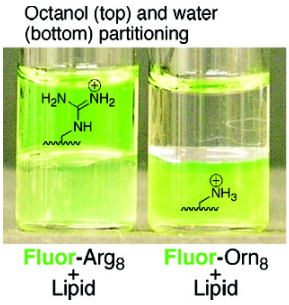
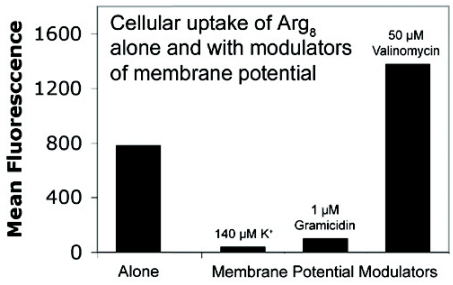
Communication

Role of Membrane Potential and Hydrogen Bonding in the Mechanism of Translocation of Guanidinium-Rich Peptides into Cells

Jonathan B. Rothbard, Theodore C. Jessop, Richard S. Lewis, Bryce A. Murray, and Paul A. Wender

J. Am. Chem. Soc., **2004**, 126 (31), 9506-9507• DOI: 10.1021/ja0482536 • Publication Date (Web): 20 July 2004





More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 18 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 07/20/2004

Role of Membrane Potential and Hydrogen Bonding in the Mechanism of Translocation of Guanidinium-Rich Peptides into Cells

Jonathan B. Rothbard,[†] Theodore C. Jessop,[‡] Richard S. Lewis,[§] Bryce A. Murray,[†] and Paul A. Wender*[,‡]

Departments of Chemistry and Molecular & Cellular Physiology, Stanford University, Stanford, California 94305, and CellGate, Inc., Sunnyvale, California 94086

Received March 26, 2004; E-mail: wenderp@stanford.edu

Biological membranes have evolved in part to prevent xenobiotics from passively entering cells.[1] Numerous organisms have developed proteins, many of which are transcription factors, that breach these biological barriers through a variety of mechanisms.[2] The protein HIV tat, for example, when used in vitro rapidly enters the cytosol (and nucleus) of a wide spectrum of cells by endocytosis.[3] However, the nine amino acid peptide required for the uptake of HIV tat, residues 49−57 (RKKRRQRRR), appears itself to utilize an additional mechanism, as evident from its uptake even at 4 °C, by a route differentiated from the intact protein.[4] We have found that guanidinium-rich oligomers enter suspension cells more effectively than the tat nonamer[5] often without the production of observable endocytotic vesicles.[6,7] We describe herein studies on the cellular uptake mechanism of guanidinium-rich transporters conjugated to small molecules (MW ca. <3000).

Our previous studies on tat 49−57 demonstrated that the guanidinium headgroups are principally responsible for its uptake into cells. Replacing all nonarginine residues in the tat nonamer with arginines provides transporters that exhibit superior rates of uptake. Charge itself is necessary but not sufficient because lysine nonamers show poor uptake.[5,6] The number of arginines is important, with optimal uptake for oligomers of 7−15 residues.[6,8] Backbone chirality is not critical for uptake. Even the position of attachment and length of the side chains can be altered as shown with guanidinium-rich peptoids that exhibit highly efficient uptake. Changes in the backbone composition and in the side chain spacing can also increase uptake.[5,9] Even highly branched guanidinium-rich oligosaccharides and dendrimers are efficient transporters.[7,10,11]

