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EFFECT OF ATRIAL NATRIURETIC FACTOR ON THE WATER PERMEABILITY OF ENDOTHELIAL CELLS

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Atrial natriuretic factor increases the water permeability of the whole endothelium. This study investigates how it would affect the transcellular osmotic water permeability of bovine artery endothelial cells. The cyclic-GMP production by the isolated cells was maximal for 10^{-6}M atrial natriuretic factor within 30 minutes at 37°C. The cyclic-GMP protein kinase cell concentration was 1.87±0.15 ng/mg protein. The control apparent water permeability of the cells measured by light scattering was 195±11 μm/sec (n=5). Membrane folding revealed by light and scanning electron microscopy indicated that their true water permeability values would be close to 20- 40 μm/sec, similar to the values for lipid membranes. The energy activation calculated from the temperature dependence of water permeability between 15°C and 37°C was 9.3 kcal/mol. This value suggests water movement through the lipid bilayer and not through water channels. Atrial natriuretic factor 10^{-6}M did not significantly increase the water permeability of the cells. Hence, atrial natriuretic factor-stimulated increase in water permeability of the endothelium is more related to changes in paracellular water pathways than in transcellular water flux.

The movement of fluid across blood vessels depends on their water permeability and the transmural gradient of osmotic and hydrostatic pressures. The balance between these parameters results in an appropriate distribution of fluid between the blood and the extracellular compartments. However, in certain circumstances, such as congestive heart failure, this balance is disturbed, resulting in pulmonary and peripheral edema. A role for atrial natriuretic factor (ANF) has been suspected, as it induces an increase in hematocrit in rats, suggesting a movement of fluid from the blood to the extracellular compartment (1,2). This cannot be explained by increased transmural hydrostatic pressure, since ANF decreases blood pressure. The reproducibility of these results in bilaterally nephrectomized and splenectomized rats also excludes the possibility of renal albumin loss and ensures that the changes in hematocrit do not result from changes in plasma protein concentration after splenic contraction. The movement of fluid from the blood to the extracellular compartment could also be due to a leak of albumin into the extracellular space which would reduce the transmural oncotic pressure of proteins, or to a change in the osmotic permeability of the endothelium, or both.

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The permeability of the endothelium to macromolecules depends on the leakiness of the intercellular junctions, whereas its permeability to water is related to the amplitude of the osmotic permeability of the respective transcellular and paracellular pathways. A "trap-door" effect (3), which can be defined as a change in the permeability of the arterial endothelium to circulating macromolecules by opening and closing of endothelial cell junctions, has been described in rats. Vasoactive peptides, including ANF have been found to regulate this transepithelial permeability to macromolecules (4). It should theoretically be part of the mechanism of edema formation, although the relationship between an increase in albumin permeability and local edema is not experimentally supported by the data of Williamson et al. (5). On the other hand, Huxley et al.(6) found an increase in endothelial water permeability by ANF. This is linked to the presence of particulate guanylate cyclase binding ANF on endothelial cells, which is stimulated by the increase in plasma ANF concentration, leading to the generation of intracellular and extracellular cyclic GMP (cGMP) (7). The formation of edema could therefore be linked to a larger water permeability of the endothelium in the presence of ANF which would facilitate the movement of fluid out of the blood compartment.

However, an increase in osmotic permeability of the whole endothelium does not indicate which of the paracellular or transcellular pathways is involved in the ANF response. We therefore looked for the presence of cGMP dependent protein kinase (G kinase) in cultured bovine pulmonary endothelial cells and tested the effect of ANF on the generation of cGMP and on the water permeability of these cells as measured by light scattering (8).

METHODS

Bovine artery endothelial cells (CCL 209) were purchased from the American Type Culture Collection (Rockville, Maryland, 20852 USA). They were received at passage 15 and used between passage 15 and 24. The cells were cultured in Eagle's minimum essential medium with Hanks' salts, 22 mM $NaHCO_3$, 1% Penicillin-Streptomycin (5000 UI/ml- 5 mg/ml) , 2 mM L-glutamine, 10 % fetal calf serum (FCS) (Boehringer, Mannheim, France) and 1 µg/l fibroblast growth factor (a gift from Dr Gospodarowitz). Cells were routinely seeded at a concentration of 30 000 cells/ml and the medium was changed twice a week. Cells were used 2 days after they had reached confluence (6 days after seeding).

