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Regulation by phorbol esters of the glycine transporter (GLYT1) in glioblastoma cells

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Abstract

The high-affinity glycine transporter in neurons and glial cells is the primary means of inactivating synaptic glycine. The effects of 12-*O*-tetradecanoylphorbol ester (TPA), a potent activator of protein kinase C (PKC), on the high-affinity Na^+-dependent glycine transport were investigated in C6 cells, a cell line of glial origin. Incubation of C6 cells with TPA led to concentration- and time-dependent decrease in the glycine transport that could be completely suppressed by the addition of the PKC inhibitor staurosporine. The TPA effect could be mimicked by oleoylacetylglycerol and exogenous phospholipase C. Northern and Western blot analysis indicate that C6 cells express the GLYT1 glycine transporter. Incubation of COS cells transiently transfected with a full-length clone of the GLYT1 transporter in the presence of TPA, produces a decrease in glycine uptake.

Keywords: Glycine uptake; Phorbol ester; Glioblastoma cell

1. Introduction

Glycine is known to have two roles in neurotransmission. In the spinal cord and brain stem glycine is an important inhibitory neurotransmitter that opens Cl^- channels in the postsynaptic cell, so keeping the membrane potential at a hyperpolarized state. In addition, glycine could potentiate the action of glutamate, the main excitatory neurotransmitter in the brain, on postsynaptic *N*-methyl-D-aspartate (NMDA) receptors.

It is generally well accepted that glial cells of the central nervous system play an important role in the modulation of neuronal excitability via control of the levels of neuroactive substances in the extracellular millieu of neurons [1–5]. The reuptake of neurotransmitter amino acids into presynaptic nerve endings or the neighbouring glial elements provides one way of clearing the extracellular space of potential neuroactive substances, and so constitutes an efficient mechanism by which postsynaptic action can be terminated. Specific high-affinity transport systems have been identified in glia for several amino acid neurotransmitters including glycine [6,7].

Within the past few years, some of the neurotransmitter transporters have been purified from mammalian brain [8–10], and more recently, cDNA clones encoding transporters for GABA, catecholamines, serotonin, glycine, glutamate and proline have been isolated [11–19]. After cDNA cloning techniques have been applied to the study of these proteins, transporters can now be classified into families and subfamilies based on topology and sequence relatedness [20–22]. To date, two different glycine transporters have been cloned. A glycine transporter (GLYT1) [18,20,23] which seems to colocalize mainly with NMDA receptors, and presents two isoforms produced by alternative splicing or alternative promoter usage [24]. More recently, a second glycine transporter (GLYT2) [25], which is present specifically in the brain stem and spinal cord, brain areas where glycine receptors are more represented, has been cloned.

Despite the rapid progress in this field, there is currently very little information available about the regulatory possibilities of these proteins by hormones or second messengers. Recently, data by our group have demonstrated the regulation of GABA and glutamate transport through the activation of second messengers systems [26–

Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PDD, 4α-phorbol 12,13-didecanoate; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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29]. These data, together with the presence of potential phosphorylation sites in the already cloned glycine carriers, led to the idea that intracellular signalling mechanisms and protein kinases may be involved in the regulation of glycine transporters in neurons or glial cells.

In the present report we describe the effect of phorbol esters on the glycine transport activity in glioma cells and on the expressed GLYT1 transporter cDNA.

2. Materials and methods

2.1. Materials

[2-^{3}H]Glycine (1.76 PBq/mol) was obtained from the Du Pont-New England Nuclear, Boston, MA, USA. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were obtained from Gibco, Paisley, UK. Dishes for tissue culture were purchased from Costar, Cambridge, MA. USA. BSA and phospholipase C from *Bacillus cereus* were from Boehringer, Manheim, Germany. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), 4α-phorbol 12,13-didecanoate (PDD), and staurosporine were purchased from Sigma, St. Louis, MO, USA. All other reagents were of the highest purity available.

2.2. Cell culture

C6 glioma cells, cloned originally from a rat glioma [30] and obtained from The American Type Culture Collection (Rockville, MD, USA), 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% CO_2. Cells were subcultured twice a week by standard procedures. The methods for harvesting and subculturing have been described previously [31]. For glycine-uptake experiments, cells were grown in 24-well tissue-culture plates to reach confluency.

