10.1071/CH10322_AC

Australian Journal of Chemistry, 2011, 64(1), 31–35

ACCESSORY PUBLICATION

Robust Electrophysiological Assays using Solid Supported Membranes: the Organic Cation Transporter OCT2

Olga Gaiko,[A,B] *Ingo Janausch,*[A,C] *Sven Geibel,*[D,E] *Henning Vollert,*[D,F] *Petra Arndt,*[D] *Sigrid Gonski,*[D] *and Klaus Fendler*[A,G]

[A]MPI for Biophysics, Max-von-Laue Str.3, 60438 Frankfurt/Main, Germany.

[B]Present address: Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, 9500 Gilman Dr, Mail Code 0718, La Jolla CA 92093-0718 USA.

[C]Present address: Universität Heidelberg, Im Neuenheimer Feld 325, 69120 Heidelberg, Germany.

[D]Sanovi-Aventis Germany GmbH, Industriepark Höchst, 65926 Frankfurt, Germany.

[E]Present address: Bayer BioScience NV, Technologiepark 38-9052 Zwijnaarde (Gent), Netherlands.

[F]Present address: BioActive Food GmbH, am Ihlsee 36a, 23975 Bad Segeberg, Germany.

[G]Corresponding author. Email: klaus.fendler@biophys.mpg.de

Materials and methods

Generation of stable FlpIn and FlpIn T-REx CHO cell lines

FlpIn and FlpIn T-REx systems (Invitrogen (Karlsruhe, Germany) were used to generate stable cell lines. The hOCT2 and rOCT2 inserts were subcloned from pcDNA3.1 via *Hind*III and *Eco*RI restriction sites into pcDNA5/FRT. The resulting expression plasmids were introduced into the FlpIn CHO host cell lines (Invitrogen) using FuGENE 6 reagent (Roche Diagnostics, Mannheim). The FlpIn CHO-OCT cells were maintained in F12-Ham with L-Glutamine (Invitrogen) supplemented with 10% tet-system-approved fetal bovine serum (FBS) (BD Biosciences, Heidelberg,

Germany) and 300 µg/ml hygromycinB (Invitrogen) at 37°C under 5% CO_2. To generate FlpIn T-REx CHO cell lines, hOCT2 as well as rOCT2 were subcloned into pcDNA5/FRT/TO vector. FlpIn T-REx CHO cells were transfected with a mixture containing hOCT2 or rOCT2 cDNA in pcDNA5/FRT/TO and pOG44 vector using FuGENE 6. The selection of the FlpIn T-REx CHO-OCT cells was carried out by incubating cells with 300 µg/ml hygromycin. After 5 days, the remaining cells were selected for five additional days in the presence of 300 µg/ml hygromycin and 30 µg/ml blasticidineS (Invitrigen) before the stable cell lines were fully established and stored in liquid nitrogen. The optimal expression of OCT in FlpIn T-REx CHO cells was achieved after 48 h treatment with 1 µg/ml *dox*ycycline (BD Biosciences). The clones were screened for OCT expression by both fluorescence and western blotting.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

For SDS-PAGE and Western blotting, cellular lysates of FlpIn and FlpIn T-REx CHO cells transfected with rOCT2 or hOCT2 were obtained as described previously[4]. Electrophoresis for 2 h at 200 V in a 4-12% NuPAGE®Novex Bis-Tris gel (Invitrogen) was followed by transfer to a nitrocellulose membrane (Invitrogen) at 200 mA for 6 min in a iBlot™Dry Blotting System (Invitrogen). The NuPAGE MOPS SDS Running Buffer (10x) and NuPAGE Transfer Buffer (20x) were both from Invitrogen. The primary antibody was polyclonal rabbit anti-rat OCT2 affinity pure IgGs (Alpha Diagnostic International, San Antonio, TX), used at a dilution 1:1000. Bound alkaline phosphatase conjugated secondary antibody (goat anti-rabbit IgG) (SantaCruz Biotechnology Inc., Heidelberg) was diluted 1:3000 and visualized by the AP conjugate substrate kit from BioRad Laboratories GmbH (München, Germany). Prestained molecular weight marker SeaBlue®Plus 2 (Invitrogen) was used to determine apparent molecular masse.

