Transporter Assays Using Solid Supported Membranes: A Novel Screening Platform for Drug Discovery

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Abstract: Transporters are important targets in drug discovery. However, high throughput-capable assays for this class of membrane proteins are still missing. Here we present a novel drug discovery platform technology based on solid supported membranes. The functional principles of the technology are described, and a sample selection of transporter assays is discussed: the H+/H11001-dependent peptide transporter PepT1, the gastric proton pump, and the Na+/H11001/Ca2+/H11001 exchanger. This technology promises to have an important impact on the drug discovery process.

Introduction

CONTROLLING THE FLUX of substrates and information across the cell membrane is one of the most challenging processes in biology. This function is accomplished by a large number of highly selective and effective proteins located in the cellular membrane. They transport molecules of all imaginable sizes and properties across the cell membrane, reaching from hydrogen atoms to macromolecules like peptides and even proteins. Transporters are involved in the uptake of nutrients, the build-up of membrane potential, the generation of muscle tension, and many other physiological processes. Consequently, membrane transport plays a crucial role in the development of disease, and transport proteins are important targets for drug discovery.1–3

Transport assays are notoriously difficult. In contrast to simple biochemical enzyme assays where the consumption of a substrate or the production of a product is monitored, transporter assays not only require a functional enzyme but also its proper incorporation into a membrane and the ability to assess the translocation of the substrates across this membrane. Among the many techniques used for the investigation of transport processes, electrical techniques play a central role. However, standard electrophysiological methods are technically challenging and time consuming and require well-trained personnel. In addition, automation is a challenging task with these techniques, and sensitivity is not sufficient to investigate low-turnover transporters with good quality.

Special techniques have been developed recently to observe charge displacements in electrically active transporters that are based on solid supported membranes (SSMs). They take advantage of the high specific capacitance and the ruggedness of a planar lipid bilayer prepared on a solid support. Liposomes in which the transporter has been incorporated (proteoliposomes) are adsorbed to the SSM, and the currents generated by the transporter activity are recorded via capacitive coupling.4–8 The compound membrane formed by the adsorbed proteoliposomes and the SSM can withstand high flow velocities, allowing for a fast solution exchange at the surface. By rapidly changing from a solution containing no substrate for the transporter to one that contains a substrate, the transporter can be activated, and a transient current can be recorded that contains information about the size and the temporal development of the transport process.9,10 This technique has been shown to

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ABBREVIATIONS: ATP, adenosine triphosphate; H+/K+-ATPase, proton- and potassium-dependent ATPase; NCX, Na+/Ca2+ exchanger; SSM, solid supported membrane.
possess a sensitivity comparable or even higher than standard electrophysiological methods like patch clamp and voltage clamp.\textsuperscript{11}

While this technique is interesting for the investigation of basic questions pertaining to the transport mechanism of transport proteins, it also contains potential for industrial applications. The SSM sensor is robust and inexpensive and can be used for many solution exchange processes. In addition, it has been shown that membrane preparations from native material or from overexpressing cell lines can be adsorbed to the SSM, and transient currents generated by the transporters in these membranes can be measured.\textsuperscript{10,11} The measurement is rapid, and automation and computer control are straightforward.

\begin{figure}
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\caption{Components of the SURFE\textsuperscript{2}R One system. (A) Schematic drawing of the SSM system. Three solution containers are used for storing of test solutions. A computer-controlled valve system allows the exchange of solutions on the sensor surface. The sensor consists of an SSM to which membrane fragments are adsorbed. The membrane fragments are capacitively coupled to the SSM, thereby enabling the measurement of transporter currents. (B) SURFE\textsuperscript{2}R One system. (C) The flow cell houses the sensor chip (inset) and connects it to the fluidic system.}
\end{figure}
The SSM has already been used in basic research for the investigation of several transport proteins belonging to different families and is expected to become a promising platform technology for drug development. Recently, the traditional SSM has been robotized and has become commercially available (SURFE²R, IonGate Biosciences, Frankfurt, Germany).