Cyclic GMP production was determined on cell monolayers cultured on a gelatin film (0.2 % in phosphate buffered saline, PBS) in 12-well plates (4.8 cm^2 per well) (Costar). For stopped-flow experiments, cells monolayers were cultured on a gelatin film in 75 cm^2 closed flasks (Costar). Both cultures were placed in a humidificated incubator at 37°C and 5% CO_2 / 95% air. For determination of cGMP generation, the cells were washed with PBS and ANF 10^{-12} -10^{-5}M was added to medium free of FCS, containing 10^{-5}M isobutyl-methylxanthine (Sigma chemicals, St Louis, USA). Each assay was performed in triplicate. Controls were done by adding fresh medium only. After 90 minutes of incubation at 37°C (time known to allow maximum egression of cGMP), cGMP was measured in the extracellular fluid with a radio-immuno assay kit (Amersham, les Ulis, France). The cells of each well were then collected for DNA measurement (9). The same experiment was performed on isolated cells. Attached cells were washed with PBS, and removed by treatment with 0.05% trypsin and 0.02% EDTA or 0.01% collagenase for 3 to 5 minutes. The cells were resuspended in complete medium, collected by centrifugation (1000 rpm / 2 minutes), resuspended in FCS-free medium and distributed in 12 well plates. Atrial natriuretic factor was added as in the former experiment and cGMP measured. The kinetic of cGMP generation was measured by incubating cells with 10^{-6}M ANF at 37°C for 10, 30 and 90 minutes. The mean diameter of the cells was calculated from their surface area (X200) using a video-camera connected to an image analyser (Samba 2005). The aspect of the cell surface was evaluated by phase contrast microscopy on isolated trypsinized cells (Nikon ELWD2/O3) and scanning electron microscopy (Jeol JSM 840A) on freshly isolated cells collected on a porous membrane (1µm).

The cell concentration of cGMP kinase was determined by enzyme linked immunosorbent assay (ELISA) with affinity-purified anti-cGMP kinase rabbit antibodies (10). Seven to nine 75 cm^2 flasks were washed with PBS (plus 2mM EDTA and 2mM benzamidin). The cells were scraped into a small amount (3 ml) of buffer, centrifuged (2000 rpm for 5 minutes at 4°C) resuspended in 500 µl buffer, sonnicated (30 seconds) and homogenized (2000 rpm for 2 minutes). Aliquots were taken for protein dosage. The suspension was centrifuged (15 000 rpm for 30 minutes at 4°C) and the supernatant frozen (-20°C).

Osmotic water permeability was determined on isolated cells resuspended (10^6 cells/ml) in Dulbecco's modified medium for chemoluminescence (Boehringer, Mannheim, France). This concentration was found to be the best cell concentration for the light scattering measurements. The osmolality of the suspending medium was 316 mOsm /kg H_2O. Cells were mixed in a stopped-flow apparatus with a hyperosmotic solution made from the same medium for chemoluminescence to which mannitol was added to produce a mean osmolality of 716 mOsm/kg H_2O. Equal volumes of cell suspension and hyperosmotic solution (0.05 ml) were flushed in the optical cell of the stopped-flow apparatus to give an inward gradient of 200 mOsm. The wavelength of the light emitted by a 150 W halogen lamp was 400 nm.

The increase in intensity of the scattered light as a function of time was recorded during 10 seconds at a data acquisition rate of 1 kHz. At least three runs were averaged. Data were stored on a Tandon AT Personal Computer for subsequent analysis. These curves were fitted to a single exponential function by the Pladé-Laplace and least square method. The exponential rate constant (k) was related to the osmotic permeability (P_f) by the equation (11):

$$P_f = k \cdot Vo\ (1-b/Vo) \times (\sigma . Vw \cdot A \cdot Cm)^{-1}$$

where k is the exponential rate constant, Vo the initial cellular volume, A the membrane area, Cm the osmolality of the hyperosmotic medium, Vw the molar volume of water, σ the reflection coefficient of the solute used for the osmotic gradient (σ mannitol =1), and b the osmotically active volume of the cells (0.86) (12).

The osmotic water permeability of the cells was measured in control conditions and after incubation with 10^{-6}M ANF at 37°C for 30 minutes. The effect of temperature on water permeability was studied between 15°C and 37°C. The energy activation (Ea) was calculated from the value of the slope of the regression line of the exponential rate constant versus the temperature.

Statistical methods:

Data are presented as means±SD. Values were compared by two-way ANOVA with the Fisher's coefficient. Statistical significance was taken as $p<0.05$.

RESULTS

The maximum egression of cGMP was produced by 10^{-6}M ANF in attached cells (Fig.1). The same dose-response curve was obtained with isolated cells. There was no difference in the egression of cGMP under ANF stimulation in cells treated with trypsin/EDTA or collagenase D.

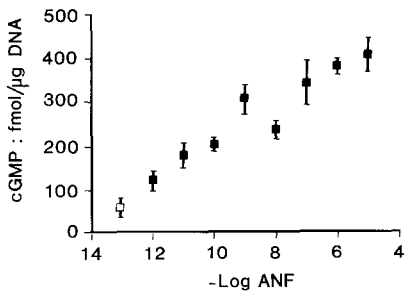

Figure 1. Release of cyclic GMP from endothelial cells following ANF stimulation (■) during 90 minutes at 37°C (□) time control.
Each point done in triplicate.

