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The regulation of glycine transporter GLYT1 is mainly mediated by protein kinase Cα in C6 glioma cells

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ABSTRACT

Glycine has been shown to possess important functions as a bidirectional neurotransmitter. At synaptic clefts, the concentration of glycine is tightly regulated by the uptake of glycine released from nerve terminals into glial cells by the transporter GLYT1. It has been recently demonstrated that protein kinase C (PKC) mediates the downregulation of GLYT1 activity in several cell systems. However, it remains to be elucidated which subtypes of PKC might be important in the regulation of GLYT1 activity. In this study, we attempted to make clear the mechanism of the phorbol 12-myristate 13-acetate (PMA)-suppressed uptake of glycine in C6 glioma cells which have the native expression of GLYT1. In C6 cells, the expression of PKCα, PKCδ, and PKCε of the PMA-activated subtypes was detected. The PMA-suppressed action was fully reversed by the removal of both extracellular and intracellular Ca^{2+}. Furthermore, the inhibitory effects of PMA or thymeleatoxin (THX), which is a selective activator of conventional PKC (cPKC), were blocked by the downregulation of all PKCs expressed in C6 cells by long-term incubation with THX, or pretreatment with GF109203X or Gö6983, which are broad inhibitors of PKC, or Gö6976, a selective inhibitor of cPKC. On the other hand, treatment of C6 cells with ingenol, a selective activator of novel PKCs, especially PKCδ and PKCε, did not affect the transport of glycine. Silencing of PKCδ expression by using RNA interference or pretreatment with the inhibitor peptide for PKCε had no effect on the PMA-suppressed uptake of glycine. Together, these results suggest PKCα to be a crucial factor in the regulation of glycine transport in C6 cells.

1. Introduction

Glycine has an important neurotransmitter function at both inhibitory and excitatory synapses in the vertebrate central nerve system. The inhibitory action of glycine is initiated by the stimulation of strychnine-sensitive glycine receptors mainly located in the spinal cord, brainstem and cerebellum (Legendre, 2001). Additionally, glycine acts as an essential co-agonist of glutamate at NMDA receptors, and its binding to these receptors triggers both the opening of ion channels and the internalization of receptors (Johnson and Ascher, 1987; Nong et al., 2003; Sershen et al., 2008). The neurotransmission mediated by glycine in the synaptic cleft is terminated by the Na^+–Cl^- dependent reuptake system. Thus far, two glycine transporters GLYT1 and GLYT2 have been cloned in the brain or spinal cord (Guastella et al., 1992; Liu et al., 1993). In general, GLYT1 is expressed in glial cells that exist close to the glycinergic nerve terminals, and plays an essential role in the clearance of glycine at synapses (Adams et al., 1995; Aragón and López-Corcuera, 2003). GLYT2 is present in axons and presynaptic terminals of inhibitory glycinergic neurons, and supplies glycine to the presynaptic cytosol for vesicular release (Liu et al., 1993; Zafra et al., 1995). In contrast, there are a report described the opposite location of GLYT1 or GLYT2 which are existed in neurons or astrocytes, respectively (Raiteri et al., 2008). It has been demonstrated that changes of GLYT1 activity caused by various factors directly affect the homeostasis of the glycinergic system, which is involved in both the excitatory neurotransmission through NMDA receptors and the inhibitory neurotransmission through glycine receptors, at synapses. For example, NMDA receptor-triggered currents were increased by the inhibition of

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GLYT1, and these events induced the antipsychotic action (Lim et al., 2004; Martina et al., 2004; Henjum and Hassel, 2007; Papp et al., 2008). Furthermore, GLYT1 inhibitors might also have the potential to enhance the efficacy of inhibitory transduction, suggesting that such drugs might improve dysfunctional motor activity such as convulsions and decrease the perception of pain at the spinal level (Gomeza et al., 2003; Harvey et al., 2004; Morita et al., 2008). Therefore, it is worth identifying the regulatory mechanisms of GLYT1.

