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International Journal of Pharmaceutics 336 (2007) 133–139
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Molecular mechanism involved in the transport of a prodrug dopamine glycosyl conjugate

A. Dalpiaz [a], R. Filosa [b], P. de Caprariis [b], G. Conte [b], F. Bortolotti [a], C. Biondi [c], A. Scatturin [a], P.D. Prasad [d], B. Pavan [c,*]

[a] *Department of Pharmaceutical Sciences, University of Ferrara, via Fossato di Mortara 19, 44100 Ferrara, Italy*
[b] *Department of Pharmaceutical Sciences, University of Salerno, via Ponte don Melillo 84084, Fisciano, Salerno, Italy*
[c] *Department of Biology, General Physiology Section, University of Ferrara, via L. Borsari 46, 44100 Ferrara, Italy*
[d] *Department of Obstetrics and Gynecology, Medical College of Georgia, Augusta, GA 30912-2100, United States*

Received 14 July 2006; received in revised form 20 November 2006; accepted 22 November 2006
Available online 28 November 2006

Abstract

We have previously demonstrated that dopamine conjugation to glucose allows it to induce therapeutic effects against Parkinson's disease after intravenous administration. In this paper we demonstrate that, unlike dopamine, the prodrug glu-dopamine is a transportable substrate of glucose transporters. Towards this, the effect of glucose-conjugation on the affinity and uptake of dopamine have been assessed *in vitro*, using human retinal pigment epithelium (HRPE) cells. Glucose transporter-mediated uptake was measured using [^{3}H]3-*O*-methylglucose ([^{3}H]3-*O*-MG) as the tracer. The uptake was found to be rapid and hyperbolically related to its concentrations ($K_t = 7.8 \pm 1.2$ mM and $V_{max} = 54 \pm 2$ nmol/min mg protein). Inhibition experiments showed that dopamine was able to interact with glucose carriers only when conjugated to glucose ($IC_{50} = 2.6 \pm 0.6$ mM). HPLC analysis of HRPE cell extracts showed that both dopamine and the prodrug permeate the cell, but only the uptake of the prodrug is inhibitable by glucose. This confirms that glucose transporters mediate the transport of the prodrug glu-dopamine, but not of dopamine. HRPE cells is therefore proposed as a promising model for *in vitro* studies involving the glucose transporter-mediated transport of drugs and their conjugates.

Keywords: Dopamine; Drug targeting; Glucose; Glucose transporters; HRPE cells

1. Introduction

Parkinson's disease is a chronic, progressive neurodegenerative movement disorder. Tremors, rigidity, slow movement, poor balance, and difficulty walking are characteristic symptoms of this disease. It results from the degeneration of dopamine-producing nerve cells in the brain, specifically in the substantia nigra and the locus coeruleus. Dopamine (Fig. 1) is the neurotransmitter that stimulates the motor neurons, the nerve cells that control muscle movement. When dopamine production is depleted, as in Parkinson disease, the patient is unable to control movement and coordination (Bernheimer et al., 1973).

Though dopamine supplementation alleviates the neurological symptoms associated with Parkinson's disease, the peripheral administration of dopamine cannot be an useful treatment since this drug can not cross the blood–brain barrier (BBB) and penetrate into the central nervous system (CNS). In addition, it induces dangerous unwanted effects in the peripheral tissues by activation of specific and ubiquitous dopaminergic receptors (Jankovic, 2002). The usual treatment for Parkinson's disease is L-dopa, a dopamine precursor able to penetrate the CNS, where it is rapidly converted to dopamine (Djaldetti and Melamed, 2001). The transport of L-dopa across the BBB is mediated by system 1, an amino acid transporter which accepts large neutral amino acids as substrates. Unfortunately, conversion of L-dopa to dopamine also occurs in peripheral tissues as well, especially in the gut, by the enzyme L-dopa decarboxylase. Therefore, only a small fraction of L-dopa is able to reach the brain, the primary site of drug action. Thus, the unwanted side effects associated with treatment with L-dopa during the therapy beginning are mainly caused by its premature conversion to dopamine in peripheral tissues (Jenner, 2003). To reduce

* Corresponding author. Tel.: +39 0532 291476; fax: +39 0532 207143.
E-mail address: pvnbbr@unife.it (B. Pavan).

