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Angiotensin II Receptors: Localization of Type I and Type II in Rat Epididymides of Different Developmental Stages

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Abstract. Previous studies from our laboratory have provided evidence for the existence of a local renin-angiotensin system in the rat epididymis. Evidence has also accumulated, indicating that locally formed angiotensin II from the rat epididymis may play a paracrine and/or autocrine role in regulating epididymal electrolyte and fluid transport. In the present study, specific anti-peptide antibodies against the second extracellular loops of angiotensin II type I (AT_1) and type II (AT_2) receptors were used to localize immunocytochemically these receptors in the rat cauda epididymides of three developmental stages, namely, immature (2-week), early mature (6-week) and fully mature (10-week). The immunostaining intensity for AT_1 receptors was found to be stronger than that for AT_2 receptors throughout rat epididymides of all stages. However, the immunostaining for both AT_1 and AT_2 receptors observed in the fully mature rat epididymis was much more intense than that observed in the epididymides of the two younger stages. While the immunostaining for both AT_1 and AT_2 receptors in the younger rat epididymides appeared to be distributed in both basal and apical regions, the immunostaining in the fully mature epididymis was predominantly localized in the basal region. The present finding of the differential patterns of angiotensin II receptor immunoreactivity in three different stages of the rat epididymis may reflect the fine tuning of rat epididymal function by angiotensin II, acting as a paracrine or autocrine agent, during the course of development.

Key words: Renin-Angiotensin System — Angiotensin II receptor — Immunocytochemistry — Antibodies — Epididymis — Epithelium

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Introduction

It is well known that the epididymis plays a crucial role in sperm physiology. During their transit in the epididymis, the spermatozoa undergo important maturation changes and it is these changes that confer their fertilizing capacity (Bedford, 1967; Orgebin-Crist, 1967). It is also believed that the epididymis may be important in regulating the fertilizing ability of spermatozoa by providing an optimal fluid microenvironment for sperm maturation (Cooper, 1986). The regulation of epididymal electrolyte and fluid secretion has been suggested to affect the sperm transport and influence sperm maturation (Wong, 1986). Previous work from our laboratory has shown that epididymal anion secretion in the rat epididymis is subject to neural and humoral regulation (Leung et al., 1992*a;* Leung & Wong, 1992; Huang et al., 1993; Chan et al., 1994). Several lines of evidence have also accumulated to indicate that anion secretion in the rat epididymis may also be under local paracrine and/or autocrine control. A number of vasoactive peptides such as endothelin (Wong et al., 1989), calcitonin gene-related peptide (Leung et al., 1992*b*), arginine vasopressin (Lai et al., 1994) and angiotensin II (Wong et al., 1990) have been shown to have an effect on anion secretion by the rat epididymal epithelium, giving rise to the notion that the fine tuning of epididymal anion secretion could be provided by vasoactive peptides, possibly through their paracrine and/or autocrine regulatory interaction. Previous findings from our laboratory have indicated the presence of a local renin-angiotensin system in rat epididymal tissues suggesting that angiotensin II could be formed locally in the epididymis (Wong et al., 1990; Wong & Uchendu, 1990, 1991). Angiotensin II has also been immunocytochemically localized in the basal cells, and to a lesser extent, the principal cells of the rat epididymal epithelium, further indicating that angiotensin II

Table 1. Comparison of amino acid sequences of the second extracellular loops in human and rat angiotensin II type I receptors in type II receptors

Species	Position	Sequences
AT_1 receptor		
Human	165–191	I-H-R-N-V-F-F-I-E-N-T-N-I-T-V-C-A-F-H-Y-E-S-Q-N-S-T-L
Rat	165–191	I-H-R-N-V-Y-F-I-E-N-T-N-I-T-V-C-A-F-H-Y-E-S-R-N-S-T-L
AT_2 receptor		
Human	171–196	A-C-L-S-S-L-P-T-F-Y-F-R-D-V-R-T-I-E-Y-L-G-V-N-A-C-I
Rat	171–196	A-C-L-S-S-L-P-T-F-Y-F-R-D-V-R-T-I-E-Y-L-G-V-N-A-C-I

See Bergsma et al., 1992; Martin et al., 1994 for detailed nucleotide sequence

may be produced locally and play a role in local regulation of epithelial transport (Zhao et al., 1996).

