CRF grant "Molecular study of lysosomal transporters involved in the cystine-depleting effect of cysteamine"

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The long-term objective of this grant is to identify the membrane transporters underlying the cystine-depleting effect of cysteamine as this would provide a rationale to improve its efficiency or discover more efficient, better tolerated drugs. According to the current model of cysteamine therapy, cystine depletion is mediated by an unknown cationic amino acid transporter, which exports a mixed disulfide of cysteine and cysteamine from the lysosomal lumen.

Paris group

(Adrien Jézégou, Christine Anne, Seana O'Regan, Christopher Ribes and Bruno Gasnier)

In previous unpublished studies, we obtained biochemical evidence for the existence of a lysosomal cationic amino acid transporter distinct from the candidates investigated thus far. The past months were devoted to the identification of this novel transporter in collaboration with a group of yeast geneticists in Brussels (B. André).

The Brussels group identified a membrane protein from the vacuole (the yeast equivalent of lysosomes) involved in lysine homeostasis. The presence of a homologous protein in purified liver lysosomes prompted us to analyze this novel candidate. The rat protein functionally complemented the yeast mutant. RT-PCR and immunofluorescence studies showed that the rat protein is ubiquitously expressed across tissues and that it localizes to lysosomes. Flux measurements showed that it transports cationic amino acids, as well as the mixed disulfide of cysteine and cysteamine formed in cystinotic lysosomes treated with cysteamine. Our current efforts are aimed at determining whether this novel transporter is required for the cystine-depleting effect of cysteamine in cultured patient fibroblasts.

Mainz group

(Ellen Closs, Jean-Paul Boissel and Isabel Jaenecke)

Our goal is to determine if members of the SLC7 sub-family of cationic amino acid transporters mediate not only plasma but also lysosomal membrane transport of these amino acids and thus are candidates to provide the salvage efflux pathway for cystine after cysteamine treatment. Our attempts to measure lysosomal transport using radioactive tracers have not been successful so far. In this grant period, we thus measured transport of cationic amino acids in lysosomal preparations using the method of solid supported membrane (SSM)-based electrophysiology.

Transporter-containing membrane fragments or vesicles are adsorbed to a SSM. The SSM consists of a lipid monolayer on a gold coated glass substrate which is coated with a long chained alkane thiol (Florin and Gaub 1993; Pintschovius, Fendler, and Bamberg 1999; Seifert, Fendler, and Bamberg 1993). After their integration into a flow system, sensor surfaces are exposed to

various solutions that can be changed rapidly. Figure 1 shows schematic pictures of the SSM principles.

Two kinds of lysosomal membrane preparations were used for the measurements. First, the preparation of the granular fraction C using a slightly modified protocol from Harms et al. and Pisoni et al. (Harms, Kern, and Schneider 1980; Pisoni et al. 1987). In this kind of preparation, non-lysosomal elements such as cell debris, nuclei, endoplasmatic reticulum and most of the mitochondria are removed by differential centrifugation at speeds between 900 and 10,000xg. Lysosomes then are pelleted at 20,000xg. Light endosomes and cytoplasmic proteins remain in the supernatant. Second, lysosomal membranes were isolated using a sucrose gradient as described by Schulz et al. (Schulz et al. 2010). According to the literature, lysosomal membranes accumulate at the border of 31% and 45% sucrose. So this fraction was used for SURFE^{2}R analysis.

Measurements were performed on the commercial system SURFE²R One (Surface Electrogenic Event Reader) from Scientific Devices Heidelberg GmbH (Heidelberg). In pre-tests with plasma membrane preparations of hCAT-2B-overexpressing CHOs and U373MG cells carried out by Scientific Devices Heidelberg GmbH, hCAT-2B exhibited electrogenic arginine transport measurable by the SURFE^{2}R. Subsequently we isolated fraction C and fraction 31/45 samples out of U373MG overexpressing hCAT-2B.EGFP, SLC7A14.EGFP or EGFP alone.

Sensors were loaded with the respective membranes and currents were measured for 1 second in non-activating buffer (140 mM NaCl, 30 mM HEPES pH 7.2; 2 mM $MgCl_2$, 1 mM Choline-Chloride), for 1 second in activating buffer (140 mM NaCl, 30 mM HEPES pH 7.2; 2 mM $MgCl_2$, 1 mM arginine) and again in non-activating buffer for 2 seconds. After 30 seconds the sensor was flushed again with non-activating buffer to wash away all remaining arginine and after a 5 minute rest the measurement was repeated. Of each cell line two Fraction C- and two fraction 31/45 preparations were made. Per fraction and preparation 2 Sensors were loaded and each sensor was measured 4 times.

Membrane preparations derived from of U373-hCAT-2B.EGFP-expressing cells showed a strong signal when arginine was present in the activating buffer, while in the measurements with EGFP- and SLC7A14.EGFP there was no amplitude visible. Statistical analysis of all data revealed an arginine-mediated amplitude in hCAT-2B.EGFP membranes that was significantly higher ($p < 0.0001$) than the one in EGFP membranes(. In contrast, the A14.EGFP membranes showed no significant increase in the arginine-mediated amplitude (18 ± 1 pA) compared to EFGP membranes.

As an alternative to the fraction C samples, we also measured arginine-mediated current changes with sensors loaded with fraction 31/45 samples derived from U373-EGFP, -hCAT-2B.EGFP und -A14.EGFP. Again, an arginine-mediated signal was only detected in membrane preparations from hCAT-2B.EGFP overexpressing cells.