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doi:10.1016/j.ijpharm.2006.11.051

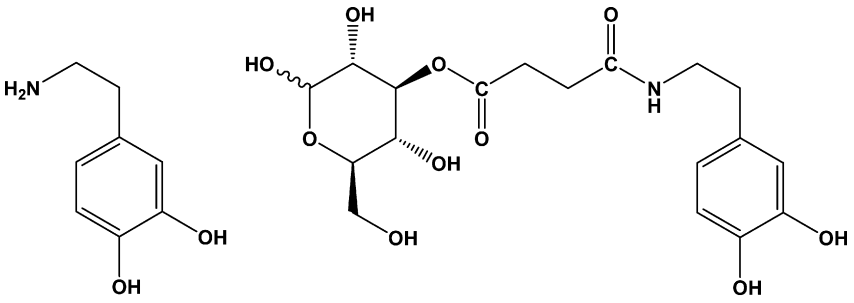

Fig. 1. Structural formulae of dopamine and its pro-drug (glu-dopamine) obtained by coupling dopamine to D-glucose via a succinic spacer.

these drawbacks, a treatment strategy for Parkinson's disease is the use of a combination of L-dopa and a dopa decarboxylase inhibitor, like carbidopa, or benserazide. The inhibitor blocks the conversion of L-dopa to dopamine in the peripheral tissues, often lessening or even preventing the side effects associated with L-dopa treatment.

Recently, an interesting strategy has been proposed to overcome the problems associated with poor absorption of drugs into CNS from the blood stream. This strategy involves the synthesis of prodrugs able to interact with specific transporters that mediate the uptake of nutrients (Pardridge, 2002) such as glucose, amino acids and vitamins (Bonina et al., 1999, 2000; Ohnishi et al., 2000; Manfredini et al., 2002; Dalpiaz et al., 2005a). The absorption of glucose into the CNS from the bloodstream in mammals has been identified as a facilitated diffusion phenomenon involving the transporter GLUT1, a glucose-carrier located in the membrane of brain capillary endothelial cells which constitute the BBB (Carruthers, 1990; Regina et al., 1997).

Taking into account the aspects reported above, a dopamine prodrug was synthetized by coupling dopamine to D-glucose via a succinic spacer (Fig. 1). It has been demonstrated that the administration of this prodrug reduced morphine-induced locomotion in mice and reverted the reserpine-induced hypolocomotion in rats (Bonina et al., 2003). Such an effect was not observed when dopamine was injected. In other words, the prodrug administration produced a CNS pharmacologic effect on classic dopaminergic models. Based on these results we hypothesize that the dopamine conjugation to glucose allows the drug to interact and be transported by the carrier GLUT1.

In this report, we have investigated the molecular mechanism by which the glu-dopamine prodrug crosses the BBB. Human retinal pigment epithelium cells (HRPE) were employed as a model for the cells lining the BBB as these cells, similar to the cells lining the BBB (Toimela et al., 2004), have been shown to express GLUT1 transcripts and protein (Takagi et al., 1994; Busik et al., 2002; Senanayake et al., 2006). [^{3}H]3-*O*-methylglucose ([^{3}H]3-*O*-MG), a non-metabolizable substrate, was used as the tracer to characterize glucose transporter-mediated transport process (Regina et al., 1997). The results obtained show that glu-dopamine is an excellent substrate of glucose transporters, and its transport across the BBB could be mediated by these transporters.

2. Materials and methods

2.1. Materials

[^{3}H]3-*O*-MG (specific activity = 6 Ci/mmol) and [^{14}C]L-glucose (specific activity = 55 mCi/mmol) were obtained from Amersham Biosciences (Milan, Italy). HRPE cell line was originally developed by Del Monte (Del Monte and Maumenee, 1981) and characterized in detail by Dutt et al. (1989). 3-*O*-MG, pyruvate, floretin and CHAPS were obtained from Sigma (St. Louis, MO, USA). FBS (foetal bovine serum), 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, streptomycin and penicillin, trypsin–EDTA and PBS (phosphate-buffered saline) were obtained from Invitrogen (Life Technologies Italia, Milan, Italy). HPLC grade solvents were purchased from Carlo Erba Reagenti (Milan, Italy). All other chemicals and solvents were obtained from standard sources.

2.2. HRPE cell culture

The cells were routinely grown in 1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 media, supplemented with 10% FBS, 50 μg/ml streptomycin and 50 IU/ml penicillin at 37 °C in 5% CO_2. Cells used for uptake measurements were seeded in 24-well tissue culture plates and grown to confluence (2–3 days).