Several mechanisms could accommodate the above structure function relationships, and some could operate concurrently. A receptor-mediated process is inconsistent with the broad range of structural modifications that promote uptake. Conventional passive diffusion across the nonpolar interior of the plasma membrane is difficult to reconcile with the polarity of the arginine oligomers and the dependency of uptake on the number of charges. However, the polarity of the guanidinium groups could be attenuated through association with cell surface groups bearing a complementary charge (phospholipids, fatty acid salts, and sulfates), thereby producing a less polar ion pair complex capable of diffusing into the membrane. To test this point, a fluoresceinated arginine octamer (Fl-aca-D-Arg_8-$CONH_2$) was added to a bilayer of octanol and water. Not surprisingly, the highly polar charged system partitioned almost exclusively into the water layer (Figure 1, inset B). When, however, a surrogate for a membrane bound fatty acid salt, namely, sodium laurate, was added to this mixture, the transporter partitioned significantly (>95%) into the octanol layer (inset D).[12] The relative partitioning was quantified by separation of the layers and analysis of the dissolved agents.

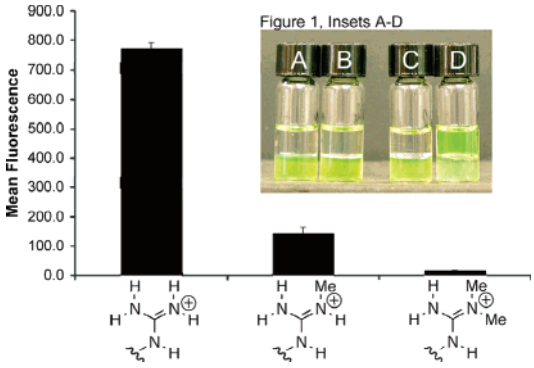


Figure 1. Uptake of Fl-aca-D-Arg_8-$CONH_2$ (left), Fl-aca-$Arg^m{}_8$-$CONH_2$ (center), and Fl-aca-$Arg^{mm}{}_8$-$CONH_2$ (right) into Jurkat cells (5 min, 50 mM); Fl = fluorescein-HNC(S)−, aca = aminocaproic acid, Arg^m = N^G-methylarginine, Arg^{mm} = N^G,N^G-dimethylarginine. Inset: Octanol/water (upper and lower phases, respectively) partitioning of Fl-aca-Orn_8-$CONH_2$ and Fl-aca-D-Arg_8-$CONH_2$ alone (A, B) and after addition of sodium laurate (C, D).

While other polycations such as short ornithine oligomers might participate in a similar ion pair mechanism, they are observed to be significantly less effective in cellular uptake than the arginine oligomers. This difference could arise in part from the more effective bidentate hydrogen bonding possible for guanidinium groups. Consistent with this analysis, when ornithine oligomers were submitted to the above two-phase partitioning experiments, they preferentially stayed in the aqueous layer even with added sodium laurate (insets A and C).

Further evidence for the importance of hydrogen bonding in uptake arises from the study of alkylated guanidinium oligomers. While incorporating the dispersed cationic charge of a guanidinium group, these alkylated guanidiniums have an attenuated ability to form hydrogen bonds. When octamers of mono- and dimethylated arginine (Fl-aca-$Arg^m{}_8$-$CONH_2$, Fl-aca-$Arg^{mm}{}_8$-$CONH_2$) synthesized from the corresponding ornithine octamer were assayed for cell entry, uptake of the former was reduced by 80% and the latter by greater than 95% when compared with an unalkylated arginine octamer (Figure 1).

While charge complementation with endogenous membrane constituents allows for entry into the membrane, it does not explain the driving force for passage through the membrane and the energy dependency of uptake observed in some studies. Given that phospholipid membranes in viable cells exhibit a membrane potential, the maintenance of which requires ATP, we reasoned that uptake of cation-rich transporters might be driven by the voltage potential across most cell membranes.[13]

[†] CellGate, Inc.
[‡] Department of Chemistry, Stanford University.
[§] Department of Molecular & Cellular Physiology, Stanford University.