A Rapid Solution Exchange Technique for the Investigation of Electrogenic Transporters

The sensor

The SSM is prepared by linking an alkanethiol (octadecyl mercaptane) monolayer to a gold electrode deposited on a glass support and covering it with a lipid monolayer. The planar membrane formed has an area of 1–8 mm². Membrane fragments or proteoliposomes containing the protein of interest adsorb to the SSM as shown in Fig. 1A. The compound membrane composed of the SSM and the adsorbed proteoliposomes/membrane fragments form a capacitively coupled system that allows measurement of charge movements in the proteoliposomes/membrane fragments via the gold electrode and a reference electrode in the solution.

The set-up

In the set-up the SSM is mounted in a flow-through cuvette. The gold electrode is connected to an amplifier; the reference electrode is connected to the solution in the outward channel. The solution is driven through the cuvette by applying pressure (0.1–1 bar) to the solution containers connected to the cuvette (Fig. 1A). The solution flow is controlled by computer-controlled electromagnetic valves. Different solution exchange protocols can be applied to the electrode. Usually, before the start of the experiment the cuvette contains the resting solution. When the experiment is started, the resting solution is replaced by the nonactivating solution (concentration jump 1). The nonactivating solution does not contain the substrate of the transporter under investigation, but it may have a different composition than the resting solution. Thus a concentration gradient that may be required for the transport activity of the transporter may be established. In a second step the activating solution rapidly replaces the nonactivating solution (concentration jump 2). Finally, the solution in the cuvette is replaced first by the nonactivating solution (concentration jump 3) and then by the resting solution (concentration jump 4), which reestablishes the initial conditions. If no concentration gradient is required, a simplified protocol without resting solution may be used.

If not otherwise indicated the experiments shown below have been performed in a SURFE²R One instrument (IonGate Biosciences) (Fig. 1B). SURFE²R One consists of a central analytical unit and a separate power supply. The analytical unit houses the sensor (SSM), the flow cell (Fig. 1C), and a low-noise amplifier. Here the solution exchange protocol is controlled by computer, allowing for a completely automatic operation of the measurement cycle. For the inhibition studies and the measurements of the substrate concentration dependence, the instrument was equipped with a computer-controlled autosampler.

FIG. 2. (A) Solution exchange protocol for PepT1 measurements. The solution notations used here are: A = activating, B = nonactivating. First the sensor is bathed in nonactivating solution. Exchange of the nonactivating solution for a dipeptide-containing solution (B → A) leads to electrical activity of the protein that is detected by the instrument. The resulting currents show substrate concentration dependence: 0 mM Gly-Gly (gray line), 3 mM Gly-Gly (dashed line), and 20 mM Gly-Gly (black line). (B) Substrate affinity determination. Comparison of different substrates at different concentrations allows the comparison of their affinities on one sensor. Here two dipeptides (Gly-Gln [●] and Gly-Gly [*]) were compared: \( K_{0.5} \) (Gly-Gln) \( = 2.6 \) mM and \( K_{0.5} \) (Gly-Gly) \( = 5.4 \) mM.
Results and Discussion

The proton-dependent peptide transporter PepT1

The peptide transporter PepT1 (SLC15A1) occurs in several mammalian tissues. It is most abundant in brush border membranes of epithelial cells from the small intestines, where it serves as the main uptake system for di- and tripeptides.12 PepT1 is capable of transporting cationic, anionic, and zwitterionic substrates in a proton-dependent, electrogenic fashion. Apart from dipeptides and tripeptides, PepT1 also transports a range of peptidomimetic compounds, among them β-lactam antibacterics as well as several peptidase and protease inhibitors.

Owing to this comprehensive spectrum of substrates, PepT1 is highly relevant in drug delivery and an important target in pharmacology. SURFE2R technology can be used for the electrophysiological characterization of PepT1, using membranes isolated from a Chinese hamster ovary cell line expressing human PepT1. This allows identification of PepT1 substrates and inhibitors.

