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Pertussis toxin-sensitive modulation of glutamate transport by endothelin-1 type A receptors in glioma cells

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Abstract

Endothelin-1 (ET-1) is a 21 amino acids peptide that exerts several biological activities through interaction with specific G-protein coupled receptors. Increased ET-1 expression is frequently associated with pathological situations involving alterations in glutamate levels. In the present study, a brief exposure to ET-1 was found to increase aspartate uptake in C6 glioma cells, which endogenously express the neuronal glutamate transporter EAAC1 (pEC_{50} of 9.89). The stimulatory effect of ET-1 mediated by ET_A receptors corresponds to a 62% increase in the V_{max} with no modification of the affinity for the substrate. While protein kinase C activity is known to participate in the regulation of EAAC1, the effect of ET-1 on the glutamate uptake was found to be independent of this kinase activation. In contrast, the inactivation of $G_{o/i}$ type G-protein dependent signaling with pertussis toxin was found to impair ET-1-mediated regulation of EAAC1. An examination of the cell surface expression of EAAC1 by protein biotinylation studies or by confocal analysis of immuno-fluorescence staining demonstrated that ET-1 stimulates EAAC1 translocation to the cell surface. Hence, the disruption of the cytoskeleton with cytochalasin D prevented ET-1-stimulated aspartate uptake. Together, the data presented in the current study suggest that ET-1 participates in the acute regulation of glutamate transport in glioma cells. Considering the documented role of glutamate excitotoxicity in the development of brain tumors, endothelinergic system constitutes a putative target for the pharmacological control of glutamate transmission at the vicinity of glioma cells.

Keywords: EAAC1; Glutamate transporter; Endothelin; Pertussis toxin; Glioma cell

1. Introduction

Endothelin-1 (ET-1) is a 21 amino acids peptide, first isolated from porcine aortic endothelial cells and identified as a potent vasoconstrictor [1]. In addition, ET-1 has been shown to exert a wide spectrum of effects on non-vascular tissues including the central nervous system [2]. In the brain and the spinal cord, ET-1 is produced by the endothelium of cerebral microvessels and by glial cells [3–5]. To induce its biological responses, ET-1 acts on two distinct high-affinity receptor subtypes, namely ET_A and ET_B, belonging to the family of G-protein coupled receptors. Importantly, increased secretion of ET-1 and expression of the ET_A receptor have been documented during gliosis frequently associated with pathologies such as trauma, ischemia, or Alzheimer's disease, suggesting that the ET-1 system may play important roles in several pathological conditions [6,7]. Furthermore, ET-1 has been proposed as an autocrine or paracrine factor in the proliferation of tumor cells, including astrocytoma [8] as both ET_A and ET_B receptors have been demonstrated to be expressed in glioma cells [9,10]. Recent studies have depicted a crucial role for glutamate in the progression of brain tumors. Indeed, glutamate, which is the major mediator of excitatory transmission in the mammalian brain, paradoxically represents a potent neurotoxin when present at high extracellular concentrations. Hence, exacerbated glutamate transmission is generally considered as a

Abbreviations: EAAC1, neuronal glutamate transporter; ET-1, endothelin-1; InsP, inositol phosphates; PKC, protein kinase C; PI3-K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PTx, pertussis toxin

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key feature in several neurological pathologies [11] as this amino acid may cause irreversible excitotoxic neuronal damage. It is suggested that insufficient uptake and enhanced release by glioma cells locally promote neuronal death [12] and thereby facilitate the progression of the tumor. Indeed, disturbed glutamate handling by tumor cells probably reflects alteration in the activity and/or cell-surface expression of the predominant glial glutamate transporters GLAST and GLT-1 [13].

Several data have highlighted the existence of a physiological crosstalk between the ET-1 and the glutamatergic systems. Among these, it has been shown that ET-1 specifically induces the efflux of glutamate and decreases its uptake in primary cultured rat astrocytes [14,15] through the modulation of specific glial transporter activity. However, less is known regarding the influence of ET-1 on the uptake of glutamate achieved by astrocytic tumor cells which have been shown to paradoxically express the neuronal glutamate transporter EAAC1 (EAAT3) [16]. The activity and/or expression of this glutamate transporter subtype have been demonstrated to be acutely altered after the direct modulation of intracellular signaling pathways [17,18] or after the activation of membrane receptors [19,20].

In the present work, we provide evidence that acute exposure to ET-1 increases the activity of the EAAC1 transporter in C6 glioma cells. We also address some aspects of the intracellular mechanisms of this regulation and demonstrate that the effect of ET-1 requires an intact cytoskeleton as it involves the EAAC1 translocation at the cell surface via a pertussis toxin (PTx) sensitive type G-protein dependent process.

