Angiotensin II receptors, AT₁ and AT₂ in the rat epididymis
Immunocytochemical and electrophysiological studies


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Abstract

Previous work from our laboratory has provided evidence for the presence of a tissue renin-angiotensin system in the rat epididymis. In the current investigation, the regional localization of angiotensin II receptors, type I (AT₁) and type II (AT₂) was studied immunocytochemically using specific anti-peptide antibodies against the second extracellular loops of AT₁ and AT₂ receptors, and pharmacologically using specific receptor antagonists in conjunction with the short-circuit current technique. The immunocytochemical results showed that AT₁ and AT₂ immunoreactivities were predominantly localized in the basal region of the epididymal epithelium. Electrophysiological studies using the short-circuit current technique demonstrated a stimulatory effect of basolaterally applied angiotensin II on the epididymal electrogenic ion transport. This effect was inhibitable by the addition of AT₁ antagonist, losartan but not by AT₂ antagonist, PD123177, indicating a functional role of AT₁ in epididymal electrolyte transport. The present finding suggests that angiotensin II receptors may play an important role in the regulation of epididymal function.

Keywords: Angiotensin II; Angiotensin II receptor; Epididymis; Epithelium; Immunocytochemistry; Electrophysiology; (Rat)

1. Introduction

Mammalian spermatozoa are formed in the testes and transported into a highly coiled duct system, the epididymis. During their transit through the histologically defined regions namely, the caput, corpus and cauda epididymidis, the spermatozoa are subject to certain physiological, biochemical and morphological changes before they become mature and achieve full fertilizing capacity [1–3]. By virtue of the regulated absorptive and secretory activities of its epithelium, the epididymis may provide an optimal microenvironment for sperm maturation and thereby influencing sperm function [4–6].

Recently, evidence has been provided for the presence of several key components of the renin-angiotensin system in the rat epididymis [7,8]. The stimulatory effect of angiotensin II on electrolyte transport has also been observed in the rat epididymal epithelium [9]. Using immunocytochemistry, angiotensin II immunostaining has been specifically localized in the basal region of the epididymal epithelium with progressively increased intensity along the rat epididymis [10]. Recently, angiotensin II receptor, type I (AT₁) has also been immunocytochemically demonstrated in the rat epididymis as well as in epididymal
sperm. However, its precise localization along the epididymal tract has not been demonstrated [11]. The present investigation aimed to demonstrate the regional localization of angiotensin II receptor subtypes, AT₁ and type II (AT₂) in the rat caput, corpus and cauda epididymidis using immunocytochemistry. Electrophysiological studies, employing the short-circuit current technique in conjunction with specific receptor antagonists, were also performed to investigate the involvement of angiotensin II receptors in mediating anion secretion in the rat epididymis.

2. Materials and methods

2.1. Production of anti-peptide antibodies

Peptides corresponding to the sequences, residues 165–191 and residues 171–196 of the second extracellular loops of the human angiotensin II receptor subtypes, AT₁ [12] and AT₂ [13] respectively were synthesized commercially (Vitrogen, Inc., Canada).

Animal model and experimental procedure have been approved by the Animal Ethical Committee of University of Gothenburg, Sweden. Two rabbits were immunized by these free AT₁ or AT₂ 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 of AT₁ and AT₂ receptor peptide 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% NH₄SO₄, and dialyzed extensively against phosphate buffered saline PBS: 10 mM 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% bovine serum albumin 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 mounted in mounting medium (Vectashield, Vector Laboratories). Sections were then examined by confocal laser scanning microscopy (MRC-1000UV confocal imaging system, Bio-Rad) equipped with an Argon-ion UV laser and connected to an inverted microscope (Nikon Diaphot).

The following controls for AT₁ and AT₂ antibodies were used: (a) substitution of primary antibodies with buffer; (b) incubation with rabbit non-immune serum; (c) preadsorption of primary antibodies with excess peptide antigens (1 mg/ml); (d) positive control using rat adrenal gland cryosections.

2.2. Immunocytochemistry

Male Sprague-Dawley rats (300–320 g) were sacrificed by cervical dislocation. The epididymides were removed from testes, rinsed in PBS and immediately frozen in iso-pentane which was pre-cooled 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 staining 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 mounted in mounting medium (Vectashield, Vector Laboratories). Sections were then examined by confocal laser scanning microscopy (MRC-1000UV confocal imaging system, Bio-Rad) equipped with an Argon-ion UV laser and connected to an inverted microscope (Nikon Diaphot).

