Characteristics and regulation of proline transport in cultured glioblastoma cells

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L-Proline transport in C6 glioblastoma cells takes place mainly via a saturable Na⁺-dependent mechanism. The uptake process can be discriminated into two components, system A and system ASC. A minor proportion of L-proline transport is carried out by the ASC system, which appears to be constitutively expressed by the cell, but most is by system A which shows adaptive responses to amino acid deprivation and sensitivity to *N*-methyl- α -aminoisobutyric acid. The transport system is inhibited by proline derivatives, such as methyl and benzyl esters, and also hydroxy-

proline, and is stereospecific. Incubation of glioblastoma cells with phorbol 12-myristate 13-acetate led to concentration- and time-dependent decreases in L-proline transport. This effect could be mimicked by exogenous phospholipase C. Proline transport is significantly stimulated in the presence of Ca²⁺-mobilization agents and strongly inhibited in the absence of Ca²⁺. The present data suggest a complex regulation of L-proline transport by different kinases in glioblastoma cells.

INTRODUCTION

L-Proline has for many years been known to exert a variety of electrophysiological actions, both excitatory and inhibitory, when applied to different areas of the mammalian central nervous system depending on the type of neuron examined (Curtis and Johnston, 1974; Zarzecki et al., 1975; Felix and Kunzle, 1974; Nistri and Morelli, 1978; Van Harreveld and Strumwasser, 1981; Nadler, 1987; Henzi et al., 1992). Proline can be synthesized from ornithine in the nerve terminals, and it has been reported that exogenously loaded radiolabelled proline can be released in a Ca²⁺-dependent manner from neocortical slices and synaptosomes (Bennet et al., 1974; Balcar et al., 1976; Nickolson, 1982). These data, among others, suggest that proline is a putative neurotransmitter or neuromodulator in the central nervous system.

The uptake of neutral amino acids such as proline by animal cells involves several transport systems with overlapping substrate reactivities (Serksen and Lathja, 1979; Christensen, 1979; Guidotti et al., 1978). Largely on the basis of the ionic-dependence (Serksen and Lathja, 1979) and the pattern of competitive interactions between neutral amino acids, the existence of two different transport systems for proline in various animal cell types has been proposed: a Na⁺-dependent system which is specific for proline and inhibited by proline analogues, and system A, which is effective for neutral amino acids, such as alanine, glycine and proline, and the non-metabolizable analogue 2-aminoisobutyric acid and its *N*-methylated derivative (MeAIB). Transport by system A is Na⁺-dependent and shows adaptive responses to amino acid deprivation (Shotwell and Oxender, 1983). A third Na⁺-dependent transport system for neutral amino acids has also been characterized in animal cells. This system, which is able to transport alanine, serine and cysteine, is designated system ASC. Recently, two cDNAs that encode the A and ASC transport systems respectively have been cloned and expressed (Arriza et al., 1993; Kong et al., 1993).

Na⁺-dependent uptake systems for proline and other putative

neurotransmitters have been described in synaptosomes (membrane vesicles derived from the plasma membrane of cultured cells) (Bennet et al., 1972; Peterson and Raghupathy, 1972; Kanner and Sharon, 1980; Rhoads et al., 1982; Hauptman et al., 1983; Nadler, 1987). More recently, the molecular cloning and expression of a high-affinity L-proline transporter from rat brain has been reported (Fremau et al., 1992). Na⁺-dependent proline uptake has been shown to take place mainly in the hippocampal formation and is associated with putative glutamatergic pathways (Fremau et al., 1992).

Several lines of evidence suggest that glial cells of the central nervous system play an important role in the modulation of neural excitability via control of the levels of neuroactive substances in the extracellular milieu of neurons (Henn and Hamberger, 1971; Henn et al., 1974; Schrier and Thompson, 1974; Henn, 1976; Hertz, 1979; Schousboe, 1981). Although the transport of proline has been fairly well characterized in nerve terminals, the possible role of glial cells in this process is not understood. Moreover, there is currently very little information available about the regulatory possibilities of the Na⁺-dependent neurotransmitter transporters by hormones or second messengers.

