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Autoradiographic Localization of Atrial Natriuretic Peptide Binding Sites in the Pig Ovary

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Specific affinity binding sites for atrial natriuretic peptide (ANP) were investigated in the pig ovarian tissues by *in vitro* autoradiographic techniques. In the pig ovary, the highest binding sites for ^{125}I-labelled rANP$_{(1-28)}$ were localized in the granulosa cell layer of the follicles. The binding sies on theca layer of the ovarian follicles were mainly localized in the external layer, but none was observed in the internal layer. In the corpus luteum, the binding site was not observed. The specific bindings of 200 pM of ^{125}I-labelled rANP$_{(1-28)}$ to granulosa and theca externa layers were reversed completely by excess concentration (1 μM) of unlabelled rANP$_{(1-28)}$ but not by 10 μM of unrelated peptides, human angiotensin II and arginine vasopressin. The binding was also displaced by 1 μM of des[Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}] ANP$_{(4-23)}$ (C-ANF) as a specific ligand of the ANP clearance receptor. Therefore these results indicate that the biological and the clearance ANP receptors exist in the theca externa and granulosa layer of the pig ovary, and suggest that the ANP receptors may be related with the regulatory function of the ovarian follicular development including oocyte maturation.

KEY WORDS: ANP Receptor, Autoradiography, Corpus Luteum, Granulosa Layer, Pig Ovary, Theca Externa Layer

It is well known that atrial natriuretic peptide (ANP) has an important role in the regulation of extracellular fluid volume and blood pressure, such as natriuresis, diuresis, relaxation of vascular smooth muscle and suppression of renin-angiotensin-aldosterone system.

It has been suggested that the endogenous ANP may affect the female reproductive process, because the specific ANP binding sites have been found in the placenta (Roy *et al.*, 1988; Salas *et al.*, 1991), endometrium (Gililland *et al.*, 1992) and umbilical cord (Salas *et al.*, 1991). Especially in the ovarian system, Vollmar *et al.* (1988) reported the presence of immunoreactive atrial natriuretic peptide (ir-ANP) in the bovine ovary. We have also reported the presence of ANP in the follicular fluid and ovary of the pig (Kim *et al.*, 1989) and rat (Kim *et al.*, 1992), respectively. The identification of mRNA encoding ANP in the granulosa cells of the pig ovary suggested that the ovarian granulosa cells could be the site for the synthesis and secretion of ANP. The presence of ANP in the oocyte (Kim *et al.*, 1993) and the inhibition of oocyte maturation by ANP have also been reported (Tornell *et al.*, 1990). Furthermore, ANP has been shown to stimulate the secretion of progesterone in the ovary (Pandey *et al.*, 1987; Musah *et al.*, 1994). It is therefore suggested that the intra-ovarian ANP system may be closely related with the ovarian functions including the follicular development and oocyte maturation.

The presence of specific binding sites for ANP

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has been found in several ovarian tissues of mammals; in the preovulatory follicles of human (Kim *et al.*, 1987), the cultured human granulosa-luteal cells (Pandey *et al.*, 1987) and the bovine corpus luteum (Vollmar *et al.*, 1988). The cellular distribution of the receptors for ANP, and the biological functions in the ovarian tissues, however, have not yet been clarified. Therefore the purpose of the present experiments was to localize the binding sites for ANP in developmental stages of the pig ovary using *in vitro* autoradiographic analysis.

Materials and Methods

Iodination of $rANP_{(1-28)}$

^{125}I-labelled $rANP_{(1-28)}$ was prepared as decribed previously (Cho *et al.*, 1988a,b). Synthetic $rANP_{(1-28)}$ (5 μg/5 μl of 0.1 M acetic acid, Peninsula Laboratories, Belmont, CA, U.S.A.) was introduced into a vial containing 25 μl of 0.5 M phosphate buffered saline (pH 7.4) followed by addition of 1 mCi of ^{125}I-Na (Amersham International, U.K.). Chloramine-T (10 μg/10 μl) was added to the reaction vial, was mixed gently, and 30 seconds later bovine serum albumin (BSA, 60 mg/200 μl) solution was added. The reaction mixture was immediately applied to a Sephadex G-25 column (1.0 × 24 cm) and was eluted with 0.1 M acetic acid containing 0.3% BSA, 0.3% lysozyme, 0.1% glycine, and 200 KIU/ml aprotinin. The iodinated $rANP_{(1-28)}$ was divided and stored at −70°C until used. Immediately before using, the iodinated $rANP_{(1-28)}$ was repurified by high performance liquid chromatography on a reversed phase μBondapak column (Waters Associates, Milford, MA, U.S.A.) with a linear gradient (20% to 60% acetonitrile) elution. The specific activity (1,500–2,000 Ci $mmol^{-1}$) of ^{125}I-labelled $rANP_{(1-28)}$ was determined by radioimmunoassay technique (Morris, 1976; Joseph *et al.*, 1988).