To establish a SURFE2R assay for studies of PepT1-mediated transport, we analyzed electrical responses to the addition of dipeptides in the presence and absence of different PepT1 inhibitors. The membranes were allowed to adsorb to the SSM, and the peptide transporter was activated by a substrate concentration jump. The solution exchange protocol is shown in Fig. 2A. Under resting conditions, the SSM flow cell contains the nonactivating solution (solution B in Fig. 2). The solution exchange consists of three flow phases of 0.5 to 1 s in duration: (1) non-activating solution, (2) activating solution (solution A), and (3) nonactivating solution. The activating solution contains a substrate of the transporter. To avoid solution exchange artifacts, the nonactivating solution contains the nontransported amino acid glycine. Transient currents due to the transport activity of PepT1 were observed (Fig. 2A) when switching from the nonactivating to the activating solution (B → A) and from the activating to the nonactivating (A → B). Only the transient current at the solution exchange B → A was used for the analysis.

The amplitude of the electrical response to both substrates, Gly-Gln and Gly-Gly, was concentration-dependent (Fig. 2). Half-saturating concentrations for Gly-Gln of 2.6 mM and for Gly-Gly of 5.4 mM were determined. The values are higher (10 times higher for Gly-Gln, five times higher for Gly-Gly) than data obtained by other groups.13,14 This difference in apparent affinities may reflect different physiological properties as well as technological parameters of the applied methods: for instance, in the studies mentioned above steady-state methods have been applied (two-electrode voltage-clamp and uptake assays), while the SURFE2R assay represents a pre–steady-state relaxation method.15

FIG. 3. Inhibition of PepT1 currents by Lys-[Z(NO2]-Val. Application of 100 μM Lys-[Z(NO2]-Val (dashed line) reduced dipeptide-induced currents compared with 3 mM Gly-Gly (gray line). Upon washout (black line) of the inhibitor the signal is restored to more than 90% of the original amplitude.

FIG. 4. Identification of PepT1 substrates. Using drugs as substrates in the SURFE2R experiment allows the identification of substrates. The relative activity induced by diverse substrates (here, captopril [dashed line] and valacyclovir [dark gray line]) is related to signals that are found upon application of Gly-Gly dipeptide to PepT1 membranes (light gray line) and to signals that are measured upon application of drugs on control membranes that are not expressing PepT1 protein (black line). For nonsubstrates, substrate application on control and PepT1 membranes yields identical results (data not shown).
Lys-[Z(NO2)]-Val and glibenclamide are inhibitors of the PepT1 transporter.14,16 Accordingly, PepT1-mediated responses were suppressed by Lys-[Z(NO2)]-Val (Fig. 3) as well as by glibenclamide (data not shown), and the inhibition was reversible (Fig. 3). Affinities of inhibitors determined using the SURFE2R technology were lower than published data by a factor of 2–8.

Uptake of pharmacologically relevant compounds in the small intestine is an important issue for the effectiveness of a drug. PepT1 plays a key role for this process. We have, therefore, investigated the transport properties of PepT1 using the antihypertensive drug captopril and the antiviral drug valacyclovir.14,17 As shown in Fig. 4, these compounds are both transported by PepT1. The results presented demonstrate that the SURFE2R technology is suitable for direct studies of the interaction between PepT1 and drug. In contrast to radioactive uptake measurements, the SURFE2R technology allows a distinction between transportable drugs and competitive inhibitors. Therefore, the SURFE2R technology is perfectly suited for the search for new substrates of human PepT1.

The gastric proton pump

Acid secretion in the stomach is mediated by a proton- and potassium-dependent ATPase (H⁺/K⁺-ATPase) of parietal cells, the gastric proton pump. This enzyme is a drug target for the treatment of ulcers and gastro-
esophageal reflux disease. Many of the so-called proton pump inhibitors belong to the group of substituted pyridinemethylenbenzimidazoles. Omeprazole (Prilosec®, AstraZeneca, London, UK), lansoprazole (Takeda, Osaka, Japan), pantoprazole (Protonix®, Altana, Bad Homburg, Germany), and pariprazole (Eisai, Tokyo, Japan) are the corresponding drugs available on the market today. Much of the early work that ultimately led to development of these drugs has been done on animal models. Those experiments are expensive and time consuming. Progress was made when isolated vesicles were used and assayed for phosphate release in the absence and presence of inhibitors. However, real-time monitoring was not possible at this stage since the phosphate determination requires the addition of further reagents that are not compatible with the enzyme activity.