A prerequisite for a local biological function of angiotensin II in the epididymis is the presence of specific receptors on the cell surfaces of epididymal epithelium. In this context, it is important to localize angiotensin II receptors in the rat epididymal epithelium to demonstrate a local paracrine and/or autocrine role of angiotensin II in regulation of epididymal functions. In addition, data on developmental variations in expression of specific angiotensin II receptors in the rat epididymis during its course of maturation could be essential for understanding the role of angiotensin II in epididymal and sperm functions. The present study employing specific anti-peptide antibodies against the second extracellular loops of angiotensin II receptors has demonstrated the presence of type I and type II of angiotensin II receptors in rat cauda epididymides of different developmental stages using immunocytochemistry in conjunction with confocal laser scanning microscopy.

Materials and Methods

Peptides

Peptides corresponding to the sequences, residues 165–191 and residues 171–196 (Table 1) of the second extracellular loops of the human angiotensin II receptor, type I (AT_1) and type II (AT_2) respectively (Bergsma et al., 1992; Martin et al., 1994) were synthesized commercially by Vitrogen (Canada). The peptides were judged to be pure by HPLC analysis on a Vydac C-18 column and by amino acid analysis, followed by mass spectral analysis.

Production and Purification of Anti-peptide Antibodies

Animal model and experimental procedure have been approved by the Animal Ethical Committee of University of Gothenburg, Sweden. Two rabbits were immunized by free human AT_1 or AT_2 receptor peptide (1 mg) which was emulsified in complete Freund's adjuvant and injected subcutaneously at multiple sites. Four weeks later, a booster injection (1 mg in incomplete Freund's adjuvant) was given. Rabbits were bled one week after the second injection. The immunoglobulin fractions were prepared from sera of rabbits by precipitation in 50% $(NH_4)_2SO_4$, and dialyzed extensively against phosphate buffered saline (PBS: 10 mM phosphate, 140 mM NaCl, pH 7.4). The antiserum was further loaded on a Sepharose 4B CNBr-activated gel (Pharmacia, Sweden) to which the human AT_1 or AT_2 receptor peptide was covalently linked. After washing of the immunosorbent with PBS, the specific anti-human AT_1 receptor peptide antibodies were eluted with 3 M potassium thiocyanate (pH 7.4) followed by extensive dialysis in PBS (Fu et al., 1995). In this study, anti-human AT_1 and AT_2 antibodies were employed in the rat because the amino acid residues 165–191 and residues 171–196 of the second extracellular loops of both human and rat AT_1 and AT_2 receptors are highly conserved (Bergsma et al., 1992; Martin et al., 1994) (Table 1).

Immunocytochemistry

Male Sprague-Dawley rats at three developmental stages: immature (2-week old), early mature (6-week old) and fully mature (10-week old), were used. Animals were sacrificed by cervical dislocation. The epididymides were then dissected out from testes, rinsed in PBS and immediately frozen in iso-Pentane which was precooled in liquid nitrogen. Cryostat sections (8 μm) was cut on Cryotome (Shandon AS 620 Cryotome). Sections were transferred onto gelatin-coated glass slides and air-dried for 20 min. Sections were then fixed with cold acetone (−20° C) for 10 min and were processed for the indirect immunofluorescence method. Residual acetone was removed by drying, followed with PBS wash (0.1 M phosphate, 140 mM NaCl, pH 7.4). Unspecific binding was blocked by 1% (w/v) bovine serum albumin (BSA) in PBS for 30 min at 37° C, followed with several PBS washes. Sections were then incubated overnight at 4° C with affinity-purified antibodies (1:200) diluted in PBS containing 0.1% BSA and 0.5% Triton X-100. After washing briefly six times with PBS, sections were incubated with anti-rabbit IgG-fluorescein $F(ab')_2$ fragment (Boehringer Mannheim, working dilution: 40 μg/ml) for 60 min at 37° C. Sections were again washed briefly six times with PBS and embedded in mounting medium (Vectashield, Vector). Sections were then examined by confocal laser scanning microscopy (Bio-Rad MRC-1000).

