Supplementary materials for:

Arginine-rich peptides destabilize the plasma membrane, consistent with a pore formation translocation mechanism of cell penetrating peptides

H. D. Herce[1,2], A. E. Garcia[1,2§], J. Litt[2,3], R. S. Kane[2,3], P. Martin[4], N. Enrique[4], A. Rebolledo[4], and V. Milesi[4].

[1]Department of Physics, Applied Physics and Astronomy, [2]Center for Biotechnology and Interdisciplinary Studies, and [3]Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY, USA. 12180

[4]Universidad Nacional de La Plata, Consejo Nacional de Investigaciones Científicas y Técnicas, La Plata, Argentina.

[§]Corresponding author: angel@rpi.edu

Simulation methods

All simulations were performed using the GROMACS package (1), on a cluster of dual Opteron processors. The simulated system consists of 4 Arg-9 peptides, 92 DOPC phospholipid molecules and 8795 water molecules. The overall temperature of the water, lipids, and peptides were kept constant, coupling independently each group of molecules at 323 K with a Berendsen thermostat (2). The pressure was coupled to a Berendsen barostat at 1 atm separately in every direction (2). The temperature and pressure time constants of the coupling were 0.2 ps and 2 ps, respectively. An external electric field of 0.05 V nm^{-1} was included pointing towards the distal side of the bilayer to qualitatively take into account the external electrostatic potential included in the experiments (3). The integration of the equations of motion was performed using a leap frog algorithm with a time step of 2 fs. Periodic boundary conditions were implemented in all systems. A cut-off of 1 nm was implemented for the Lennard-Jones and the direct space part of the Ewald sum for Coulombic interactions. The Fourier space part of the Ewald splitting was computed using the particle-mesh Ewald method (4), with a grid length of 0.11 nm on the side and a cubic spline interpolation. Periodic boundary conditions and Ewald summations that do not include the surface term ensure system electro neutrality (5). We used the SPC/E model for water (6), the parameters from Berger et al. for the lipids (7), and the parameters from the GROMACS force field (1) for the peptide.

Experimental Methods

Experiments on Planar lipid bilayers

The basic idea of the *planar lipid bilayer* method setup (more details are described in Fig. 1s) is that the bilayer is made across a 100 μm diameter hole that separates two chambers filled with a saline solution. In each chamber an electrode is introduced to generate an electrostatic

potential across the membrane and measure ionic currents. The chamber where the active electrode is introduced is conventionally called the *cis* chamber and the other chamber is called the *trans* chamber. The *cis* chamber is held at 50 mV relative to the *trans* chamber. The peptides are added to the *cis* chamber because according to the model it is expected that they would be more likely to cross the bilayer from the higher to the lower electrostatic potential, which corresponds to the potential difference between the exterior and interior of the cell. Addition of micromolar concentrations of Arg-9 to the aqueous solution bathing a planar bilayer membrane led to an increase of membrane conductance in all experiments ($n > 50$, where n is the number of experiments).

