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Pericardium of the Frog, *Rana esculenta,* Is Morphologically Designed as a Lymphatic Space

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ABSTRACT The importance of the pericardium and the pericardial fluid (PF) in the control of cardiac function has emerged over the past few years. Despite the acknowledgment that amphibians are exposed to both dehydration and excessive water accumulation, nothing is known about their pericardial structure and the morphological basis of the PF formation. We have studied the parietal pericardium (PP) morphology in *Rana esculenta* by electron microscopy. SEM images of the inner surface, which lines the pericardial cavity, revealed the presence of large vesicles and many small circular openings. TEM observations showed that the PP is made up of an inner mesothelial lining, often constituted by two layers of very flat cells lying on a basal membrane and of regularly oriented collagen bundles. The PP outer surface is lined by a layer of flat cells, without a basal membrane. The mesothelial cells had overlapping boundaries with complex intercellular connections and a rich pool of caveolae opened in the direction of both the pericardial cavity and intercellular spaces. These cells indicate an intense intracellular and/or intercellular transfer of fluids and substances. The intraperitoneal injection of the idromineral hormone, Val^5-ANG II, induced PP modifications, particularly evident at the level of the structures involved in the transmesothelial traffic. These lymphatic-like traits suggest that the frog PP represents a large lymphatic sac, subject to paracrine-endocrine remodeling, which can actively adjust the PF, influencing the composition and volume of the myocardial interstitial fluid. J. Morphol. 257:72–77, 2003.

KEY WORDS: *Rana esculenta*; pericardial mesothelium; fluid transfer; Angiotensin II

Due to their aquatic lifestyle, many anurans, including frogs, experience difficulties in water and electrolyte balance. It is acknowledged that the lymphatic drainage of their two body cavities, i.e., the peritoneal and the pericardial, plays a crucial role in preventing dehydration and excessive water accumulation (Wentzell et al., 1993; Toews and Wentzell, 1995). The peritoneal cavity is drained by a number of lymphatic vessels interconnected with the lymph sacs, which contribute to regulation of the peritoneal fluid content and thus the interstitial fluid (IF) composition of the abdominal and thoracic organs, i.e., the liver, the gastrointestinal tract, the kidney, and the lungs (Toews and Wentzell, 1995). In contrast, nothing is known about the morphological bases of the pericardial fluid (PF) formation and drainage, particularly in relation to the structure of the pericardial envelope. Knowledge of this aspect may be of importance for understanding PF formation and dynamics in those vertebrate hearts, such as the frog heart, that are fully trabeculated and avascular (Tota et al., 1983).

In mammals, the serous pericardium is made up of the visceral pericardium (epicardium), i.e., the inner layer inseparable from the heart, and the parietal pericardium (PP) that is reflected over the heart. The PP represents the external parietal wall, facing on one side the fibrous pericardium and on the other side the pericardial cavity (PC) in which the PF is contained. This fluid is an ultrafiltrate of plasma and is believed to originate through epicardial transudation as an overflow of the myocardial interstitial fluid (MIF) (Gibson and Segal, 1978; Stewart et al., 1997). Over the past few years the PF, responsible for the lubrication of the heart movements during the cardiac cycle, has been proposed to play a role in short- and long-term cardiac regulation via several paracrine or autocrine substances, including the atrial natriuretic peptide (ANP) (Szokodi et al., 1997), endothelin-1 (Horkay et al., 1998; Mebazaa et al., 1998), angiotensin II, and several polypeptide growth factors (Corda et al., 1997). The lymphatic drainage of mammalian PF is physiologically important because it can prevent liquid accumulation, preserving the colloid osmotic equi-

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librium of the MIF (Miller et al., 1988). In fact, the impaired action of the cardiac lymphatic system causes myocardial edema, increased epicardial transudation, and increased concentration of interstitial proteins (Stewart et al., 1997). In mammals, both pleural and peritoneal fluids are reabsorbed from the serous cavities through the lymphatic system following either the vesicular intracellular drainage of the mesothelial cells or using the so-called "units of lymphatic drainage" (Tsilibary and Wissig, 1987) directly connecting the cavity with the submesothelial lymphatic vessels (Azzali, 1999).