Fluorescence measurements

Functional expression of hOCT2 or rOCT2, was verified by fluorescence measurements using a fluorescent substrate of organic cation transporters ASP^+ (4-(4-(dimethylamino) styryl)-N-methylpyridinium iodide (4-Di-1-ASP)) (Molecular Probes, Eugene, OR, USA). The experiments were performed using the Fluorometric Imaging Plate Reader ($FLIPR^{96}$) (Molecular Devices Corporation, Sunnyvale, U.S.A.). The FlpIn CHO-OCT cells were seeded at a density of ~150^3 cells/ml (200µl/well) in Costar 96-well uncoated, clear-bottomed, black-walled plates and allowed to proliferate overnight. On the day of experiment, the culture medium was aspirated from cell plate using an EMBLA plate washer (MDS Analytical Technologies). The

cells were washed twice with PBS DULBECCO'S with Ca^{2+} & Mg^{2+} (90 µl/well) followed by addition of 1 µM 5-(and-6)-carboxy SNARF (Invitrogen) and incubating at 37°C/5% CO^2 for 20 minutes. After the plate was placed in the $FLIPR^{96}$ experiments were conducted at 37°C using excitation at 450-490 nm, emission at 605 nm and a slit width of 25 nm. A baseline was collected for 10 s, after which 10 µM ASP^+ (30 ± 5 µl per measurement/sample) were added to each well. Sampling continued for another 10 min. The integration time of signal detection was set to 5 s from Scan 72 to Scan 96 and to 10 s (Scan 96-114). Transport assays with FlpIn T-REx CHO-OCT cells were carried out 24 h and 48 h after induction with 1 µM *dox*ycycline. Non-transfected FlpIn and FlpIn T-REx CHO cells were used as control.

The specific uptake of ASP^+ was calculated by subtracting the unspecific uptake in non-transfected FlpIn as well as FlpIn T-REx CHO cells from the total uptake in transfected (CHO-OCT) cells. Practically, the total uptake of ASP^+ in (CHO-OCT) cells was set to 100%, and the ASP^+ uptake of the same number of non-transfected cells was assumed to be unspecific uptake.

Large-scale cell growth and harvesting

FlpIn CHO-OCT or FlpIn T-REx CHO-OCT cells were seeded in 150 mm diameter Petri dishes and grown under conditions as described above to ~80-95% confluence. Cultures were confluent within 3 days of seeding. For induction of hOCT2 or rOCT2 expression in FlpIn T-REx CHO cells, medium was replaced with fresh medium containing 1 µM doxycycline, and cultures were continued for another 48 hours. For harvesting, cell monolayers were washed once with ice cold PBS (10 ml/Petri dishes) and 10 ml PBS with protease inhibitors (Protease Inhibitor Cocktail, 1 tablet/50 ml)

were added. Cells were scraped, collected and spun down for 10 min at 1000x*g*. Pellets were resuspended in 5 ml PBS buffer, combined and centrifuged until the number of tubes was reduced to one. After the final wash, the cell pellet was resuspended in 5 ml ice-cold sucrose buffer (250 mM Sucrose, 5 mM Tris, 2 mM DTT, 0.5 µL/mL ethanolic solution saturated with phenylmethanesulfonyl fluoride (PMSF), 1 tablet/50 ml Complete Protease Inhibitor Cocktail, pH 7.5), centrifugated (10 min at 1000x*g)*, frozen in liquid nitrogen and stored at -80°C until use. To prepare cell membranes, 4 to 6 g of cells were collected.