In the present paper we describe the characteristics of proline uptake by C6 cells, a cell line of glial origin, and its regulation by amino acid availability and second-messenger systems.

EXPERIMENTAL

Materials

L-[5-³H]Proline (17 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were obtained from Gibco, Paisley, Scotland, U.K. Dishes for tissue culture were purchased from Costar, Cambridge, MA, U.S.A. BSA and phospholipase C from *Bacillus cereus* were from Boehringer, Mannheim, Germany. Phorbol 12-myristate 13-acetate (PMA), 4 α -phorbol 12,13-didecanoate (PDD), *N*-(6-aminohexyl)-5-

Abbreviations used: MeAIB, *N*-methyl-2-aminoisobutyric acid; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol 12-myristate 13-acetate; PDD, 4 α -phorbol 12,13-didecanoate; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide.

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chloro-1-naphthalenesulphonamide (W7) and the calcium ionophore A23187 were purchased from Sigma, St. Louis, MO, U.S.A. All other reagents were of the highest purity available.

Cell culture

C6 glioma cells, cloned originally from a rat glioma (Benda et al., 1968) and obtained from The American Type Culture Collection (Rockville, MD, U.S.A.), were maintained in monolayer culture in DMEM containing 10% (v/v) horse serum and 2.5% (v/v) fetal calf serum in 100 mm-diameter plastic dishes. They were maintained at 37 °C in humidified air with 5% CO₂. Cells were subcultured twice weekly by standard procedures. The methods for harvesting and subculturing have been described previously (Volpe and Marasa, 1975). For proline-uptake experiments, cells were grown in 24-well tissue-culture plates to confluency.

Transport assays

Assays were performed at 37 °C in Hepes-buffered saline (150 mM NaCl, 10 mM Hepes, 1 mM CaCl₂, 10 mM glucose, 5 mM KCl, 1 mM MgCl₂, pH 7.4). All solutions used in the uptake experiments were prepared with distilled deionized water and filtered through Millipore filters (0.45 µm pore size) to avoid bacterial contamination. The osmolarity of all solutions was kept constant during the uptake experiments. Growth medium was removed by aspiration and after washing with Hepes-buffered saline medium, uptake was started by adding 300 µl of Hepes-buffered saline medium containing radioactive proline (0.1 µCi). Cells were incubated for the indicated time and the experiments were terminated by washing the cells with 3 × 500 µl of ice-cold PBS medium (0.9 mM CaCl₂, 2.68 mM KCl, 1.47 mM KH₂PO₄, 0.49 mM MgCl₂, 136.89 mM NaCl, 7.37 mM Na₂HPO₄, pH 7.4). Cells were solubilized with 200 µl of 0.1 M NaOH. Samples (125 µl) were placed directly in microvials and their radioactivity was measured in a liquid-scintillation counter (LKB 1219 Rackbeta). All incubations were carried out in triplicate. Each experiment was repeated at least three times with different cell cultures. Statistical differences were estimated by comparing the data using Student's *t* test; differences at the 0.05 level were considered statistically significant.

Protein determination

Proteins were determined by the method of Bradford (1976).

RESULTS

Figure 1 shows the time course of L-proline uptake in C6 cells. It was almost completely Na⁺-dependent and increased linearly with time up to approx. 10 min. This uptake decreased to very low levels when Na⁺ in the external medium was iso-osmotically replaced by Li⁺.

Using a wide range of concentrations of proline (0.1 µM–3 mM), a saturable system for proline accumulation by C6 cells could be observed. Saturation kinetics were even clearer when values were corrected for the rates observed in the absence of Na⁺ (Figure 2a). This Na⁺-independent component is non-saturable, and probably represents substrate diffusion. The experiments were performed by measuring the initial rates of proline transport corrected for non-saturable diffusion. Analysis of the kinetic data for this Na⁺-dependent uptake system by the Eadie–Hofstee method (Figure 2b) suggested the existence of a unique transport system for proline in C6 glioma cells with a *K_m* of 1.1 mM and a *V_{max}* of 67 nmol/5 min per mg of protein.

