pm) for 60 min. Shown are arithmetic mean ± SEM of ecto-ALP activity (3–17 determinations/group) and ^{125}I-insulin internalization (4–26 determinations/group) in the presence of the drug relative to the control.
**Significantly different from the respective control ($P < 0.05$).

there was an increase in *p*-NPPase activity to 2.1 ± 0.7 nmol mg^{-1} protein min^{-1}. So, progesterone seems to be modulating only the enzyme activity and not, for example, the synthesis de novo of the enzyme.

Relationship Between PI-PLC-Sensitive Ecto-ALP Activity and ^{125}I-Insulin Internalization Into RBE4 Cell Monolayers

In order to study the role of ecto-ALP activity on the receptor mediated insulin transport into the blood-brain-barrier, ^{125}I-insulin internalization into RBE4 cell monolayers was investigated.

The acid-resistant radioactivity rate, representing internalized ^{125}I-insulin, was 0.563 ± 0.024 fmol mg^{-1} protein, after 60 min of incubation (Fig. 5). To determine whether the association of ^{125}I-insulin with RBE4 cells is hormone-specific, the effect of insulin on ^{125}I-insulin internalization was studied. Nonspecific association of ^{125}I-insulin was found to be < 5% of the total cell-associated ^{125}I-insulin (data not shown).

Orthovanadate (100 μM), L-arginine (1 mM), and adenosine (100 μM) were found to reduce ^{125}I-insulin internalization by RBE4 cells (Table I). Additionally, the ALP inhibitor levamisole (500 μM) was able to reduce ^{125}I-insulin internalization to $69.1 \pm 7.1\%$ of control ($n = 8$).

Of the steroids tested, progesterone and beta-estradiol increased ^{125}I-insulin internalization, but testosterone (100 μM) and corticosterone (100 μM) had no effect (Table I). A maximal increase to 286 and 160% of control was obtained with 200 μM of progesterone and 100 μM of beta-estradiol, respectively. The effect of progesterone (10–200 μM) on ^{125}I-insulin internalization was concentration-dependent (Fig. 2b).

Table I shows that PAO (100 μM) increased ^{125}I-insulin internalization, but cytochalasin D (10 μg ml^{-1}) had no effect. It also shows that the bioflavonoid kaempferol (100 μM), the phorbol

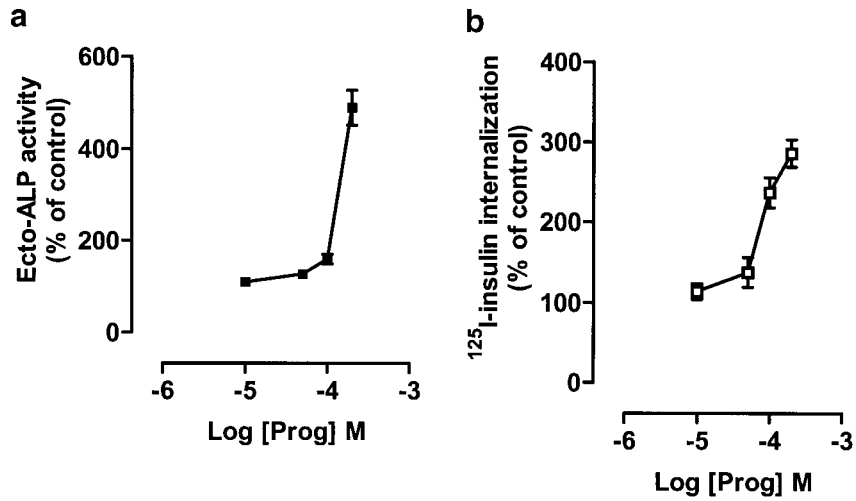

Fig. 2. **a:** Effect of progesterone on ecto-ALP activity in RBE4 cells. Cells were incubated at 37°C with *p*-NPP (2.86 mM) for 60 min, and the enzyme activity was measured in the absence or presence of increasing concentrations (10–200 μM) of progesterone. **b:** Effect of progesterone on ^{125}I-insulin internalization by RBE4 cells. Cells were incubated at 37°C with ^{125}I-insulin (100 pM) for 60 min, and ^{125}I-insulin internalization was measured in the absence or presence of increasing concentrations (10–200 μM) of progesterone. Symbols are means of three experiments in triplicate and vertical lines show SEM.

ester PMA (80 nM) and IBMX (1 mM) increased ^{125}I-insulin internalization (Table I).