DOPC, and 1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (DOPG) were purchased from Avanti Polar Lipids, Tat peptide, penetratin and Arg-9 were purchased from Global Peptides, Dap-9 was purchased from Chi Scientific, Protegrin-1 (PG-1) was purchased from the core facility at Emory University, and n-hexadecane was purchased from Fisher Scientific. All reagents were used as received with no further purification. The lipid was dissolved in n-hexadecane at a concentration of 200 mg/ml, and was used immediately. Compositions used were either 100% DOPC, or 3:1 DOPC:DOPG. Bilayers were formed over a 100 micron diameter aperture in a polystyrene cup (Warner Instruments). The chambers and the cup were washed in *sodium* dodecyl sulfate (SDS) and distilled water before each experiment. The cup was then dried under a stream of nitrogen. Lipid solution was applied over the area surrounding the aperture using a thin glass rod. The lipid solution was then spread evenly over the surface using a stream of nitrogen. The cup was inserted into a thermally conductive chamber (Warner Instruments) and the cup and chamber sides were both filled with 1 mL of 0.1M KCl solution, pH 7.4. Ag/AgCl electrodes were inserted in small electrode wells containing 3M KCl which were then connected to the cup and chamber liquid using salt bridges containing 3M KCl with 2.5% agar. The chamber was then placed in a BLM-TC thermocycler (Warner) and allowed to reach 37°C. Before forming a bilayer, we checked that the current was greater than 1000 pA, indicating that there was no obstruction blocking the aperture. The lipid was then painted over the aperture using a glass rod as applicator. The formation of a bilayer was indicated by a sharp drop in current accompanied by an increase in bilayer capacitance. Current and capacitance were measured using a BC-535 patch clamp amplifier (Warner Instruments). Bilayers were required to reach a capacitance value greater than 40 pF to be used in our experiments. Once these conditions had been met and the capacitance and current had remained stable for at least 15 minutes, a transmembrane potential of 50 mV with respect to the *cis* side was applied across the bilayer, which was then allowed to equilibrate until the capacitance and current were stable for 15 minutes, while making a baseline recording. Finally, the peptides were added to the *cis*-side to a final concentration of 7 μM. The data was then collected from the amperimeter through a Digidat 1440 data digitizer (Axon Instruments) at a sampling rate of 150 kHz and analyzed using the Clamplex 10.0 software (Molecular Devices Corp.). Signal filtering was achieved using the 4-pole Bessel filter built into the amplifier unit, at a setting of 2 kHz.

Cell isolation procedure

Umbilical cords obtained after vaginal and cesarean delivery were placed in saline solution containing 130 mM NaCl, 4.7 mM KCl, 1.17 mM KH_2PO_4, 1.16 mM $MgSO_4$, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, pH 7.4 at 4°C and immediately transported to the laboratory (in La Plata, Argentina) and stored at 4°C. The arteries were dissected from the Wharton´s jelly just before the

cell isolation procedure. Human umbilical artery (HUA) smooth muscle cells were obtained following a method described by Klockner (8) and later modified in our laboratory at La Plata in order to diminish the enzyme content in the dissociation medium (DM) (9). Briefly, a segment of HUA was cleaned of any residual connective tissue, cut in small strips and placed for 15 min in a DM containing 130 mM NaCl, 1.2 mM KH_2PO_4, 5 mM $MgCl_2$, 5 mM HEPES, and 6 mM glucose. The pH was adjusted to 7.4 with NaOH. The strips were then placed in DM with 2 mg/ml collagenase type I during 25 min, with gentle agitation, at 35°C. After the incubation period the strips were washed with DM and single HUA smooth muscle cells were obtained by a gentle dispersion of the treated tissue using a Pasteur pipette. The remaining tissue and the supernatant containing isolated cells were stored at room temperature (~20°C) until used.

Patch-clamp recordings

HUA smooth muscle cells were allowed to settle onto the cover glass bottom of a 3 ml experimental chamber. The cells were observed with a mechanically stabilized, inverted microscope (Telaval 3, Carl Zeiss, Jena, Germany) equipped with a 40X objective lens. The chamber was perfused for 15 min, at 1 ml min^{-1} by gravity, with the appropriate saline solution (described below) before the patch-clamp experiment was started. Application of test solutions was performed through a multi barreled pipette positioned close to the cell being investigated. After each experiment on a single cell, the experimental chamber was replaced by another one containing a new sample of cells. Only well-relaxed, spindle-shaped smooth muscle cells were used for electrophysiological recordings. Data were collected within 4-6 h after cell isolation. All experiments were performed at room temperature (~20°C).

The standard tight-seal whole-cell configuration of the patch-clamp technique was used. In this configuration the patch pipette containing a saline solution of a composition similar to the intracellular medium was tightly sealed to the membrane; after a soft suction the membrane patch under the pipette tip was ruptured and the solution comes in contact with the intracellular medium. An electrode placed inside the pipette measured the current across the whole plasma membrane of the cell and, at the same time, was able to control the voltage across the membrane. In this case the cells were clamped at a holding potential of -50 mV, hence evoking a macroscopic holding current, which we measured before and after adding Arg-9 to a final concentration of 0.07 μM or 7 μM to the bath solution.

