Biochemical and Biophysical Research Communications 384 (2009) 530–534

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Glycine transporter 1 associates with cholesterol-rich membrane raft microdomains

Xin Liu, Ann D. Mitrovic, Robert J. Vandenberg *

Transporter Biology Group, School of Medical Sciences (Pharmacology), Bosch Institute, University of Sydney, Sydney, NSW 2006, Australia

ARTICLE INFO

Article history:
Received 4 May 2009
Available online 8 May 2009

Keywords:
Glycine transporter
Membrane rafts
Fluorescence resonance energy transfer
Fluorescence life time imaging
Cholesterol
Co-localization
Glycine transport
GLYT1

ABSTRACT

Membrane rafts, the highly-ordered, cholesterol-rich microdomains of the plasma membrane play important roles in cellular functions. In this study, GLYT1-CFP and GLYT2-CFP were constructed, followed by investigation of whether the tagged transporters associate with a fluorescence probe that labels membrane rafts (DiIC16) by using Fluorescence Resonance Energy Transfer. A close association was observed between DiIC16 and GLYT1-CFP, but not for GLYT2-CFP. The glycine transport ability of GLYT1 is also highly dependent on the integrity of this area. Together, the results suggest that GLYT1 and membrane rafts are co-localized in the membrane, and that this influences the rate of glycine transport.

Glycine is an inhibitory neurotransmitter in the spinal cord and brain stem [1]. Glycine transporters 1 and 2 mediate the uptake of the inhibitory neurotransmitter glycine from the extracellular space into glial cells (GLYT1) and glycinergic neurons (GLYT2), respectively [2,3]. Studies on GLYT knock out mice have shown that, GLYT1 is required for removing synaptic glycine [4], whereas GLYT2 is required for the reuptake of glycine into the presynaptic terminal [5]. In addition, GLYT1 has been shown to be involved in the regulation of glutamatergic neurotransmission by modulating the occupancy of the glycine binding site of the *N*-methyl-D-aspartate subtype of glutamate receptors [6–9]. Therefore changes in transport activities and surface expression levels of GLYTs can affect the efficacy of both excitatory and inhibitory neurotransmission.

Plasma membrane organization is emerging as a new way of regulating membrane protein function [8–10]. Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, cholesterol- and sphingolipid-enriched domains that compartmentalize cellular processes. They can influence neurotransmitter signaling, selectively incorporate and segregate proteins and are required for the function and correct trafficking of some proteins [8–10]. In this study, Fluorescence Resonance Energy Transfer (FRET) was employed in combination with glycine transport studies before and after the disruption of membrane rafts. The results demonstrate that GLYT1 is co-localized with plasma membrane rafts in CHO-K1 cells and that glycine transport activity is sensitive to changes in the lipid environment.

Materials and methods

Materials. DiIC16 and cell culture media and solutions were supplied by Invitrogen, Australia (Mt Waverly, VIC, Australia) unless otherwise stated. Chinese hamster ovary (CHO) cells stably transfected with human GLYT1 and GLYT2 were a gift from John Morrow at Organon Laboratories Ltd. (Cambridge, England). All other chemicals were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia) unless otherwise stated.

Plasmid construction and preparation of RNA. Stop codons of GLYT1-pcDNA3.1 and GLYT2-pcDNA3.1 cDNA were removed by mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The GLYT plasmids and the cerulean fluorescent protein cDNA (CerCFP) were then linearised with the restriction enzyme XbaI. The ligation of linearised vectors containing GLYT1 and GLYT2 with CFP was performed by using Fast-Link™ DNA Ligation Kit (Epicentre Biotechnologies, Madison, WI, USA) and the sequences were confirmed by DNA sequencing. The GLYT1 and GLYT2 cDNAs were also subcloned into the pOTV vector for production of RNA and injection into *Xenopus laevis* oocytes. RNA was synthesized from linearized pOTV-GLYT1 and pOTV-GLYT2 using the mMessage mMachine (Ambion, Inc., Austin, TX, USA) kit according to the manufacturer's instructions.