10.1021/ja0482536 CCC: $27.50

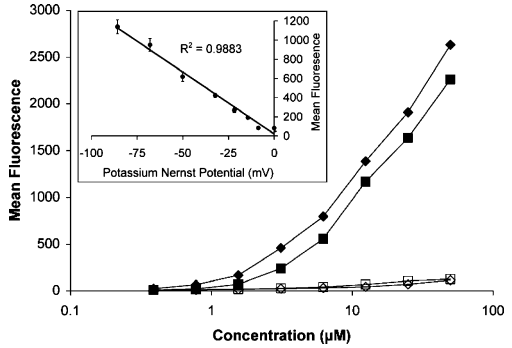


Figure 2. Uptake of Fl-aca-D-Arg_8-$CONH_2$ (diamonds) and Fl-aca-tat_{49-57}-$CONH_2$ (squares) into Jurkat cells in PBS (solid markers) and K^+PBS (outlined markers). Inset: At various extracellular concentrations of K^+, uptake of Fl-aca-D-Arg_8-$CONH_2$ was measured and plotted as a function of calculated potassium Nernst potential.

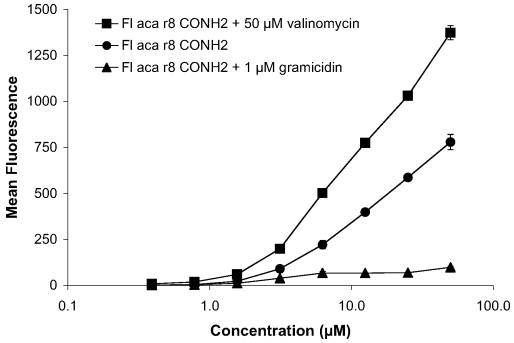


Figure 3. Cellular uptake of Fl-aca-D-Arg_8-$CONH_2$ alone and with or without preincubation of cells with valinomycin or gramicidin A.

To test this hypothesis, the membrane potential was reduced to close to zero by incubating the cells with an isotonic buffer with potassium concentrations equivalent to that found intracellularly. The intracellular concentration of K^+ in lymphocytes is ~140 mM, whereas the extracellular concentration is ~5 mM.[14] Replacement of a portion of the sodium salts in PBS with equimolar amounts of the equivalent potassium salts afforded what was called K^+PBS. To test whether the membrane potential in lymphocytes was a factor in transport of guanidinium-rich transporters, fluorescently labeled tat 49−57 (Fl-aca-tat_{49-57}-$CONH_2$) and an octamer of D-arginine (Fl-aca-D-Arg_8-$CONH_2$) were incubated individually with Jurkat cells for 5 min in either PBS or K^+PBS. The cells were washed and analyzed by flow cytometry. Uptake was reduced by greater than 90% at all concentrations when the assay was done in the presence of a buffer with a high concentration of potassium (Figure 2). The observed inhibition of uptake was equivalent to that seen when the cells were pretreated with sodium azide.

Repeating the uptake experiment with a series of buffers whose K^+ concentration varied between 140 mM and zero showed that uptake decreased with an increase in the external concentration of potassium. The uptake as measured by cellular fluorescence varied linearly with the K^+ Nernst potential[15] calculated across the range of extracellular K^+ concentrations (Figure 2, inset).

To explore whether high potassium buffers inhibited uptake by modulating the membrane potential or by an alternative effect, lymphocytes were pretreated with gramicidin A, a pore-forming peptide known to reduce membrane potential,[16] prior to the addition of Fl-aca-D-Arg_8-$CONH_2$. This procedure reduced cellular uptake by more than 90% (Figure 3). The reciprocal experiment, hyperpolarizing the cell to increase uptake, was accomplished with valinomycin, an antibiotic that selectively shuttles potassium ions across the membrane.[17] When Jurkat cells were preincubated with 50 μM valinomycin, the uptake of Fl-aca-D-Arg_8-$CONH_2$ was significantly increased (Figure 3).