The protein kinase C (PKC) family can be divided into three groups, conventional, novel, and atypical (Nishizuka, 1988; Hug and Sarre, 1993; Mellor and Parker, 1998). The conventional PKCs (PKCα, PKCβ, and PKCγ) are activated by diacylglycerol (DAG) or phorbol 12-myristate 13-acetate (PMA), and require Ca^{2+} as a co-factor. The novel PKCs (PKCδ, PKCε, PKCη, and PKCθ) are also activated by DAG or PMA, but do not require Ca^{2+} for their activation. The atypical PKCs (PKCζ and PKCλ) are insensitive to DAG, PMA, or Ca^{2+}. Because several studies have demonstrated a cell- or organelle-specific distribution of different PKC subtypes (Way et al., 2000; Newton, 2001), each enzyme might have a distinct role in cell signaling. Therefore, it is possible that specific subtypes of PKC serve specific cell functions.

Recently, analyses of amino acid sequences in glycine transporters have identified the consensus sites of phosphorylation by several protein kinases such as PKC, protein kinase A, and Ca^{2+}/calmodulin-dependent protein kinase (Liu et al., 1993; Gomeza et al., 1995; Gadea et al., 2002). It has been previously suggested that the activation of PKC by PMA induced a decrease in the uptake of glycine via GLYT1 (Gomeza et al., 1995; Sato et al., 1995). However, it remains unclear which subtypes of PKC contributed to the PMA-sensitive transport of glycine. The aim of the present study is to determine whether specific subtypes of PKC contribute to the regulation of GLYT1 activity. In this study, by using subtype-specific pharmacological reagents or the down-regulation of the different subtypes of PKC in C6 glioma cells, which possess potentiality with astrocytes-type characteristics, and express GLYT1 but not GLYT2, we found evidence that PKCα is mainly involved in the regulation of GLYT1-mediated transport.

2. Experimental procedures

2.1. Materials

Ham's F12K medium, PMA, and ingenol were obtained from Sigma Chemical Co. (St. Louis, MO). Thymeleatoxin (THX), GF109203X, Gö6976, Gö6983, and PKCε translocation inhibitor peptide were from Calbiochem (La Jolla, CA). Rottlerin was purchased from Tocris Cookson (Bristol, UK). BAPTA-AM was from Dojindo Laboratories (Kumamoto, Japan). [^{14}C]-glycine (3.74 GBq/mmol) was from PerkinElmer Life Sciences (Boston, MA). All other reagents used were of analytical grade and from Katayama Chemical (Osaka, Japan) or GibcoBRL (Gaithersburg, MD). PMA and THX were dissolved in ethanol. PKCε translocation inhibitor peptide was dissolved in distilled H_2O. GF109203X, Gö6983, Gö6976, ingenol, rottlerin and BAPTA-AM were dissolved in DMSO. The final concentrations of all solvents for treatment of the cells were maintained at 0.25%.

2.2. Cell culture

Rat C6 glioma cells were acquired from the American Type Tissue Collection (CCL-107), and were maintained in Ham's F12K medium supplement with 15% horse serum, 2.5% fetal calf serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin in an atmosphere of 5% CO_2/95% air at 37 °C. Twenty-four hours before each experiment, the medium was exchanged with serum-free medium to exclude the undesirable effects of serums. C6 cells between passage numbers 40 and 60 were used for all experiments.