The ecto-ALP inhibitor orthovanadate (100 μM) produced a significant reduction in the progesterone (200 μM) effect on ^{125}I-insulin internalization. On the other hand, cytochalasin D (10 μg ml^{-1}) and PAO (100 μM), did not significantly modify the effect of progesterone (Fig. 4b). When we compare the effect of cytochalasin D and PAO both on ecto-ALP activity and ^{125}I-insulin internalization, we verify that there is a discrepancy in the effect of these compounds alone and in the presence of progesterone.

As shown in Table I, the internalization of ^{125}I-insulin by RBE4 cells was significantly increased in the presence of RA (10 μM).

Chronic treatment of RBE4 cells. Treatment of RBE4 cells for 24 and 48 h with RA (10 μM) increased internalization to 116 and 180% of control, respectively (Table I). When RBE4 cells were treated with $FeSO_4$ (100 μM) for 24 h, the internalization of ^{125}I-insulin was increased. Purified bovine kidney ALP (4 U ml^{-1}), on the other hand, significantly reduced ^{125}I-insulin internalization, and this effect was not affected by 24 h treatment with levamisole (500 μM) (Table I).

Influence of extracellular pH on ^{125}I-insulin internalization. Alkalinization of the incubation medium (to pH 7.8 or pH 8.2) produced an increase on ^{125}I-insulin internalization (Fig. 6a).

Finally, Figure 7 shows that there is a significant positive correlation between the effect of compounds on ecto-ALP activity and ^{125}I-insulin internalization by RBE4 cells ($P < 0.0001$, $r = 0.82$, $n = 18$).

Relationship Between PI-PLC-Sensitive Ecto-ALP Activity and Membrane-Bound ^{125}I-Insulin in RBE4 Cells Monolayers

In order to define the role of ecto-ALP activity in the insulin transport into the BBB, membrane-bound ^{125}I-insulin (acid-sensitive radioactivity) of cell monolayers was measured.

As shown in Figure 8, progesterone (100 and 200 μM) and RA (10 μM) significantly increased membrane-bound ^{125}I-insulin. Orthovanadate (100 μM), PAO (100 μM), and cytochalasin D (10 μg ml^{-1}) had no effect (data not shown) but were able to significantly reduce the progesterone effect on membrane-bound ^{125}I-insulin. Purified bovine kidney ALP (4 U ml^{-1}) decreased membrane-bound ^{125}I-insulin (Fig. 8). The other tested drugs had no significant effect on membrane-bound ^{125}I-insulin.

Chronic treatment of RBE4 cells. The presence of purified bovine kidney ALP or levamisole (500 μM) in culture medium for 24 h had no effect on membrane-bound ^{125}I-insulin. However, treatment of the cells with

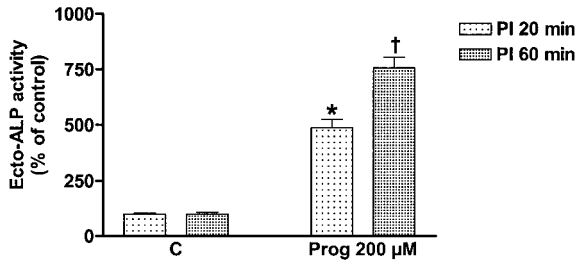

Fig. 3. Effect of different preincubation periods in the effect of progesterone (Prog, 200 μM) on ecto-ALP activity on RBE4 cells. Cells were preincubated for 20 min ($n = 15$) or 60 min ($n = 3$) at 37°C and incubated at 37°C with *p*-NPP (2.86 mM) for 60 min. Ecto-ALP activity was measured in the absence ($n = 18$) or presence of progesterone 200 μM. Shown are arithmetic mean ± SEM of the enzyme activity in the presence of the drug relative to the control. *Significantly different from respective control and † significantly different from PI 20 min ($P < 0.05$).

