Real-Time Transmembrane Translocation of Penetratin Driven by Light-Generated Proton Pumping

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ABSTRACT Cell penetrating peptides (CPPs) are small peptides that are able to penetrate the plasma membrane of mammalian cells. Because these peptides can also carry large hydrophilic cargos such as proteins, they could potentially be used to transport biologically active drugs across cell membranes to modulate in vivo biology. One characteristic feature of the CPPs is that they typically have a net positive charge. Therefore, a key issue associated with the transport mechanism is the role of the transmembrane electrochemical potential in driving the peptides across the membrane. In this study, we have reconstituted bacteriorhodopsin (bR) in large unilamellar vesicles (LUVs) with fluorescein-labeled CPP penetratin enclosed within the LUVs under conditions when the fluorescence is quenched. Illumination of the bacteriorhodopsin-containing LUVs resulted in creation of a transmembrane proton electrochemical gradient (positive on the inside). Upon generation of this gradient, an increase in fluorescence was observed, which shows that the proton gradient drives the translocation of penetratin. The mechanism most likely can be generalized to other CPPs.

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The cell penetrating peptides (CPPs) are peptides commonly composed of <30 amino acids. They do not share common sequence motifs; however, all CPPs are amphiphilic and carry a net positive charge at physiological pH (1). These peptides are either model peptides designed to have specific properties or they are small parts of larger proteins. The CPPs can also carry large molecules across membranes and could therefore be used to transport, e.g., drugs across cell membranes (2–6). The CPP penetratin, derived from the third helix of the homeodomain of the *Drosophila melanogaster* transcription factor Antennapedia, belongs to the latter class of peptides. It is composed of 16 amino acids, with the sequence RQIKIWFQNRRMKWKK; i.e., it contains seven positively charged residues; three Arg and four Lys (7). For CPPs in general, it has been suggested that transmembrane translocation may be mediated through endocytosis; however, translocation of a wide range of CPPs has been observed also under conditions when endocytosis is inhibited (for a recent review, see Magzoub and Gräslund (8)).

In the case of an HIV-1 Tat protein-derived CPP, it has been demonstrated that the translocation is initiated by binding to the membrane surface followed by penetration through macropinocytosis, a special form of endocytosis. (9). According to this proposed mechanism, after the initial binding to the membrane surface, an invagination is formed such that eventually the peptide is found inside a large vesicle, a macropinosome, on the opposite side of the membrane. In the next step, the peptide has to escape from the macropinosome before it reaches the lysosome. It has been suggested that the peptide penetration across the macropinosome membrane is driven by enzymatic activity inside the macropinosome that leads to a decrease in the pH (10–12), resulting in creation of a proton electrochemical gradient that renders the inside of the macropinosome positive. This mechanism would indicate that the membrane potential drives the CPP transport in the final step of the translocation process. If this is the case, the CPPs could in principle also translocate directly across cell membranes carrying a transmembrane electrochemical gradient. Thus, controlling and observing the membrane potential during CPP translocation is of key importance when understanding the mechanism by which peptides like the Tat-derived segment or penetratin are transported and in the design of new cell-specific CPPs.

In this study, we present a novel method that was used to investigate the influence of a transmembrane electrochemical gradient on the peptide transport in a well-defined in vitro system using bacteriorhodopsin (bR), which is a light-driven proton pump (see Heberle (13)), inserted into large unilamellar vesicles (LUVs, 100 nm diameter (14)). The bR was oriented in the membrane such that it pumped protons into the vesicles upon illumination, creating an inside positive electrochemical proton gradient across the vesicle membrane. Fluorescein-labeled penetratin (flu-penetratin) was enclosed within the bR-LUVs either at a high enough concentration to self-quench the fluorescence, or together with potassium iodide, which is a fluorescence quencher. Thus, any translocation of the peptide out of the LUVs is expected to result in an increase in the fluorescence intensity (Fig. 1).