Glass pipettes were drawn from WPI PG52165-4 glass on a two-stage vertical micropipette puller (PP-83, Narishige Scientific Instrument Laboratories, Tokyo, Japan) and pipette resistance ranged from 2 to 4 Mohms. Ionic currents were measured with an Axopatch 200 A amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 2 kHz, digitized (Digidata 1200 Axon Instruments, Foster City, CA) at a sample frequency of 20 kHz. The experimental recordings were stored on a computer hard disk for later analysis.

Solutions for whole cell experiments: The extracellular saline solution used for recording whole-cell ionic currents contained 130 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 6 mM glucose, and 5 mM HEPES. The pH was adjusted to 7.4 pH using KOP. The composition of the intracellular pipette solution contained 130 mM KCl, 5 mM Na_2ATP, 1 mM $MgCl_2$, 0.1 mM EGTA, and 5 mM HEPES. The pH was adjusted to 7.3 with NaOH.

In the *cell-attached* configuration the tip of the pipette was sealed to the cell membrane without rupturing it. Therefore, the pipette-solution was in contact with the extracellular face of the cell-

membrane patch, and was maintained in this condition during the recording, while the intracellular cell content remained intact. The tip of the pipette was filled with a pipette solution without peptide, while the rest was back-filled with the same solution containing the peptide. This produces a time delay (a few minutes) before the peptide slowly diffuses into the tip and comes into contact with the membrane. The membrane potential of the cell was kept near 0 mV by bathing them with a high K^+ extracellular solution, but the presence of the seal allowed the application of a transmembrane voltage of 50 mV (relative to the interior of the cell) across the patch under the pipette, which was maintained constant during all the recoding time.

Solutions for cell-attached experiments: The tip of the peptide was filled by capillarity with a solution composed of 140 mM KCl, 0.5 mM $MgCl_2$, 10 mM HEPES, 10 mM Glucose, and 1 mM $CaCl_2$. The pH 7.4 was adjusted using KOH. The rest of the pipette was filled with the same solution plus Arg-9 peptides at a concentration of 7 μM. The bath solution was composed of 140 mM KCl, 0.5 mM $MgCl_2$, 10 mM HEPES, 10 mM Glucose, and 1 mM EGTA. The pH was adjusted to 7.4 using KOH.

Solutions for inside-out experiments: Both bath and pipette solutions were the same composed of 140 mM KCl, 0.5 mM $MgCl_2$, 10 mM HEPES, 10 mM Glucose, and 1 mM EGTA. The pH 7.4 was adjusted using KOH.

References:

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Figure 1s Schematic representation of the planar lipid bilayer setup used to measure ionic currents across the bilayer induced by peptides added to the *cis* chamber.

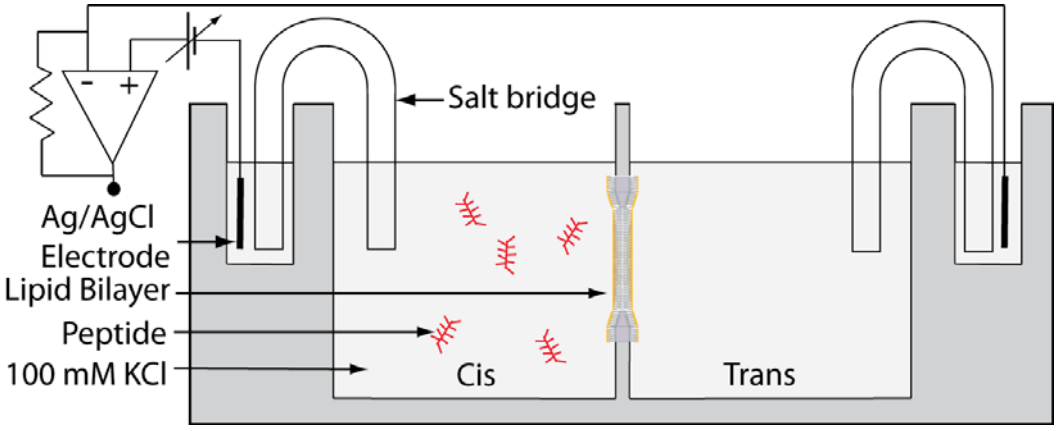
Figure 2s Schematic representations of the patch clamp configurations used to measure ionic currents across mammalian cell membranes induced by the Arg-9 peptides: (a) *Cell attached* (b) *Whole-cell* and (c) *Inside out*.