2.3. Expression in COS cells

Transient expression of COS cells was carried out using DEAE-dextran with dimethyl sulfoxide according to the method of Kaufman [32] with minor modifications. COS cells were grown in 24-well plates at 37°C and 5% CO_2 in high glucose Dulbecco's modified Eagle's medium supplemented with 10% bovine fetal serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Cells were routinely used 2 days after transfection for transport studies.

2.4. Transport assays

Assays were performed at 37°C in Hepes-buffered saline (150 mM NaCl, 10 mM Hepes, 1 mM $CaCl_2$, 10 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 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 possible 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 glycine (0.2 μM). Cells were incubated for the indicated time and then the experiments were terminated by washing the cells with three times 500 μl of ice-cold PBS medium (0.9 mM $CaCl_2$, 2.68 mM KCl, 1.47 mM KH_2PO4, 0.49 mM $MgCl_2$, 136.89 mM NaCl, 7.37 mM Na_2HPO_4, 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. For estimating statistical differences, the data were compared using the Student's *t*-test; differences at the 0.05 level were considered statistically significant.

2.5. cDNA cloning

A cDNA corresponding to the 664 nucleotides close to the carboxyl terminus of the glycine transporter cDNA [18] was obtained by PCR using rat GLYT1 cDNA as template. The 5′ sense strand oligonucleotide (5′-GGTGGGGAATGAGTGGATTCTGCAG-3′) correspond to bases 1257 to 1281. The 3′ antisense oligonucleotide (5′-TGCTCATATCCGGGAGTCCTGGAGG-3′) corresponds to bases 1896 to 1920. The 664 bp amplified cDNA was blunt-end ligated to a Bluescript plasmid previously digested with *Sma*I. Inserts were sequenced by standard methods.

2.6. RNA preparation and Northern blot analysis

RNA was extracted from cells as described [33]. 10 μg of total cellular RNA per lane were electrophoresed through a 1.3% agarose gel and transferred to Hybond N filters [34]. After blotting, the filters were hybridized with ^{32}P-labelled cDNA probes generated using the hexanucleotide random priming method [35]. Hybridization was carried out at 42°C for 12–16 h in a solution containing 50% formamide, 5 × SSC (0.15 M NaCl and 0.015 M sodium citrate), 5 × Denhardt's solution and 0.1% SDS (sodium dodecylsulfate). The membranes were washed with 0.2 × SSC, 0.1% SDS at 42°C and exposed to X-ray film for 3–5 days. Radioactivities of the positive bands were measured by Molecular Dynamics Image Quant v. 3.0.

2.7. Immunoblotting

Samples were transferred by electroblotting onto a nitrocellulose membrane in a semidry electroblotting sys-

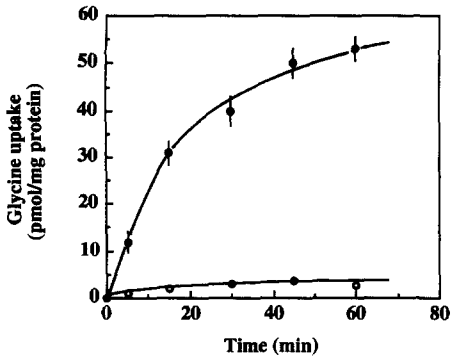

Fig. 1. Time course of glycine uptake in glioblastoma cells. C6 cells grown to confluence were incubated at 37°C in the presence of 0.2 μM of [2-^{3}H]glycine in the following media: Hepes-buffered saline, control (with 150 mM NaCl) (●), and Hepes-buffered saline in which NaCl has been iso-osmotically replaced by LiCl (○). Values represent means ± S.E. of three duplicate experiments.

tem (LKB) at 1.2 mA/cm^2 for 2 h. The transfer buffer consisted of 192 mM glycine and 25 mM Tris-HCl, pH 8.3. Nonspecific protein binding to the blot was blocked by the incubation of the filter with 3% non-fat milk protein in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl overnight at 25°C. The blot was then probed with 1:200 dilution of antiserum produced against a fusion protein containing 76 amino acids of the C-terminus of GLYT1 transporter, for 4 h at 25°C. After washing, the blot was then probed with an antirabbit IgG peroxidase linked, and bands were visualized with the α-chloronaphthol method [36].