In the frog, lymphatic fluid has a volume equal to or more than the total blood volume and is a filtrate of plasma formed from the balance between colloid osmotic and blood capillary hydrostatic pressures. It contains salts and blood proteins in concentrations similar to those found in the plasma (Conklin, 1930a,b). Lymph circulates in a complex lymphatic system composed of rhythmically beating lymphatic hearts, large subcutaneous lymph sacs divided by connective tissue septa, and an extensive network of vessels that drain fluids from the deep interstitial spaces and the two body cavities (Conklin, 1930a; Baldwin et al., 1990). The back-and-forth movement of the lymphatic fluid through the pleural-peritoneal cavity and the PC is important for the maintenance of blood and IF composition and volume in amphibians (Hillman et al., 1987). Although the mechanisms for the regulation of the lymphatic fluid are not understood, there is some evidence that physical stimuli, circulating hormones (i.e., arginine vasotocin), and autocrine-paracrine substances could be involved (Parsons et al., 1994).

The present study was performed to obtain morphological information on the PP of the frog *Rana esculenta*, with particular reference to the structures that are potentially relevant for the PF turnover. In addition, the sensitivity of these structures to angiotensin II (ANG II), the potent hormone that modulates idromineral homeostasis and vascular permeability (Risau and Flamme, 1995; Kim and Iwao, 2000), was evaluated.

MATERIALS AND METHODS

Untreated Animals

Rana esculenta specimens of both sexes (18–26 g; n = 5) were obtained from a local breeder in the middle of June and maintained in aerated aquaria for 2 weeks at room temperature. After decapitation and spinal pithing, the parietal pericardium (PP) was removed and processed for transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The SEM observations were performed on the complete PP inner surface, while the TEM investigations were carried out on the anterior zone, devoid of peritoneal and pleural connections. After removal, the PP was flushed in a saline-specific medium (in mM): NaCl 115, KCl 2.5, $CaCl^2$ 1.0, Na_2HPO_4 2.15, NaH_2PO_4 0.85, anhydrous glucose 5.6, pH 7.2 (Gattuso et al., 1999) and then fixed in 2.5% glutaraldehyde in the same saline for 2 h at 4°C.

TEM

Parietal pericardial fragments obtained from all five frogs were postfixed in 1% OsO_4 for 1 h at 4°C, dehydrated in a graded series of alcohols, cleared in propylene oxide, and then embedded in EPON 812 (Fluka, Buchs, Switzerland). Ultrathin sections (20 sections from each animal), cut with an ultramicrotome (Ultracut E – Reichert-Jung), were mounted on copper grids, stained with uranyl acetate and lead citrate, and subsequently observed with a JEOL (JEM 1220) TEM at 120 kV.

SEM

Parietal pericardial fragments obtained from all five frogs (three fragments from each animal) were postfixed for 2 h in 1% OsO_4 at 4°C, dehydrated in increasing concentrations of acetone, and dried by the critical point method using CO_2 as the transitional fluid. Samples were then mounted on aluminum stubs, sputter-coated with gold/palladium, and examined with a JEOL (JSM-6301F) SEM operated at 10 kV.

Treatments

To test the effects of Val^5-ANG II (ANG II) on the PP of the frog, seven *Rana esculenta* specimens (both male and female, 18–26 g), randomly selected, were divided into two groups and treated as follows.

The first group (three frogs) was injected peritoneally with Val^5-ANG II (Peninsula Lab, Belmont CA) diluted to a final concentration of 10^{-7} M in phosphate buffer saline (PBS), pH 7.3, containing 0.1% bovine serum albumin (BSA) (Dako, Carpinteria, CA). 0.1 ml per 10 g body weight was injected. After injections, frogs were maintained for 3 h in separate aerated aquaria and then sacrificed as described above. The PP was removed from the three frogs and processed for SEM and TEM.

In order to test the effects of the saline, the second group (four frogs) was injected with PBS, pH 7.3, containing 0.1% BSA, and processed as described for the first group.

RESULTS

In the frog *Rana esculenta* the PP is a thin membrane (10 $\pm$ 1.2 μm) in which two surfaces are evident. The inner surface that lines the PC is covered by mesothelial cells lying on a submesothelial layer of loose connective tissue constituted by five or six layers of collagen fibers arranged in parallel, fibroblasts, and pigment cells. The outer pericardial surface is constituted by only one layer of flat cells, resembling mesothelial cells. This cellular layer and the connective tissue are not separated by a basal membrane (images not shown). The fact that we did not find evidence of pericardial lymphatic or blood vessels confirms the avascular arrangement of the frog heart (Staley and Benson, 1968) also at the pericardial level.

