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Abundant Expression of Sodium-Potassium–Activated Adenosinetriphosphatase Alpha 1 Subunit in Corpus Luteum of Porcine Ovary

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Follicular development is accompanied by the accumulation of follicular fluid. During corpus luteum formation, follicular fluid is diminished and antrum is replaced by lutein cells. These dynamic changes in fluid distribution suggest the existence of control mechanism of fluid transport and membrane permeability. One of the major factors regulating membrane permeability is the sodium-potassium–activated adenosinetriphosphatase (Na^+-K^+-ATPase). To elucidate the possible involvement of Na^+-K^+-ATPase in follicular growth and luteinization, immunohistochemical localization of Na^+-K^+-ATPase α1 subunit and enzyme activity in porcine ovary were investigated. In primordial follicles, Na^+-K^+-ATPase α1 subunit immunostaining was localized only in the oocyte and the surrounding stromal cells. In preantral follicles, immunostaining for Na^+-K^+-ATPase α1 subunit became apparent in granulosa and theca cells. As the follicle matured, the staining intensity in the oocyte, theca, and granulosa cells increased, which corresponded with the enzyme activity. Na^+-K^+-ATPase α1 subunit immunostaining became most abundant in granulosa and theca lutein cells in corpus luteum, and decreased in the regressing corpus luteum. Enzyme activity in corpus luteum was significantly higher than that in the follicles. This is the first study indicating that Na^+-K^+-ATPase α1 subunit expression is augmented in granulosa cells by follicular growth and most abundant in lutein cells in the corpus luteum, suggesting its possible involvement in corpus luteum formation.

Key Words: Na^+-K^+-ATPase; ovary; granulosa; theca; follicular growth; corpus luteum.

Introduction

The growth of ovarian follicles, ovulation, and the subsequent transformation into corpora lutea is strictly regulated by specific set of hormones and growth factors *(1)*. Follicular development is accompanied by the granulosa cell proliferation and subsequent differentiation into granulosa lutein cells. The other characteristic phenomenon during follicular growth is the accumulation of follicular fluid *(2)*. The presence of follicular fluid testifies to its potential importance in ovarian physiology, including steroidogenesis, growth of the follicle, maturation of the oocyte, ovulation, and oocyte transport to the oviduct *(3)*. After ovulation, follicular fluid is diminished and antrum of the follicle is occupied by differentiated granulosa lutein cells, which result in the corpus luteum formation. These dynamic changes in fluid distribution over the course of follicular development and corpus luteum formation suggest that the control mechanism of fluid transport and membrane permeability may exist.

One of the major factors responsible for membrane permeability is the sodium potassium–activated adenosinetriphosphatase (Na^+-K^+-ATPase; the sodium pump) *(4)*. Na^+-K^+-ATPase plays a housekeeping role in the maintenance of intracellular ion composition by transporting internal Na^+ and external K^+ against their concentration gradients across the cell membrane. Thus, this enzyme regulates and maintains several cellular processes, such as regulation of cell volume *(5)*, contractility of muscle cells *(6)*, and excitability of neurons *(7)*. To perform these cellular functions, Na^+-K^+-ATPase utilizes 5–40% of the steady-state cellular energy, which is generated by hydrolysis of cytosolic ATP *(8)*.

Na^+-K^+-ATPase is a heterodimer that consists of one α subunit and one glycosylated β subunit *(8)*. α subunit (MW = 112,000) is a catalytic subunit that contains the binding site for Na^+, K^+, and ATP. Three different isoforms of α subunit (α1, α2, α3) are reported *(8)*. α1 is widely distributed in the plasma membrane of most cells. In contrast, α2 is expressed in the muscle, heart, adipose tissue, and brain, and α3 is present only in the heart and brain. β subunit (MW = 35,000) contains a highly glycosylated extracellular domain, facilitates the formation of the active $\alpha\beta$ complex, and regulates the levels of enzyme transported to the plasma membrane *(8)*. Three different isoforms of β subunit (β1, β2, β3) are also reported *(8)*.