2.8. Protein determination

Membrane proteins were determined according the method of Bradford [37].

3. Results and discussion

Fig. 1 is a time course showing uptake of glycine in C6 glioblastoma cells at a concentration (0.2 μM) which serves as substrate for the high-affinity glycine transport system with little involvement of the low-affinity components. The experiments were performed by measuring the initial rates of glycine transport corrected for non-saturable diffusion (non-specific transport). To make this correction, we used the experimental corrections described previously [38]. The uptake was almost completely Na^+-dependent and increased linearly with time up to approx. 10 min.

We have described a short-term regulation in the function of GABA and glutamate transporters by phorbol esters acting through PKC [26,26]. Moreover, we have demonstrated that a glial glutamate transporter can be phosphorylated both in vitro and in vivo by PKC and that this phosphorylation affects the transport of glutamate activity [29]. A long-term regulation of the GABA transporter by cAMP has also been reported [28]. All these data, together

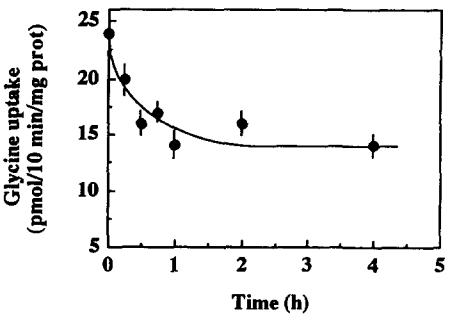

Fig. 2. Effects of TPA on Na^+-dependent glycine uptake in glioblastoma cells. C6 cells were incubated in Hepes-buffered saline at 37°C in the presence of 0.1 μM TPA at the indicated times. Transport activity was measured for 10 min in the same medium containing 0.2 μM of labelled glycine. Values represent means ± S.E. of three duplicate experiments.

with the fact that the glycine transporters already cloned have several consensus sequences for protein kinase-mediated phosphorylation, led us to investigate a possible regulation of the high-affinity glycine uptake through PKC activation.

The effect of the phorbol ester TPA on high-affinity glycine uptake was tested in C6 cells. Incubations of cells with TPA decreased glycine uptake in a time-dependent manner until approx. 1 h (Fig. 2). Exposure for additional time did not result in any further significant effect of TPA. A dose-response relationship for the TPA effect on the high-affinity glycine transport in glioma cells demonstrates that uptake decreases with TPA concentration, reaching a maximum of inhibition at approx. 0.1 μM TPA (Fig. 3).

To test the possibility that TPA-induced decrease in glycine uptake might be exerted through activation of Na^+/H^+ exchange, a known effect of PKC activation in many cell types [39], the effect of TPA was also studied in the presence of amiloride, an inhibitor of the Na^+/H^+ exchange. The results show that the presence of amiloride does not significantly influence by itself or in the presence of TPA the uptake of glycine (29.2 ± 1.5 pmol/10 min per mg of protein in the controls versus 30.1 ± 1.2 pmol/10 min per mg of protein in the presence of 100 μM amiloride).

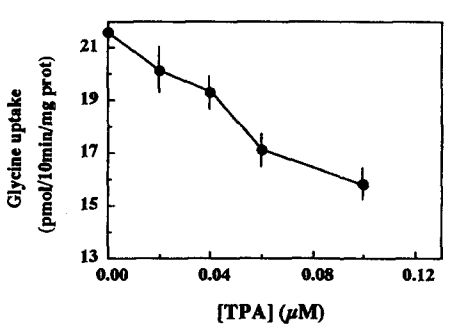

Fig. 3. Effects of increasing concentrations of TPA on Na^+-dependent glycine uptake in glioblastoma cells. C6 cells were incubated for 45 min at 37°C with TPA at the indicates concentrations. Transport activity was measured in the presence of 0.2 μM of [2-^{3}H]glycine. Values represent means ± S.E. of three duplicate experiments.