* Corresponding author.
E-mail address: rvan9242@mail.usyd.edu.au (R.J. Vandenberg).

0006-291X/$ - see front matter Crown Copyright © 2009 Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.05.014

Cell culture and transient expression of GLYTs. Stably transfected CHO-K1 cells were cultured in Dulbecco's modified Eagle's medium: F-12 (DMEM-F12) media supplemented with 8% foetal bovine serum (FBS) and 0.6 mg/ml geneticin. Untransfected CHO-K1 cells were cultured in DMEM-F12 media supplemented with 8% FBS and 5 μg/ml penicillin. The CHO-K1 cells were transfected with the plasmid encoding GLYT1-CFP and GLYT2-CFP mediated by Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions and the clones were confirmed on the basis of CFP fluorescence. For DiIC16 labeling experiments, cells were pre-labelled with 5 μg/ml DiIC16 [11,14,15], and then rinsed and incubated with Leibovitz's L-15 (Invitrogen) Medium for observation. The membrane cholesterol was measured using the Amplex Red cholesterol assay kit (Invitrogen).

[^{3}H]-glycine uptake measurements. CHO-K1-GLYT1 and CHO-K1-GLYT2 cells were passaged and plated into 24 well plate at a density of 4×10^5 cells per well and left to settle for 3 h. Media was then removed and the cells were washed once with PBS buffer. Uptake was carried out at 37 °C in the presence of 30 μM ^{3}H-glycine (GE Healthcare, Buckinghamshire, England) for 20 min with and without *N*[3-(4-fluorophenyl)-3-(4′-phenylphenoxy)] propylsarcosine (NFPS), ALX 1393, and methyl-β-cyclodextrin treatment. Radiation from ^{3}H-glycine was counted using Packard TopCount Liquid Scintillation Counter (GMI, Ramsey, MN USA).

FRET and FLIM. FRET between CFP and DiIC16 was measured by the lifetime reduction of donor (CFP) using fluorescence lifetime imaging (FLIM). The images were obtained using NikonC1 confocal microscope equipped with a picosecond pulsed 440 nm laser and a high-speed gated detection system for lifetime imaging (Nikon Instruments, Melville, NY, USA). This system accommodates a time-gated detection technique using four windows representing gates. Each window is decayed by a different time relative to the excitation pulse, allowing a fluorescence decay curve to be recorded, and then lifetime is calculated by the Nikon C1 LIMO software. CHO-K1 cells expressing GLYT1-CFP and GLYT2-CFP were labelled with DiIC16. Levels of labelling were determined by observing fluorescence under the 488 nm laser. The cells displaying similar levels of labelling throughout the plasma membrane were selected for the FRET experiments. Images were collected at 512×512 or 256×256 pixel resolutions and regions of interest in the membrane with photon counts per pixel greater that 500 were chosen for FLIM measurements. FLIM measurements were done by using a 60× H_2O objective and the fluorescence lifetime was determined after excitation with a 440 nm pulsed laser and a 470/20 nm bandpass emission filter and quantified with LIMO software. All FLIM experiments were carried out in live, transiently-transfected cells cultured in 8-well culture slides (Becton Dickinson, North Ryde, NSW, Australia).

The FRET efficiency (E) was calculated using,

$$E = 1 - (\tau_{DA}/\tau_D), \quad (1)$$

where τ_{DA} represents the donor lifetime in the presence of acceptor and τ_D represents the lifetime of donor in the absence of acceptor.