Figure 3s Side chain structures of the α-amino acids Dap, Lys, and Arg.

Figure s4 Histogram of the current amplitude of discrete current jumps.

Figure s5 Permeabilization of phospholipid bilayers composed of a lipid mixture of DOPC:DOPG (3:1) (a) control measurement, (b) after the addition of 7μM of penetratin, (c) Tat, and (D) PG-1 to the *cis* chamber. The arrow's origin indicates the time at which the peptides or the $CaCl_2$ were added to the solution. The potential of the *cis* chamber relative to the *trans* chamber (the holding potential) is 50mV. The ionic concentration is 100mM of KCl and the pH is 7.4. It can be seen that after addition of the peptides the membrane permeabilizes progressively following two types of kinetic behavior a seemingly continuous increase in the ionic permeability reflected in a continuous increase of the current and discrete current jumps. The addition of 100 mM of $CaCl_2$ reduces the permeability of the membrane induced by the peptides in every case.

Figure 1s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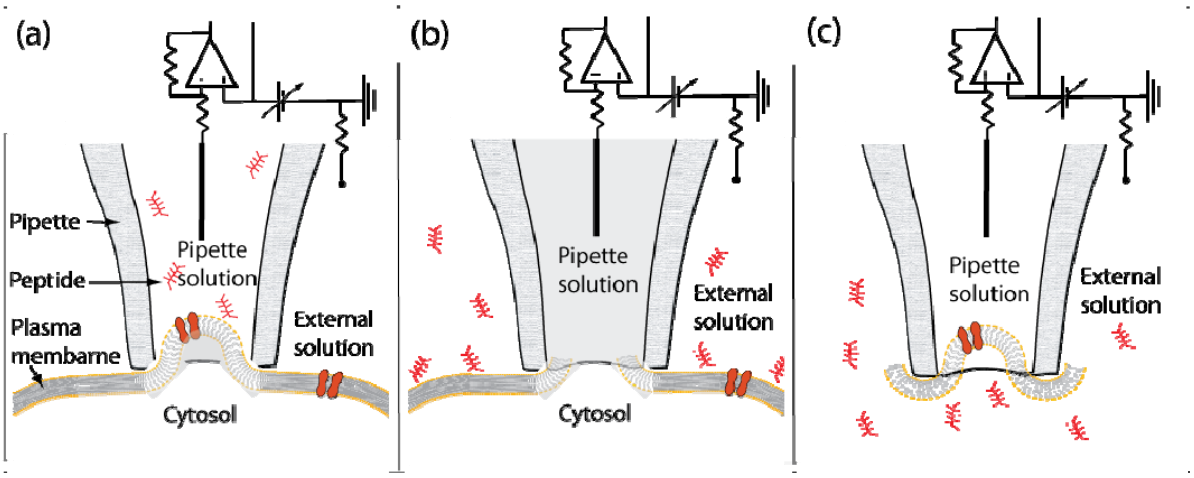
Electrode
Lipid Bilayer
Peptide
100 mM KCl
Cis
Trans

Figure 2s


(a)
Pipette
Peptide
Plasma membarne
Pipette solution
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Cytosol
(b)
Pipette solution
External solution
Cytosol
(c)
Pipette solution
External solution