2.3. GLUT1 transporter interactions

The uptake into HRPE cells was measured with the employ of 3-*O*-MG in the presence of [^{3}H]3-*O*-MG used as tracer. [^{14}C]L-glucose was used as a marker for non-specific uptake, extracellular binding and any remaining extracellular fluid (Takakura et al., 1991).

Time course experiments were performed as follows: the culture plates containing the cell monolayers were maintained at 37 °C in a shaking water bath. Cells were first washed three times with 1 ml of warm (37 °C) glucose-free buffer (GFB: 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2 mM pyruvate, 25 mM HEPES, pH 7.4) and preincubated in 1 ml GFB for 30 min at 37 °C. All solutions were removed by aspiration. After the preincubation cells were incubated with 0.5 ml GFB containing 5 mM 3-*O*-MG plus [^{3}H]3-*O*-MG (2 μCi/ml) and [^{14}C]L-glucose (0.2 μCi/ml) in a shaking bath for time intervals

ranging from 15 s to 5 min. Uptake was stopped with the addition of 1 ml ice-cold phosphate buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 0.25 mM floretin. Cell monolayers were washed twice with 1 ml PBS blocking solution. The cells were subsequently solubilized in 0.25 ml of 0.2N NaOH containing 0.5%, w/v CHAPS and transferred to vials containing 3 ml of scintillation cocktail (Ultima Gold, Packard, Milan, Italy). The radioactivity associated with the cells was determined by dual counting using a liquid scintillation counter (Beckmann LS 6000). Protein content of cell monolayers was determined by the method of Lowry et al. (1951). Aliquots of the incubation solution were also taken in order to measure the exact specific activity of the solution used. A spreadsheet was used to calculate the total [^{3}H]3-*O*-MG uptake, the non-specific [^{14}C]L-glucose uptake, the specific [^{3}H]3-*O*-MG uptake in the cells and the 3-*O*-MG uptake per mg of protein.

The kinetics of 3-*O*-MG uptake into HRPE cells was investigated according to the experimental protocol above described above, however, with the following modifications: the cells were incubated for 30 s with 3-*O*-MG concentrations ranging from 0.5 to 80 mM in the presence of 4 μCi/ml [^{3}H]3-*O*-MG and 0.2 μCi/ml [^{14}C]L-glucose. Data were then analysed by non-linear regression and confirmed by Eadie–Hofstee linear regression.

Inhibition of 3-*O*-MG transport was determined by adding the indicated concentrations of unlabelled compounds to plated cells along with 5 mM 3-*O*-MG, 4 μCi/ml [^{3}H]3-*O*-MG and 0.2 μCi/ml [^{14}C]L-glucose (30 s incubation). The concentrations of the inhibitors displacing 50% of 3-*O*-MG uptake (IC_{50} values) were obtained by non-linear regression analysis.

All the values obtained are means of three independent experiments performed in duplicate.

Linear and non-linear regression analyses were performed using the computer program Graph Pad Prism (GraphPad, San Diego, USA).

2.4. Uptake in HRPE cells

HRPE cells were washed and preincubated as described above. Intracellular accumulation of dopamine or prodrug, in the absence or in the presence of 10 mM glucose, were measured by incubating the cells with the compounds at 10 mM concentration in GFB for 90 s at 37 °C. Following the incubation, the transporter-mediated uptake was stopped with the addition of 1 ml ice-cold PBS containing 0.25 mM floretin. Cell monolayers were then washed, first with 1 ml PBS containing floretin, followed by 1 ml PBS without floretin. Finally, the cells were lysed by adding deionized water (0.3 ml per well of a 24-well plate) and freezing them at −80 °C for 30 min. The cells were then thawed on ice, and the lysate was centrifuged (12,500 × *g*, 10 min) to remove cell membranes. The supernatant (40 μl) was used to measure the levels of the test substrate by HPLC.

All the values obtained are means of four independent experiments.

2.5. HPLC analysis

HPLC apparatus consisted of a modular chromatographic system (Model 1100 series pump and diode array detector; Agilent, Waldbronn, Germany) linked to an injection valve with 50 μl sample loop (Model 9125; Rheodyne, Cotati, CA, USA). Analyses were all performed at room temperature.

Data acquisition and processing were accomplished with a personal computer using Chem Station software (Agilent). The identity peaks was assigned by co-chromatography with the authentic standards. Quantification was performed by integration of peak areas using external standardization.