To establish the formation and decay rates of the proton electrochemical gradient, we measured absorbance changes of the pH-sensitive dye phenol red added on the outside of

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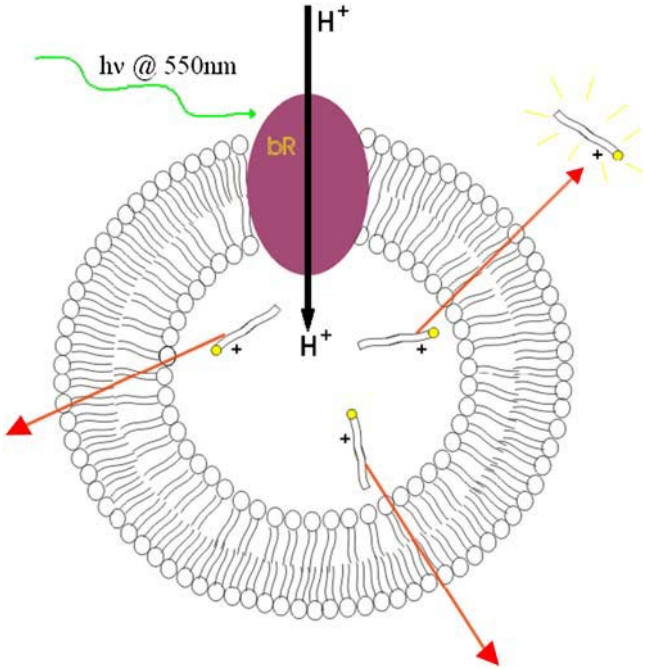

FIGURE 1 Experimental system. Fluorescein (yellow)-labeled penetratin (positively charged) was enclosed within large unilamellar vesicles (LUVs) containing bacteriorhodopsin (bR) oriented such that upon illumination (hν) it pumps protons into the LUVs. The transfer of penetratin out of the LUVs (*red arrows*) results in an increase in fluorescence.

the bR-LUVs. As seen in Fig. 2, upon initiation of illumination, the dye absorbance increased and reached a maximum level after ~5 min. The increase in absorbance is consistent with an increase in the pH on the outside of the LUVs, due to proton pumping by bR from the outside into the LUVs. Upon cessation of illumination, the pH decreased slowly due to proton leakage across the membrane out of the vesicles. After return of the absorbance to the original level in the dark, the same absorbance changes were observed after a repeated illumination period, which indicates that the vesicles were stable in the presence of an electrochemical gradient. The formation and decay kinetics of the gradient were also detected using the fluorescent probe Oxonol VI (not shown), which responds to changes in the electrical potential across the phospholipid bilayer. The fluorescence increased by ~15%, and then decreased to the initial level with about the same kinetics as that observed with phenol red.

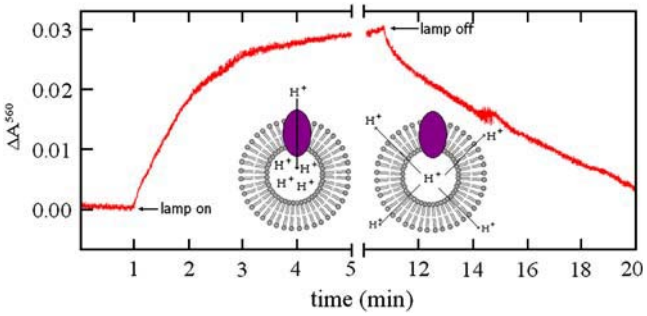

FIGURE 2 Light-induced proton pumping across bR-containing LUVs. The increase in absorbance is associated with proton pumping into the vesicles. Conditions: 1.5 μM bR, 100 mM KCl, and 20 mM potassium phosphate buffer inside, and 120 mM KCl and 17 μM phenol red outside of LUVs; pH 7.4, 20°C.

Fig. 3 shows fluorescence changes of a sample containing bR reconstituted in LUVs composed of the zwitterionic lipid 1-palmitoyl-2-oleoyl-phosphatidylcholine with flu-penetratin and the fluorescence quencher potassium iodide on the inside of the vesicles. In the dark, we observed a small increase in the fluorescence intensity, which is presumably due to a slow leakage of flu-penetratin out of the LUVs. The same leakage rate was observed when flu-penetratin was enclosed in LUVs without bR (not shown), which indicates that in the dark the leakage rate is independent of the presence of the protein reconstituted in the membrane.

Upon illumination of a bR-LUV sample containing flu-labeled penetratin, a significantly faster increase in fluorescence intensity was observed (Fig. 3), which indicates that the migration of penetratin out of the LUVs accelerates upon establishment of an electrochemical proton gradient across the membrane. After ~180 min, the intensity reached a value corresponding to transport of ~20% of penetratin out of the LUVs. In these measurements, 100% release was taken as the fluorescence intensity measured after addition of detergent (0.3% Triton X), which breaks up the LUV membrane.