Tissue preparation

Ovaries were obtained from the pigs within 5 min after slaughter, and were immediately snap frozen by liquid nitrogen. Sections (16 μm) were cut in a cryostat, thaw-mounted on gelatin-chrom-alum coated slides, and then dried in a desiccator overnight at 4°C.

in vitro autoradiography

The incubation conditions of ^{125}I-labelled $rANP_{(1-28)}$ were followed as other reports (Brown *et al.*, 1990., Brown and Zuo, 1992). Briefly, the sections were washed with 150 mM NaCl-0.5% acetic acid (pH 5.0) at room temperature for 10 min in order to remove the endogenous ANP, and then preincubated with 30 mM phosphate buffer (pH 7.2) containing 120 mM NaCl and 1 mM phenanthroline at room temperature for 8 min. They were then incubated with 200 pM of ^{125}I-labelled $rANP_{(1-28)}$ in fresh preincubation buffer containing 40 μg/ml bacitracin, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 0.5% BSA at room temperature for 60 min. After incubation, the sections were rinsed and washed with fresh preincubation buffer for 5 min at 4°C. Subsequently, they were rinsed three times in cold distilled water at 4°C and quickly dried under a stream of cold air. For analysis of the distribution of binding sites, the competitive inhibition of the binding of ^{125}I-labelled $rANP_{(1-28)}$ was examined on consecutive sections by incubating with the incubation mixture containing the radioligand plus 1 μM concentration of unlabelled $rANP_{(1-28)}$, C-ANF, porcine brain natriuretic $peptide_{(1-26)}$ ($pBNP_{(1-26)}$), or $ANP_{(5-25)}$ (Peninsula Laboratories). To test the specificity of ^{125}I-labelled $rANP_{(1-28)}$ binding, the adjacent sections were incubated in the presence of the unrelated peptides, human angiotensin II or arginine vasopressin (all 10 μM, Sigma Chemical Co., St. Louis, MO, USA). Autoradiographic images were generated by exposing the slides to Hyperfilm-^{3}H (Amersham International) in X-ray cassettes for 2 - 3 days. Autoradiograms were developed in Kodak D-19 at room temperature for 5 min. The slides were then counterstained with hematoxylin and eosin for tissue localization.

Results

Specific ^{125}I-labelled $rANP_{(1-28)}$ binding sites

were demonstrated in the pig ovarian tissues using *in vitro* autoradiographic technique.

As shown in Fig. 1, the comparison of autoradiograms with the corresponding hematoxylin-eosin stained sections revealed that the high concentration of ^{125}I-labelled rANP$_{(1-28)}$ binding sites was localized in the granulosa cell layer of the maturing follicles in the pig ovary. A low density of binding sites was revealed in the theca externa layer of the maturing follicles, while the binding was not observed in the theca interna layer and the interstitial region of the ovary. Fig. 2 shows the localization of specific ^{125}I-labelled rANP$_{(1-28)}$ binding sites in several developmental stages of follicles. Firstly specific ^{125}I-labelled rANP$_{(1-28)}$ binding site in the primary follicles was only noticed in granulosa layer. Following the follicles were surrounded by the theca folliculi, specific ^{125}I-labelled rANP$_{(1-28)}$ binding sites were found in the theca externa layer as well as in the granulosa layer. In these cases, specific ^{125}I-labelled rANP$_{(1-28)}$ bindings were revealed to be different, in which the specific binding affinity for ^{125}I-labelled rANP$_{(1-28)}$ in the granulosa and theca externa layers of the maturing follicles including antral and vesicular follicles was much higher than that in the growing follicles. In the Graafian follicle, specific ^{125}I-labelled rANP$_{(1-28)}$ binding was also found in the cumulus oophorus, a hillock of granulosa cells. But ^{125}I-labelled rANP$_{(1-28)}$ binding sites were absent in the ovum. The corpus luteum was shown not to contain the binding sites