Histochemical localization of ATPase active sites in the ovary has been studied in several mammalian species, e.g.,

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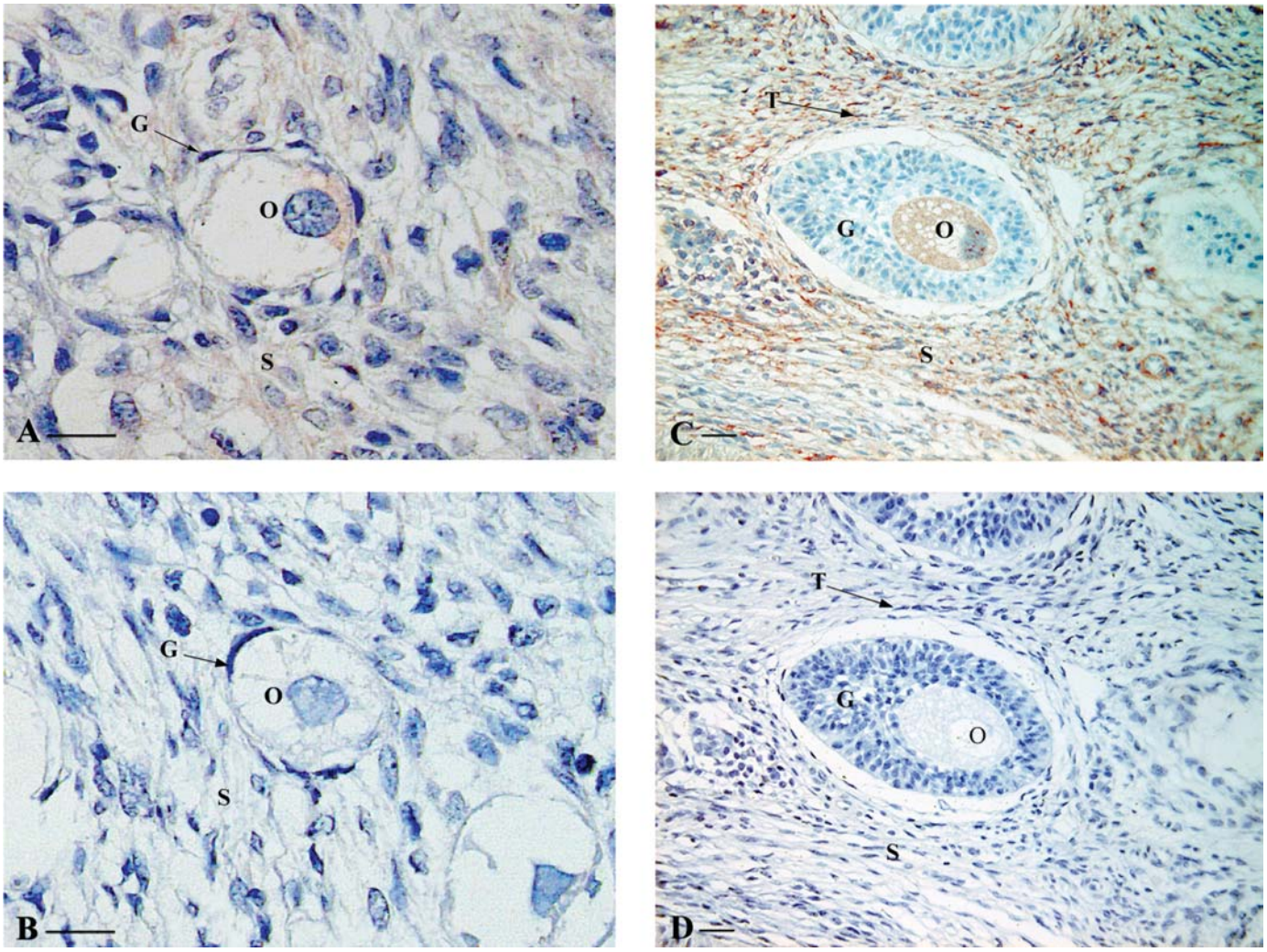

Fig. 1. Immunohistochemical localization of Na^+-K^+-ATPase α1 subunit in primordial (**A**) and preantral follicles (**C**) within the porcine ovary. Abbreviations represent as follows: oocyte (O), granulosa cells (G), surrounding stromal cells (S), and theca cells (T). In **B** and **D**, replacement of the primary antibody with non-immune rabbit serum showed a lack of positive staining. Bars represent 10 μm. Original magnification ↔400 for **A** and **B**, ↔200 for **C** and **D**.