Collectively, these studies provide a mechanistic hypothesis for how short oligomers of arginine can migrate across the plasma membrane of a cell. The water-soluble, positively charged guanidinium headgroups of the transporter form bidentate hydrogen bonds with H-bond acceptor functionality on the cell surface. The resultant ion pair complexes partition into the lipid bilayer and migrate across at a rate proportional to the membrane potential. The complex dissociates on the inner leaf of the membrane and the transporter enters the cytosol. This mechanism is consistent with the highly permissive structure−function relationships, the apparent lack of cell-type specificity and uptake being slowed but not inhibited at 4 °C. This hypothesis does not preclude competing uptake by other mechanisms including endocytosis, which is likely to dominate with large cargos.[3,18] Further studies and applications are in progress.

Acknowledgment. Support of this work by grants from the National Institutes of Health (CA31841, CA31845) is gratefully acknowledged.

Supporting Information Available: Experimental results and procedures for the synthesis and assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Alberts B. B. D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J. D. *Molecular Biology of the Cell*; 3rd ed.; Garland: New York, 1994.
(2) Joliot, A.; Prochiantz, A. *Nat. Cell Biol.* **2004**, *6*, 189.
(3) Mann, D. A.; Frankel, A. D. *Embo J.* **1991**, *10*, 1733.
(4) Silhol, M.; Tyagi, M.; Giacca, M.; Lebleu, B.; Vives, E. *Eur. J. Biochem.* **2002**, *269*, 494.
(5) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003.
(6) Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B. *J. Pept. Res.* **2000**, *56*, 318. (See also Supporting Information)
(7) Luedtke, N. W.; Carmichael, P.; Tor, Y. *J. Am. Chem. Soc.* **2003**, *125*, 12374.
(8) Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2001**, *276*, 5836.
(9) (a) Wender, P. A.; Rothbard, J. B.; Jessop, T. C.; Kreider, E. L.; Wylie, B. L. *J. Am. Chem. Soc.* **2002**, *124*, 13382. (b) Rothbard, J. B.; Kreider, E.; VanDeusen, C. L.; Wright, L.; Wylie, B. L.; Wender, P. A. *J. Med. Chem.* **2002**, *45*, 3612. (c) Umezawa, N.; Gelman, M. A.; Haigis, M. C.; Raines, R. T.; Gellman, S. H. *J. Am. Chem. Soc.* **2002**, *124*, 368. (d) Rueping, M.; Mahajan, Y.; Sauer, M.; Seebach, D. *ChemBioChem* **2002**, *3*, 257.
(10) (a) Futaki, S.; Nakase, I.; Suzuki, T.; Youjun, Z.; Sugiura, Y. *Biochemistry* **2002**, *41*, 7925. (b) VanDeusen, C. Ph.D. Thesis, Stanford University, Stanford, CA, 2003.
(11) Chung, H.; Harms, G.; Seong, C. M.; Choi, B. H.; Min, C.; Taulane, J. P.; Goodman, M. *Biopolymers* **2004**, *76*, 83.
(12) For work on Arg_6 and polyarginine, see: Sakai, N.; Matile, S. *J. Am. Chem. Soc.* **2003**, *125*, 14348.
(13) For related work, see: Terrone, D.; Sang, S. L. W.; Roudaia, L.; Silvius, J. R. *Biochemistry* **2003**, *42*, 13787.
(14) Aidley, D.; Stanfield, P. *Ion Channels*; Cambridge University Press: Cambridge, England, 1996.
(15) Weiss, T. F. *Cellular Biophysics*; MIT Press: Boston, MA, 1996; Vol. 1, p 487.
(16) Urban, B. W.; Hladky, S. B.; Haydon, D. A. *Biochim. Biophys. Acta.* **1980**, *602*, 331.
(17) Harada, H.; Morita, M.; Suketa, Y. *Biochim. Biophys. Acta.* **1994**, *1220*, 310.
(18) Wadia, J.; Stan, R.; Dowdy, S. *Nat. Med.* **2004**, *10*, 331.

JA0482536