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2.3. Epididymal cell culture

The culture procedure for epithelial cells from the rat cauda epididymides was described previously [15,16]. Briefly, the cauda epididymides of male Sprague-Dawley rats (200–300 g) were removed, chopped into pieces and digested with trypsin. Tissue was collected by centrifugation and treated with col-
Isolated cells were plated at a density of about $1.4 \times 10^6$ cells/ml onto Millipore filters floating on Eagle’s Minimum Essential Medium (EMEM; ICN Biomedicals Inc., Costa Mesa, CA, USA). Cultures were incubated at 37°C in 5% CO$_2$ and 95% air atmosphere for four days.

Fig. 1. Immunofluorescent localization of AT$_1$ receptors in cryosections from different regions of the rat epididymis. Histologically, the epididymis displays multiple tubules in a cross-section. Basally localized immunostaining was found less distinct in the caput (1a) than that in the corpus (1b) and cauda (1c) epididymal tubules. Specificity of the AT$_1$ immunostaining was confirmed by the negative control experiment obtained in the cauda region when AT$_1$ antibody was preadsorbed with its receptor antigen in excess (1d). $\times 400$. 
2.4. Short-circuit current measurement

The measurement of short-circuit current ($I_{SC}$) has been described previously [16,17]. Monolayer cultures grown on filters after four days in culture were clamped between the two halves of the Ussing chambers. The sample was short-circuited (Voltage clamped at zero potential) using a W-P. Instruments Dual Voltage Clamp Amplifier (DVC 1000; World Precision Instruments Inc., New Haven, CT, USA).

Fig. 2. Immunofluorescent localization of AT$_2$ receptors in cryosections from different regions of the rat epididymis. AT$_2$ immunostaining was also predominantly localized in the basal region and exhibited less intensity in the caput (2a) and the corpus (2b), as compared to that in the cauda (2c) epididymal tubules. Negative immunostaining for AT$_2$ was obtained in cauda region when the respective antibody was omitted (2d). ×400.
and the $I_{SC}$ displayed on a pen recorder (Kipp and Zonen, Delft, The Netherlands). Monolayers were bathed at 37°C in both sides with Krebs-Henseleit solution consisting of the following composition (mM): NaCl, 117; KCl, 4.5; CaCl$_2$, 2.5; MgSO$_4$, 1.2; NaHCO$_3$, 24.8; KHPO$_4$, 1.2; glucose, 11.1. The solution was bubbled with 95% O$_2$ and 5% CO$_2$ to maintain the pH at 7.4. Drugs could be added directly to the basolateral or apical sides of the epithelium. The change in $I_{SC}$ was defined as the maximal rise in $I_{SC}$ upon agonist or antagonist application.

3. Results

3.1. Regional localization of AT$_1$ and AT$_2$ in rat epididymis

Positive immunoreactivity for AT$_1$ and AT$_2$ was consistently observed throughout the caput, corpus and cauda epididymidis. While there was no substantial difference in AT$_1$ immunostaining intensity observed in various regions of the epididymis, the outline of AT$_1$ immunostaining in the corpus and cauda appeared to be more distinct as compared to that in the caput (Fig. 1a-1c). The immunostaining was predominantly localized to the basal region throughout different regions of the rat epididymis. Immunostaining for AT$_2$ was also observed throughout all regions of the epididymis but the distribution of AT$_2$ immunostaining was less uniform as compared to that of AT$_1$ immunostaining (Fig. 2a-2c). AT$_2$ immunostaining observed in the caput and corpus was less intense when compared to that in the cauda.

The specificity of the immunocytochemical staining was demonstrated by the negative control when the AT$_1$ antiserum was preadsorbed with excess AT$_1$ receptor antigen (Fig. 1d) or omission of the AT$_2$ antiserum (Fig. 2d). Negative control experiments including substitution of primary antibodies with buffer and incubation with rabbit non-immune serum also exhibited consistently negative results (data not shown). Positive control experiments were conducted

![Fig. 3](image_url). Positive control immunofluorescent staining for AT$_1$ and AT$_2$ antibodies in cryosections from the rat adrenal gland. Positive immunostaining for AT$_1$ (3a, ×400), and AT$_2$ (3b, ×600), was observed in the secretory cells within the zona glomerulosa of the rat adrenal cortex region. The arrows indicate the relative region of zona glomerulosa.
using rat adrenal gland showing positive immunostaining for AT₁ and AT₂ (Fig. 3a and 3b) within the zona glomerulosa of the cortex as reported by previous studies [18,19].