The specificity of the immunostaining was checked by the following control experiments: (a) substitution of primary antibodies with buffer, (b) replacement of primary antibodies with rabbit nonimmune serum; (c) preadsorption of primary antibodies with excess peptide antigens (1 mg/ml); (d) negative control using cultured horse sweat gland epithelial cells; (e) positive control using rat kidney cryosections.

Results

The immunoreactivity of AT_1 and AT_2 antibodies was compared in the rat cauda epididymides of three devel-

opmental stages: immature (2-week), early mature (6-week) and fully mature (10-week).

Immature Epididymis

It was noted that the histological morphology of the immature rat epididymal tubules was distinctly different from that of mature rat epididymides. The mature rat epididymal tubules were small and composed of a thick pseudostratified epithelial layer with a small irregular lumen when compared to those of older rat epididymal tubules. In the immature epididymis, immunostaining for AT_1 receptors was distributed throughout the epididymal epithelium. AT_1 immunostaining was also observed in the smooth muscle layer surrounding the epididymal tubules. The basal region of epithelial tubules displayed intense AT_1 immunoreactivity. Moreover, immunostaining was also found within the epididymal epithelial layer but it was difficult to distinguish whether the immunostaining was intracellular or at the basolateral membrane of the principal cells of the epithelium (Fig. 1*A*). A similar pattern of immunostaining for AT_2 receptors was also observed in the immature epididymis but with much weaker intensity throughout all regions (Fig. 1*B*).

Early Mature Epididymis

At this early mature stage, the epididymal lumen became larger when compared to that of the immature epididymis, but still with an irregular shape (Figs. 2*A–D*) compared to the rounded lumen of the fully mature epididymis (see below). In the early mature epididymis, intense AT_1 immunoreactivity was observed predominantly in the basal border of the epididymal epithelium as shown in Fig. 2*A*. However, moderate AT_1 immunoreactivity was also occasionally localized in the apical region (Fig. 2*B*). AT_2 immunoreactivity in the early mature epididymis was also observed in both the basal (Fig. 2*C*) and apical regions of the epididymal epithelium (Fig. 2*D*).

Fully Mature Epididymis

At the fully mature stage, the epididymal lumen was rounded and filled with spermatozoa and the epithelial layer became thinner and regularly shaped when compared to that of the epididymides of the two younger stages. The fully mature epididymis exhibited intense bandlike patterns of staining in the basal region of the epididymal epithelium when immunostained with the AT_1 antibody (Fig. 3*A*). In contrast, no AT_1 immunostaining was observed in the apical region of the epididymal epithelium. Similarly, intense AT_2 immunoreactivity, though less strong than that of AT_1 immunostaining, was found to be localized only in the basal border but not in the apical region of the epididymal epithelium (Fig. 3*B*). Both AT_1 and AT_2 immunostaining in the fully mature epididymis was found to be more intense than that found in the epididymides of the two younger stages. A comparison of the immunostaining results obtained in the rat cauda epididymides of the three developmental stages is given in Table 2.

Specificity of the AT_1 and AT_2 Antibodies

Specificity of the AT_1 and AT_2 immunostaining was confirmed by control experiments. Figure 4*A* shows the absence of immunostaining in the fully mature epididymis when AT_1 antibody was preadsorbed with its receptor peptide antigen in excess. Figure 4*B* shows the negative staining obtained in the fully mature epididymis when AT_2 antibody was preadsorbed with its receptor peptide antigen in excess. Substitution of primary antibodies with buffer or with rabbit pre-immune serum gave consistently negative results (*data not shown*). Using the same antibodies, negative staining of AT_1 and AT_2 were also obtained in horse sweat gland epithelial cells as shown in Fig. 4*C* and *D* respectively, excluding the possibility of crossreactivity. Positive staining of AT_1 in renal blood vessel was observed (Fig. 4*E*), which is consistent with the tissue distribution as reported by others.