The catalytic cycle of the H+/K+-ATPase is electroneutral. However, proton transport in the absence of K+ is electrogenic, giving rise to a detectable current. To demonstrate the potential of the SURFE2R-based screening system, the current response to an adenosine triphosphate (ATP) concentration jump has been recorded using microsomes with H+/K+-ATPase prepared from pig stomach (Fig. 5A). The preparation of the sensor and the measurement procedure are similar to that described in the previous section. Large transient currents were observed, and incubation with 10 μM activated omeprazole led to a complete inhibition of the response (Fig. 5B). This demonstrates the specific response of the SURFE2R assay and its application also with tissue-based preparations. In addition, it is worth noting that using appropriate conditions, electroneutral transporters can also be investigated.

Complete and irreversible inhibition with omeprazole is found after an incubation time of ~15 min. As a comparison, the reversible proton-pump inhibitor SCH28080 was also tested. The result is shown in Fig. 5C. Here we demonstrate inhibition and washout of the drug on the same sensor. An investigation of activity and mode of action gives first results within 1–2 h compared with much longer experiments using conventional approaches.

The Na+/Ca2+ exchanger (NCX)

The cardiac NCX (NCX1), a plasma membrane antiporter, is almost certainly the major Ca2+ extrusion mechanism in cardiac myocytes. Enhanced expression of NCX1 has recently been recognized as one of the molecular mechanisms that contribute to reduced Ca2+ release, impaired contractility, and an increased risk of arrhythmias during the development of cardiac hypertrophy and failure. The NCX1 has also been implicated in the mechanism of cellular injury associated with ischemia and reperfusion. Thus, the NCX1 plays a key role in the Ca2+ homeostasis of cardiomyocytes and is discussed as a potential drug target. In addition, all transporters of the NCX family may play a role with regard to potential side effects of drugs and could be used as an in vitro assay to characterize pharmacological profiles of new leads.

Rapid generation of stably transfected cell lines was achieved using the Flp-In™ T-Rex™ expression system (Invitrogen, Carlsbad, CA). Chinese hamster ovary cells were cultivated, and membranes were harvested as described. The membranes were allowed to adsorb to the SURFE2R sensor as shown in Fig. 1A. In contrast to the above-mentioned systems, NCX needs a Na+ gradient as an energy source for the transport of Ca2+. The NCX solution exchange protocol, therefore, is an additional phase where the Na+ gradient is established. Before and after the experiment, the sensor is in contact with resting solution (solution C in Fig. 6), which ensures that the buffer in the compartment between the membrane and the SSM contains Na+. Now a Na+ gradient is built up by exchanging the Na+ -containing resting solution by a Na+-free nonactivating solution (C → B) as shown in Fig. 6. In the next step, transport is initiated by providing Ca2+-containing activating solution (B → A), and the charge translocation is recorded. The transient currents observed approximately 1 s after the C → B and the B → C exchanges represent exchange artifacts due to nonbalanced solutions. They are useful because they allow estimation when the gradient is fully established but are not used for the analysis of NCX activity.

FIG. 6. Solution exchange protocol for NCX experiments. The solution notations used here are: A = activating, B = nonactivating, and C = resting solution. For detection of NCX activity a sodium gradient is established in a first concentration jump (C → B). The second concentration jump (B → A) activates the transport cycle by adding Ca2+ ions to the solution. After activation the solution is switched back to resting solution to re-establish the initial experimental conditions.
A direct comparison of the signals generated by the SURFE2R technology with standard electrophysiology is shown in Fig. 7. A Ca\(^{2+}\)-induced transient current obtained on a SURFE2R electrode is shown in Fig. 7A. Whole-cell patch-clamp recordings of human embryonic kidney 293 cells heterologously expressing the human NCX1 were performed as previously described (Fig. 7B). Inhibition of NCX was tested using the NCX-specific inhibitor A (Fig. 8).\(^{10}\) In the SURFE2R measurement the 50% inhibitory concentration value for compound A was determined to be 0.9 ± 0.2 \(\mu\)M with a Hill coefficient of 0.7 ± 0.03. Subsequent experiments in the absence of inhibitor A resulted in NCX peak currents with up to 100% of the amplitude of the initial signal, proving the inhibitor’s reversibility. In the patch-clamp experiment, the 50% inhibitory concentration value determined was 1.4 ± 0.4 \(\mu\)M, with a Hill coefficient of 0.43 ± 0.03.