guinea pig, rabbit, and human *(9–11)*. Sangha et al. *(12)* reported the histoenzymological changes in general ATPase activity during folliculogenesis, and corpus luteum formation and regression in the rat ovary. ATPase activity in small follicles in rat ovaries was weak in the oocyte, mild in granulosa cells, but strong in the theca cells. As the follicle grew, ATPase activity decreased in the oocyte and in the granulosa cells, whereas in corpus luteum, moderate to strong ATPase activity was observed. Ge and Spicer *(13)* showed positive immunostaining for Na^+-K^+-ATPase only in interstitial cells and theca interna cells in the rat ovary. However, to our knowledge, no systematic investigations have been achieved to determine the changes in Na^+-K^+-ATPase expression in the porcine ovary during folliculogenesis and corpus luteum formation.

In this study, we focused on the immunohistochemical localization of Na^+-K^+-ATPase α1 subunit in the ovary because this subunit is a functional catalytic subunit and the Na^+-K^+-ATPase α1 subunit expression is associated with the enzymological activity of Na^+-K^+-ATPase (see Results section). This is believed to be the first to demonstrate the changes in immunolocalization of Na^+-K^+-ATPase α1 subunit during follicle development and corpus luteum formation and regression in the porcine ovary.

Results

Immunohistochemical Localization of Na^+-K^+-ATPase α1 Subunit in the Follicles

In primordial follicles, immunostaining for Na^+-K^+-ATPase α1 subunit was moderate in the oocyte and weak in the surrounding stromal cells (Fig. 1A). In preantral follicles, immunostaining for Na^+-K^+-ATPase α1 subunit was weak in granulosa cells (Fig. 1C). In antral follicles, immunostaining