The ability of phorbol esters to inhibit glycine uptake in glioblastoma cells correlates with the degree of specificity of TPA to stimulate PKC [40]. On the other hand, the concentrations of phorbol esters are consistent with their potency in binding and promoting PKC-stimulated events in neuronal cells and other tissues, and would imply that the effects of phorbol esters on glycine uptake in C6 cells are mediated through the activation of PKC.

The effect of preincubation with staurosporine, a well known inhibitor of PKC [41], in the absence and the presence of TPA, on high-affinity glycine uptake in C6 cells was examined (Table 1). The results show that the presence of staurosporine does not by itself affect the uptake of glycine; however, it prevents the induced decrease of glycine uptake in the presence of TPA. Results in Table 1 also show that preincubation of cells with 4α-PDD, an inactive phorbol ester, had no effect on the glycine uptake. We also evaluated the effect of OAG, a permeant DAG analog which has been reported to stimulate PKC in several cell types [42], on glycine transport in glioma cells. As shown in Table 1, OAG exerted a similar inhibitory effect to that observed in the presence of phorbol esters. The addition of exogenous phospholipase C (0.02 units/ml) mimic the inhibitory effect achieved in the presence of TPA or OAG. It has been reported that phospholipase C generates DAG from membrane phospholipids when added exogenously to a variety of cell types [43,44]. This would result in an increase in intracellular Ca^{2+} and activation of PKC through second messengers inositol trisphosphate and DAG, respectively.

C6 cells seem to express mainly the GLYT1 transporter system, as we have shown previously that high-affinity uptake of glycine in C6 is competitively inhibited by sarcosine [38], a characteristic that is not shown by the GLYT2 transporter subtype [26]. To define the presence or not of the mRNA encoding the glycine transporter GLYT1 we carried out Northern blot analysis of total RNA isolated from C6 glioblastoma cells. A single transcript of 3.8 kb which hybridized at high stringency with the GLYT1 cDNA probe can be observed in the Fig. 4A. Fig. 4B shows immunoblotting data with a polyclonal antibody raised against a fusion protein containing 76 amino acids of the C terminus of GLYT1 transporter (L. Olivares, C. Aragón, C. Giménez and F. Zafra, unpublished data). When a membrane fraction from glioblastoma cells was probed with this antibody, a broad band of approx. 70–100 kDa lip up.

Table 1
Effects of TPA, staurosporine, 4α-PDD, OAG, and phospholipase C on sodium-dependent glycine uptake in glioblastoma cells

Conditions	Sodium-dependent glycine uptake (pmol/mg protein per 10 min)
Control	27.1 ± 1.5
Staurosporine	29.0 ± 0.4
TPA	15.8 ± 1.0 [a]
TPA + staurosporine	26.9 ± 0.5
4α-PDD	27.0 ± 1.7
OAG	16.7 ± 0.9 [a]
Phospholipase C	9.4 ± 1.0 [b]

C6 glioblastoma cells were incubated for 30 min at 37°C in the presence of either TPA (1.0 μM), 4α-PDD (1.0 μM), OAG (50 μg/ml) or phospholipase C (0.02 U/ml) or its solvent at an equivalent concentration. When staurosporine was used, was added and incubated for 30 min at 37°C before the addition of TPA. The glycine transport assay was carried out for 10 min in Hepes-buffered saline in the presence of 0.2 μM labelled glycine. Values represent the means ± S.E. of three different duplicate experiments. They were compared with the control value by Student's t-test. Values [a] $P < 0.01$; [b] $P < 0.001$.