ALP + levamisole (500 μM) decreased membrane-bound ^{125}I-insulin to 75% of control ($n = 6$).

Influence of extracellular pH on membrane-bound ^{125}I-insulin. Alkalinization of the incubation medium (to pH 7.8 or pH 8.2) produced an increase on the amount of membrane-bound ^{125}I-insulin (Fig. 6b). This result is in agreement with the increased ^{125}I-insulin internalization observed after extracellular alkalinization (Fig. 6a).

DISCUSSION

Most research effort concerning phosphorylation/dephosphorylation mechanisms has focused on understanding the biochemical regulation and physiological importance of intracellular phosphorylation pathways. However, during the past few years, numerous reports described the presence of ecto-kinases on the outer cell surface of a wide variety of cells. These ecto-enzymes were shown to phosphorylate both extracellular (soluble) substrates and cell-surface proteins [Redegeld et al., 1999]. Thus, they might play an important role in the regulation of ligand binding, signal transduction, and cell-to-cell interactions. It has been suggested that both protein kinases and phosphatases are required for reversible control of extracellular phosphorylation processes, acting in a manner similar to that in which they control cytoplasmatic phosphorylation/dephosphorylation systems. However, although activation by protein kinases has been studied in some detail, the dephosphorylation step has received little attention.

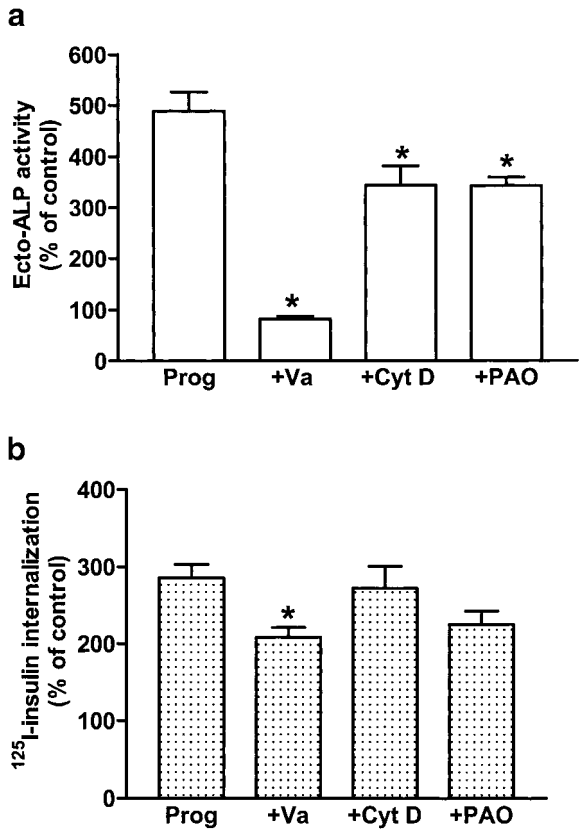

Fig. 4. **a:** Effect of drugs on ecto-ALP activity in RBE 4 cells. Cells were incubated at 37°C with *p*-NPP (2.86 mM) for 60 min, and ecto-ALP activity was measured in the absence ($n = 18$) or in the presence of progesterone 200 μM (Prog; $n = 15$) alone and with 100 μM orthovanadate (+Va; $n = 8$), 10 μg/ml cytochalasin D (+Cyt D; $n = 8$) or PAO (+PAO; $n = 8$). Shown are arithmetic means ± SEM of the enzyme activity in the presence of the drug relative to the control. **b:** Effect of drugs on ^{125}I-insulin internalization by RBE4 cells. Cells were incubated at 37°C with ^{125}I-insulin (100 pM) for 60 min, and ^{125}I-insulin internalization was measured in the presence of progesterone 200 μM ($n = 27$) alone and with 100 μM orthovanadate (+Va; $n = 8$), 10 μg/ml cytochalasin D (+Cyt D; $n = 8$) or PAO (+PAO; $n = 12$). Shown are arithmetic means ± SEM of the internalization in the presence of the drug relative to the control. *Significantly different from 200 μM progesterone ($P < 0.05$).