The structural characteristics of the frog PP of untreated animals were similar to those observed in the frogs injected with BSA; thus, the morphological descriptions hereafter reported are common to both groups, collectively considered controls.

With SEM, the inner PP surface exhibits long folds that are organized in parallel series (Fig. 1A). These folds are covered by irregular polygonal mesothelial cells whose boundaries appear well defined

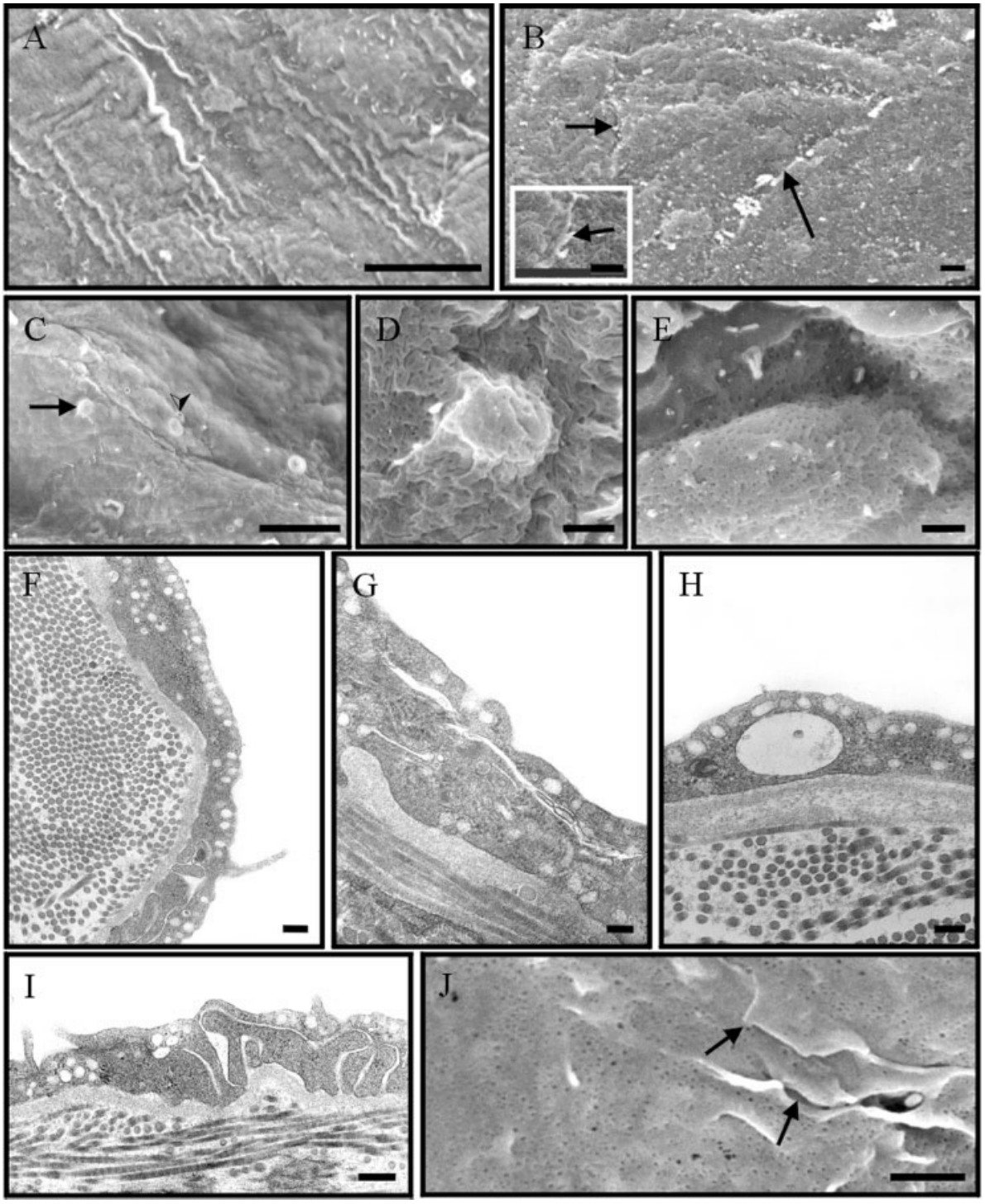

Fig. 1. Morphology of the parietal pericardium (PP) of the frog, *Rana esculenta,* as shown by SEM (**A–E,J**) and TEM (**F–I**). A: The mesothelial suface facing the pericardial cavity (PC) exhibits undulate parallel folds (scale bar = 10 μ). **B:** Short and rare microvilli (short arrow) and well-defined cellular boundaries (long arrow) (scale bar = 1 μ). **C:** Round blebs (arrow), sometimes opened toward the PC (arrowhead), are evident on the cellular surface (scale bar = 10 μ). **D:** Magnification of a single bleb (scale bar = 1 μ). **E:** On the cavity surface small round openings are observed (scale bar = 1 μ). **F:** Two or more layers of mesothelial cells lie on the basal lamina and on the submesothelial connective tissue. Cytoplasmic caveolae are present in the mesothelial cells, especially toward the luminal surface (scale bar = 200 nm). **G:** Thin intercellular spaces divide the superficial cells from the deepest cellular layers. Rare intercellular junctions can be found on the contact regions of adjacent mesothelial cells (scale bar = 200 nm). **H:** A round cavity (containing fine granular material) delimited by the plasmalemmae of two mesothelial cells (scale bar = 200 nm). **I:** Observe the complexity of the mesothelial intercellular connections (scale bar = 200 nm); note the tortuous intercellular spaces limited by many interdigitations. **J:** The connections between the mesothelial cells are characterized by laminar overlapped extroflessions (scale bar = 1 μ). *The inset (scale bar = 1 μ) shows the prominent cellular margins.

(Fig. 1B). Along the protruding cellular borders some few, short microvilli can be observed (Fig. 1B, inset). The cellular surface is characterized by voluminous round vesicles, hereafter called "blebs," sometimes opened into the PC (Fig. 1C,D) and by numerous small circular openings, homogeneous in size, which are regularly present on the whole surface (Fig. 1E).