2. Materials and methods

2.1. Cell culture

Rat C6 glioma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 μg/ml proline, 100 U/ml penicillin and 100 μg/ml streptomycin. At confluence, cells were routinely dispersed in trypsin-EDTA and plated at a 1:10 dilution. All cultures were maintained at 37 °C in water-saturated atmosphere containing 5% CO_2. All culture media and consumables were from Invitrogen (Merelbeke, Belgium).

2.2. Measurement of [^{3}H]inositol phosphates (InsP) and mobilization of intracellular Ca^{2+}

The ability of ET-1 to mediate phospholipase C (PLC) activation was evaluated by measuring the accumulation of the [^{3}H]InsP in intact cells in the presence of 10 mM LiCl. Sample extraction and analysis of the [^{3}H]InsP fraction were performed as previously described [21]. ET-1 induced Ca^{2+} mobilization was measured on cells grown on coverslips and loaded with 5 μM Fura-2 AM for 60 min at room temperature in Krebs buffer (25 mM HEPES pH 7.4, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 6 mM Glucose and 140 mM NaCl). Rinsed coverslips were then mounted in a thermostatized (37 °C) and perfused 100 μl microscope chamber (Warner Instrument Corporation, Hamden, CT, USA). Loaded cells were excited alternatively at 340 and 380 nm (excitation lights obtained from a Xenon lamp coupled to a monochromator) and emitted fluorescence was monitored at 510 nm using a CDD camera coupled to an inverted Olympus IX70 microscope (T.I.L.L. photonics, Martinsried, Germany). Fluorescence intensities from single cell excited at the two wavelengths were recorded separately and corrected for the background using the software TILLvisION v3.3.

2.3. Measurement of Na^+-dependent transport activity

For uptake assays, cells were grown on 24-well plates. At 80% confluence, plates were placed at the surface of a 37 °C water bath, rinsed twice with preheated Krebs buffer and then treated with drugs or vehicles. For saturation studies, D-[^{3}H]-aspartate (30 nM; Amersham Pharmacia Biotech, Roosendaal, Netherlands) was diluted with unlabeled L-aspartate (Sigma, Bornem, Belgium) to achieve final aspartate concentrations of 1–200 μM. Inhibitors were added 15 min before the addition of ET-1 (Peninsula laboratories Europe LTD, Meyerside, U.K.). Unless stated, the uptake was stopped after 6 min by 3 rinses with ice-cold Na^+-free Krebs buffer in which NaCl was substituted with equi-osmolar choline chloride (120 mM). In these experimental conditions, uptake was found to be linear up to 20 min. The cells were lysed with 500 μl of 1 N NaOH and the radioactivity of 200 μl of the lysate was determined by liquid scintillation counting. A fraction of the lysate was also used for protein determination. The specific activity of the glutamate transporters (expressed as the uptake velocity per mg of protein) was estimated after subtracting the data obtained using the Na^+-free Krebs buffer. Statistical differences were determined by Student's *t* test for two-groups comparison or by one-way ANOVA followed by the Tukey's test for multiple comparisons between more than two groups.

2.4. Cellular trafficking of glutamate EAAC1 transporter

Biotinylation experiments were performed as previously described with minor modifications [20]. After rinsing with PBS containing 0.1 mM Ca^{2+} and 0.1 mM Mg^{2+}, cells were incubated with 0.8 ml of sulfo-NHS-biotin (Pierce, Perbiosceicne, Erembordegem-Aalst, Belgium) (1.5 mg/ml in PBS–Ca^{2+}/Mg^{2+}) for 30 min at 4 °C with mild shaking. Thereafter, the cells were rinsed twice with cold PBS–Ca^{2+}/Mg^{2+} containing 100 mM glycine and incubated with the same solution for 45 min at 4 °C to quench the unbound biotin reagent. Cell lysis was performed with radio-

immunoprecipitation assay (RIPA) buffer (100 mM Tris–HCl, pH 7.4, 150 mM.7 NaCl, 1mM EDTA, 1% triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with proteases inhibitors (250 μM phenylmethylsulfonyl fluoride and proteases inhibitors cocktail from Sigma, Bornem, Belgium) for 1 h at 4 °C with brisk shaking. After centrifugation at 16,000×*g* at 4 °C for 20 min, 150 μl supernatant were incubated with an equal volume of streptavidin beads (Pierce, Perbio-science, Erembordegem-Aalst, Belgium) suspension for 1 h at room temperature. After centrifugation (15 min at 16,000×*g* at 4 °C), the supernatant was collected for analysis of the non biotinylated intracellular fraction while the pelleted biotinylated cell surface proteins were washed four times with 0.8 ml of RIPA buffer and resuspended in 50 μl of Laemmli buffer containing 50 mM dithiothreitol. After a 15 min centrifugation at 16,000×*g*, the supernatants were stored at −20 °C until analyzed.