Figure 1 Time course of L-proline uptake in glioblastoma cells

C6 cells grown to confluence were incubated at 37 °C in the presence of 0.2 µM L-[5-³H]proline in the following media: Hepes-buffered saline, control (with 150 mM NaCl) (●) and Hepes-buffered saline in which NaCl had been iso-osmotically replaced by LiCl (○). Values represent means ± S.E.M. for three duplicate experiments.

Figure 2 Kinetics of L-proline uptake in glioblastoma cells

(a) C6 cells were incubated for 5 min in the presence of labelled L-proline at concentrations ranging from 0.1 µM to 3 mM in Hepes-buffered saline (with 150 mM NaCl) (●) or Hepes-buffered saline in which NaCl had been iso-osmotically replaced by LiCl (○). ■, Difference between the uptake in the presence and absence of Na⁺. (b) Kinetic parameters were calculated by using Eadie–Hofstee plots.

Table 1 Pharmacological specificity of proline uptake in glioblastoma cells

C6 glioblastoma cells were incubated for 5 min in Hepes-buffered saline containing 200 μ M [3 H]proline and inhibitors (10 mM). Results were corrected for diffusion. Values are means $\pm$ S.E.M. for three replicate determinations. Values in parentheses represent percentage of control values in the absence of inhibitors. Values were compared with control values by Student's *t* test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Addition	Uptake of proline (nmol/5 min per mg of protein)
None	10.9 $\pm$ 0.4 (100)
L-Proline	1.0 $\pm$ 0.2*** (9)
L-Alanine	0.5 $\pm$ 0.1*** (0.5)
L-Serine	0.7 $\pm$ 0.1*** (0.7)
Glycine	3.4 $\pm$ 0.3*** (31)
L-Cysteine	0.5 $\pm$ 0.1*** (0.5)
D-Proline	9.8 $\pm$ 0.5 (90)
MeAIB	2.7 $\pm$ 0.1*** (25)
Proline methyl ester	4.1 $\pm$ 0.1** (38)
Proline benzyl ester	1.9 $\pm$ 0.2*** (17)
<i>cis</i> -Hydroxyproline	5.3 $\pm$ 0.3* (49)
<i>trans</i> -Hydroxyproline	3.3 $\pm$ 0.2** (30)
3,4-Dehydroproline	4.6 $\pm$ 0.2** (42)
Prolinol	12.0 $\pm$ 0.6 (110)

Table 2 Effect of amino acid deprivation on proline uptake in glioblastoma cells

C6 glioblastoma cells were grown to confluence and then incubated for 12 h in DMEM (non-deprived cells), in amino acid-free DMEM (deprived cells) or in amino acid-free DMEM except for the amino acid indicated in parentheses. Transport activity was measured for 5 min in Hepes-buffered saline containing 200 μ M [3 H]proline and in the presence or absence of 10 mM MeAIB. Results were corrected for diffusion. Values are means $\pm$ S.E.M. for three replicate determinations. Values in parentheses represent percentage of control values (non-deprived cells in the absence of MeAIB). Values were compared with control values by Student's *t* test: ***P* < 0.01; ****P* < 0.001.

Culture conditions	Addition	Proline uptake (nmol/5 min per mg of protein)
Non-deprived	None	11.4 $\pm$ 0.5 (100)
	MeAIB	3.9 $\pm$ 0.3*** (34)
Deprived	None	82.7 $\pm$ 5.9*** (725)
	MeAIB	5.3 $\pm$ 0.1** (46)
Deprived (+ Ala)	None	12.3 $\pm$ 0.4 (108)
Deprived (+ Pro)	None	13.0 $\pm$ 0.4 (114)
Deprived (+ Cys)	None	11.3 $\pm$ 0.5 (99)

The possible inhibition of proline uptake by various amino acids and derivatives was investigated (Table 1). Na⁺-dependent proline transport in C6 cells was completely abolished in the presence of serine, alanine and cysteine and it was also inhibited, although to a lesser extent, by MeAIB and glycine, suggesting that L-proline is mainly transported by system A. Proline derivatives, such as methyl and benzyl esters, as well as hydroxyproline also inhibited its transport. D-Proline had no effect on L-proline transport.