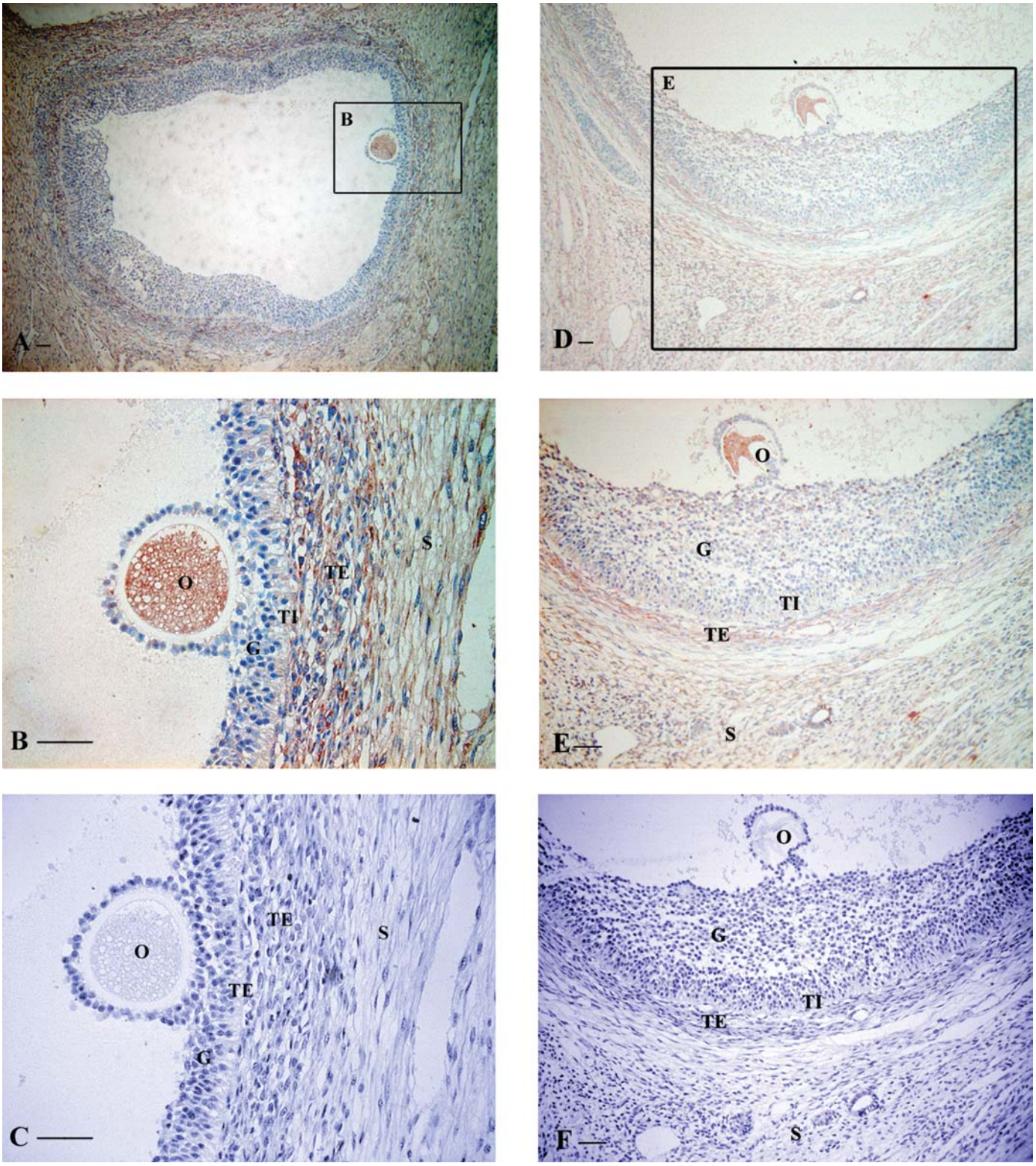

Fig. 2. Immunohistochemical localization of Na^+-K^+-ATPase α1 subunit in antral (**A,B**) and preovulatory follicles (**D,E**) within the porcine ovary. Abbreviations represent as follows: oocyte (O), granulosa cells (G), surrounding stromal cells (S), theca interna cells (TI), and theca externa cells (TE). The control staining in antral follicle (**C**) and preovulatory follicle (**F**). See Fig. 1 for explanation of the control. Bars represent 10 μm. Original magnification ↔100 for **A** and **D**, ↔400 for **B** and **C**, and ↔200 for **E** and **F**.

for Na^+-K^+-ATPase α1 subunit was intense in the oocyte and theca externa cells, moderate in theca interna cells and the surrounding stromal cells, while it remained weak in granulosa cells (Fig. 2A, B). In preovulatory follicles, Na^+-K^+-ATPase α1 subunit immunoreactivity increased in granulosa cells, which was comparable with that in the surrounding

Table 1
Immunostaining for Na^+-K^+-ATPase α1 Subunit in Follicular and Stromal Compartments in the Different Stages of Follicular Growth

Follicular Stage	Oocyte	Granulosa cells	Theca cells		Surrounding stromal cells
			Interna	Externa	
Primordial (n = 10)	++	–			+
Preantral (n = 15)	++	+	++		++
Antral (n = 18)	+++	+	++	+++	++
Preovulatory (n = 15)	+++	++	++	+++	++

Grading by intensity: – not detectable; + weak but definitely detectable; ++ moderate staining; +++ intense staining.

Table 2
Immunostaining for Na^+-K^+-ATPase α1 Subunit in Luteal Tissues and the Surrounding Stromal Cells in the Different Luteal Stages

Luteal stage	Granulosa lutein cells	Theca lutein cells	Surrounding stromal cells
Corpus luteum (n = 10)	+++	+++	++
Regressing corpus luteum (n = 8)	++	++	+
Corpus albicans (n = 7)	+	+	+

Grading by intensity: – not detectable; + weak but definitely detectable; ++ moderate staining; +++ intense staining.

stromal cells (Fig. 2D, E). Immunostaining for Na^+-K^+-ATPase α1 subunit in the stromal cells surrounding the follicles was weak to moderate over the course of follicular growth (Table 1). The replacement of primary antibody with non-immune rabbit serum showed a lack of positive immunostaining of the oocyte, granulosa, theca, and surrounding stromal cells (Fig. 1B, 1D, 2C, 2F).

Immunohistochemical Localization of Na^+-K^+-ATPase α1 Subunit in the Corpus Luteum

Table 2 summarizes the immunohistochemical localization of Na^+-K^+-ATPase α1 subunit in corpora lutea and the surrounding stromal cells over the course of the luteal development and regression. Corpora lutea showed intense immunostaining for Na^+-K^+-ATPase α1 subunit in granulosa lutein cells and theca lutein cells (Fig. 3A). In regressing corpora lutea, immunostaining for Na^+-K^+-ATPase α1 subunit was moderate in granulosa lutein cells and theca lutein cells (Fig. 3B). In corpus albicans, Na^+-K^+-ATPase α1 subunit staining decreased (Fig. 3C). The surrounding stromal cells showed weak to moderate immunostaining for Na^+-K^+-ATPase α1 subunit over the course of luteal development and regression. The replacement of primary antibody with non-immune rabbit serum resulted in the lack of positive staining of the luteal cells and stromal cells (Fig. 3D).