2.5. Immunoblot analysis

The samples were thawed and boiled for 5 min. Cell extracts were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli. Proteins were electroblotted to nitrocellulose membranes with a Bio-Rad minitransblot electrophoretic transfer cell. After electroblotting, the nitrocellulose membranes were treated with 10% dry milk in TBS (50 mM Tris pH 8.1, 150 mM NaCl) buffer containing Tween-20 0.05% for 1 h with a gentle shaking at room temperature and probed with an affinity-purified anti-EAAC1 (0.5 μg/ml) (Alpha Diagnostic, San Antonio, TX, USA) diluted in a blocking solution for 18 h at 4 °C. The antigen–antibody complex was visualized with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:3000) (Sigma, Bornem, Belgium) and immuno-reactive proteins were detected with enhanced chemiluminescence (ECL) reagents. Densitometric analysis of the signal detected by autoradiography was performed using an MCID-M4 imaging system (Imaging Research, Ontario, Canada).

2.6. Immunofluorescent confocal microscopy

C6 glioma cells were plated and grown on sterile glass coverslips. After treatment with or without ET-1, the cells were washed twice in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Following wash, the cells were permeabilized and blocked with 0.1% triton X-100 and 5% dry milk in TBS for 1 h at room temperature. The cells were incubated with anti-EAAC1 primary antibody (2 μg/ml) in the blocking solution 1 h at room temperature. After three washes with TBS, the cells were further incubated with a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody for 1 h at room temperature (1/500, Sigma, Bornem, Belgium). The cells were washed with blocking solution three times, 1 time with PBS and the coverslips were mounted on glass slides in Mowiol with 2.5% DABCO overnight at room temperature and examined with an Axiovert confocal microscope (Zeiss, Oberkochen, Germany) coupled to MRC 1024 confocal scanning equipment (Bio-Rad, Richmond, CA). Immunostained cells were sectioned optically at 0.5 μm intervals with a 63× oil objective and images were examined using the software Confocal Assistant.

3. Results

In accordance with the literature concerning ET-1 [4,10,22–24], preliminary studies conducted on C6 glioma cells revealed that ET-1 induces the production of InsP and triggers rapid intracellular Ca^{2+} mobilization in intact cells (Fig. 1). In cell homogenates, the peptide also increases the binding of guanylyl nucleotides to G-proteins (data not shown). Together, these results suggest a dual coupling of ET-1 receptors to $G_{q/11}$ and $G_{i/o}$ type G-proteins as already documented in other cellular models [25].

The effect of short-term activation of ET-1 receptors on EAAC1 transporter activity was evaluated by measuring the velocity of aspartate uptake. As shown in Fig. 2A, non linear analysis of the specific uptake of isotopic dilutions of D-[^{3}H]-aspartate/L-aspartate revealed that a 5 min ET-1 (100 nM) treatment resulted in a substantial increase in the uptake velocity (V_{max} values of 0.50±0.02 and 0.81±0.03 nmol/mg prot/min for control and ET-1-treated cells, respectively; n=3) with no modification of the affinity for the substrate (Km values of 7.2±1.7 μM for control and 6.7±1.5 μM for ET-1 treated cells, n=3). The effect of ET-1 on the uptake was concentration-dependent (Fig. 2B). Thus, a significant response was detected with 0.1 nM ET-1 and the maximal stimulation was observed with 100 nM ET-1. Non linear

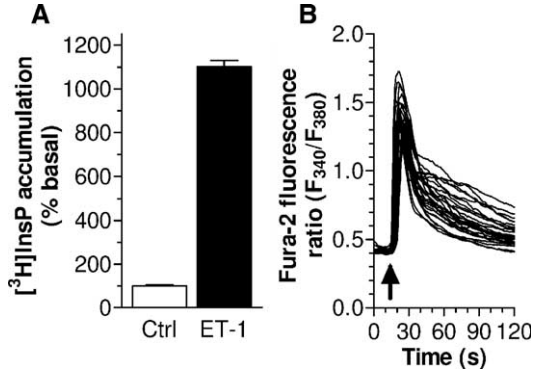

Fig. 1. Effect of ET-1 on [^{3}H]InsP accumulation and $Ca^{2+}i$ mobilization in intact C6 glioma cells. (A) ET-1 (100 nM)-induced [^{3}H]InsP accumulation in C6 cells. Data shown are mean values and S.E. from three independent experiments performed in triplicate. (B) Effect of ET-1 on $[Ca^{2+}]_i$ in single C6 cells. Data shown are representative of at least three independent experiments. Variations in $[Ca^{2+}]_i$ were monitored as changes in the Fura-2 excitation spectra and data shown correspond to the fluorescence ratio F340/F380. Each trace represents the experimental recording from a single cell. The arrow indicates the application of ET-1 (100 nM).