Discussion

The present study has demonstrated the presence and localization of both AT_1 and AT_2 receptors in rat epididymides of three developmental stages. This finding may have a number of implications.

Involvement in Paracrine/Autocrine Regulation of Epididymal Anion Secretion

Previous studies from our laboratory have indicated the presence of a local renin-angiotensin system in the rat epididymis (Wong et al., 1990; Wong & Uchendu, 1990; 1991; Zhao et al., 1996). Cultured rat cauda epididymal epithelial cells have been shown to exhibit reninlike and angiotensin-converting enzyme activities, and contain immunoreactive angiotensin I and angiotensin II (Wong & Uchendu, 1990; 1991). Angiotensinogen, which is obligatory for an intrinsic renin-angiotensin system, has also been shown to be present in epididymal cells, presumably synthesized and processed in the cell cytosol by intracellular renin. A more recent immunocytochemical study from our laboratory has shown predominant localization of angiotensin II in the basal cells, and to a less extent, in the principal cells of the rat epididymal epi-

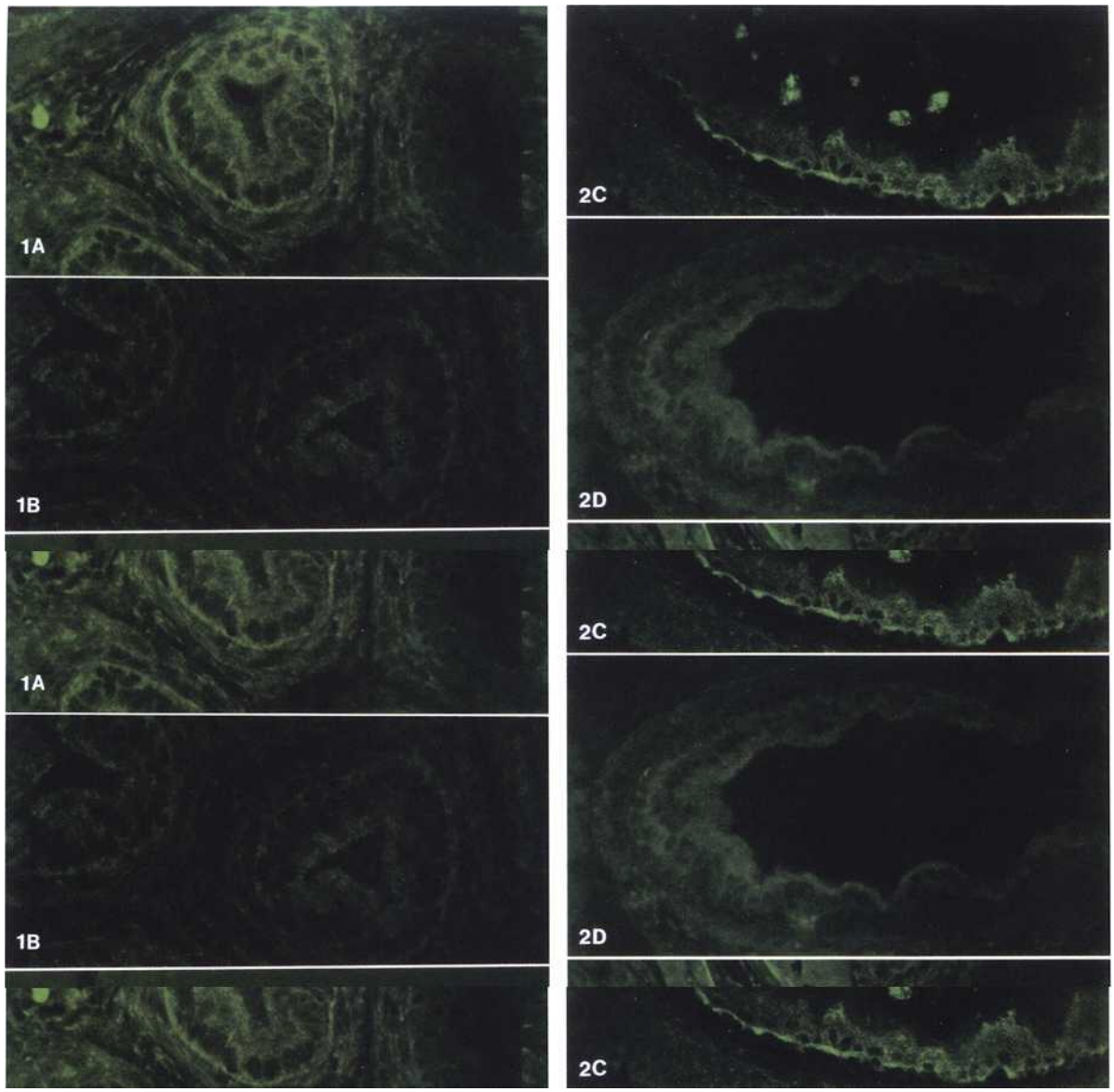

Fig. 1. Immunofluorescence localization of AT_1 and AT_2 receptors in cryostat sections from 2-week old rat cauda epididymis. ×400 (*A*). Intense and diffused AT_1 immunoreactivity was observed throughout the epithelial layer. It was noted that both basal and principal cells were immunolocalized with AT_1 staining. (*B*). Similar pattern but less intense AT_2 immunoreactivity was also observed in plasma membrane both basolaterally and apically from pseudostratified epithelium.

Fig. 2. Immunofluorescence localization of AT_1 and AT_2 receptors in cryostat sections from 6-week old rat cauda epididymis. ×400. (*A*). AT_1 immunoreactivity was predominantly localized in basal plasma membranes of the epididymal epithelial cells as demonstrated in the figure. (*B*). Moderate AT_1 immunoreactivity was also observed in apical plasma membranes of the epithelial cells. (*C*). AT_2 immunoreactivity was localized in basal region of epididymal epithelium. (*D*). Immunoreactivity for AT_2 was also localized in apical plasma membranes in this early mature epididymis.