Chromatography was performed on a reversed-phase column (Hypersil BDS C-18 5U cartridge column, 150 mm × 4.6 mm i.d.; Alltech, Milan, Italy) equipped with a guard column packed with Hypersil C-18 material (Alltech). The detector was set at 280 nm. The mobile phase consisted of a mixture of 0.2% TFA and methanol with a ratio of 70/30 (v/v). The flow rate was 0.8 ml/min. The retention times were 2.6 and 2.9 min for dopamine and its prodrug, respectively.

2.5.1. Statistical analysis

Unpaired *t* tests between the uptake rates of dopamine and glu-dopamine in the absence and in the presence of glucose were performed using the computer program GraphPad Prism (GraphPad, San Diego, CA, USA). Differences were considered statistically significant when *p*-values were less than 0.05.

3. Results

The time course of specific uptake of 3-*O*-MG is shown in Fig. 2. Uptake was rapid for 1 min during the initial time period, following which the slope reduced until a plateau was reached by 5 min. When the uptake obtained in the initial time periods was analysed by linear regression (Fig. 2 inset) it was linear during the initial 60 s and fit to the equation:

$$3-O-\text{MG uptake (nmol/mg protein)} = 0.1(\pm 0.5) + 0.30(\pm 0.01)\ \text{time(s)}.$$

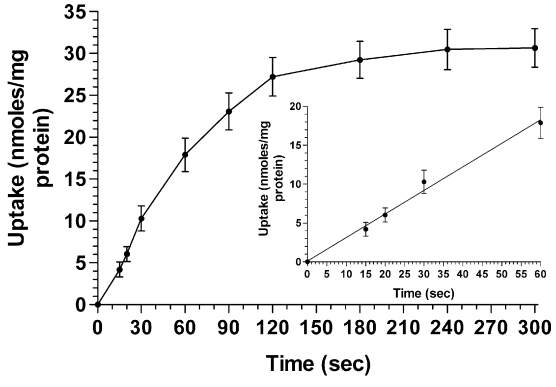

Fig. 2. Time course of 3-*O*-MG uptake into HRPE cells. Inset: linear regression of the 3-*O*-MG uptake within 1 min ($r = 0.995$, $p < 0.001$, $n = 5$). Data are mean ± S.E. ($n = 3$).

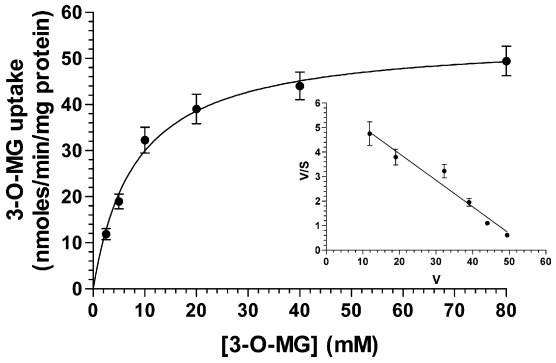

Fig. 3. Kinetics of 3-*O*-MG uptake in HRPE cells measured by 30 s incubation at 37 °C, pH 7.4. 3-*O*-MG concentrations were in the range 2.5–80 mM. Curve was fitted to the Michaelis–Menten equation using a non-linear least-squares method. Inset: Eadie–Hofstee transformation of the same data. V, 3-*O*-MG uptake (nmol/min mg protein); S, 3-*O*-MG concentration (mM). Data are mean ± S.E. ($n = 3$).

Subsequent experiments were therefore done with a 30 s incubation time. As seen in Fig. 3, the uptake rate was found to be hyperbolically related to the 3-*O*-MG concentrations, indicating that the transport process is saturable. The related Eadie–Hofstee linear regression of data is represented in the inset. This plot was linear in the concentration range investigated ($n = 6$, $r = 0.980$, $p < 0.001$) and, analogously, computer analysis of the saturation experiment suggested a one-site, rather than a two-site model. The Michaelis–Menten constant (transport constant, K_t) and V_{max} values for the transport process were found to be 7.8 ± 1.2 mM and 54 ± 2 nmol/min mg protein, respectively.