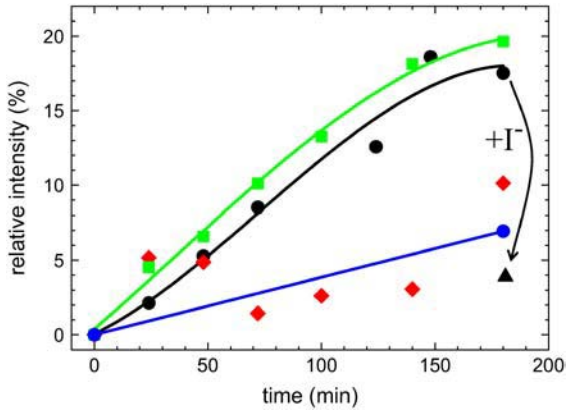

FIGURE 3 Fluorescence changes of the bR-containing LUVs with fluorescein-labeled penetratin on the inside. Changes in fluorescence at 537 nm (excitation wavelength 494 nm) were monitored in the absence of illumination (*blue*, ●) and in the presence of illumination (*black*, ●). Also shown are fluorescence changes upon addition of FCCP (*red*, ♦) and valinomycin (*green*, ■) in the presence of illumination. The arrow shows the fluorescence change upon addition of KI to a sample after illumination. Conditions: 100 mM KCl, 20 mM KP_i buffer, pH 7.4, 20°C, 1.5 μM bR, 250 μM flu-penetratin, and 50 mM potassium iodide inside LUVs; 100% is taken as the fluorescence in the presence of 0.3% Triton X, which breaks up the vesicles.

A <100% saturation level has been observed previously in investigations of the leakage of calcein from vesicles (see e.g., Magzoub et al. (12)).

No measurable changes in fluorescence were observed in the presence of iodide added to the solution on the outside of the LUVs before illumination. When iodide was added after an illumination period, the fluorescence level decreased to a level slightly above the initial level (Fig. 3). These results further support the conclusion that the increase in fluorescence observed upon illumination is due to transport of the flu-labeled penetratin out of the LUVs and not, for example, due to swelling of the LUVs as a result of proton pumping. No changes in fluorescence were observed upon illumination of bR-LUVs in the absence of flu-labeled penetratin.

As a further control, the proton ionophore FCCP was added to the bR-LUVs containing flu-labeled penetratin before illumination. Because the ionophore carries protons across the membrane, there is no proton electrochemical potential gradient established upon illumination. In the presence of FCCP upon initiation of illumination, we first observed a rapid increase in the fluorescence level after which the rate was about the same as that in the absence of illumination. Fluorescence changes were also investigated in bR-LUVs in the presence of the potassium ion ionophore valinomycin, which dissipates the electrical potential by transporting K^+ ions out of the LUVs. In this case, there was no significant effect of the ionophore addition, which indicates that a proton-concentration gradient is sufficient for transport of penetratin out of the LUVs.

As pointed out above, it has been discussed that the initial interaction of the peptide with the membrane surface may govern the transport efficiency. Structural studies of peptides interacting with vesicles have indicated only weak interactions with zwitterionic lipids, whereas much stronger interactions were observed with negatively charged membranes (15) Here, we have used zwitterionic LUVs, and found that penetratin translocates across the membrane aided by a proton gradient. Significant translocation was also induced in partly negatively charged bR-LUVs composed of 30% of the negatively charged lipid 1-palmitoyl-2-oleoyl-phosphatidylglycerol and 70% 1-palmitoyl-2-oleoyl-phosphatidylcholine (data not shown). Because translocation was observed with both zwitterionic and negatively charged bilayers, the strength of the initial binding of the peptide to the membrane surface appears not to be critical for the penetration process.

In summary, the results show that establishment of a transmembrane proton electrochemical gradient significantly accelerates translocation of penetratin across the membrane. Because most CPPs identified to date carry a net positive charge, a potential may be important for the translocation of most such peptides. Future studies will be important to quantify these effects and to be able to predict sequence and structural characteristics required for membrane penetration.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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