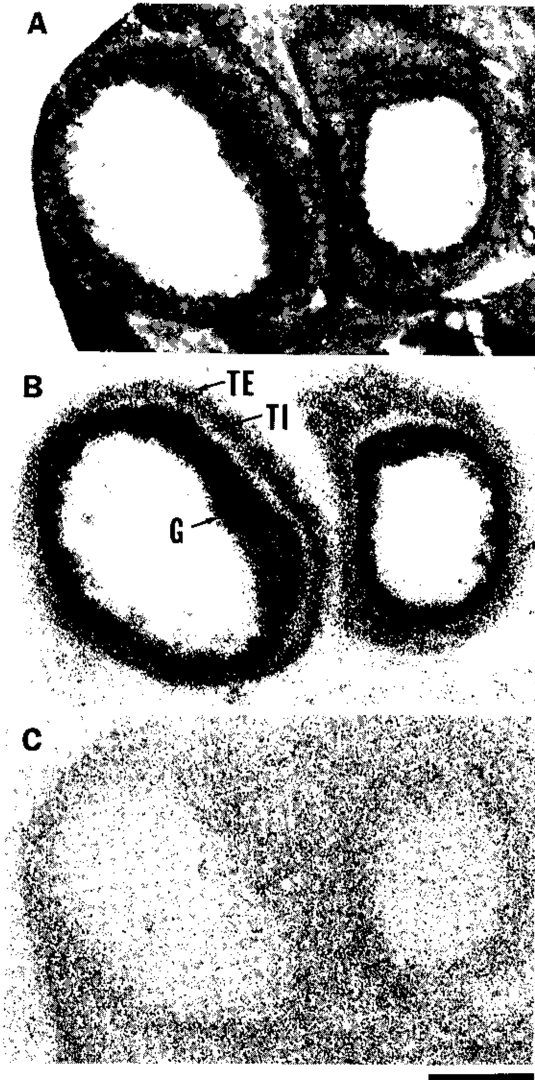

Fig. 1. Distribution of specific ^{125}I-labelled rANP$_{(1-28)}$ binding sites in maturing follicle of the pig ovary. Hematoxylin and eosin stained section (A). Autoradiogram of ^{125}I-labelled rANP$_{(1-28)}$ binding to adjacent section; total binding (B) and nonspecific binding in the presence of 1 μM unlabelled rANP$_{(1-28)}$ (C). Specific ^{125}I-labelled rANP$_{(1-28)}$ binding sites were located in the granulosa (G) and the theca externa (TE) layers of the follicles, but none in the theca interna (TI) layer. Bar = 500 μm.

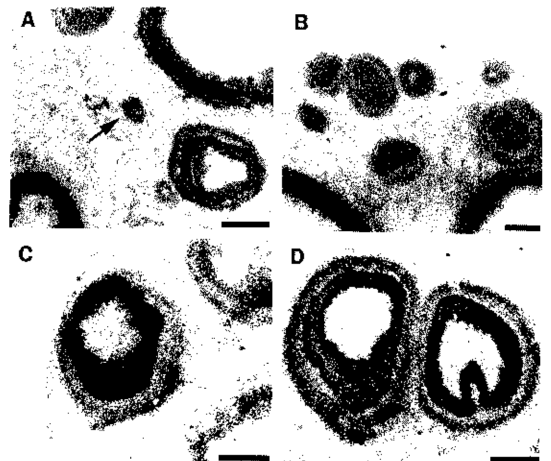

Fig. 2. Typical autoradiograms of ^{125}I-labelled rANP$_{(1-28)}$ binding to several developmental stages of follicles in the pig ovary. (A) primary follicle (arrow), (B) growing follicle, (C) maturing follicle, and (D) Graafian follicle with cumulus oophorus. Bar =500 μm.

(Fig. 3).