One characteristic that seems to be specifically different between systems A and ASC is the adaptive response of system A to amino acid deprivation in the culture medium. The experiment presented in Table 2, with cells maintained for 12 h in modified DMEM (without amino acids), shows the effect of amino acid deprivation on the Na⁺-dependent proline-transport system. Amino acid deprivation markedly enhanced the uptake

Figure 3 Effects of PMA on Na⁺-dependent L-proline uptake in glioblastoma cells

C6 cells were incubated in Hepes-buffered saline at 37 °C in the presence of 100 nM PMA. Transport activity was measured for 10 min in the same medium containing 0.2 μ M labelled L-proline. Values represent means $\pm$ S.E.M. for three duplicate experiments.

of proline (approx. 10-fold). The induced component was inhibited when the transport assay was carried out in the presence of MeAIB, which specifically suppresses the operation of system A. The component of transport that was not inhibited by MeAIB scarcely changed on amino acid deprivation. Furthermore, as the uptake of proline in the absence of Na⁺ is negligible (thus ruling out any substantial contribution of Na⁺-independent systems to its total uptake), the Na⁺-dependent fraction that is not inhibited by MeAIB is likely to reflect the activity of system ASC. This is further reinforced by the fact that the strongest inhibition of L-proline transport was observed in the presence of alanine, serine or cysteine. The activity of system A in the controls (normal culture medium) can be estimated by the difference between total and MeAIB-non-inhibitable uptake, and represented more than 75% of total uptake. The deprivation-enhanced activity of the low-affinity transport of proline (system A) was prevented by amino acid repletion of one of the amino acid substrates of system A (namely alanine, proline or cysteine).

After characterizing the L-proline transporter in glioblastoma cells, we investigated the regulation of the transport. We have previously described short-term regulation of γ -aminobutyric acid and glutamate transporters by phorbol esters acting via protein kinase C (PKC) (Casado et al., 1991; Gomeza et al., 1991). More recently we have also shown long-term regulation of the γ -aminobutyric acid transporter by cyclic AMP (Gomeza et al., 1994). These data, together with the fact that the recently cloned system A transporter has several consensus sequences for protein kinase-mediated phosphorylation, led us to investigate the possible regulation of proline uptake via PKC or Ca²⁺/calmodulin activation.

The effect of the phorbol ester PMA on Na⁺-dependent proline uptake in C6 glioma cells is shown in Figure 3. Incubation of cells with PMA decreased proline uptake in a time-dependent manner up to approx. 1 h. Exposure for a longer time did not result in any further significant effect.

A dose-response relationship between PMA and Na⁺-dependent proline transport in glioma cells was demonstrated (Figure 4), uptake decreasing with PMA concentration, reaching a plateau at approx. 0.1 μ M. Incubation of cells with PDD, a permeant inactive phorbol ester, did not influence proline uptake (10.60 $\pm$ 0.3 nmol/5 min per mg of protein in the controls compared with 10.45 $\pm$ 0.4 nmol/5 min per mg of protein in the presence of 100 μ M PDD).

Figure 4 Effects of increasing concentrations of PMA on Na⁺-dependent L-proline uptake in glioblastoma cells

C6 cells were incubated for 45 min at 37 °C with PMA at the indicated concentrations. Transport activity was measured in the presence of 0.2 μM L-[5-³H]proline. Values represent means ± S.E.M. for three duplicate experiments.

Table 3 Effect of PMA on A and ASC components of L-proline transport

C6 glioblastoma cells were incubated at 37 °C in the presence or absence of 100 nM PMA. Transport was measured for 10 min in HEPES-buffered saline containing 0.2 μM [³H]proline in the absence or presence of 10 mM MeAIB. Values in parentheses represent percentage of control values in the absence of PMA. Values were compared with control values by Student's *t* test: **P* < 0.05; ***P* < 0.01.