By TEM it was seen that the PP is comprised of the mesothelium and underlying orthogonal bundles of parallel collagen fibers (Fig. 1F–I). The thin mesothelium (500 ± 150 nm) is made up of a continuous lining in which one, two, or three layers of flat cells lie on the basal membrane (Fig. 1G). The superficial cells often show some short, thin protrusions towards the PC (Fig. 1F). Intercellular connections are notably complex. In fact, the lateral surfaces of the mesothelial cells exhibit irregular processes that are intertwined, with similar processes belonging to neighboring cells. Tight junctions between cells can sometimes be observed. The tortuous intercellular spaces are delimited by the interdigitations between the mesothelial cells and the basal membrane (Fig. 1I). When observed with SEM, the areas of intercellular connections have thin overlapping laminar cellular protrusions which often delimit small openings, covered in part by the protrusion itself (Fig. 1J).

The mesothelial cells are characterized by sparse cytoplasmic organelles and numerous caveolae. Caveolae, which are very numerous in the cells of the superficial layer, open either at the cellular surface facing the PC or into the exiguous intercellular spaces or on the basal surfaces of the cells that form the deepest layer (Fig. 1F,G). Thin empty spaces sometimes divide the mesothelial layers (Fig. 1G). Rounded caveolae containing fine granular material open towards these spaces (Fig. 1H).

In contrast to the morphological arrangement observed in untreated and in BSA-injected animals, the treatment with ANG II 10^{-7} M remarkably modifies the morphology of the frog PP. It becomes clearly edematous, with intercellular spaces that appear dilated, highlighting the complexity of the intercellular connections (Fig. 2A,B). Moreover, the number of caveolae are increased in both the cellular surface facing the PC and the deep mesothelial layer (Fig. 2A,B). These structures are more frequently open in comparison with those observed in non-treated frogs. Larger and more numerous blebs, sometimes open into the PC, are also evident (Fig. 2C). High magnification SEM images reveal that their inner surface resembles that of the cells belonging to the superficial layer, being characterized by small circular openings (Fig. 2D). When observed with TEM, the large blebs are delimited by mesothelial cells which exhibit cytoplasmic caveolae, often opening into the bleb itself (Fig. 2E). The mesothelial lining of the blebs appears interrupted towards the PC in those regions where it becomes very thin (Fig. 2F).