In the presence of 1 μM unlabelled $rANP_{(1-28)}$ the bindings to the granulosa and theca externa layers of follicles were completely displaced, but the diffuse background bindings were not affected (Fig. 4B). Ten micromolar unrelated peptides including human angiotensin II and arginine vasopressin did not displace the binding of ^{125}I-labelled $rANP_{(1-28)}$ at either granulosa or theca externa layers of follicles (data not shown).

The displacement of ^{125}I-labelled $rANP_{(1-28)}$ binding to the granulosa and theca externa layers by C-ANF, a specific ligand for the ANP clearance receptor, was also investigated (Fig. 4D). Unlabelled 1 μM C-ANF displaced completely ^{125}I-labelled $rANP_{(1-28)}$ binding in these specific regions. To test the displacement of ^{125}I-labelled $rANP_{(1-28)}$ binding to the granulosa and theca externa layers by other ligands, unlabelled $pBNP_{(1-26)}$ or unlabelled $ANP_{(5-25)}$ was added to the incubation mixture. Either 1 μM of $pBNP_{(1-26)}$ or $ANP_{(5-25)}$ displaced completely ^{125}I-labelled rANP $_{(1-28)}$ binding to the granulosa and theca externa layers of the follicles (Fig. 4C).

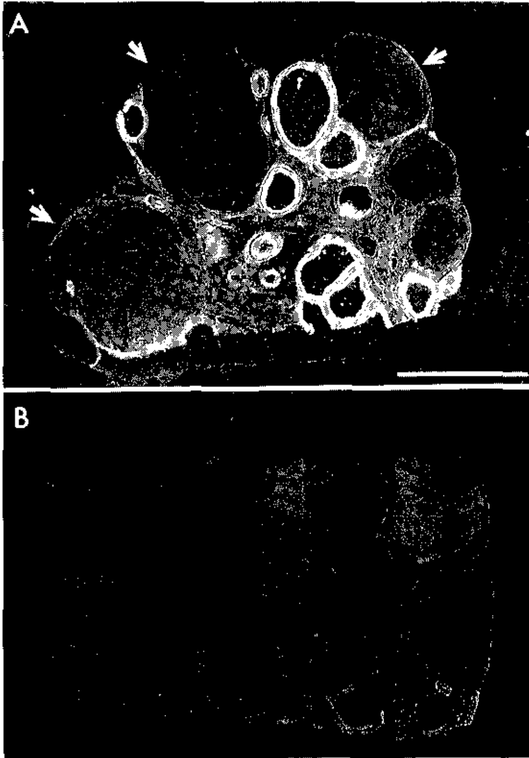

Fig. 3. Dark-field photomicrograph of autoradiograms of the pig ovarian sections with corpus luteum (arrow) incubated in the presence of 200 pM ^{125}I-labelled rANP $_{(1-28)}$ (A), and its adjacent sections incubated in 200 pM ^{125}I-labelled $rANP_{(1-28)}$ plus 1 μM unlabelled $rANP_{(1-28)}$ (B). ^{125}I-labelled $rANP_{(1-28)}$ binding sites appear as white silver grains. Bar =700 μm.

Discussion

Our results clearly provide evidence for the existence of specific binding sites of ^{125}I-labelled $rANP_{(1-28)}$ in the granulosa and theca externa layer of the maturing follicles in pig ovary as well as in the granulosa cell layer of the primary follicles. No significant binding sites of ^{125}I-labelled $rANP_{(1-28)}$ in the theca interna layer of any