ALP belongs to a class of proteins that are anchored to plasma membrane via covalent linkage to glycosylphosphatidylinositol. The fact that ALP can be released by phospholipase C or D has provided evidence of its ecto-orientation [Low, 1987]. The physiological function of ALP as an ecto-phosphatase remains controversial, mainly because early studies with purified ALP describe the enzyme as exhibiting a non-physiological alkaline pH optimum [McComb et al., 1979]. However, a

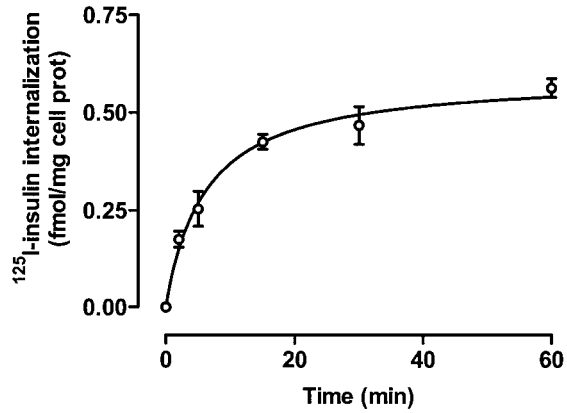

Fig. 5. Time course of ^{125}I-insulin internalization in RBE4 cells. Cells were incubated at 37°C with 100 pM ^{125}I-insulin in the presence and absence of 10 μM unlabeled insulin. Each value is corrected for nonspecific binding. Shown are arithmetic means ± SEM ($n = 3$).