Na^+-K^+-ATPase Activity in the Follicles and Corpus Luteum

Na^+-K^+-ATPase activity in granulosa cells gradually and significantly increased as the follicle became larger (Fig. 4). Na^+-K^+-ATPase activity [μmol Pi·(mg protein)$^{-1}$·min^{-1}] (see definition in method section) was 15.77 ± 0.07 in small follicles, 16.73 ± 0.06 in medium follicles, and 19.28 ± 0.06 in large follicles. Na^+-K^+-ATPase activity in corpus luteum was significantly higher (24.50 ± 0.06) than that during follicular growth (Fig. 4).

Discussion

Like other somatic cells, functional Na^+-K^+-ATPase αβ complexes exist in the oocyte's plasma membrane *(14)*. A histoenzymological study *(12)* showed that ATPase activity in the rat oocyte was weak during the early stages of follicular development and decreased as the follicle matured. In contrast, Na^+-K^+-ATPase α1 subunit expression in the porcine oocyte was weak in the primordial follicle but increased as the follicle reached to the preovulatory phase. This gradual increase in Na^+-K^+-ATPase α1 subunit expression might be due the changes of the oocyte permeability and metabolism during the oocyte maturation. When comparing our findings with those previously obtained in rat *(12)*, although difference of animal species should be taken into consideration, it must be emphasized that the ATPase activity in rat was a sum of the several ATPase activities, such as Na^+-K^+-ATPase, Ca^{2+}-ATPase, Mg^{2+}-ATPase, and H^+-ATPase. On the other hand, our study specifically demonstrated the Na^+-K^+-ATPase α1 subunit expression, which is associated with the Na^+-K^+-ATPase activity (Fig. 4).

Follicular fluid is first seen as a secretion accumulating between the layers of granulosa cells *(15)*. This secretion is an extracellular product of the granulosa cells, as judged by autoradiographic study *(16)*. Follicular fluid is separated from the blood circulation by two layers of tissue; namely, membrana proplia lies between granulosa cells and theca interna cells *(15)*. The basement membrane of the granulosa cell layer appears to be very permeable, especially that of the growing follicle *(3)*. Therefore, follicular fluid is composed partly of secretion from granulosa cells *(15)* and partly of exudates from plasma *(16)*. As the follicle grew, immunostaining for Na^+-K^+-ATPase α1 subunit in porcine granulosa cells became abundant. Higher Na^+-K^+-ATPase activity in granulosa cells in growing follicles creates the ion gradients across the cell membrane, which may gener-