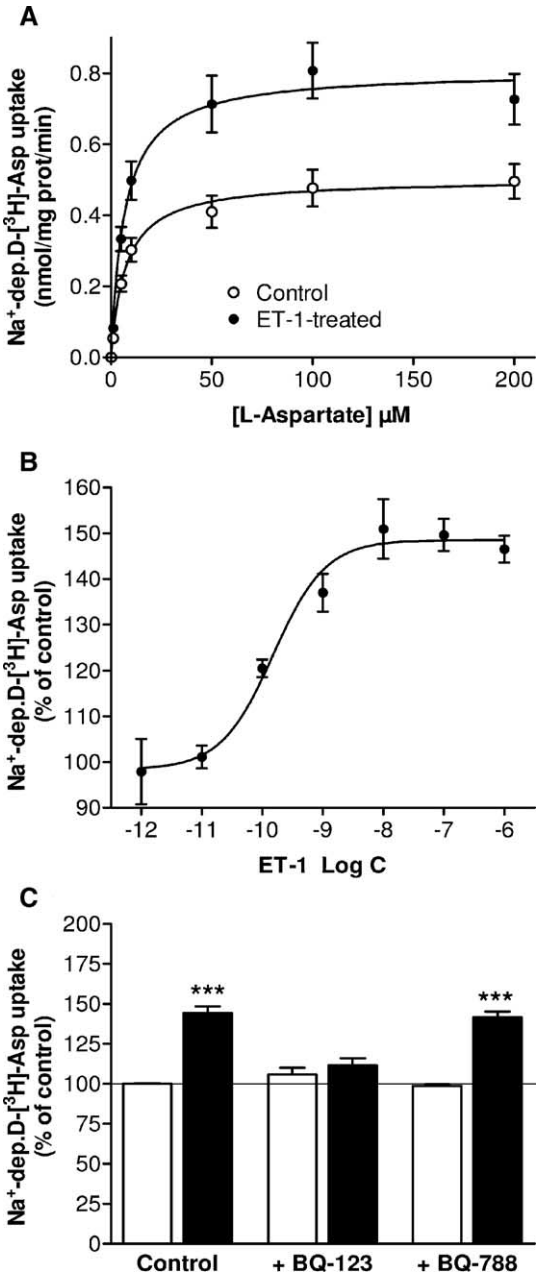

Fig. 2. Effect of ET-1 on Na^+-dependent D-[^{3}H]-aspartate uptake in C6 glioma cells. (A) Saturation curve for D-[^{3}H]-aspartate uptake measured in C6 cells treated for 5 min with 100 nM ET-1 (closed symbols) or vehicle (open symbols). Data shown correspond to mean values±S.E. of typical experiments performed 3 times in duplicate. (B) Effect of pretreating C6 cells with increasing concentrations of ET-1 for 5 min on the D-[^{3}H]-aspartate (30 nM) uptake. Data are expressed as percent of control and correspond to mean values±S.E. of 6 independent experiments performed in triplicate. (C) Effect of BQ-123 and BQ-788 on ET-1-stimulated aspartate uptake in C6 cells. Cells were pretreated with 10 µM BQ-123 or 10 µM BQ-778 for 15 min. Thereafter, 100 nM ET-1 was added and [^{3}H]-aspartate (30 nM) uptake assay was performed after 5 min. Data (open bars for non-treated and closed bars for ET-1 treated cells) are expressed as percent of non-treated cells and correspond to mean values and S.E. of 3 different experiments performed in triplicate. ***P<0.001 as compared to the corresponding control, one-way ANOVA followed by the Tukey's test for multiple comparisons.

analysis revealed a pEC_{50} value 9.89±0.17 which correlates with the nanomolar potency of ET-1 at endothelin receptors [26]. In order to determine the receptor subtype involved in the effect observed in C6 cells, we used BQ-123 and BQ-788 as selective ET_A and ET_B receptor antagonists, respectively. The effect of ET-1 was specifically blocked by BQ-123 only, indicating that ET-1 potently and efficiently increases the activity of EAAC1 in C6 cells through an ET_A receptor mediated process (Fig. 2C).

ET-1 is known to trigger the activation of the PLC with subsequent activation of protein kinase C (PKC) and mobilization of Ca^{2+} [27,28]. Because activation of PKC modulates the activity of EAAC1 in C6 cells [17], we evaluated whether the effect of ET-1 on aspartate uptake was dependent of the activation of this kinase. For this purpose we treated C6 glioma cells with ET-1 (100 nM) or PMA (500 nM) in the presence or in the absence of the PKC inhibitor Ro-31-8220 (500 nM). As shown in Fig. 3A, both PMA and ET-1 induced an increase in the specific aspartate uptake (91% and 74% increase respectively as compared to control, P<0.001) whereas Ro-31-8220 alone was without effect. While in cells pre-incubated with Ro-31-8220, PMA failed to induce an increase in aspartate uptake, this inhibitor did not prevent the ET-1-mediated response, suggesting that PKC is not the pathway required for ET-1 to modulate the EAAC1 activity in C6 glioma cells.

