Expression and Location of the Bradykinin B2 Receptor in Rat Testis

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ABSTRACT

To investigate the possible role of the local tissue kallikrein-kinin system in spermatogenesis, we analyzed gene expression and cellular distribution of the bradykinin subtype-2 receptor (B2 receptor) in the rat testis. Reverse transcription-polymerase chain reaction revealed B2 receptor expression in testis and primary cultures of Sertoli cells and peritubular cells isolated from immature and mature rats. In situ hybridization of the B2-receptor mRNA showed intense labeling of cells on the base of the seminiferous tubule, whereas the autoradiographic signals gradually decreased toward the lumen. Immune histochemistry using testicular sections of pubertal and adult rats showed specific staining for the B2-receptor protein in cells of the adluminal compartment of the seminiferous tubules, especially on pachytene spermatocytes and spermatids. This immunostaining varied with the stages of the seminiferous cycle. The receptor protein was also observed on peritubular cells of pubertal rats. In conclusion, we demonstrated a stage-specific expression of the bradykinin B2 receptor in different cells of the seminiferous tubules of the rat testis. The results point to a possible function of the tissue kallikrein-kinin system in the local regulation of spermatogenesis.

male reproductive tract, polypeptide receptors, Sertoli cells, spermatogenesis, testis

INTRODUCTION

Kinins are small peptide hormones released from kininogens by controlled activity of specific proteases (kallikreins). Kinins are involved in multiple physiological processes, such as vasodilatation, smooth muscle contraction, and cell proliferation. Certain pathological conditions, including induction of pain, inflammation, and hypertension, are also caused by the kinins. Their effects are mediated by at least two types of membrane-bound receptors, which belong to the G protein-coupled receptor family characterized by seven membrane-spanning helices [1]. Most of the biological activities observed are mediated by constitutively expressed membrane-spanning helices [1]. Various reports have demonstrated a possible function of the B2 receptor in different types of cells, including immune cells, smooth muscle cells, and neuronal cells [2]. The B1 subtype is mostly absent but can be expressed under several pathological conditions. It shows high affinity for the carboxyl terminally truncated kinins, [desArg9]bradykinin and [des-Arg10]kallidin. The B2 receptor subtypes are present in low copy numbers in most natural cells [2]. Bradykinin is systemically released into the plasma via limited proteolysis of high-molecular-weight kininogen by plasma kallikrein. On the other hand, local production of kallidin (or bradykinin in the rat) also occurs on the surface of target cells by the action of tissue kallikrein on the precursor low-molecular-weight kininogen [3]. Kallidin acts on the B2 receptor, either directly or after conversion to bradykinin via proteolytic cleavage by aminopeptidases [4]. The liberated kinins are rapidly inactivated by peptidases, which are called kininases and are located on the cell membrane. Exposure to kinins alters the response of bradykinin receptors to agonists. This desensitization process may result from receptor endocytosis preceded by agonist-induced dimerization and phosphorylation of the B2 receptor [5–7]. Internalization of the B2 receptor seems to be a prerequisite for desensitization and receptor recycling to the plasma membrane [8, 9].

Peritubular cells, Sertoli cells, and germ cells form the seminiferous tubules of the testis. Sertoli cells play a key role in spermatogenesis by protecting and controlling the immediate environment around the germ cells. The contractile peritubular myoid cells are embedded within the lamina propria surrounding the seminiferous tubules. We detected several components of the tissue kallikrein-kinin system (tKKS) in the rat testis: The kinin-liberating protease, tissue-kallikrein, was present on the acrosomal cap of round and elongated spermatids [10], whereas kinin-degrading proteases, kininases, were located on the surface of Sertoli cells [11–13]. The B2-receptor mRNA was identified in testis and Sertoli cell extracts isolated from immature rats [10].

Several reports have demonstrated a possible function of the tKKS in mammalian reproduction. In vitro, kinins can enhance sperm motility in human [14], ram [15], and bull [16]. Moreover, components of the tKKS, including kininogen, tissue kallikrein, kinins, and kininases, have been identified in human seminal plasma [17, 18]. The sperm motility-enhancing effect is probably not mediated by a bradykinin receptor located on the spermatozoon [19]. In the rat, tissue kallikrein has been demonstrated to increase the relative number of spermatocytes [20] and the concentration of Sertoli cell-derived androgen-binding component [21]. Using in vitro organ cultures from rat testicular fragments, Atanasova et al. [22] showed that physiological concentrations of bradykinin stimulated prepubertal germ cell proliferation.

