Channel-forming peptide allows diffusion of riboflavin and dextran into the sea urchin embryo blastocoel and inhibits blastula spinning and sperm movement

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A synthetic peptide (NC-1059) that creates a non-selective ion channel in the membranes of living epithelial cells also loosens cell-cell contacts between them, transiently opening the "paracellular" pathway between cells, allowing molecules to diffuse across an epithelial cell layer, all without killing cells. Here, we observed that the same peptide, at similar concentrations, allows sea urchin blastulae (which are essentially a single-layered epithelium arranged as a hollow sphere) to take up molecules of at least 500 MW to diffuse into the blastocoel. In addition, also in a concentration-dependent manner, the peptide inhibits blastula rotation/spinning, a cilia-dependent activity, and also inhibits sea urchin sperm cell motility, a flagella-dependent activity. The mechanisms by which the peptide affects ion channels, cell-cell adhesion, and cilia/flagella movements are not known, nor whether they are related to one another.

Keratoconus, an eye disorder that alters the shape of the cornea, affects 1 in 2,000 people. It causes the cornea to elongate into a more conic shape that will eventually perforate, allowing dirt and bacteria to enter the anterior chamber facing the lens, thus necessitating rapid corneal transplantation. Currently, there is a procedure that stops the progression of this disorder by shining ultraviolet A light onto the cornea where the epithelium has been scraped away purposely, and where riboflavin (vitamin B2) is then dripped onto its surface and allowed to diffuse into the corneal stroma, where it catalyzes the formation of strong covalent bonds in response to the UVA light. This causes the cornea to stiffen, concomitantly halting the progression of keratoconus. However, removal of the epithelium requires an inconveniently long period of rest for patients after the riboflavin/UVA treatment (while awaiting epithelial cells from the edge of the cornea and limbal stem cells to cover the "debrided" corneal stroma again). To obviate epithelium removal would require designing a treatment that would allow riboflavin to cross the intact corneal epithelium to enter the corneal stroma. Recently, such a treatment was devised, involving exposure of the intact epithelium to a synthetic peptide, NC-1059[5].

Originally synthesized as a possible treatment to allow airway epithelial cells of cystic fibrosis patients to secrete chloride ions[1], NC-1059 forms non-selective channels on the apical surface of epithelia exposed to it, with the effect of modulating epithelial tight junctions in such a way that the paracellular pathway opens between cells in monolayers and multilayered epithelia[3,4]. As a positive result of this effect, peptide NC-1059 is now a candidate for use in the "EPI-ON" method of treating keratoconus corneas, in which the epithelium is not scraped off, but instead is left in place on the corneal surface; in the presence of the peptide, riboflavin is dripped onto the surface and then diffuses directly across the epithelium into the stroma[5]. Other effects of this peptide on epithelial cells remain under study. Here, we have examined the effects of the peptide on sea urchin embryos at the blastula phase, when embryos are composed entirely of cells arranged as a monolayer epithelium whose apical surfaces face outward into the surrounding seawater, to determine their ability to allow riboflavin and dextran to cross into the blastocoel cavity in response to exposure to peptide NC-1059.

Riboflavin 5'-monophosphate sodium salt (here referred to as "riboflavin")(FW 478.33) was purchased from Sigma-Aldrich Fluka, dextran Alexa Fluor-546 (MW 10,000) from Life Technology, and *p*-aminobenzoic acid (PABA)(4-aminobenzoic acid) (FW 159.12) from Aldrich. Peptide NC-1059 (FW 2722.4) was synthesized at Kansas State University. For each experiment, it was prepared in 7 different concentrations: 400 μM, 200 μM, 100 μM, 50 μM, 25 μM, 12.5 μM, and 6.25 μM; solutions were stored on ice when in use, and frozen (-20°C) when not in use. Shedding of gametes from sea urchins (*Lytechinus pictus*) was triggered by injection of 0.5 M KCl solution into the gonad regions of each organism. Sperm were collected "dry" and stored on ice. Eggs were collected and washed in 10 mM PABA, pH 8, in seawater. Sperm were activated when mixed with filtered natural sea water (FNSW) at a 1:1000 dilution; 1 mL of activated sperm was added to 15 mL of eggs suspended in PABA-FNSW. Eggs and sperm, mixed together, were kept gently in suspension for 2 min, washed into PABA-FNSW, and strained through 80 μm nitex mesh to remove the fertilization envelope. Fertilized eggs were

brought up to volume with FNSW until a monolayer of eggs covered the bottom of the petri dish and then incubated at 12˚C overnight. Embryos reached blastula stage 10-21 h after fertilization. Once embryos reached mid-blastula stage, one embryo was transferred with as little FNSW as possible onto a glass slide with 3 or more chips of crushed cover slips to act as a "spacer". A cover slip was then placed on the chips and a mixture of 1 part dextran: 2 parts riboflavin: 3 parts peptide was added to the corners to fill the space left in the chamber. At 40x magnification, images were captured using three filters per time point: bright field, green fluorescence, and red fluorescence. Photomicrographs using each such filter were taken at 5-minute intervals for one hour.

Epifluorescence micrographic images taken of embryos in the presence of peptide concentrations of 200 μM indicated both riboflavin and dextran diffused into the blastocoel of the blastula stage embryo. In contrast, neither molecule entered the blastocoel of embryos in control FNSW in the absence of peptide. This suggested the peptide opened the paracellular pathway in this system as well, allowing molecules to pass between the cells and into the cavity of the blastocoel. However, the uptake of riboflavin appeared to be much greater than that of dextran, most likely a reflection of the respective relative sizes of the two molecules: "riboflavin" (FW 478.33) *vs*. dextran Alexa Fluor-546 (MW 10,000). In addition, uptake of each fluorescent molecule was determined by the incubation time in the peptide-fluorescent solution. Over the course of an hour, the blastocoel became progressively more fluorescent. Another variable that determined the amount of passage into the blastocoel was the peptide concentration. At the lower peptide concentrations, less fluorescence entered the blastocoel; at higher peptide concentrations, *e.g.,* 200 μM, more fluorescence entered the blastocoel. Therefore, uptake of both riboflavin and dextran was based on molecular size, incubation time, and peptide concentration.

Photographing embryos in the control group was challenging because the cells composing normal blastula stage embryos form cilia that beat, causing the embryos to rotate ("spin") freely in seawater, keeping them in suspension. However, in the presence of 200 μM peptide NC-1059, embryos did not spin, suggesting that the peptide inhibited cilia beating movement. To test this hypothesis further, activated sperm were suspended in a range of concentrations of peptide to determine their effects on the sperm flagella movement. Observation of living sperm indicated that in any concentration of peptide at or above 12.5 μM, sperm rapidly stopped moving.

With these added data, peptide NC-1059 now can be said to cause three known effects: creating ion channels[1,2], transiently opening the paracellular pathway[3-5], and hindering movement of cilia and of flagella (here). Currently, the mechanisms of these three effects are not known, nor if any are related to one another.

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