Membrane preparation for the electrophysiological assay

Stored cell pellets were thawed at 37°C and diluted with 2 ml ice-cold sucrose buffer. Cells were then pottered in a Wheaton glass/glass dounce-homogenizer. The cell nuclei, unbroken cells and mitochondria were removed by centrifugation 10 min at 700x*g* (rotor 3744, Sigma 3K-1) and 6100x*g*, (rotor 3744). Supernatant was centrifuged 45 min at 100000x*g* using a Beckman ultracentrifuge L60 (rotor SW41). Pellets were filled up with 5 mM Tris, pH 7.5 and 70% (w/v) sucrose to a concentration of 51% sucrose. The suspended cell fragments in 51 % sucrose were stacked subsequently with 45 % sucrose, 31 % sucrose and 9 % sucrose in 5 mN Tris, pH 7.5 and separated in sucrose density gradient for 1h 30 min at 100000*g* in rotor SW41 (Beckman L60). Fraction 9/31 % was diluted with at least two times their volume with the basic solution (30 mM Hepes/NaOH, 300 mM NaCl, 5 mM $MgCl_2$, 0.2 mM DTT, pH 7.4) and ultracentrifuged for 30 min at 150000*g* in a Ti 70.1 rotor. Pellets were resuspended in 1 ml of basic medium, frozen in liquid nitrogen and stored at -80°C. Protein was determined by the BCA method (BCA^{TM}Protein Assay Kit, Pierce International GmbH, Bonn) with bovine serum albumin as standard.

Insect cell expression

Two different gateway adapted baculovirus systems from Invitrogen were used (BactoBac and BaculoDirect). hOCT2 and rOCT2 were cloned in pENTRIa vector (Invitrogen Cat No. 11813011) via PCR and blunt end cloning. Constructs were used directly for infection of insect cells via the BaculoDirect system (Invitrogen Cat. No. 12562-054 or 12562-013). Constructs were also indirectly used with the BacToBac system (Invitrogen, Cat. No. 11827-0ll) to generate a recombinant virus. The OCT inserts in the pENTR-vector were switched into pDESTS (Cat. No. I1804-010) or pDEST1O (Cat. No. 11804-015) vectors using gateway technology. Then, a baculovirus shuttle vector propagated and recombined with pDEST vectors in E.coli were used for the transfection of insect cells. The resulting recombinant virus stocks were used for infection of Mimic Sf9 or HighFive cells for protein expression. Cells were harvested after three days of incubation and membrane fractions were prepared as described for CHO cells.

Data analysis

For the determination of the Michaelis constant K_M of the substrates, a linear rundown correction was applied. During each test series, repetitive reference measurements with saturating substrate concentration were performed, and the signal I_{peak} was corrected according to the following equation:

$$I = \frac{I_{R1}}{I_R} I_{peak} \qquad I_R = I_{R1} - \frac{I_{R1} - I_{R2}}{t_2 - t_1}(t - t_1) \qquad \text{(Equation 1)}$$

where: I_{R1}, I_{R2} are reference values measured at time t_1 *and* t_2, respectively, I_R is the calculated reference value at time t, the time of the measurement of the peak current I_{peak} corresponding to a given substrate concentration. I is the corrected peak current.

For the analysis of the nonreversible inhibitor phenoxybenzamin (PbA) an exponential rundown correction was used based on an independent measurement of rundown at 30 mM choline chloride in the absence of inhibitor performed every 10 minutes over 12 cycles (a typical inhibition measurement had a duration of 120 minutes) and the time constant of rundown τ = 128 min was determined. Using these data a reference value for the uninhibited transporter I_R could be generated and a corrected value for inhibition was calculated according to:

$$Inhibition(\%) = 100 - (\frac{I_{peak}}{I_R}100) \qquad I_R = I_{R(0)}e^{-\frac{t}{\tau}} \qquad \text{(Equation 2)}$$

Experiments were repeated at least three times. Data are presented as mean ± SEM (standard error mean) and n refers to the number of experiments. For determination of K_M values the Hill coefficient was set to 1 (Michaelis-Menten kinetics). For determination of IC_{50} values of the inhibitors the Hill coefficient was variable and V_{max} was set to100%. The inhibition constant K_i for competitive inhibitors was calculated according to the equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_M}}$$

with the substrate concentration [S], the Michaelis constant K_M of the substrate and the measured IC_{50} of the compound.