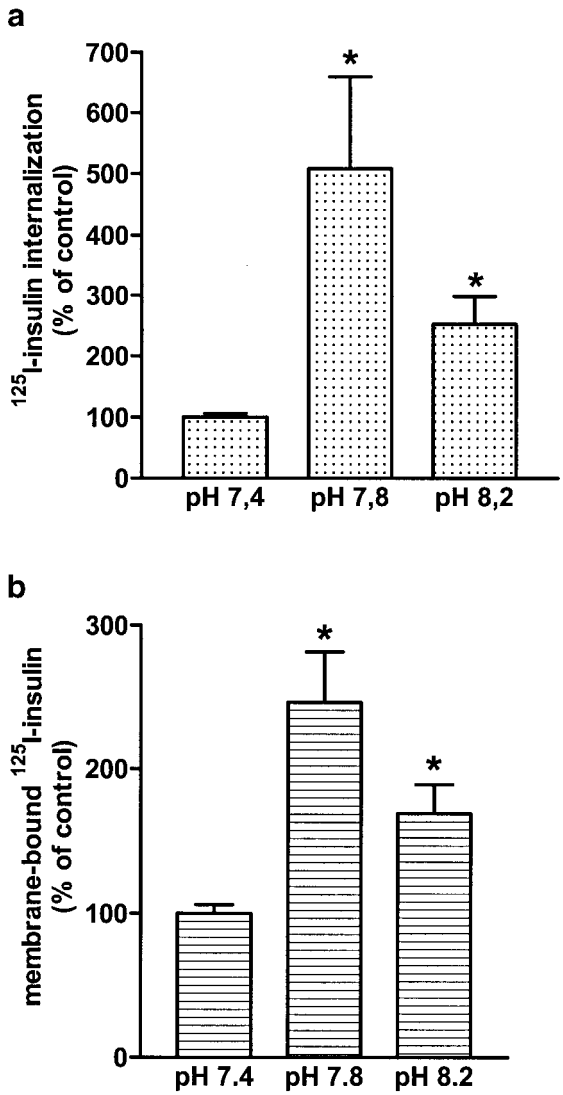

Fig. 6. **a:** Influence of extracellular pH on ^{125}I-insulin internalization by RBE4 cells. Cells were incubated at 37°C with ^{125}I-insulin (100 pM) for 60 min at pH 7.4 (control), pH 7.8 and pH 8.2 ($n = 6$). Shown are arithmetic means ± SEM of the percentage of control. **b:** Influence of extracellular pH on membrane-bound ^{125}I-insulin in RBE4 cells. Cells were incubated at 37°C with ^{125}I-insulin (100 pM) for 60 min at pH 7.4 (control), pH 7.8, and pH 8.2 ($n = 6$). Membrane-bound ^{125}I-insulin was measured after separation from cell surface by exposure to ice-cold acidified medium (pH 2.8) for 10 min. Shown are arithmetic means ± SEM of the percentage of control. *Significantly different from control ($P < 0.05$).

pH optimum of 7–8 was determined for purified and plasma membrane-bound human liver ALP [Chan and Stinson, 1986], as well as for purified bovine hepatic and calf intestinal ALP [Swarup et al., 1981].

The involvement of ALP as an ecto-enzyme regulating membrane transporters was first reported by Becq et al. [1993, 1994]. Additionally, it is known that high levels of ecto-phosphatase activity are correlated with the dephosphorylation state of plasma membrane proteins and of a 98-kDa surface phosphoprotein in RA-treated HL-60 and P19 cells, respectively [Scheibe et al., 2000]. More recently, Ohkubo et al. [2000] suggested that ecto-ALP plays an essential role in the P_1 antagonist-sensitive ATP-induced cAMP accumulation in NG108-15 cells, as AMP phosphohydrolase.

This study demonstrates that cultured RBE4 cells possess a phosphatase activity able to hydrolyze *p*-NPP in the external medium under physiologic pH conditions. The experimental conditions used in this study allow us to characterize this activity as an ecto-phosphatase activity, mainly because: (1) the substrate was present in the external medium; (2) the substrate used, *p*-NPP, does not penetrate into plasma membranes [DePierre and Karnovsky, 1974]; (3) the *p*-NPPase activity was not detected in the external medium, what rules out the possible enzymatic action of soluble contaminants or the leakage of intracellular enzymes; (4) substrate hydrolysis was linear with time, demonstrating that cell breakage did not occur during the incubation.

It has been shown that PI-PLC removes the glycosylphospholipid-linked ALP from membranes, and that solubilization of ALP does not affect its ability to hydrolyze *p*-NPP [Low and Finean, 1977]. In agreement with Roux et al.

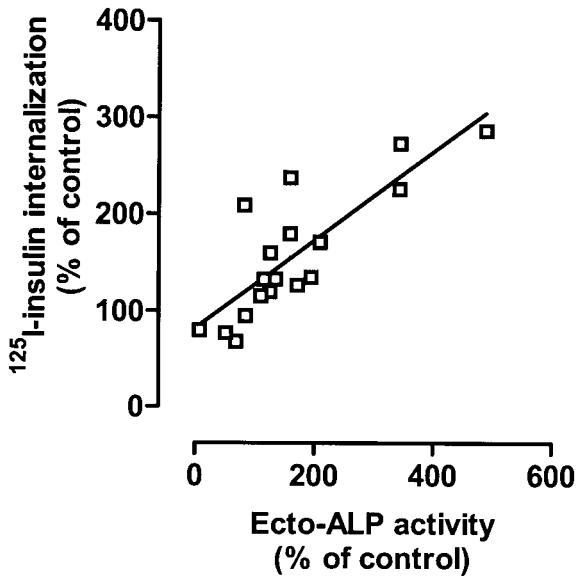

Fig. 7. Correlation between the effect of compounds on ^{125}I-insulin internalization into RBE4 cells and ecto-ALP activity in these cells. Drugs used were: adenosine, vanadate, kaempferol, progesterone, beta-estradiol, testosterone, corticosterone 100 μM, D- and L-arginine and IBMX 1 mM, progesterone 200 μM (in the absence and in the presence of cytochalasin D 10 μg ml^{-1}, vanadate 100 μM or PAO 100 μM), RA 10 μM (acutely, 24 and 48 h treated cells), $FeSO_4$-treated cells (100 μM, 24 h). Each value represents arithmetic mean ± SEM of the percentage of control. There is a significant positive correlation ($P < 0.0001$; $r = 0.82$; $n = 18$).