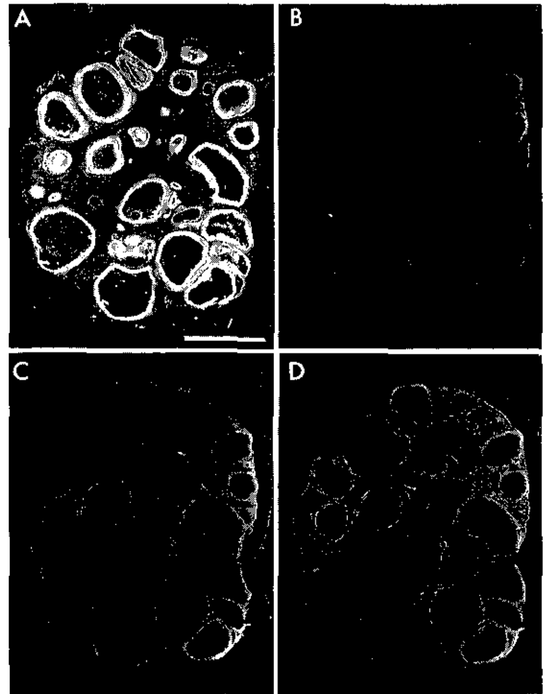

Fig. 4. Dark-field photomicrograph of autoradiograms of the pig ovarian sections incubated in the presence of 200 pM ^{125}I-labelled $rANP_{(1-28)}$ (A), and its adjacent sections incubated in 200 pM ^{125}I-labelled $rANP_{(1-28)}$ plus 1 μM unlabelled $rANP_{(1-28)}$ (B), 1 μM unlabelled $pBNP_{(1-26)}$ (C) or 1 μM unlabelled C-ANF (D). ^{125}I-labelled $rANP_{(1-28)}$ binding sites appear as white silver grains. Bar =500 μm.

developmental stages of the follicles, ovum, corpus luteum and the other structure of the interstitium were found in this experiments using *in vitro* autoradiographic technique.

The presence of specific ANP receptors in the ovarian tissues was reported. Pandey *et al.* (1987) have demonstrated that the binding of ^{125}I-ANP to the human granulosa-lutein cell membranes was competed by unlabelled ANP in a dose-dependent manner. Kim *et al.* (1987) have reported that high-affinity binding of ^{125}I-rANP was found in ovarian follicular tissues by membrane binding assay. Previous studies have suggested that ovarian ANP might be involved in the regulation of follicular growth (Kim *et al.*, 1989, 1992; Sundfjord *et al.*, 1989), steroidogenesis (Steegers *et al.*, 1990), and ovulation (Kim *et al.*, 1993; Tornell *et al.*, 1990). It has also been reported that ANP could stimulate progesterone secretion and cyclic guanosine monophosphate (cGMP) accumulation *via* its receptors (Pandey *et al.*, 1987). Therefore, these results imply that specific binding sites for ANP located in the ovarian tissues may directly participate in the intra-ovarian ANP system.

In the present studies, *in vitro* autoradiography was applied in an attempt to detect the localization of specific binding sites for ^{125}I-labelled $rANP_{(1-28)}$ and to characterize the conservative structural requirements with analogues of $ANP_{(1-28)}$ for ANP receptors in the pig ovary with developmental stages of follicles. The binding of ^{125}I-labelled $rANP_{(1-28)}$ in the theca externa and granulosa cell layers of growing and maturing follicles as well as in the granulosa layer of primary follicles in pig ovary was apparently specific, because it was displaced by the excess concentration (1 μM) of unlabelled $rANP_{(1-28)}$ but not at all by unrelated peptides, angiotensin II and arginine vasopressin (all 10 μM). The finding that the specific binding sites for $ANP_{(1-28)}$ with high affinity exist in the granulosa cell layer of the pig ovarian follicles are in consistent with the results obtained from the same structure of human (Pandey *et al.*, 1987). Kim *et al.* (1987) have also demonstrated the high affinity binding of ^{125}I-rANP in ovarian follicular tissues of human using membrane binding assay. They also suggested that the binding capacity may be different by developmental stages. But, in that case, the examined cell type was not clear.

It is worthy to notice that the specific binding sites for ^{125}I-labelled $rANP_{(1-28)}$ in the theca layer of the pig ovarian follicles were observed in the external layer but not in the internal layer. It is well known that the theca layer during the developments of the follicles in mammalian ovary differentiate into two layers, the internal and external layers, and has been considered very important sites for steroidogenesis. Especially, ANP and angiotensin coexist in the ovary (Usuki *et al.*, 1993), and angiotensin II (AII) receptors also locate in the theca interna layer as well as in the granulosa cell layer of the follicles (Speth *et al.*, 1986; Husain *et al.*, 1987). Therefore, our results suggest that ANP and AII may play a possible antagonistic action within the ovarian structures.

Our results show that the specific binding sites for ^{125}I-ANP in the Graafian follicle are also localized in the cumulus oophorus but not in the ovum. Tornell *et al.* (1990) have reported that ANP could inhibit dose-dependently spontaneous rat oocyte maturation, and increase cGMP accumulation in oocyte-cumulus complexes without elevating cyclic adenosine monophosphate levels. Our finding could give good evidence for ANP functions related to the oocyte maturation in the cumulus oophorus.