The effect of the presence of increasing concentrations of dopamine and the prodrug glu-dopamine on the uptake of 3-*O*-MG is shown in Fig. 4. It can be observed that dopamine was not able to inhibit the 3-*O*-MG uptake into HRPE cells in the concentration range investigated (0.3–30 mM). On the other hand, the conjugate glu-dopamine was able to inhibit the uptake of 3-*O*-MG in a concentration-dependent manner. Complete inhibition was observed at a prodrug concentration of 30 mM. By non-linear regression analysis, the IC_{50} value for the prodrug was found 2.6 ± 0.6 mM. These results indicate that dopamine is unable to interact with the glucose transport systems expressed in HRPE cells, while its prodrug glu-dopamine is able to interact with relatively high affinity.

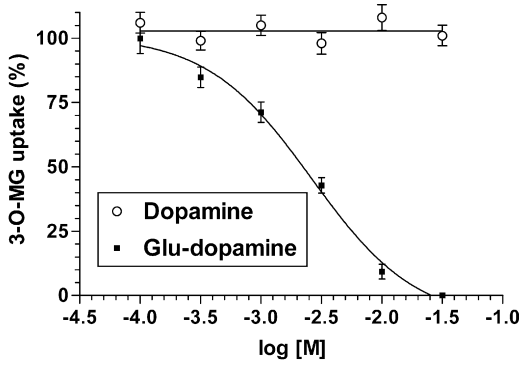

Fig. 4. Inhibition of 5 mM 3-*O*-MG uptake by dopamine and its prodrug glu-dopamine. 3-*O*-MG uptake in the presence of inhibitors was measured at 37 °C, pH 7.4. Data are mean ± S.E. ($n = 3$).

While the above studies do show that glu-dopamine is able to interact with the glucose transporters expressed in HRPE cells, it does not necessarily demonstrate that the prodrug is indeed transported into the cells. To demonstrate that the prodrug is transported into the cells, we measured intracellular levels of glu-dopamine after incubating the cells with the prodrug in the absence and presence of D-glucose. The concentrations of glu-dopamine detected before and immediately after the incubation were not significantly different (data not shown). Fig. 5 depicts the chromatograms of the cytosolic fraction of HRPE cells after their incubation with dopamine and glu-dopamine (10 mM) in the absence and in the presence of 10 mM D-glucose. The LQD value for dopamine was 0.37 nmol/ml = 0.14 nmol/(mg protein min) (0.015 nmol/injection) and for glu-dopamine 1.12 nmol/ml = 0.42 nmol/(mg protein min) (0.045 nmol/injection) with a signal-to-noise ratio 10:1. It can be observed that both the compounds are able to permeate into the cells, but only the uptake of the prodrug was significantly inhibited in the presence of 10 mM D-glucose. Fig. 6 summarizes the uptake of dopamine and its prodrug detected into the cytosol of HRPE cells after their incubation in the presence and absence of D-glucose. Similar uptake was observed for both the compounds (9.6 ± 2.4 and 10.0 ± 2.1 nmol/mg protein min for dopamine and glu-dopamine, respectively) in the absence of glucose. However, in the presence of glucose only the uptake of the prodrug was significantly reduced (12.8 ± 2.9 versus 1.0 ± 0.4 nmol/mg protein min for dopamine and glu-dopamine, respectively). These results indicate that only glu-dopamine shows a glucose transporter-mediated uptake into HRPE cells.

4. Discussion

A typical problem associated with treatment using neuroactive drugs, such as the receptor ligands, is the unwanted and often dangerous side effects caused due to the interaction of the drug with peripheral receptors. Another problem frequently encountered is the poor passage of the drug across the BBB into the brain. Both these problems are seen when dopamine is used as a treatment for certain neurological problems such as Parkinson's disease.

It has been demonstrated recently using animal dopaminergic models that the prodrug glu-dopamine is able to induce central effects in the absence of the classical peripheral vascular phenomena evoked by dopamine (Bonina et al., 2003). The prodrug has been designed and synthesised following a new strategy, which involve the conjugation of neuroactive drugs with native compounds that are known to be transported into the CNS by specific carriers located on the physiological barriers between the blood and CNS. Since the expression of GLUT1 in the BBB of mammals has been reported by Regina et al. (1997), it was hypothesised that the facilitative glucose carriers, especially GLUT1 would mediate the transport of the prodrug glu-dopamine across the BBB.