Fig. 3. Immunofluorescence localization of AT_1 and AT_2 receptors in cryostat sections of 10-week old rat cauda epididymis. ×400. (*A*). Very intense AT_1 immunostaining with bandlike pattern was localized in basal membrane border of epididymal epithelial cells. (*B*). Less intense AT_2 immunostaining with similar pattern was also observed in the basal membranes of epithelial cells.

Table 2. Immunoreactivities of angiotensin II receptors, AT_1 and AT_2, in rat cauda epididymides of three different developmental stages

Epididymal stages	Immunoreactivity			
	AT_1		AT_2	
	Basal	Apical	Basal	Apical
2-week	+++	++	+	+
6-week	+++	++	+++	++
10-week	++++	–	+++	–

Immunoreactivity: ++++ = very intense; +++ = intense; ++ = moderate; + = weak; – = negative
The assessment of intensity was confirmed by three assessors

thelium, further indicating that angiotensin II could be produced locally and play a paracrine/autocrine role (Zhao et al., 1996). The evidence for a role for angiotensin II in epididymal functions was provided by demonstrating that angiotensin II, when added to the basolateral as well as the apical surfaces, elicited transient increases in the short-circuit current which was mediated by Cl^- secretion (Wong et al., 1990). Thus, a local renin-angiotensin system and its role in the regulation of epididymal anion secretion could be pertinent for sperm transport and maturation.

The present finding of both AT_1 and AT_2 receptors on both apical and basal surfaces of the epididymal epithelium suggests that these epithelial membrane-bound receptors are the primary site of action for angiotensin II. Thus, locally produced and secreted angiotensin II could act in a paracrine or autocrine fashion to regulate the anion secretion across the epididymal epithelium through the epithelial membrane-bound AT_1 and AT_2 receptors.