As this method is very sensitive to contamination with plasma membrane we analyzed all the fractions on Western blots using antibodies against GFP, LAMP-1 and Na^+/K^+-ATPase. The EGFP fusion proteins were enriched in fraction C together with the lysosomal marker LAMP-1, while the content of the cytoplasmic localized EGFP was reduced in fraction C. However, the content of the plasma membrane marker Na^+/K^+-ATPase was also increased in all fraction C samples compared to the total cell homogenate, indicating a marked contamination with plasma

membrane. Similar results were obtained with membrane preparations using a sucrose gradient: The EGFP fusion proteins enriched in the fraction 31/45 together with LAMP-1, but also with Na^+/K^+-ATPase.

Our results using SSM indicate that hCAT-2B, but not SLC7A14 mediates lysosomal transport of cationic amino acids. Due to the contamination of the lysosomal preparations with plasma membrane proteins, we can however not be certain that the arginine-mediated signal seen with preparations from hCAT-2B.EGFP-overexpressing cells was derived from transporter localized in lysosomes. Membrane preparations from cells overexpressing the exclusively lysosomal localized SLC7A14.EGFP, gave no arginine-induced signal. This could be due to a lack of electrogenicity of arginine transport mediated by SLC7A14. Further experiments using a bona fide lysosomal transporter with electrogenic properties (such as cystinosin, (Ruivo et al. 2012)) are thus necessary to further establish this method. SSM has the big advantage to directly measure the activity of intracellular localized transporters within their physiological membrane environment and thus could be an excellent tool to screen for cysteamine analogues more suitable for treatment. We will thus further pursue our aim to improve the methods to purify intact lysosomes and measure transport directly.

References

Florin, E, and H Gaub. 1993. “Painted supported lipid membranes.” Biophysical Journal 64 (2): 375-83.

Harms, E, H Kern, and J Schneider. 1980. “Human lysosomes can be purified from diploid skin fibroblasts by free-flow electrophoresis.” Proceedings of the National Academy of Sciences of the United States of America 77 (10): 6139-43.

Pintschovius, J, K Fendler, and E Bamberg. 1999. “Charge translocation by the Na+/K+-ATPase investigated on solid supported membranes: cytoplasmic cation binding and release.” Biophysical Journal 76 (2): 827-36.

Pisoni, R L, K S Flickinger, J G Thoene, and H N Christensen. 1987. “Characterization of carrier-mediated transport systems for small neutral amino acids in human fibroblast lysosomes.” The Journal of Biological Chemistry 262 (13): 6010-7.

Ruivo, R, G C Bellenchi, X Chen, G Zifarelli, C Sagné, C Debacker, M Pusch, S Supplissone, and B Gasnier. 2012. “Mechanism of proton/ substrate coupling in the heptahelical lysosomal transporter cystrinosin.” Proceedings of the National Academy of Sciences of the United States of America 109 (5): E210-7.

Schulz, P, J Werner, T Stauber, K Henriksen, and K Fendler. 2010. “The G215R mutation in the Cl-/H+-antiporter ClC-7 found in ADO II osteopetrosis does not abolish function but causes a severe trafficking defect.” PloS one 5 (9): e12585.

Seifert, K, K Fendler, and E Bamberg. 1993. “Charge transport by ion translocating membrane proteins on solid supported membranes.” Biophysical Journal 64 (2): 384-91.

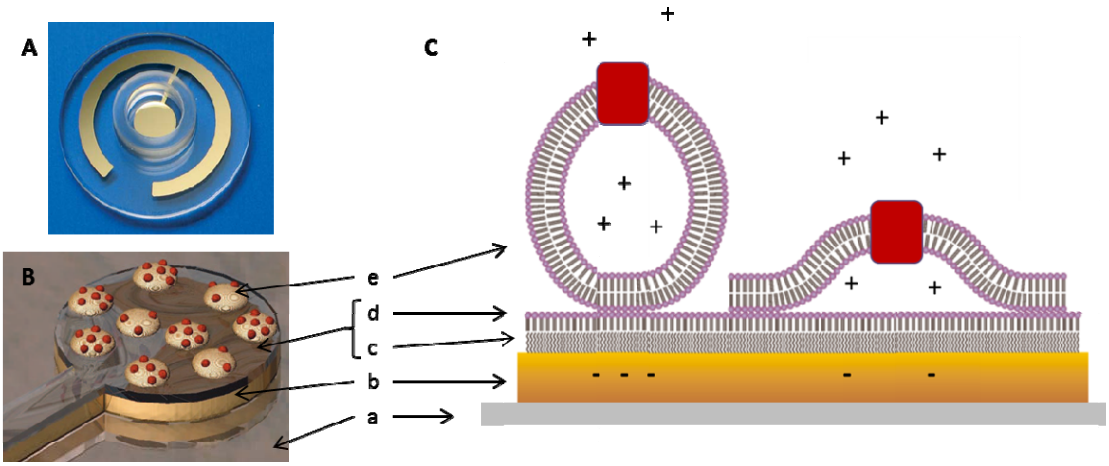

Figure 1: Principle of SSM-based electrophysiology

A) Glass substrate with gold surface. B+C) Schematic picture of a membrane fragment and a vesicle adsorbed to the SSM. Red rectangles indicate transport proteins. A gold layer (b) immobilized on a glass substrate (a) is made hydrophobic by alkane thiols (c). Binding of phospholipids (d) allows adsorption of membranes or vesicles (e). Selective, transporter-mediated movement of a charged substrate over a membrane leads to a change of the current. Pictures from the SURFE^{2}R manual.