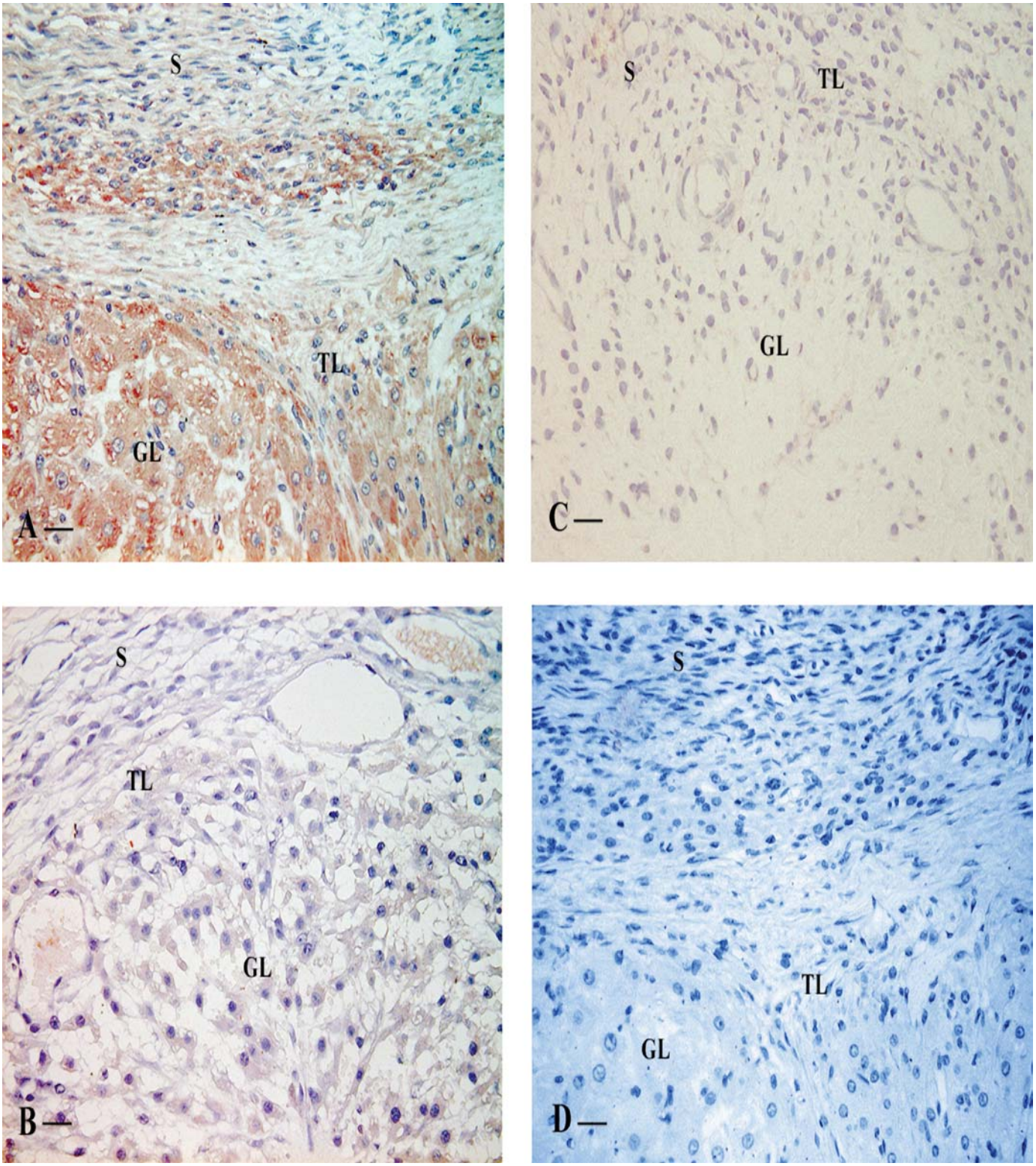

Fig. 3. Immunohistochemical localization of Na^+-K^+-ATPase α1 subunit in corpus luteum (**A**), the regressing corpus luteum (**B**), and the corpus albicans (**C**) within the porcine ovary. Abbreviations represent as follows: granulosa lutein cells (GL), theca lutein cells (TL), and the surrounding stromal cells (S). The control staining in corpus luteum (D) is the serial tissue section that corresponds to A. See Fig. 1 for explanation of the control. Bars represent 10 μm. Original magnification ↔400.

ate the secretion force for the follicular fluid accumulation. The other possibility is that the difference in Na^+-K^+-ATPase activity between surrounding stromal cells and granulosa cells creates Na^+ and K^+ ion gradients. Because the basement membrane of the granulosa layer appears to be permeable, ion gradient might be immediately equilibrated by the