Besides the documented effect on PLC activity, ET_A receptors are known to activate multiple subtypes of G-proteins leading to additional signaling cascades, including the modulation of adenylyl cyclase [29]. To check whether a G_i type G-protein depending pathway is involved in the increase of EAAC1 activity observed after acute ET-1 receptors stimulation, this regulation was examined on C6 cells previously exposed to 100 ng/ml pertussis toxin (PTx). As shown in Fig. 3B, such treatment considerably prevented the modulatory effect of 100 nM ET-1 on EAAC1 activity suggesting the role of ET-1-mediated $G_{i/o}$-protein activation in this effect.

Previous studies have indicated the possible involvement of phosphatidylinositol 3-kinase (PI3-K) in the regulation of EAAC1 in C6 cells [19]. Because this enzyme is putatively activated by ET-1 [30], we also evaluated its possible implication in the modulation of aspartate uptake by ET-1. As shown in Fig. 3C, the inhibition of this PI3-K with wortmannin (100 nM) decreases aspartate uptake to 62% but when wortmannin pretreated cells were exposed to ET-1, the peptide increases the residual aspartate uptake (65% increase as compared to the respective control wortmannin treated cells). Similarly, PP2 (10 µM), an inhibitor of Src kinases, failed to prevent the increased aspartate uptake observed in ET-1 treated C6 cells (Fig. 3C). These results indicate that PI3-K and Src kinase activities are not involved in the rapid effect of ET-1 on aspartate uptake in C6 cells.

As previously reported using the model of C6 glioma cells, most of the intracellular signaling pathways mediating acute regulation of the activity of EAAC1 have been shown

to also modulate its cell surface expression. Therefore, we investigated whether subcellular redistribution of EAAC1 could explain the effect of ET-1 on aspartate uptake. As shown in Fig. 4, pretreatment of C6 cells with 30 μM cytochalasin D, an inhibitor of actin polymerization, was without effect on basal uptake but significantly impaired the stimulatory effect of ET-1. This observation indicates that the rapid modulation of aspartate uptake induced by ET-1 requires an intact cytoskeleton, suggesting that this effect could involve the trafficking of EAAC1 from an intracellular compartment to the cell surface. Therefore the effect of ET-1 on the subcellular localization of EAAC1 was examined in cell surface protein biotinylation and confocal microscopy studies. For biotinylation studies, membrane proteins of intact cells were specifically biotinylated and

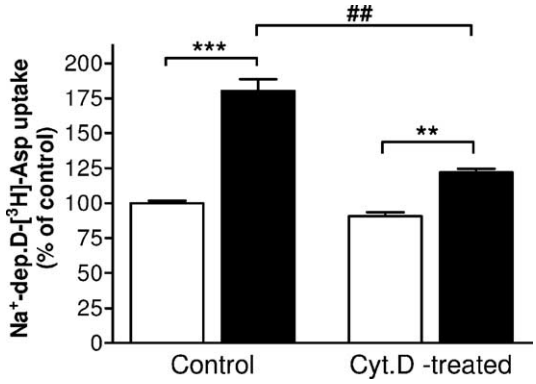

Fig. 4. Effect of cytochalasin D (30 μM) on basal (open bars) and ET-1 (closed bars)-stimulated aspartate uptake in C6 cells. Cells were pretreated with the drug for 15 min. Thereafter, ET-1 (100 nM) was added and [^{3}H]-aspartate (30 nM) uptake assay was performed after 5 min. Results (percent of control) correspond to mean values and S.E. of 2 independent experiments performed in hexaplicate. Statistical analysis was performed by one-way ANOVA followed by the Tukey's test for multiple comparisons. ** and *** denote $P<0.01$ and $P<0.001$, respectively (comparison with the corresponding control) and $^{\#\#}P<0.01$ denotes a difference between cells treated or not with cytochalasin D.

thereafter, cellular extracts were analyzed for EAAC1 immunoreactivity. As presented on Fig. 5A, the band corresponding to the biotinylated EAAC1 was identified with an apparent molecular weight of ~68 kDa. The incubation of C6 cells with 100 nM ET-1 for 5 min resulted in an increase in the intensity of EAAC1 immunoreactivity (about 75% as compared to control values) in this biotinylated fraction (cell surface proteins) that correlated