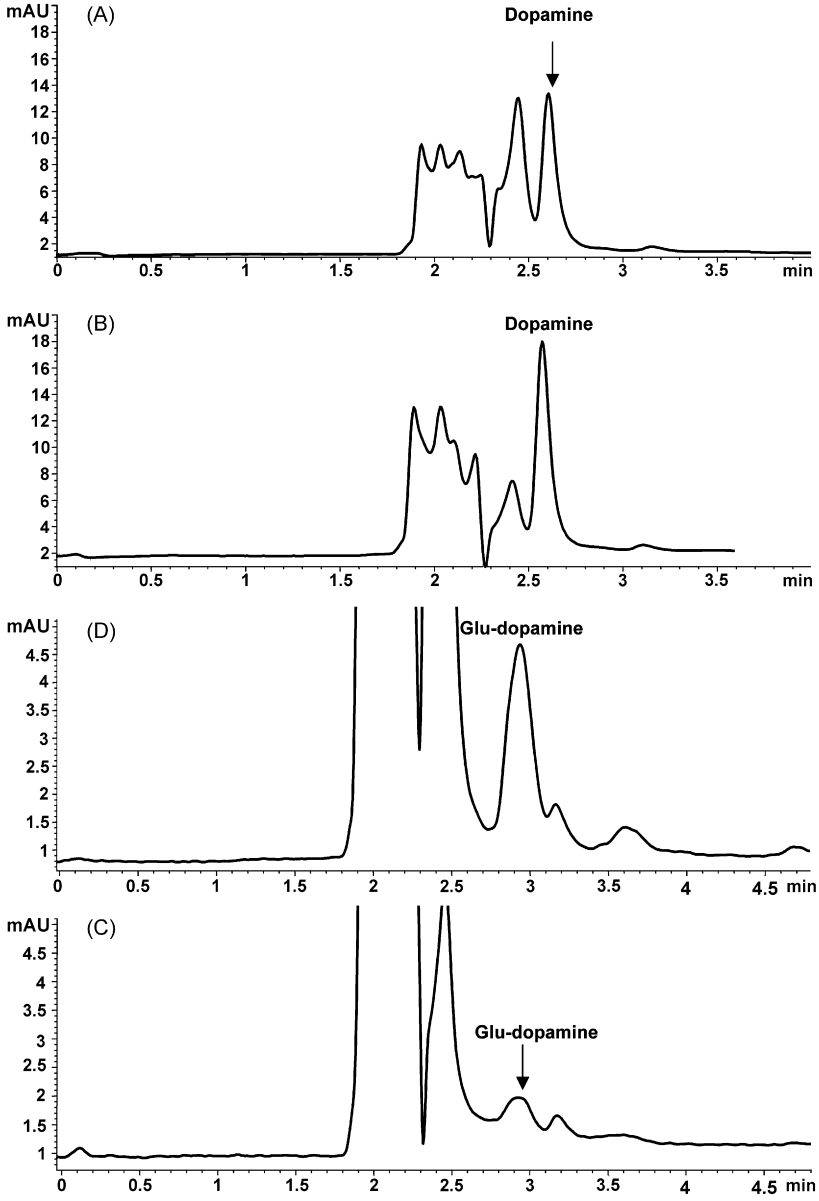

Fig. 5. HPLC chromatograms for intracellular fluid of HRPE cells after their incubation for 90 s with 10 mM dopamine in the absence (A) and in the presence of 10 mM glucose (B) and with 10 mM glu-dopamine in the absence (C) and in the presence of 10 mM glucose (D).

Our studies presented here demonstrate that, unlike dopamine, the prodrug glu-dopamine is able to interact with the glucose transporters expressed on HRPE cells and gains entry into the cells through these transporters. The HRPE cells have been shown to express GLUT1 both at the mRNA and the protein level (Takagi et al., 1994; Busik et al., 2002; Senanayake et al., 2006). Differently from retina cells, the mammalians retinal pigment epithelium cells show a GLUT activity sodium-independent (Vilchis and Salceda, 1996). Our HRPE cell line was previously utilized by us to study the involvement of the Vitamin C transporter SVCT2 in mediating the transport of Vitamin C-conjugated prodrug of several neuroactive agents into the CNS (Manfredini et al., 2002; Dalpiaz et al., 2004, 2005a,b).

Although HRPE cells are not endothelial cells of brain microvessels, one of their main functions is to form barrier structures (the natural blood–retinal barrier) inside the eye and, therefore, many of their properties are similar to the BBB of the brain. Indeed, a previous study (Toimela et al., 2004), reported a strict similarity between rat RBE4 barrier (a model widely used for BBB *in vitro* studies) and human retinal pigment epithelial ARPE-19 barriers. Moreover, some properties, particularly useful for studies of penetration selectivity, were found more appropriate in the case for ARPE-19 cells with respect to RBE4 lines.