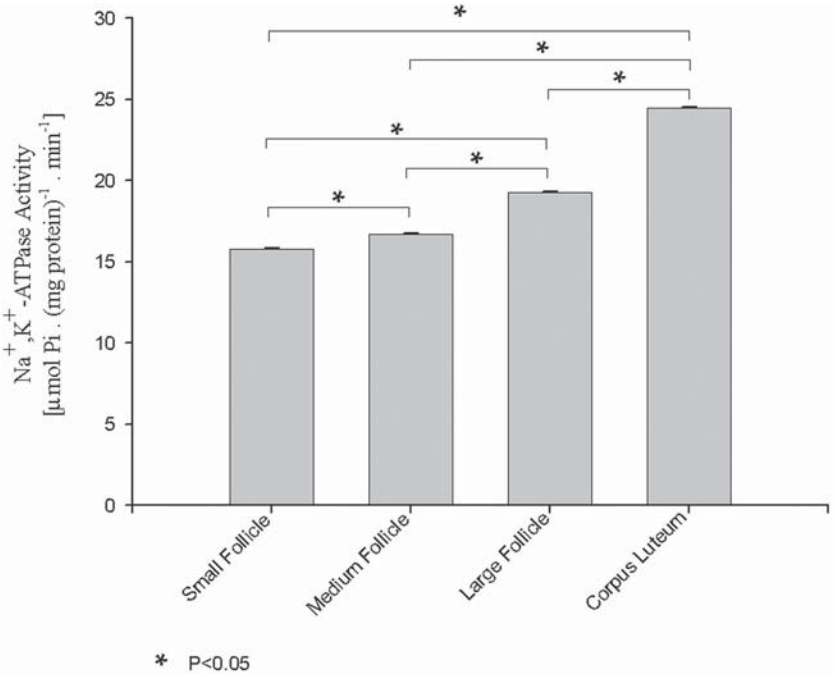

Fig. 4. Na^+-K^+-ATPase activity in small, medium, large follicles and corpus luteum of porcine ovary. Data are the mean ± SD of five independent experiments, with error bars too close to distinguish from the columns.

ion diffusion and subsequent water distribution. However, this ion gradient hypothesis is supported by the fact that more potassium was found in bovine follicular fluid than in the serum *(17)*.

During the process of luteinization, the follicular fluid is diminished and follicular antrum is replaced by the hypertrophied granulosa lutein cells. Na^+-K^+-ATPase α1 subunit expression became more abundant in granulosa lutein cells and theca lutein cells than in the surrounding stromal cells. We speculate that the increase of Na^+-K^+-ATPase expression in luteal cells relative to that in the surrounding stromal cells creates ion gradients that may lead to the disappearance of follicular fluid.

There is an increasing body of evidence indicating that granulosa cells have an electrical property. Changes at the level of the plasma membrane potential in granulosa cells may participate in the transduction mechanism for endocrine signals, and the hormonal stimulation may set the cell into a new functional status. Mattioli et al. *(18)* demonstrated that the depolarization of porcine granulosa cells has been recorded under the influence of luteinizing hormone, and this depolarization seems to be dependent on the inhibition of outward delayed rectifier potassium current *(19)*. The upregulation in Na^+-K^+-ATPase expression could be related to the changes of depolarization in granulosa cells *(18)*. Na^+-K^+-ATPase has an important role in driving a variety of secondary transport processes such as Na^+-dependent glucose and amino acid transport *(20,21)*. Luteal cells synthesize progesterone utilizing the energy generated by the glucose metabolism *(22)*. Thus, abundant expression of Na^+-K^+-ATPase in the corpus luteum may contribute to the progesterone production.

Ultrastructural studies uncover another hypothesis to explain the possible involvement of Na^+-K^+-ATPase in follicular growth and ovulation. Motta and DiDio *(23)* demonstrated that rabbit ovarian follicles are not passive organs, but contain granulosa cells with a contractile feature in developing, ovulating, and post-ovulatory follicles. Cavallotti et al. *(24)* demonstrated the abundant expression of microfilaments in granulosa cells in antral follicles and granulosa lutein cells in the corpus luteum. Microfilaments including smooth muscle actin and myosin are immunohistochemically localized in the wall of follicle and corpus luteum in the rat ovary *(25,26)*, especially in the theca externa cells *(27)*. Granulosa and theca externa cells may have some of the characteristics of smooth muscle cells. Moreover, adrenergic nerve fibers were distributed between the theca externa cells *(25)*. These results suggest that there is a possible involvement of neuromuscular mechanism in the development and rupture of the ovarian follicles *(28)*. The actin-based cytoskeleton is a dynamic structure that plays an active role in a multitude of cellular functions and events including stability of cell shape, the onset of motility, and the control of hormone action *(8)*.

In this study, Na^+-K^+-ATPase α1 subunit was immunohistochemically localized in the theca externa cells. Actin cytoskeleton interacts with the Na^+-K^+-ATPase via the protein ankyrin and spectrin *(29)*. On the other hand, Na^+-K^+-

ATPase α1 subunit is shown to have direct binding domain with the actin cytoskeleton *(8,30)*. The interaction between actin cytoskeleton and the Na^+-K^+-ATPase suggests a novel functional role of the cytoskeleton in the regulation of Na^+-K^+-ATPase-mediated Na^+ and K^+ transport *(8)*.