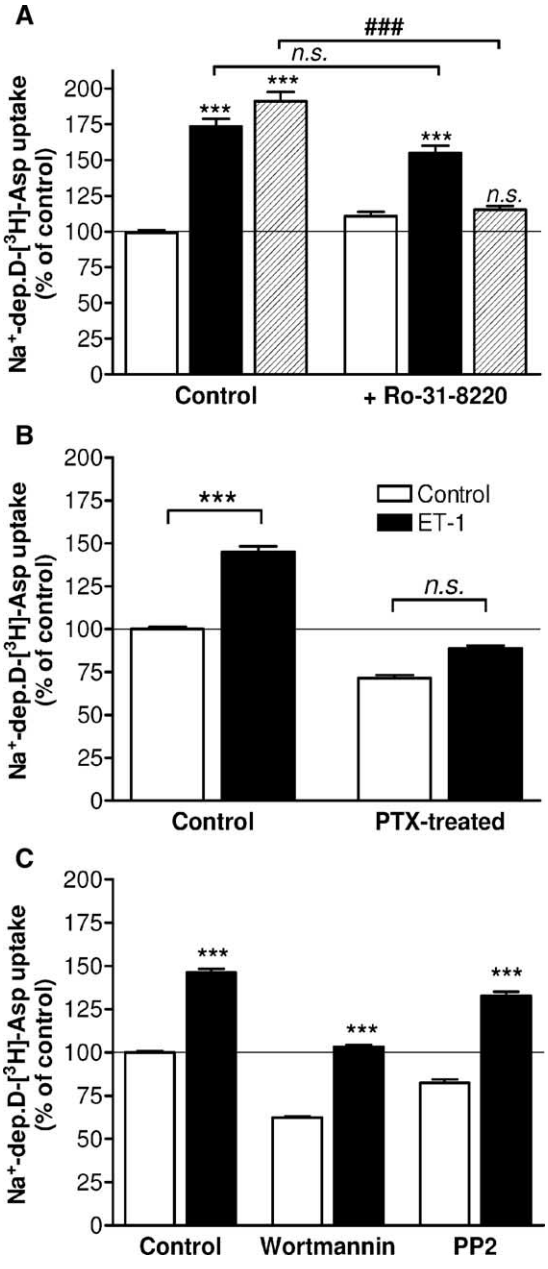

Fig. 3. (A) Role of the PKC pathway in the modulation of stimulated EAAC1 activity by ET-1 in C6 glioma cells. Cells were pretreated with 500 nM Ro-31-8220 or vehicle for 15 min. Thereafter, 100 nM ET-1 (closed bars) or 500 nM PMA (hatched bars) were added and [^{3}H]-aspartate (30 nM) uptake assay was performed 5 min later. Results are expressed as percent of uptake in untreated C6 cells (open bars) and represent mean values and S.E. of 3 independent experiments performed in triplicate. One-way ANOVA followed by Tukey's test for multiple comparisons revealed that ET-1 and PMA significantly increased D-[^{3}H]-aspartate uptake in control cells (***$P<0.001$ as compared to control). While Ro-31-8220 was effective in blocking the response to PMA ($^{\#\#\#}P<0.001$ as compared to PMA alone), it failed to inhibit the effect of ET-1 on aspartate uptake (n.s.). (B) Effect of PTx on ET-1-mediated increase in EAAC1 activity. C6 glioma cells were pretreated with PTx (100 ng/ml) for 20 h. Thereafter, ET-1 (100 nM, closed bars) was added for 5 min before the [^{3}H]-aspartate (30 nM) uptake assay. Results expressed as percent of uptake in untreated cells (open bars) are mean values and S.E. of 4 independent experiments performed in triplicate. n.s. and *** denote lack of difference or a significant difference ($P<0.001$) as compared to the corresponding control, respectively (one-way ANOVA followed by Tukey's test). (C) Effect of Wortmannin and PP2 on ET-1-induced EAAC1 activity. C6 cells were pretreated with either vehicle (control) or these inhibitors for 15 min before exposure to ET-1 (100 nM). The [^{3}H]-aspartate (30 nM) uptake assay was performed 5 min later (open bars for control and closed bars for ET-1 treated cells). Both wortmannin and PP2 had a noticeable effect on the aspartate uptake. However, in the presence of these inhibitors, ET-1 was shown to significantly induce the uptake activity, and the intensity of this induction was similar to that measured in the absence of inhibitors. *** denote significant ($P<0.001$) effect of ET-1 as compared to the corresponding control (one-way ANOVA followed by Tukey's test).