[1994], who described the presence of ALP activity in RBE4 cell membrane, our results suggest that the *p*-NPPase activity released into the incubation buffer after incubation with PI-PLC may be attributable to the soluble form of ALP. Additionally, the absence of *p*-NPPase activity in our incubation buffer before incubation with PI-PLC demonstrates that ALP bound to RBE4 plasma membranes was not released spontaneously as observed in osteosarcoma cells [Fukayama and Tashjian, 1990] and that intracellular ALP did not leak out into the reaction buffer.

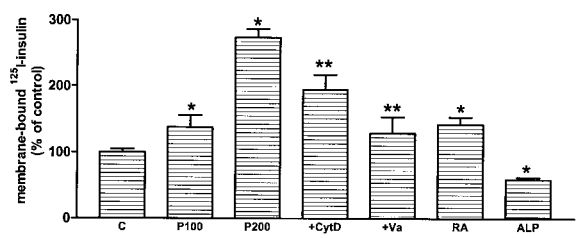

Fig. 8. Effect of several drugs on membrane-bound ^{125}I-insulin in RBE4 cells. Cells were incubated at 37°C with ^{125}I-insulin (100 pM) for 60 min, in the absence ($n = 27$) and presence of progesterone 100 and 200 μM (P100 and P200; $n = 16$ and $n = 27$, respectively), progesterone 200 μM with 10 μg ml^{-1} cytochalasin D (+CytD; $n = 8$) or with 100 μM orthovanadate (+Va; $n = 4$), RA (10 μM; $n = 6$) and purified bovine kidney ALP (4 U ml^{-1}; $n = 11$). Membrane-bound ^{125}I-insulin (acid sensitive) was measured after separation from cell surface by exposure to ice-cold acidified medium (pH 2.8) for 10 min. Shown are arithmetic means ± SEM of the percentage of control. *Significantly different from control and **significantly different from 200 μM progesterone ($P < 0.05$).

Signals that regulate long-term energy balance have been difficult to identify. Increasingly strong evidence indicates that insulin, acting on the CNS, in part through its effect on neuropeptide Y, inhibits food intake. This fact was first reported by Woods and coworkers more than 20 years ago [Woods et al., 1979]. Since then, it has been demonstrated that insulin is transported across the BBB. Recently, Brüning et al. [2000] showed that insulin signalling in the brain is essential for normal regulation of adiposity. So, compounds able to modify the transport of insulin across the BBB could modify the rate of insulin effects on CNS/periphery. Our results showed that delivery of plasma insulin into the BBB is saturable and probably dependent on an insulin-receptor mediated transport process. Moreover we show that there is a positive correlation between the effect of drugs on ALP activity and ^{125}I-insulin internalization.

Acute or chronic treatment (for 48 h) of RBE4 cells with RA, one of the biologically active forms of vitamin A, increased the amount of internalized ^{125}I-insulin, and also increased ecto-ALP activity in these cells. The effect of RA on ecto-ALP activity is in agreement with a previous report [El Hafny et al., 1996]. These results suggest that RA may play an important role in the CNS, namely in the effects of insulin, by interfering with its transport at the BBB.

Orthovanadate, an inhibitor of all protein tyrosine phosphatases, including ALP [Seargeant and Stinson, 1979], strongly inhibited ecto-ALP activity and significantly reduced ^{125}I-insulin internalization in RBE4 cells. Thus, it seems that ^{125}I-insulin endocytosis is dependent on the dephosphorylated status of the receptor. Adenosine was used because this compound was previously found to increase ALP activity in primary cultured hepatocytes [Calhau et al., 2000]. Interestingly enough, adenosine decreased both ^{125}I-insulin internalization and ecto-ALP activity.