Surgery/harvesting of X. laevis oocytes and electrophysiology recording. X. laevis were obtained from NASCO (Fort Atkinson, WI, USA). Oocytes were harvested from *X. laevis*, as described previously [12,13], and injected with 10–50 ng of wild-type or mutant cRNA and incubated at 16 °C in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.55), supplemented with 0.1% gentamicin, 2.5 mM pyruvate and 0.5 mM theophylline. Two to five days later, whole-cell currents were measured using the standard two-electrode voltage-clamp technique with a Geneclamp 500 amplifier (Molecular Devices, Sunnyvale, CA, USA) interfaced with a MacLab2e chart recorder (AD Instruments, Castle Hill, NSW, Australia).

To measure the cholesterol content of oocyte membranes, oocytes were incubated with 1 mM MCD in ND96 and ND96 alone (control experiment) for 45 min and then washed. Two-phase sucrose gradients were prepared with 1.5 and 0.3 M sucrose buffers (20 mM Tris–HCl, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.6) at a 1:2 ratio in 1.5 mL microtubes (30 oocytes/microtube). The oocytes were manually disrupted with forceps, layered at the upper phase of the separation medium, and then centrifuged for 20 min at 12,000*g* (4 °C). The interface between the phases containing the membranes was removed, washed (5.0 mM HEPES, 160 mM NaCl, pH 7.4), and centrifuged for 10 min at 3000 *g* (4 °C). The pellets were resuspended in 50 μl of working buffer (from Amplex Red cholesterol assay kit, Invitrogen) and sonicated. The Amplex Red cholesterol assay kit was then used to estimate the cholesterol content.

Data analysis. Concentration-dependent transport currents were fitted to a derivation of the Michaelis–Menten equation using PRISM software (GraphPad Software, La Jolla, CA, USA).

$$I/I_{max} = [S]/([S] + EC_{50}), \quad (2)$$

where I is the current, I_{max} is the maximal current, [S] is the substrate concentration and EC_{50} is the concentration of substrate that generates half-maximal current. Significance tests were performed using two-tailed Student's t-test. $p = 0.05$ was taken as statistically significant.

Results

Depletion of membrane cholesterol affects the transport activity of GLYTs

^{3}H-glycine uptake by CHO-K1 cells stably expressing GLYT1 and GLYT2 was examined in the presence and absence of the GLYT inhibitors, NFPS and ALX1393 to confirm the pharmacological profile of the transporters. One μM NFPS completely blocked ^{3}H-glycine uptake by GLYT1 expressing cells and 1 μM ALX1393 inhibited ^{3}H-glycine uptake by GLYT2 expressing cells by 83 ± 1%. To investigate the role of lipid rafts on glycine transporter function, we treated the cells with 1 mM methyl-β-cyclodextrin (MCD) for 45 min. This treatment depleted 65 ± 12% ($n = 6$) of the cell cholesterol of the plasma membranes of CHO-K1 cells without causing any apparent changes in cell morphology. In CHO-K1 cells expressing GLYT1, the MCD treatment reduced ^{3}H-glycine uptake by 36 ± 4% ($n = 5$, $p < 0.05$), whereas for CHO-K1 cells expressing GLYT2, the MCD treatment caused an increase of 40 ± 12% ($n = 5$, $p < 0.05$) (Fig. 1). MCD treatment had no effect on background levels of ^{3}H-glycine uptake by CHO-K1 cells not expressing GLYT1 or GLYT2.

Co-localization of GLYT1 and membrane rafts in the plasma membrane determined by FRET

We have investigated whether the effects on transporter function caused by depletion of cholesterol are due to disruption of an association between the transporters and lipid rafts. FRET is a spectroscopic process that allows a measure of the distance between two fluorophores. We have used CFP-labelled transporters as FRET donors and a fluorescent raft marker, DiIC16, as the FRET acceptor to measure the association between glycine transporters and rafts. Lipids differ in their intrinsic physical properties such as the length and degree of unsaturation of their acyl chains and partition into regions of different rigidity. DiIC16 has a long and saturated acyl chain that roughly matches the thickness of the relatively ordered membrane regions, and has been demonstrated to be selectively incorporated into lipid rafts [11,14,15]. FRET be-