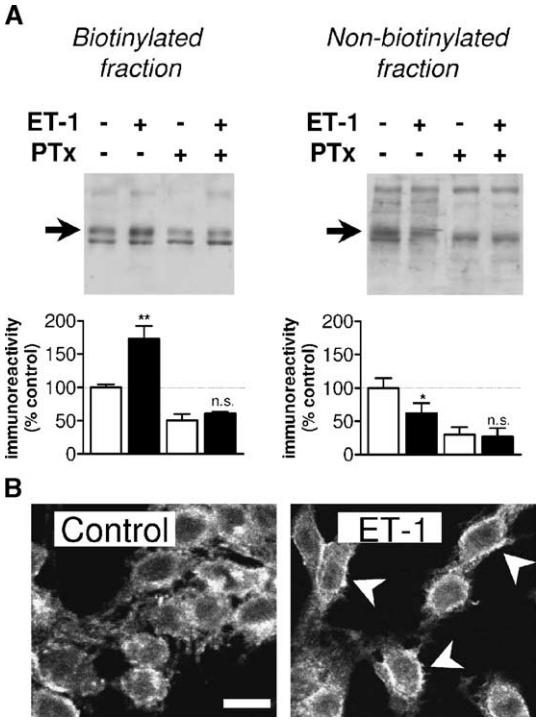

Fig. 5. Effect of ET-1 treatment on cell surface expression of EAAC1 in C6 cells. (A) Cell membrane impermeant biotinylation reagent was used to distinguish cell surface (left panels) and intracellular proteins (right panels). Cell extraction, solubilization, recovery of biotin-labelled proteins, separation by SDS-PAGE and immuno-blotting using a specific antiserum were performed as described under Materials and methods. Typical autoradiograms are shown in the upper panels (arrows indicate the band corresponding to the EAAC1 transporter) whereas lower panels show the quantification by grey-scale densitometry of the EAAC1 immunoreactivity (mean values±S.E. from three different experiments; open bars for control and closed bars for ET-1 treated cells). *P<0.05; **P<0.01; as compared to the control (one-way ANOVA followed by Tukey's test). (B) The effect of ET-1 on the cell surface distribution of EAAC1 immunostaining was analyzed by confocal microscopy. Cells were treated with 100 nM ET-1 or vehicle for 5 min, fixed and incubated with anti-EAAC1 primary antibody. Immunoreactivity was visualized using a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. Images (single optical slides through the cells) were representative of several fields examined from 3 independent experiments. Scale bar, 20 μM.

with a significant decrease (about 40% as compared to control values) in the corresponding non-biotinylated fraction (intracellular proteins) (Fig. 5A). The total expression of EAAC1 protein was not affected by the short treatment of the cells with ET-1 (data not shown). This transfer of EAAC1 immunoreactivity between biotinylated and non-biotinylated fractions was not observed when the C6 cells were exposed for 20 h to 100 ng/ml PTx, confirming that $G_{i/o}$ type G-proteins participate in the alteration of the cell surface expression of EAAC1 induced by ET-1. In parallel, confocal microscopic detection of EAAC1 immunoreactivity allowed to visualize the modulation of the cell surface expression of EAAC1 in intact C6 cells after ET-1 treatment. Labeled cells were scanned under 488 nm wavelength excitation and images were acquired as single transcellular optical sections. As shown in Fig. 5B, under control conditions, EAAC1 immunoreactivity appeared intracellularly in a perinuclear distribution with very low immunostaining at the membrane. In agreement with biotinylation studies, ET-1 treatment was found to induce an increase in the intensity of immunostaining at the periphery of the cells confirming the ET-1 mediated translocation of EAAC1.

4. Discussion

Glutamate clearance at the vicinity of responding neurons is a critical aspect of the physiological glutamate transmission. This involves high-affinity specific transporters expressed on both neuronal and glial membranes, preventing abnormal rises of extracellular glutamate concentrations to excitotoxic levels. Accordingly, alterations in the glutamate transporters activity and/or expression have been documented in many neurodegenerative diseases. Therefore, through an indirect influence on the regulation of glutamate transmission, these transporters play a critical role in the physiology and pathologies of the CNS. Hence, several studies have been focused on the mechanisms regulating the activity and expression of glutamate transporters and is now well accepted that membrane transporters are subject to physiological, pharmacological and pathological regulation processes [31].

Considering the perturbed glutamate handling by astrocytic cells undergoing neoplastic transformation [12,13] and considering the putative increased activity of ET-1 systems in brain tumors [8,9], we have examined the influence of ET-1 on the glutamate transporter subtype EAAC1. Indeed, the ET-1 receptor belongs to the family of G-protein coupled receptors, activating intracellular pathways that have been demonstrated to modulate glutamate transporters activity and/or expression. Using the model of rat glioma C6 cells, which endogenously express the glutamate transporter subtype EAAC1, we demonstrate that in vitro, ET-1 induces a rapid increase in aspartate uptake. The nanomolar potency of ET-1 measured in this study as well as the inhibition achieved with the selective antagonist BQ123 indicate that ET-1 acts through the activation of ET_A receptors, which indeed, constitute the predominant ET-1 receptor subtype expressed in C6 cells [22].