The Significance of AT_1 and AT_2 Receptors

The present immunocytochemical investigation has demonstrated the presence of angiotensin II receptor subtypes, namely AT_1 and AT_2, in the rat cauda epididymides. The specificity of AT_1 and AT_2 immunoreactivity has been demonstrated by the consistently negative staining observed in control experiments including preadsorption with their respective specific antigens. The specificity of immunostaining was further confirmed by positive staining in the rat kidney sections and negative staining in the horse sweat gland epithelial cells. The present finding of both AT_1 and AT_2 receptors in the rat epididymis is consistent with a previous observation using radioligand binding assay (Grove & Speth, 1989). It has been shown that the rat epididymis exhibits two functional angiotensin II binding sites which are localized in the circumference of the epididymal tubule and concentrated mainly within the cauda region of the epididymis. This has led to the suggestion that angiotensin II may be involved in epididymal function acting through two different receptors, although the angiotensin receptor subtypes have not been specified (Grove & Speth, 1989).

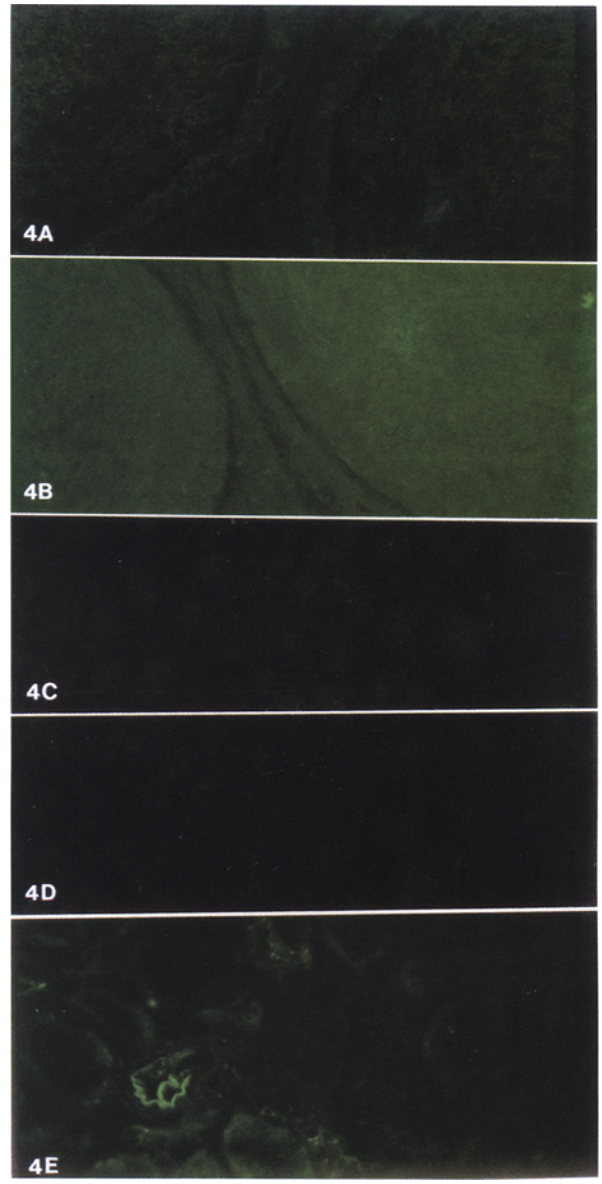

Fig. 4. Control experiments for AT_1 and AT_2 antibodies as demonstrated by immunofluorescent staining. ×400. (*A*). Negative immunostaining was detected in cryostat section of 10-week old rat cauda epididymis when AT_1 antibody was preadsorbed with its purified peptide antigen in excess. (*B*). Negative immunostaining was also obtained in cryostat section of 10-week old rat cauda epididymis when AT_2 antibody was preadsorbed with its purified peptide antigen in excess. (*C*) and (*D*). Negative results were observed when cultured horse sweat gland epithelium grown on coverslips was immunostained with AT_1 and AT_2 antibodies. (*E*). Positive staining of AT_1 was observed in blood vessel entering or leaving the renal corpuscle from cryostat section of rat kidney.