Vollmar *et al.* (1988) have reported the presence of specific binding site for ^{125}I-ANP in the bovine corpus luteum by membrane binding assay with the crude homogenates of total corpus luteum, and also the increased production of cGMP by synthetic ANP in the corpus luteum membrane. In the present experiments, however, the specific binding site of ^{125}I-labelled $rANP_{(1-28)}$ in the corpus luteum of pig ovary was not found. Further study remains to be confirmed whether the different characteristics of the binding is related to the species difference, or not.

We also found that $pBNP_{(1-26)}$ (1 μM) displaced ^{125}I-labelled $rANP_{(1-28)}$ from all of the binding sites in theca externa and granulosa cell layers of the follicles. It is well known that $pBNP_{(1-26)}$ is a powerful agonist for guanylate cyclase-coupled ANP receptors (ANPR-A and B) in various tissues

of different species (Song *et al.*, 1988; Hirata *et al.*, 1988; Maeda *et al.*, 1990). Therefore, these results suggest that endogenous BNP may be the natural ligands for the ANP receptors in the theca externa and granulosa cell layers of the ovarian follicles. However, the existence of BNP or BNP-like peptide in the ovarian tissues has not yet been reported. As shown in the present experiments, C-ANF and $ANP_{(5\text{-}25)}$ selectively displaced the binding of ^{125}I-labelled $rANP_{(1\text{-}28)}$ in the theca externa and granulosa cell layers. It has been known that C-ANF and $ANP_{(5\text{-}25)}$ bind to the clearance receptors but not to the biological receptors coupled to guanylate cyclase (Schenk *et al.*, 1987; Leitman *et al.*, 1988; Porter *et al.*, 1988). Thus these results indicate that the theca externa and granulosa cell layers of the pig ovarian follicles may possess the biological and the clearance receptors for ANP, although their proportional distribution of ANP receptor subtypes in these structures are not yet defined.

In conclusion, we have provided autoradiographic evidence for the specific binding sites of ANP in pig ovarian follicular structures; theca externa as well as granulosa cell layers. Our results suggest that the intra-ovarian ANP may have roles for the ovarian functions including the follicular development. Quantitative and biochemical studies are necessary to characterize the proportion and affinity of the ANP receptor subtypes.

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돼지 난소 Atrial Natriuretic Peptide 결합 부위의 자가방사법에 의한 검증

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돼지의 난소 조직내에 존재하는 심방이뇨 호르몬(atrial natriuretic peptide, ANP) 수용체의 분포를 알아 보기 위하여, 자가방사법을 통해 ^{125}I로 표지한 rANP$_{(1-28)}$의 특이적 결합 부위를 관찰하였다. 난소 조직 중 ^{125}I-rANP$_{(1-28)}$의 강한 결합 부위는 난포의 과립막 세포층이었으며, 외난포막층에서도 ^{125}I-rANP$_{(1-28)}$의 결합 부위가 관찰되었다. 그러나 내난포막층을 포함한 난소내의 다른 조직과 특히 황체에서는 ^{125}I-rANP$_{(1-28)}$의 결합 부위가 나타나지 않았다. 난포의 과립막 세포층과 외난포막층에서의 이러한 ^{125}I-rANP$_{(1-28)}$의 결합은 다량의 rANP$_{(1-28)}$에 의하여 완전히 전위되었지만, 펩티드 호르몬인 angiotensin II 및 arginine vasopressin에 의해서는 과량의 농도에서도 전위되지 않아 ^{125}I-rANP$_{(1-28)}$의 결합이 특이적임을 확인하였다. 또한 이러한 특이적 결합은 심방이뇨 호르몬의 생물학적 수용체 외에, 다른 기능을 담당하는 clearance 수용체의 특이적 ligand인 C-ANF에 의해서도 전이되었다.

이상의 결과는 돼지의 난소에 있어서 난포의 과립막 세포층 및 외난포막에 심방이뇨 호르몬의 생물학적 또는 clearance 수용체가 존재함을 보여주며, 이는 심방이뇨 호르몬의 수용체가 난자의 성숙에 관련된 난포의 발달과정에 관여할 수 있음을 시사한다.