As frequently reported, the effect of ET-1 was shown to involve increased maximal transport velocity, without modification in the affinity for the substrate, which is compatible with an increased availability of active transporter molecules at the surface of the cells. As expected, using two complementary approaches aimed at localizing the EAAC1 immunoreactivity—cell surface protein biotinylation studies and confocal microscopy—we demonstrated that ET-1 mediates the translocation of transporter molecules to the cell surface of the C6 cells. Accordingly, this effect was prevented after pharmacological disruption of cytoskeleton organization using cytochalasin D.

In view of the coupling of ET_A receptors with $G_{q/11}$ type G-proteins mediating PLC activation and since PKC is a well established modulator of EAAC1 activity [32], including in C6 cells, one would suggest that ET-1 mediates its influence through this conventional signaling cascade. However, the use of a specific inhibitor of PKC clearly revealed that this enzyme is not a key element in the functional crosstalk between ET_A receptors and EAAC1. This observation is consistent with previous studies revealing that other signaling pathways could participate in the regulation of EAAC1 (for review, see [33]). Besides the activation of PLC, which putatively leads to the stimulation of PKC, it is also demonstrated that ET_A receptors modulate the cyclic AMP pathway through functional coupling with $G_{i/o}$-type G-proteins [29]. The possible involvement of this alternate coupling in the modulation of EAAC1 activity and cell distribution by ET_A receptors was examined after the inactivation of the corresponding G-proteins with PTx. Both ET-1-induced increase in substrate uptake and translocation of EAAC1 transporters to the cell surface were considerably impaired in PTx-treated cells, suggesting that the effect of ET-1 is $G_{i/o}$-type G-protein mediated. Nevertheless, neither PI3-K nor Src kinase, downstream effectors of the $G_{i/o}$-type G-proteins pathways, appeared to be involved since none of the inhibitors tested influenced the regulation of EAAC1 by ET-1. Similarly, while the involvement of Gi-type G-proteins has been recently demonstrated in the regulation of the glucose transporter GLUT4, the signaling cascade remains obscure [32]. In the same model of C6 glioma cells, we have recently reported the involvement of G-proteins in the regulation of EAAC1 transporter activity and cell surface distribution after the activation of the G-protein coupled neurotensin receptors whose expression was obtained after cell transfection [20]. These previous data had highlighted the possible involvement of the early intracellular cascades of G-protein coupled receptors in the acute regulation of EAAC1 transporter expression. The study of the regulation observed after the activation of the endogenous ET-1 receptor confirms this hypothesis and suggests a possible common pathway for both neurotensin and ET-1 and probably other membrane receptors regulating the EAAC1 transporter via PKC independent pathways [34]. It is noteworthy that agonists of G-protein coupled receptors have been shown to directly influence the reorganization of the cytoskeleton through G-proteins mediated processes [35]. Similarly, activated G-proteins could directly participate in the acute regulation of the EAAC1.

While further studies are required to elucidate the molecular mechanism that contributes to the functional crosstalk between ET_A receptors and the EAAC1 transporter, the present results indicate that besides a possible modulation of glutamate release, ET-1 may also influence glutamate transmission through the acute regulation of its specific uptake. At variation with the present study focused on the EAAC1 transporter, ET-1 was previously found to decrease the activity of the glial GLAST transporter in primary cultured astrocytes [14]. While EAAC1 weakly contributes to glutamate uptake in normal astrocytes, it constitutes the predominant glutamate transporter in C6 cells as well in other brain tumors where the activity of other transporters is dramatically decreased. Therefore our data suggest that ET-1 differentially affects the activity/expression of the glutamate transporters subtypes. Such difference could be of importance, taking into account the altered expression profile of these glutamate transporters in glial tumors [16]. Many glioma cells express ET-1 and its receptors, suggesting that this peptide could act as an autocrine or paracrine modulator of tumor growth and tumoral angiogenesis. Indeed, ET-1 has been shown to induce the expression of several growth factors such as PDGF [36]. In C6 glioma cells, PDGF has been demonstrated to increase the activity and cell surface translocation of the EAAC1 transporter [19], suggesting a possible indirect pathway for the transmission of ET-1 signals to the glutamate system. Finally, the EAAC1 transporter subtype has been proposed to play a metabolic role [37], providing glutamate as a precursor of several other molecules as for instance, glutamine, which plays an important role in cell proliferation. Therefore, in this context of glial tumors, an increased EAAC1 transporter activity and cell surface translocation by ET-1 could participate in an adaptative response to support the unlimited growth of these cells.

In conclusion, the present study demonstrates that ET-1 increases aspartate uptake in C6 glioma cells via an increase in EAAC1 cell surface translocation. This acute regulation seems to imply PTx-sensitive type G-proteins pathway and an intact cytoskeleton. Moreover, these data may also shed new light on the regulatory crosstalk between G-protein coupled receptors and glutamate uptake systems in the context of metabolism and/or cell signaling in the central nervous system of mammalians.

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