Figure 3s

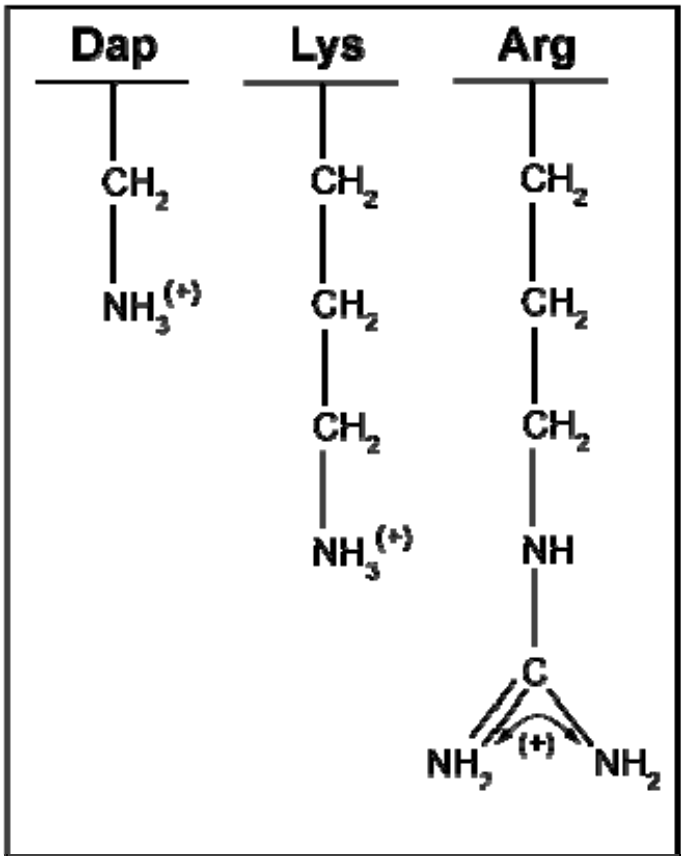


Figure s4

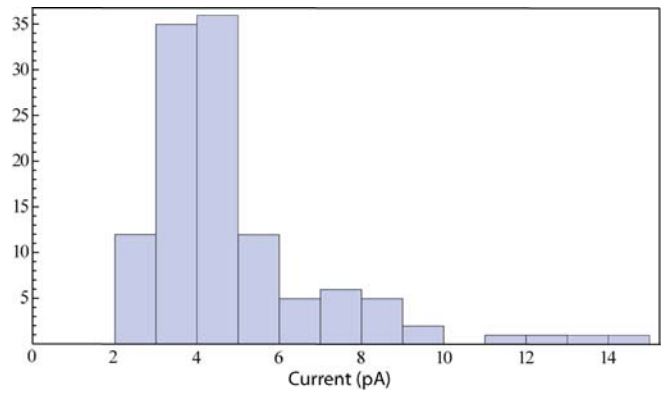
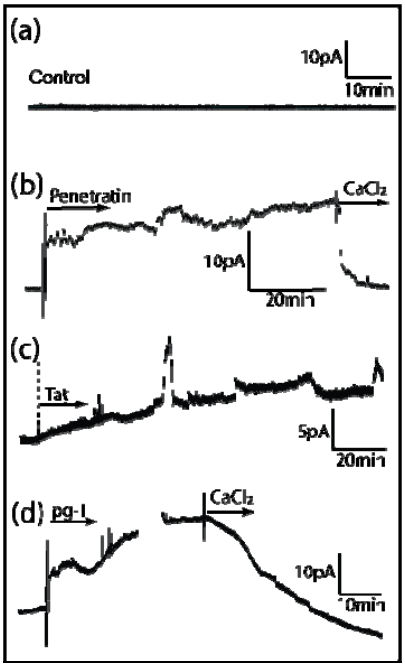


Figure s5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10min
(b)
Penetratin
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pg-I
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10pA
10min