2.3. ^{14}C-glycine uptake assay

Krebs Ringer HEPES (KRH)-buffered solution containing NaCl 125 mM, KCl 4.8 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, HEPES 25 mM, and glucose 5.6 mM, adjusted to pH 7.4 with NaOH was used in the normal uptake assay. Ca^{2+}-deficient buffer was prepared by omitting $CaCl_2$ and adding 1 mM EGTA. Cells were pretreated with PMA, and then glycine uptake assays were initiated by incubation with KRH buffer containing 5 μM of ^{14}C-glycine at 37 °C. In the Ca^{2+}-deficient assay, cells were incubated in the KRH buffer without Ca^{2+} throughout the uptake assays, including wash out. Uptake was terminated by three washes with ice-cold KRH buffer followed by immediate lysis in 2 M NaOH for the measurement of radioactivity with a liquid scintillation counter. Non-specific uptake was determined in the presence of 10 mM of unlabeled glycine.

2.4. Western blot analysis

Cells were washed with ice-cold PBS, and solubilized in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors (100 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The cell lysates were further added to Laemli's buffer and boiled for 5 min. Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. The membranes were blocked in blocking buffer for 1.5 h at room temperature, and subsequently incubated with purified monoclonal antibody against each type of PKC (PKCα, 1:1000; PKCβ, 1:250; PKCγ, 1:1000; PKCδ, 1:500; PKCε, 1:1000; PKCη, 1:250; PKCθ, 1:250; BD Biosciences, San Jose, CA), or antibody against β-actin (1:10,000; Sigma) overnight at 4 °C. After being washed, the membranes were incubated with a horseradish peroxidase-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Then, the membranes were rinsed, and incubated with luminescence reagent (Wako, Osaka, Japan). Finally, the membranes were exposed to X-ray film to detect the protein.

2.5. Transfection with small interfering RNA (siRNA)

C6 cells were seeded into 35-mm diameter dishes for Western blotting at 2.0×10^5 cells or 24-well plates for uptake assays at 4.0×10^4 cells/wells. After 24 h, the culture medium was replaced with Opti-MEM (Gibco BRL, Rockville, MD), and then cells were transfected with 50 nM of the siRNA targeting rat PKCδ or AllStars Negative Control siRNA (QIAGEN, Valencia, CA) as a nonsilencing siRNA by using HiPerFect Transfection Reagent (QIAGEN). The siRNA for rat PKCδ was synthesized by QIAGEN and annealed according to the manufacturer's directions. The sequences of siRNA for PKCδ were as follows (sense and antisense, respectively): 5′-r(CAUUCGACGCCCACAUCUA)dTdT-3′ and 5′-r(UAGAUGUGGGCGUCGAAUG)dTdT-3′. At 24 h after transfection, cells were washed with KRH three times, and analyzed for the expression of each PKC subtype by Western blotting or for the uptake of ^{14}C-glycine.

2.6. Statistical analysis

Data are expressed as the mean ± S.E. of at least three independent experiments. Differences between means were determined using a one-way analysis of variance (ANOVA) with a pairwise comparison by the Bonferroni method. Differences were considered to be significant when the P value was less than 0.05.

3. Results

3.1. PKCα, PKCδ, and PKCε are expressed in C6 glioma cells

Previous studies have indicated complicated results for the expression pattern of PKC subtypes in C6 cells because of different sources of cells, different conditions of culture, or some uncontrolled factor (Anji et al., 2001; González et al., 2005). Therefore, we have first investigated which subtypes of PKC were expressed in our cells. As a result, immunoreactive bands for PKCα, PKCδ, and PKCε were detected in C6 cells (Fig. 1). These expression patterns of PKC subtypes were similar with those described by González et al. (2005). These bands indicated the same position as those observed in the rat cortex or lung as a positive control and were consistent with the predicted size. However, there was no expression of PKCβ, PKCγ, PKCη, or PKCθ in C6 cells, although those enzymes were observed in the lysates from the cortex (PKCβ, PKCγ, and PKCθ) or lung (PKCη) (Fig. 1).

3.2. PMA-induced suppression of glycine uptake was completely reversed by the removal of both extracellular and intracellular Ca^{2+}

C6 cells have been shown to express the high affinity glycine transporter GLYT1, but not GLYT2, and are used as a model for the