Addition	Proline uptake (pmol/10 min per mg of protein)
None	31.2 ± 0.4 (100)
PMA	16.1 ± 0.3** (52)
MeAIB	9.9 ± 0.2 (100)
MeAIB + PMA	7.5 ± 0.1* (76)

Figure 5 Effect of increasing concentration of exogenous phospholipase C (*Bacillus cereus*) on Na⁺-dependent L-proline uptake in glioblastoma cells

C6 cells were incubated for 30 min with the indicated concentrations of phospholipase C. Transport activity was measured in the presence of 0.2 μM labelled L-proline for 10 min. Values represent means ± S.E.M. for three duplicate experiments.

In order to clarify whether the observed effect of PMA is exerted through the A or the ASC component of proline transport, we studied the effect of PMA in the presence of

Figure 6 Time course of the effect of A23187 on L-proline uptake in glioblastoma cells

C6 cells were incubated with 0.2 μM A23187. Transport activity was measured in the presence of 0.2 μM labelled L-proline for 5 min. Values represent means ± S.E.M. for three duplicate experiments.

Table 4 Effect of Ca²⁺ on L-proline transport

C6 cells were incubated for 30 min in the presence or absence of 0.2 μM A23187 in HEPES-buffered saline (HBS medium), a free-Ca²⁺ medium, or a free-Ca²⁺ medium containing (3 mM) EGTA (EGTA medium). When indicated, 10 mM MeAIB was added. When W7 was used, it was added 15 min before the transport assay. Treatments were maintained during the transport assay. Transport activity was measured in the presence of 0.2 μM [³H]proline for 15 min. Values represent means ± S.E.M. of three duplicate experiments. Values in parentheses represent percentage of control values. Values were compared with control values by Student's *t* test: ***P* < 0.01; ****P* < 0.001.

Conditions	Uptake of proline (pmol/15 min per mg of protein)
HBS medium	29.7 ± 0.3 (100)
HBS medium + A23187	40.7 ± 0.4** (137)
Ca ²⁺ -free medium	17.8 ± 0.3** (60)
Ca ²⁺ -free medium + A23187	17.2 ± 0.2** (58)
EGTA medium	11.6 ± 0.4*** (39)
EGTA medium + A23187	11.6 ± 0.2*** (39)
HBS medium + W7	27.0 ± 0.3 (91)
HBS medium + W7 + A23187	26.7 ± 0.2 (90)
HBS medium + MeAIB	9.2 ± 0.2 (100)
EGTA medium + MeAIB	5.4 ± 0.2** (58)

MeAIB. Results in Table 3 show that, whereas a 48% inhibition by PMA is observed in the absence of MeAIB, a 24% inhibition is achieved in its presence.

Exogenous phospholipase C exerted a similar inhibitory effect on proline uptake, in a dose-dependent manner, to that observed in the presence of PMA (Figure 5). Phospholipase C at the concentrations used did not alter the viability of the cells, as determined by Trypan Blue exclusion.

The possibility of regulation by Ca²⁺ was investigated by increasing the intracellular Ca²⁺ level with the Ca²⁺ ionophore A23187. Figure 6 shows a time course of the effect of 1 μM A23187 on proline transport. This ionophore produces a consistent stimulation of the initial rate of proline transport. This effect was maximum (approx. 30% stimulation) after 30 min of preincubation but was already significant after 5 min. Moreover, the importance of Ca²⁺ as an activator of proline transport is supported by the experiment shown in Table 4. The initial rate of proline uptake was reduced to 60% in medium that was

nominaly Ca^{2+} -free and to 39% of the control values in a Ca^{2+} -free EGTA-containing medium. No stimulation by A23187 was observed in the presence of Ca^{2+} -free medium. Similar results were obtained with ionomycin (1 μM), another Ca^{2+} ionophore (results not shown).

The possible participation of Ca^{2+} /calmodulin-dependent protein kinase in the activation of proline transporter was studied using the specific calmodulin antagonist W7 (Hidaka et al., 1991). Preincubation for 15 min with 30 μM W7 before ionophore addition (W7 was also maintained after ionophore addition), prevented the stimulatory effect of ionophore observed in the control cells (Table 4). The effect of Ca^{2+} on the ASC component of proline transport was studied in the presence of MeAIB; the absence of Ca^{2+} produces a 42% inhibition in the ASC component.