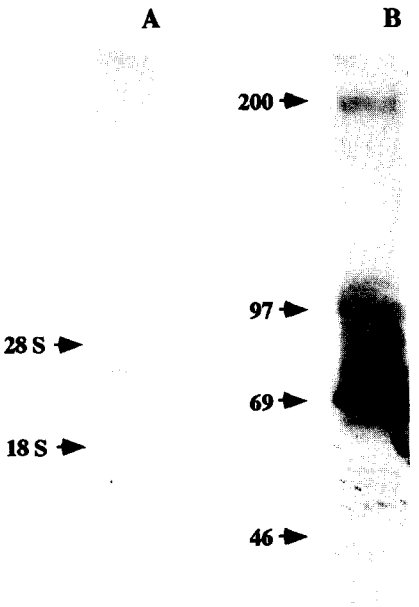

Fig. 4. Expression of the GLYT1 transporter in C6 glioblastoma cells. (A) Total RNA (10 μg) from C6 cells was extracted and analyzed by Northern blot as indicated in Materials and methods. The membrane was probed with a cDNA from the GLYT1 transporter. (B) A membrane fraction from glioblastoma cells was analyzed by immunoblotting as indicated in Materials and methods.

Next, we tested the possible modulatory action of phorbol esters in COS cells transiently transfected with rB20a (a full-length clone of GLYT1 in the eukaryotic expression vector pSVL) [18]. COS cells transiently transfected with rB20a accumulated more glycine than nontransfected control cells (Fig. 5). The same figure shows that incubation of transfected COS cells in the presence of the phorbol ester TPA produced a decrease in glycine uptake in a time-dependent form until approx. 1 h. Exposure for additional time did not result in any further significant effect of TPA.

A dose-response relationship for the TPA effect on the high-affinity glycine transport in COS-transfected cells (Fig. 6) demonstrates that uptake decreased with TPA concentration, reaching a plateau at approx. 0.1 μM TPA.

Taking together all these data, it is possible to establish that the high-affinity glycine uptake in glioblastoma cells can be regulated by second messenger systems. Results

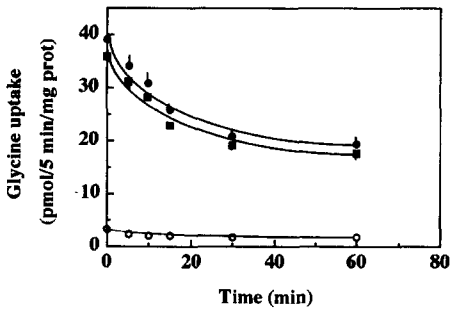

Fig. 5. Effect of TPA on glycine transport in COS cells transfected with clone rB20a. Nontransfected COS cells (○) or COS cells transfected with rB20a (●) were preincubated for the indicated time in the presence of 0.1 μM TPA. The transport assay was carried out during 5 min at 37°C in the presence of 0.2 μM labelled glycine. (■) Represents the difference between transport in transfected and nontransfected COS cells. Values represent means ± S.E. of three duplicate experiments.

demonstrating that neurotransmitter transporters can be regulated are important because there is currently very little information available on the regulatory aspects of these proteins. Apart of the role of PKC on the GABA and glutamate shown by our group [26,27,29], a similar modulatory effect has been shown in the serotonin transporter function in endothelial cells by PKC [45]. On the other hand, it has been reported the involvement of cAMP in the long-term modulation of GABA (GAT-1) transporter from rat brain [28], and a high-affinity serotonin transporter in the human placental choriocarcinoma cell line [46].

The exact mode of glycine transport regulation by PKC remains to be elucidated. Although a direct phosphorylation has been demonstrated in a glial glutamate transporter by PKC [29], other alternative mechanisms cannot be ruled out. The observed effect could be indirectly mediated by changing the activity of ion channels or the Na^+/K^+-ATPase function which could alter ion concentration both inside and outside of a cell [47].

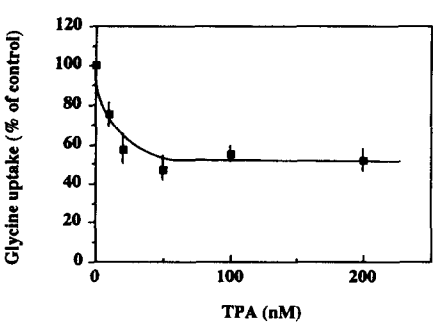

Fig. 6. Effect of increasing concentration of TPA on glycine transport in COS cells transfected with clone rB20a. COS cells transfected with rB20a were incubated for 30 min at 37°C with TPA at the indicated concentrations. Transport activity was measured in the presence of 0.2 μM labelled glycine. Values represent means ± S.E. of three duplicate experiments.

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