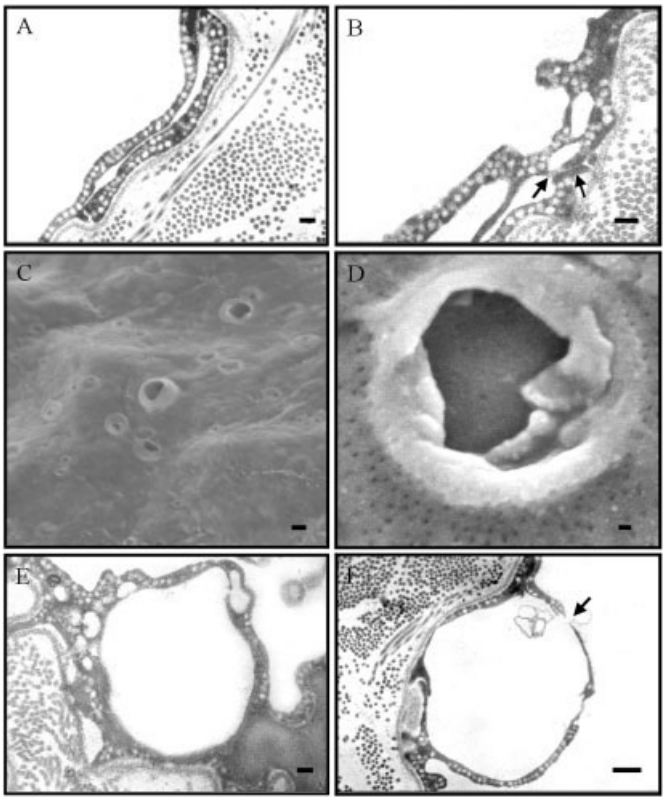

Fig. 2. Morphological aspects of the parietal pericardium (PP) of *Rana esculenta* after intraperitoneal injection of 10^{-7} M Val^5-Ang II, as revealed by TEM (**A,B,E,F**) and SEM (**C,D**). **A,B:** The edematous submesothelial connective tissue exhibits enlarged intercellular spaces (scale bar = 200 nm). In **B**, intercellular junctions (arrows) are evident between the cellular processes. **C:** The luminal surface shows numerous round blebs, some of which appear on voluminous and often opened toward the PC (scale bar = 1 μ), **D:** Note at high magnification the inner surface of an open bleb showing small round openings comparable to those observed on the luminal surface (scale bar = 100 nm). **E:** The structure of a representative bleb. The bleb wall is comprised of the plasmalemma of a mesothelial cell, whose cytoplasm contains numerous caveolae open to the bleb cavity (scale bar = 200 nm). **F:** The thin mesothelial surface of the bleb sometimes appears interrupted (arrow; scale bar = 500 nm).

DISCUSSION

This morphological study of the parietal pericardium (PP) of *Rana esculenta* illustrates structures of functional interest in relation to the transmesothelial movement of the fluids of the parietal cavity (PC).

We have demonstrated that the frog PP is made up of a thin mesothelium with unique morphological traits that distinguish it from other mesothelia. In fact, the cellular surface that faces the PC consists of flat polygonal cells arranged in one or more layers; the lateral surfaces of these cells show processes extensively interdigitated and connected by tight