It seems clear that in the case of the NCX1, SURFE2R signals yield up to 10 times higher amplitudes than those from patch-clamp recordings (Fig. 7). Moreover, a better signal-to-noise ratio is obtained. The obvious reason for these two effects is the large amount of membranes bound to the biosensor chip containing the target molecules. Thus, the biosensor is able to collect signals from a higher number of ion transporters. An additional cause for a higher signal-to-noise ratio, however, may be the triggered release of transporter substrate. The electronegic step of all proteins is started in a synchronized fashion within an activation time of 5–10 ms. This is most obvious in the case of the \(\text{H}^+/\text{K}^+\)-ATPase, where no stationary response at all is expected because of its electroneutrality but where nevertheless a transient current is observed (see above). This technological feature not only increases the signal-to-noise ratio but also allows investigation of rate-limiting steps in the transport cycle of transporters and enables mode-of-action approaches in the drug discovery process.

Another striking observation is the shifted 50% inhibitory concentration, with higher sensitivity in SURFE2R experiments. A comparison of SURFE2R and patch-clamp inhibition is given in Fig. 8. It is obvious that, especially for low inhibition, the inhibitory effect is larger in the SURFE2R experiments. A straightforward explanation for this effect can be found in the different realization of patch-clamp and SURFE2R experiments. The signals generated with the sensors in the SURFE2R are stable for several hours, showing only a moderate linear rundown. In contrast, the patch-clamp experiments have to be performed as quickly as possible because of the limited stability of the patch and rundown of the observed currents during the experiment. These two effects limit the experimental time to 20–30 min. Thus, equilibration of the cell with the given inhibitor concentration may not be perfect, leading to an underestimation of the inhibitory potency of the compound.\(^{11}\) Note that error bars in SURFE2R experiments are approximately five times smaller than in patch-clamp experiments, leading to a higher significance of the experiments.

![Fig. 7. Comparison of NCX traces recorded with the SURFE2R technology (A) and patch clamp (B). The signals found with the SURFE2R are approximately one order of magnitude higher than patch-clamp currents and show a very good signal-to-noise ratio.](image1)

![Fig. 8. Correlation of inhibition by 14 different inhibitors tested at 10 \(\mu\)M concentration in SURFE2R and patch-clamp experiments. Note that error bars in SURFE2R experiments are approximately five times smaller than in patch-clamp experiments, leading to a higher significance of the experiments. All errors are quoted as standard error of the mean; small error bars are hidden by the symbol.](image2)
Conclusions

The SURFE\textsuperscript{2}\textsuperscript{R} technology reviewed here enables efficient research on a broad spectrum of transporters. More than 20 different transporters from mammalian and bacterial origin could be investigated up to now using this technique, among them ATPases, cotransporters, exchangers, and uniporters. The most versatile preparations are probably membranes from stably infected cell lines, but also preparations from tissue and protein reconstituted in liposomes can be investigated.\textsuperscript{25} Existing bottlenecks in drug discovery projects for transporters can be overcome by detecting transporter function directly with high quality and robustness. Optimization cycles during the hit-to-lead process can be improved with respect to efficiency and success.\textsuperscript{5} Existing bottlenecks in drug discovery projects for transporters can be overcome by detecting transporter function directly with high quality and robustness. Optimization cycles during the hit-to-lead process can be improved with respect to speed and reliability using the currently available bench-top system: SURFE\textsuperscript{2}\textsuperscript{R} One. For higher throughput a standard multiwell plate-based version, the SURFE\textsuperscript{2}\textsuperscript{R} Workstation, is available.

References

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