DISCUSSION

The present results clearly show that L-proline is actively accumulated by C6 glioma cells. The ionic-dependence and kinetic characteristics of the proline-transport system in glioma cells are also established. We have found a saturable transport system in addition to a non-saturable diffusion component. In contrast with previous kinetic studies of proline uptake in rat brain synaptosomes demonstrating the existence of two components of transport, one with high affinity and one with low affinity (Hauptman et al., 1983; Nadler, 1987), we have found a unique transport system for L-proline in glioma cells. This uptake mechanism shows mixed characteristics with respect to previously described proline-transport systems (Balcar et al., 1976; Kanner and Sharon, 1980), i.e. a saturable system which is strictly dependent on the presence of Na^+ in the external medium.

The transport of proline in glioma cells can be discriminated into two components, system A and system ASC, by using general criteria given by Christensen (1984) to differentiate between transport systems for amino acids in intact cells. On the basis of sensitivity to inhibition by MeAIB and substrate specificity, it can be demonstrated that a minor proportion of proline transport in glioma cells is carried out by the ASC system, which appears to be constitutively expressed by the cell (Shotwell et al., 1983; Zafra and Giménez, 1989).

The adaptive response of the Na^+ -dependent transport of proline to amino acid deprivation was identified with system A, via which most proline is transported, as demonstrated by ionic-dependence and inhibition analysis. The blockade of the deprivation-induced increase in proline transport by amino acid repletion is mediated by system A-reactive substrates cysteine and alanine. The regulation of system A transport activity by amino acid supply has been described in several cell types including glioma cells (Heaton and Gelehrter, 1977; Guidotti et al., 1978; Kelley and Potter, 1979; Shotwell et al., 1983; Schenerman and Kilberg, 1986; Zafra and Giménez, 1989). This phenomenon requires synthesis of both RNA and protein, and is regulated by a repression/derepression mechanism acting at the transcriptional level (Gazzola et al., 1981; Quian et al., 1991).

The results included here demonstrate that proline transport in glioblastoma cells can also be regulated by second-messenger systems. Na^+ -dependent proline transport is inhibited in the presence of the phorbol ester PMA, a tumour-promoting agent known to stimulate PKC (Nishizuka, 1986). Although PMA affects both A and ASC components of proline transport, it has a more pronounced effect on system A. PDD, an inactive phorbol ester, had no effect on the transport process. Exogenous phospholipase C mimics the action of phorbol esters. It has been

reported that phospholipase C generates diacylglycerol from membrane phospholipids when added exogenously to a variety of cell types (Blackmore et al., 1986; Farese et al., 1988). This would result in an increase in intracellular free Ca^{2+} and activation of PKC via the second messenger inositol trisphosphate and diacylglycerol respectively.

On the other hand, the fact that proline transport is significantly stimulated by the Ca^{2+} ionophore A23187 and strongly inhibited in the absence of Ca^{2+} suggests that Ca^{2+} plays an important role in the regulation of L-proline transport. Moreover, we have found that the Ca^{2+} -dependent stimulation of proline transport is prevented by the Ca^{2+} /calmodulin kinase inhibitor W7.

Taken together, these data suggest that a complex regulation of L-proline transport by different kinases exists in glioblastoma cells. The exact mechanism of proline-transport regulation by PKC or Ca^{2+} /calmodulin kinases remains to be elucidated. Although direct phosphorylation by PKC has been demonstrated in a glial glutamate transporter (Casado et al., 1993), the observed effect could be indirectly mediated by changing the activity of ion channels or the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ function which could alter ion concentration both inside and outside of the cell (Clark and Amara, 1993).

This work was supported by grants from the Spanish DGICYT (PB92-0131), Boehringer Ingelheim España, S.A. and an institutional grant from the Fundación Ramón Areces.

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Received 13 January 1994/12 April 1994; accepted 25 April 1994