In this study, we found that L-arginine significantly inhibited both ecto-ALP activity

and ^{125}I-insulin internalization. On the other hand, the inactive amino acid D-arginine had no effect on both ^{125}I-insulin internalization or ecto-ALP activity. We think that the effect of L-arginine upon ecto-phosphatase activity may be related to the recently described effect of an L-arginine peptide upon drug internalization [Rothbard et al., 2000; Martins et al., 2001].

Ben-Shachar et al. [1988] observed that iron-deficient rats have an increase in brain uptake of insulin. Additionally, ALP activity may be decreased in the presence of iron chelators [Naves et al., 1998]. So, we decided to test the effect of chronic treatment of cells with $FeSO_4$ on both ^{125}I-insulin internalization and ecto-ALP activity. Interestingly enough, we observed an increase in both parameters after treatment of the cells with $FeSO_4$.

In this study, we found that progesterone, β-estradiol and kaempferol (a phytoestrogen), increased ^{125}I-insulin internalization and ecto-ALP activity in RBE4 cells. Recently, it was demonstrated that insulin and estrogen improve memory in both healthy women and female patients with Alzheimer's disease [Wickelgren, 1997, 1998]. Additionally, it is known that both insulin and estrogens inhibit appetite [Bonavera et al., 1994]. Thus, based on our results, we can speculate that part of the central effects of estrogens (eg., on memory and food intake) could be mediated by insulin.

Flavonoids are a group of compounds occurring in fruits, vegetables, red wine, and tea. According to Middleton [1984], certain flavonoids are able to affect endocytotic as well as exocytotic functions. We verified that kaempferol increased ^{125}I-insulin internalization and the ecto-ALP activity. This effect on insulin transport into the BBB could reduce peripheral and increase central effects of insulin, thus contributing for the beneficial effects of flavonoids on health. Since people may ingest up to 1 g/per day of these substances, flavonoids may prove to have significant effects on normal ecto-ALP physiology.

In addition, we have demonstrated that after treatment with purified bovine kidney ALP for 24 h, the enzyme activity in plasma membrane increased, but ^{125}I-insulin internalization and membrane-bound ^{125}I-insulin significantly decreased. We cannot advance any explanation for this fact, and underline the fact that this increase on ecto-enzyme activity is levamisole-insensitive.

Altogether, our results show that drugs that increased or decreased ^{125}I-insulin internalization had the same effect on membrane-bound ^{125}I-insulin, although some drugs modify only ^{125}I-insulin internalization.

We did not find any correlation between the amount of membrane-bound ^{125}I-insulin and ecto-ALP activity. Thus, only the ^{125}I-insulin endocytosis step seems to be dependent on the phosphorylated/dephosphorylated status at the membrane. These findings are in agreement with those of Maggi et al. [1995] who showed that an extracellular mutation of the insulin receptor did not affect insulin binding, but decreased insulin–insulin receptor internalization. These authors observed an increase in the autophosphorylation of the mutated receptor. So, we can speculate that the extracellular serine phosphorylation/dephosphorylation status could be an important target for regulation of insulin receptor autophosphorylation and consequently, of insulin internalization by the cells.

In conclusion, our data show that RBE4 cells express an ALP activity, characterized by an ecto-oriented active site, and functionally active at physiological pH. Moreover, they evidence the release of the ecto-enzyme by phosphatidylinositol-specific phospholipase C. Finally, our results reveal a positive correlation between ecto-ALP activity and ^{125}I-insulin incorporation into cultured rat brain endothelial cells, pointing to a relationship between ecto-ALP activity and BBB transport of insulin.

We can speculate that compounds able to modify the delivery of plasma insulin into the CNS can regulate food intake. In other words, nutritional or pharmacological interventions that impair/increase CNS insulin uptake can predispose to weight gain/loss, respectively. So, membrane-associated phosphatase activity may be a potential therapeutic target for insulin-related brain diseases, and the phosphorylation/dephosphorylation status of ecto-domains of functionally important cell-surface proteins may provide attractive and novel pharmacological targets.

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