Using a monoclonal antibody against a decapeptide (amino acid residues 8–17) corresponding to the N-terminus of rat AT_1 receptors, the presence of AT_1 receptor in the rat epididymis as well as in epididymal sperm has recently been demonstrated but the precise location has not been clearly shown (Vinson et al., 1995). The present study, employing specific antibodies against the second extracellular loops of AT_1 and AT_2 receptors, has demonstrated the presence of AT_2 receptors, in addition to AT_1 receptors, on the luminal as well as the basal surface of the rat cauda epididymal epithelium for the first time. The advantages of our antibodies against 27-mer26-mer peptides of either AT_1 or AT_2 receptors are: (i) the longer peptide carries a sufficient number of B cell and T cell epitopes, which makes it possible to use free peptide as an immunogen and to produce high titre of antibodies with high affinity; (ii) the longer peptide can mimic in some way the conformation of the target AT receptors which makes the antibody very specific; (iii) the antibodies against second extracellular loop of G-protein coupled receptors with seven transmembrane domains has been repeatedly demonstrated to contain functional epitopes.

The localization of both receptors in the rat epididymis is similar throughout all developmental stages examined (*see below*) although the AT_1 receptor appears to be much more abundant than AT_2 judged by immunostaining intensity. The presence of both AT_1 and AT_2 receptors has also been demonstrated in the brain with different concentrations in 2-week old and 8-week old rat brain (Saavedra et al., 1993). However, it has been shown that the AT_1 receptor mediates all or most of the known effects of angiotensin II whereas a functional role for the AT_2 receptor has yet to be elucidated (Catt, 1993). One of the possible physiological roles of the angiotensin receptors in the epididymis is to mediate the regulatory action of angiotensin II on anion secretion across the epididymal epithelium (*see above*), although it is unclear if one or both types of receptors are involved. Further study employing specific receptor antagonists in conjunction with short-circuit current measurements will help to elucidate the role of AT_1 and AT_2 receptors in the regulation of epididymal anion secretion.

Developmental Differences

Both AT_1 and AT_2 immunoreactivities are more abundant in the fully mature epididymis than in the two younger stages, indicating an increased involvement of the angiotensin receptors in epididymal functions at later stages of development. While both AT_1 and AT_2 receptors appear to be abundantly located in the basal region of the epididymal epithelium throughout all developmental stages, these receptors in the epididymides of the two younger stages, but not in the fully mature epididymis, are also found to be present in the apical region, although with less abundance. This may reflect differential roles of these receptors in epididymal functions during various stages of development. Cultured epididymal epithelium from early mature rats (6-week) responded to exogenous angiotensin II, either apical or basolateral addition, with an increase in the short-circuit current which was mediated by Cl^- secretion. A more potent response was observed when angiotensin II was added to the basolateral side. These results could be explained by the presence of angiotensin receptors on both surfaces of the epithelium but of greater abundance in the basal region. Although all angiotensin receptors could be involved in the regulation of anion secretion, differentially located populations of receptors may have differential functional roles during development. On the other hand, the intense bandlike immunostaining for AT_1 and AT_2 receptors observed in the basal region of the fully mature epididymis may indicate a possible functional role of the receptors other than regulating anion secretion. For example, the angiotensin receptors in the basal region may also be involved in the regulation of tubular smooth muscle contraction to facilitate ejaculation of sperm.

The pattern of immunostaining in the immature epididymis is distinctly different from that observed in the two mature stages in that it was uniformly distributed throughout the epithelial layer. The widespread immunostaining could be associated with intracellular organelles. Nuclear angiotensin receptors have been reported in various tissue organs (*see* review by Phillips, Speakman & Kimura, 1993), which implies that the angiotensin receptors may be involved in the regulation of cellular and nuclear processing. It has been suggested that angiotensin II affects cell replication by increasing DNA content and plays a role in growth and development (Geisterfer, Peach, & Owens, 1988; Berk et al., 1989) and also stimulates the expression of a number of growth factors such as platelet-derived growth factor and growth-related oncogenes (Norman et al., 1987; Taubman et al., 1989; Viard et al., 1992). Therefore, the angiotensin receptors in the immature epididymis could also play a role in somatic maturation of the epididymis.

In summary, the present finding of angiotensin II receptors in the epididymis further supports a paracrine/autocrine role of angiotensin II in regulation of electrolyte and fluid transport in the rat epididymis. However, the precise role of each specific angiotensin receptor in epididymal development and functions still awaits further investigation.

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