Taken together, this is the first study to demonstrate that Na^+-K^+-ATPase α1 subunit expression in granulosa cells, which corresponds with Na^+-K^+-ATPase activity, was augmented as the follicle matured and became most abundant in granulosa lutein cells in the corpus luteum, suggesting its biological role in follicular maturation and corpus luteum formation. Further study will be needed to elucidate the molecular mechanism in the participation of Na^+-K^+-ATPase in follicular growth and subsequent corpus luteum formation.

Materials and Methods

Porcine ovaries were obtained from a local slaughterhouse. The ovarian tissues were fixed in 10% buffered neutral formalin, dehydrated, and embedded in paraffin. Sections of 4 μm were deparaffinized and followed by standard histologic techniques.

Immunohistochemical Staining

Immunohistochemical staining was performed by the avidin/biotin immunoperoxidase method with the use of a polyvalent immunoperoxidase kit (Omnitags, Lipshaw, MI) as previously described *(31)*. A rabbit polyclonal antibody against Na^+-K^+-ATPase α1 subunit (Upstate, Lake Placid, NY) was used as the primary antibody. The first incubation with primary antibody diluted at 1:100 was followed by the incubation with biotinylated secondary antibody. After washing with PBS, avidin-horseradish peroxidase was applied and chromogenic reaction was developed by incubation with tetrahydrochloride diaminobenzidine. The sections were counterstained with Harris hematoxylin, mounted with Geltol mounting media supplied with Omnitags kit, and examined microscopically. The following control procedures were undertaken to ensure specificity of the immunological reaction. Adjacent control sections were subjected to the same immunoperoxidase method, except that the primary antibody against Na^+-K^+-ATPase α1 subunit was replaced by non-immune rabbit serum (Sigma, St. Louis, MO) at the same dilution. In the above-mentioned controls, no positive staining was observed. The intensity of immunostaining was evaluated by more than two observers, and was graded as (–) for no immunostaining, (+) for weak but definitely detectable immunostaining, (++) for moderate immunostaining, and (+++) for intense immunostaining on comparative basis.

Measurement of Ouabain-Sensitive Na^+/K^+-ATPase Activity

Follicular fluids were harvested aseptically by the needle aspiration method *(32)* from small (1–2 mm), medium (3–5 mm), and large (6–11 mm) follicles and from corpus luteum as previously described *(33,34)*. Granulosa and theca cells were separated from follicular fluids by centrifugation at 500*g* for 5 min at 4°C, and washed twice with Dulbecco's PBS. Protein extraction was performed according to the method of Jorgensen *(35)*. One gram of cells were homogenized in 10 mL of 0.25 *M* sucrose, 30 m*M* histidine, pH 7.2, with five strokes in a glass homogenizer with a tight-fitting Teflon pestle operated at 1000 rpm. The homogenate was centrifuged at 6000*g* for 15 min. The sediment was resuspended by homogenization in the original volume of fresh sucrose-histidine medium and centrifuged again at 6000*g* for 15 min. The combined supernatants from the two centrifugations were centrifuged again at 48,000*g* for 30 min. The pellet was resuspended in sucrose-histidine medium and protein determination was conducted by the method of Lowry et al. *(36)* using bovine serum albumin as a standard. Na^+-K^+-ATPase activity was assayed according to the method of Esmann *(37)*. Na^+-K^+-ATPase activity was defined as the ouabain-sensitive hydrolysis of ATP in the presence of Na^+, K^+, and Mg^+. Nine hundred microliters of the reaction mixture for Na^+-K^+-ATPase assay containing 130 m*M* NaCl, 4 m*M* $MgCl_2$, 3 m*M* Na_2ATP, 20 m*M* KCl, and 30 m*M* histidine was brought to 37°C, and 100 μL of the 48,000*g* pellet of the cells (5 μg protein in 20 m*M* histidine and 25% glycerol) was added with or without 1 m*M* ouabain. After 5 min incubation, the reaction was terminated by the addition of 100 μL of 50% trichloroacetic acid (TCA) and the test tubes were cooled to 4°C. Inorganic phosphate (Pi) released from Na_2ATP was measured according to the method of Baginski et al. *(38)*. Na^+-K^+-ATPase activity was expressed as $\mu mol\ Pi \cdot (mg\ protein)^{-1} \cdot min^{-1}$. Each value is the mean ± SD of five independent experiments.

Statistical Analysis

The statistical significance of difference between group means was determined by Student's *t*-test. A statistical significant difference was considered to be present at $p < 0.05$.

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