junctions. The morphological aspects of the frog pericardial mesothelium can be compared to those of the lymphatic channels of the mammalian diaphragmatic peritoneum (Azzali, 1999). In the latter, the mesothelial cells have large openings called "lymphatic stomata" that are considered direct connections between the serous cavity and the lymphatic vessels, through which substances are drained into the lymphatic system (Azzali, 1999). Although in amphibians mesothelial perforations similar to the mammalian "lymphatic stomata" have been described at the peritoneal level (Baldwin et al., 1990), in the pericardial mesothelium of *Rana esculenta* we did not find any evidence indicating the presence of structures resembling such lymphatic openings. However, the remarkable pool of intramesothelial caveolae, the presence of large circular blebs open into the PC, together with the characteristic arrangement of the cellular contacts, provide the morphological basis for theorizing an extensive transit of fluids through the pericardial mesothelium. Conceivably, such transit can be achieved via the different-sized vesicular structures and/or the intercellular pathways. Unfortunately, the direction of this traffic cannot be determined on the basis of the results of the present study because open caveolae have been described on both the mesothelial luminal surfaces (i.e., the surface facing the PC) and the abluminal one (i.e., the surface facing the intercellular spaces). In mammals, the extreme permeability of the mesothelial tissues, which offer little resistance to water and solute flux and are able to remodel themselves in response to the osmotic requirements of internal compartments, is well recognized (Payne et al., 1988; Michailova et al., 1999). At the cardiac level, exchanges between myocardial interstitial fluid (MIF), pericardial fluid (PF), and the cardiac lymph contribute to maintain the intrapericardial pressure, the equilibrium of ions, such as sodium, chloride, and potassium (Gibson and Segal, 1978), and the concentrations of active molecules such as paracrine hormones and growth factors (Corda et al., 1997), potentially important for cardiac function. In amphibians, the importance of the fluid turnover between the variably hydrated external environment, the interstitial compartments, and the blood is well known (Parsons et al., 1994; Fenoglio et al., 1998) but, surprisingly, nothing is known about the mechanisms involved in the control of the PF. On the basis of the lymphatic-like morphological characteristics described for the pericardial mesothelium, we suggest that in the frog the PP may exert an active role in the regulation of the PF and therefore of MIF drainage, acting as a large lymphatic space. This function may be crucial, given the absence of both lymphatic and blood drainage conduits in the avascular type of heart characteristic of amphibians (Staley and Benson, 1968).

The second purpose of this study was to explore whether these lymphatic-like features of the PP were influenced by a hormonal challenge with ANG II. Indeed, after the intraperitoneal administration of Val^5-ANG II, the molecular form of ANG II identified in the frog (Hasegawa et al., 1983), we observed relevant modifications of the PP that appeared highly edematous, with intracellular spaces larger than those described in untreated and in BSA-injected frogs. A notable modification of the intramesothelial pool of caveolae paralleled this finding. In fact, in ANG II-treated frogs these structures increased greatly in number (see Fig. 2A,B; quantitative data not shown) and frequently joined together, hence opening into very large blebs. Of note is the fact that caveolae are the location for many proteins involved in signal transduction cascades, including muscarinic cholinergic receptors, platelet-derived growth factor receptors, G-protein-linked receptors, G-proteins, calcium channels, and, in endothelial cells, also acylated nitric oxide synthase (cNOS) (Andries et al., 1998; Balligand, 2000). Convincing evidence indicates that the colocalization of several of these proteins and some receptors in restricted spaces of the caveolae represents spatially and temporarily "delimited" pathways for signal transduction. In mammals, in addition to its main hypertensive action, ANG II affects vascular permeability and lymphatic flow, being one of the most important idromineral and cardiovascular homeostatic factors (Valenzuela-Rendon and Manning, 1990). In amphibians, lymphatic-regulated salt and water balance is controlled by several substances such as the antidiuretic hormone and arginine vasotocin (Wentzell et al., 1993). Recently, ANG II was shown to exert an influence on amphibian osmotic equilibrium through the control of several physiological mechanisms (i.e., thirst-related behavior, cutaneous water gain, and renal handling of ions and water), all directly and/or indirectly modulating lymphatic system function (Hillyard, 1999; Johnson and Propper, 2000). The changed morphological profile observed in the frog PP after ANG II treatment is consistent with an ANG II-dependent stimulation of the pericardial mesothelial transport of fluids. We suggest that in the presence of increased concentrations of circulating ANG II, for example, those occurring in dehydrated conditions (Hillyard, 1999), intercellular and intracellular ANG II-mediated changes affect pericardial permeability, thus modulating PF characteristics and cardiac function.

In conclusion, this preliminary morphological study of the PP of the frog *Rana esculenta* indicates that this delicate serous membrane is a dynamic envelope structurally designed to adjust transmesothelial fluid transfer. The plasticity demonstrated by the frog pericardial mesothelium following ANG II stimulation highlights the putatively important role of the PP in the hormonal-dependent regulation of both the PF and the MIF. Future investigations, including immunolabeling and permeability studies,

will be necessary to clarify whether and to what extent the frog PP is a paracrine–autocrine source, and at the same time a target of peptidic hormones (Kuwara et al., 1992), cytokines, nitric oxide (Owens and Grisham, 1993), and growth factors (Gerwin et al., 1987; Corda et al., 1997), as suggested in mammals.

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