3.2. Involvement in epididymal anion secretion

Previous electrophysiological studies using the short-circuit current technique have demonstrated a stimulatory effect of angiotensin II on epididymal anion secretion [9]. In the present study, we further investigated the involvement of angiotensin II receptor subtypes in mediating the angiotensin II effect using specific antagonists for AT₁ and AT₂. Angiotensin II (300 nM), when added to the basolateral surface, elicited an increase in the Iₜsc, but much higher concentration of angiotensin II (1 μM) had to be applied to the apical surface in order to obtain a significant Iₜsc response (Fig. 4). The subsequent experiments were focused on the basolateral Iₜsc response. When losartan, a specific AT₁ antagonist [20] was added prior to angiotensin II, the angiotensin II-induced Iₜsc was greatly reduced throughout the different concentrations of angiotensin II examined (Fig. 5). However, PD123117, a specific antagonist for AT₂ [20], exerted insignificant effect on the angiotensin II-induced Iₜsc and the concentration-response curve of angiotensin II in the presence of PD123117 was not significantly different from that in the absence of PD123117 (Fig. 5).

4. Discussion

The present immunofluorescent data have demonstrated the localization of the angiotensin II receptor subtypes AT₁ and AT₂ in the rat epididymis. Both AT₁ and AT₂ immunostaining were found to be localized to the basal region of the epithelium. The presence of angiotensin II receptors has been previously demonstrated in the rat epididymis by radioligand binding assay [21]. In that study, angiotensin II binding sites were found to be present throughout the caput, corpus to the cauda epididymidis, but most concentrated in the cauda region of the epididymis. Recently, AT₁ receptor subtype has been immunocytochemically demonstrated in the rat sperm as well as in the rat epididymal epithelium using a monoclonal antibody against AT₁ [11]. However, the precise location of AT₁ was not demonstrated, and the data for localization of AT₂ was not available in that study. In the present investigation, anti-peptide antibodies against the second extracellular loops of AT₁ and AT₂ receptor subtypes were employed. Not only AT₁ but also AT₂ were predominantly immunolocalized to the basal surface of the epididymal epithelium, indicating that delicate epididymal functions could be
regulated by angiotensin II through at least two epithelial membrane-bound angiotensin II receptor subtypes. The specificity of the anti-peptide antibodies were demonstrated by the negative control experiments, including the preadsorption test, substitution with pre-immune serum or with buffer. Positive staining of AT₁ and AT₂ was observed within the zona glomerulosa of the cortex from the rat adrenal gland. This is the region of the adrenal gland responsible for aldosterone production in response to angiotensin II [18].

The presence of angiotensin II receptors and their involvement in epididymal anion secretion has also been demonstrated by present electrophysiological studies using specific antagonists for AT₁ and AT₂. Consistent with the immunocytochemical finding, angiotensin II exerted greater effect on the $I_{SC}$ when applied to the basolateral surface as compared to that when applied to the apical membrane, indicating predominant localization of angiotensin II receptors in the basolateral membrane. The stimulatory effect of angiotensin II could be blocked by AT₁ antagonist, losartan but not AT₂ antagonist, PD123177, indicating predominant involvement of AT₁ but not AT₂ in epididymal anion secretion. Although the involvement of AT₂ in anion secretion was not demonstrated in the electrophysiological studies, the presence of basally bound AT₂ receptor, as demonstrated by the present immunocytochemical study, suggests that it may play a role in some other epididymal functions.

The present immunocytochemical and electrophysiological results lend further support for the presence of a tissue renin-angiotensin II system in the rat epididymis. Previous studies have shown the presence of several key components of the renin-angiotensin II system in the rat epididymis [7,8]. The evidence for a role for angiotensin II on electrolyte transport in epididymis has also been provided [9]. Recently, angiotensin II has been immunocytochemically localized in the basal cells, and to a less extent, in the principal cells of the rat epididymal epithelium [10], indicating that angiotensin II could be produced locally in the tissue and act in a paracrine or autocrine fashion to regulate epididymal function, possibly through membrane-bound angiotensin II receptors. The present study has indeed demonstrated, both immunocytochemically and pharmacologically, the presence of AT₁ in the basal regions of the rat epididymis. The close proximity of immunolocalization for angiotensin II observed previously [10] and angiotensin II receptors observed in the present study in the rat epididymis further indicates a paracrine/autocrine role of angiotensin II in regulation of epididymal functions. In addition to regulating epididymal fluid, angiotensin II may also act on smooth muscle overlying the epididymal tubules, thus facilitating the transit of sperm through the epididymis.

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References