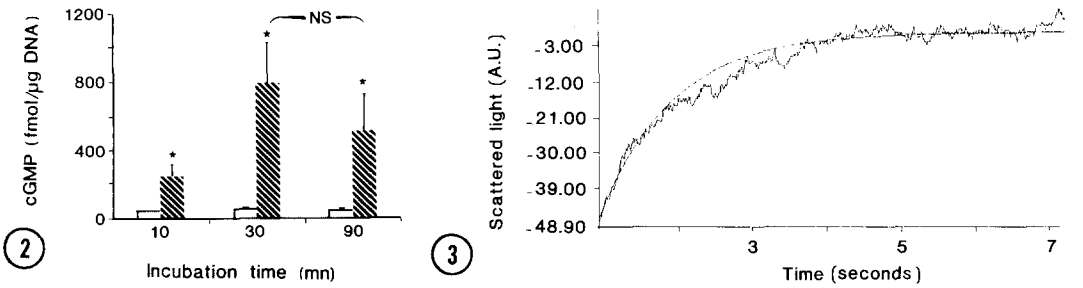

Figure 2. Comparison of cyclic GMP egression from isolated endothelial cells after ANF stimulation (10-6M at 37°C) for three different incubation periods of time.
Each point done in triplicate.
* p< 0.05 different from controls.

Figure 3. Change in light scattering intensity consecutive to the mixing of isolated endothelial cells and hyperosmotic solution.
Mean of three experimental curves and its exponential fit.

Cells treated with trypsin/EDTA were more regularly spherical than cells treated with collagenase D. Trypsin/EDTA treatment was therefore kept for further experiments. The maximum rate of cGMP generation in the presence of 10^{-6}M ANF was 30 minutes (Fig. 2) with a slight but non-significant decrease at 90 minutes. An incubation time of 30 minutes was therefore chosen for stopped-flow experiments.

The cell cGMP protein kinase cell concentration was 1.87±0.15 ng/ mg protein (n=3). The diameter of the isolated endothelial cells was 12.9±1.7 µm (n=464).

In stopped-flow experiments, mixing the cells with isoosmotic solution did not change the scattered light intensity for at least 20 seconds. In contrast, the intensity of light scattered by cells mixed with hyperosmotic solution increased during the first 5 msec. This increase could be fitted by a single exponential (Fig.3).

The osmotic water permeability (Pf) of the cells was determined with or without incubation of ANF 10^{-6}M for 30 minutes at 37°C. The mean values of Pf, calculated with an apparent diameter of 12.9 µm, were 195±11 (n=5) in control conditions and 214±22 µm/sec (n=5) in the presence of ANF.

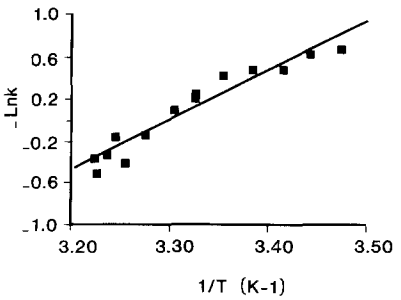

Figure 4. Temperature dependence of water permeability of isolated endothelial cells.
The logarithm of the exponential rate constant is plotted versus temperature in Kelvins -1.
Regression line (r=-0.96, p<0.001).

These values were not statistically different. The optical and scanning electron microscopy pictures showed protuberances and villi (0.8 to 1 μm) on the surface of the spherical isolated cells, resulting in increased membrane area. The calculation of Pf assuming a smooth membrane overestimated the Pf values 5-10 fold. The true Pf values per membrane area would therefore be in a range of 20 to 40 μm/sec.

The effect of the temperature on the exponential rate constant (k) is shown in figure 4. The activation energy (Ea) calculated from the slope of the regression line of -Ln k versus 1/T (kelvins^{-1}) was 9.3 kcal/mole.

DISCUSSION

The production of cGMP by both monolayers and isolated bovine artery endothelial cells incubated with 10^{-6}M ANF indicates that ANF receptors are present and functionnal in this experimental model of vascular endothelium. Moreover, cGMP protein kinase (effector of cGMP) was present in these cells, in a smaller amount than in smooth muscle cells (10) but clearly detectable. The mean Pf value of isolated endothelial cells at 37°C is 195±11 μm/sec assuming a 12.7 μm diameter. This value is in the same order of magnitude as those for erythrocytes and alveolar macrophages (520 μm/sec and 110 μm/sec respectively) and closed to that obtained by Garrick et al. (12) for calf endothelial cells (311 μm/sec). However, these Pf were calculated assuming that isolated endothelial cells were spherical without villi. The finding of protuberances at the surface of the cells indicated that the apparent Pf values would be overestimated. The true osmotic permeability of the endothelial cells is probably in the range of 20- 40 μm/sec, comparable to the water permeability of liposomes made from phospholipids and cholesterol (8). The temperature dependence of endothelial cell water permeability was investigated to determine whether water moved across the membrane lipids. Low values of Ea (below 4 Kcal/mole) would suggest the presence of water channels in the cell membrane, whereas high values would reflect the diffusion of water molecules through lipids (13). The calculated Ea of 9.3 Kcal/mole in this study indicates that water crossed the endothelial cell membrane mainly by diffusion through the lipid bilayer and not through water channels.

The Pf values with or without incubation with ANF were not statistically different in the present experiment despite ANF-stimulated cGMP production and the presence of G-kinase within the cells. Atrial natriuretic factor would not alter the transcellular water pathway of the endothelium. This supports the view that the increase in endothelial water permeability following ANF stimulation results from changes in water permeability of the paracellular water pathway rather than changes in the transcellular pathway.

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