As a first step towards the characterization of the molecular mechanism involved in the transport of glu-dopamine, we have studied the basic characteristics of the uptake of 3-*O*-MG, a metabolically inactive glucose substrate, into HRPE cells. 3-*O*-MG specific uptake into HRPE cells was found to be rapid (Fig. 2) and hyperbolically related to its concentrations (Fig. 3).

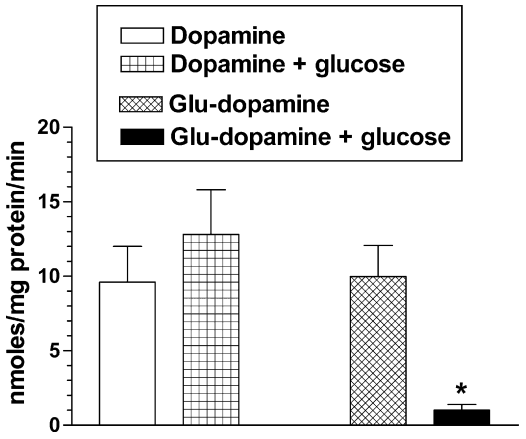

Fig. 6. Uptake into HRPE cells of 10 mM dopamine and 10 mM glu-dopamine obtained in the absence and in the presence of 10 mM glucose. Data are mean ± S.E. ($n = 4$). $^{*}p < 0.05$ vs. glu-dopamine.

The value for the affinity constant (K_t) (7.8 ± 1.2 mM) is similar to the value reported for GLUT1 expressed in the rat brain endothelial cells (Regina et al., 1997).

As a second step, we have evaluated if dopamine and its glu-dopamine prodrug were potentially able to interact with the glucose transporters expressed in HRPE cells. Towards this, we have verified the ability of the substrates to inhibit the uptake of 3-*O*-MG. While dopamine failed to interact with the glucose transporters of the cells, its prodrug showed appreciable interaction (Fig. 4). The inhibition IC_{50} value for this interaction was found to be in the mM range (2.6 ± 0.6 mM). Glu-dopamine is characterized by the amino group of dopamine linked to C-3 of the sugar trough a succinyl linker. It is interesting to note that an homologous of glu-dopamine, characterized by the amino group of dopamine linked to C-3 of glucose through a carbamate bond, appears as a weaker ligand of GLUT transporters with respect to our prodrug; in particular, their affinities differ by one order of magnitude (Fernandez et al., 2003). These data indicate that the length of the link between dopamine and the position 3 of glucose can sensibly influence the affinity of this type of prodrugs towards glucose transporters.

Finally, on the basis of affinity results, we have analysed the permeation modalities of dopamine and its conjugate into HRPE cells. When glucose was not included into the extracellular fluid, both the compounds were found able to permeate into HRPE cells (Figs. 5 and 6). On the other hand, the uptake of the prodrug was drastically reduced in the presence of glucose, whereas any significant change was detected for dopamine permeation (Figs. 5 and 6). Dopamine has several important functions in the retina of mammalians, including a possible role in controlling photoreceptor disk shedding to the RPE. A previous study provided the first report on the expression and function of the organic cation transporter, OCT3, in the cultured human ARPE-19 cells (Rajan et al., 2000). In that report it was hypothesized that the organic cation transporter OCT3 participates in the clearance of dopamine from the subretinal space. On the other hand the expression of OCT3 mRNA cells was not detected in BBB (Kang et al., 2005). The clearance process in subretinal space could explain the dopamine uptake observed by us into HRPE cells and why dopamine is not able to cross BBB.

The permeation modalities of dopamine and its conjugate indicate that the saturation of glucose transporters hinders the prodrug permeation into the cells. Accordingly, we can conclude that the conjugation of dopamine to glucose enables the prodrug to interact and to be transported by glucose transporters. Again, our results show that dopamine is not able to interact and to be transported by glucose transporters. This behaviour seems to explain why glu-dopamine, unlike dopamine, is able to be absorbed into the CNS from the bloodstream.

The above conclusions suggest that HRPE cells can be useful for *in vitro* studies characterizing molecular mechanisms involved in the transport of prodrugs obtained by conjugation with glucose or its derivatives.

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