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1832
To further clarify the possible role of the tKKS in spermatogenesis, we analyzed gene expression and cellular distribution of the bradykinin B2-receptor protein within the rat testis.

MATERIALS AND METHODS

Isolation and Culture of Primary Sertoli Cells and Peritubular Cells

Sertoli cells and peritubular cells were isolated from juvenile Sprague-Dawley rats (18–21 days postnatal) as described previously [11,23] with minor modifications. In brief, decapitated testes were rinsed twice in Dulbecco modified Eagle medium:Ham F-12 medium (DHM; 1:1 [v/v]; Gibco BRL, Karlsruhe, Germany) containing 1 ml of gentamycin solution (Sigma, Deisenhofen, Germany), minced into small fragments, and incubated in DHM containing collagenase (1 mg/ml; Sigma) and DNase (20 μg/ml; Sigma) for 15 min at 37°C with constant shaking. Thereafter, the tubule fragments were allowed to settle for 7 min at room temperature. The pellet was resuspended in DHM containing soybean trypsin inhibitor (400 μg/ml DHM supplemented with 2 mg of BSA; Sigma) to stop enzymatic cleavage, and the tubule fragments were again allowed to settle for 7 min. Then, the pellet was resuspended in a solution of collagenase (2 mg/ml), hyaluronidase (2 mg/ml; Sigma), and DNase (20 μg/ml) in DHM and incubated for 30 min at 37°C. Thereafter, the cell suspension was centrifuged for 30 sec at 35 × g. The supernatant containing the peritubular cells was removed for further use and diluted with the 1.5 volume of 10 mM PBS. Trypsin inhibitor was added to the pellet, and the tubule fragments were allowed to settle for 20 min.

Peritubular cells from the supernatant were centrifuged for 10 min at 50 × g. The pellet was resuspended in Nutrient Mixture F-10 (Gibco BRL) supplemented with 15% (v/v) fetal calf serum (Gibco BRL), 2 mM L-glutamine (Biochrome, Berlin, Germany), and 1% (v/v) penicillin/streptomycin solution (Gibco BRL). Peritubular cells were seeded in 250-ml culture flasks and incubated at 37°C. After two passages, the purity of the peritubular cells was >98% as estimated by histochemical staining for α-smooth muscle isoactin [24] and morphological examination for germ cells.

For isolation of Sertoli cells, the remaining pellet containing the seminiferous tubule fragments was washed three times in DHM before incubation in a solution of collagenase (2 mg/ml), hyaluronidase (2 mg/ml), and DNase (20 μg/ml) in DHM for 15 min at 37°C under constant shaking. Sertoli cells were collected by centrifugation for 45 sec at 63 × g, resuspended in DHM, and allowed to settle for 20 min. The pellet was then resuspended in DHM, and the Sertoli cells were washed three times with DHM. Remaining Sertoli cell clusters were broken off by gentle homogenization using a pipette with subsequent filtration through a 70-μm nylon mesh (Falcon, Becton Dickinson, Franklin Lakes, NJ). Sertoli cells (500 000 cells/cm²) were cultured in six-well plates (Primaria; Falcon) using DHM supplemented with 2 mM L-glutamine (Gibco BRL), 100 U/ml of penicillin, 100 μg/ml of streptomycin sulfate, 10 μg/ml of epidermal growth factor, 5 μg/ml of human transferrin, 2 μg/ml of insulin, 10 nM hydrocortisone, 200 ng/ml of vitamin A, 200 ng/ml of vitamin E, 10 nM testosterone, and 2 μg/ml of cytosine arabinoside (all from Sigma).

After 3 days, contaminating germ cells were lysed by hypotonic shock treatment in 20 mM Tris-HCl (pH 7.5) for 5 min. Sertoli cells were used for further analysis 2–4 days after this handling. The medium (now lacking cytosine arabinoside) was replaced every other day. The purity of the Sertoli cells was >99%, with peritubular cells (<5%) and germ cells (<3%) as minor contaminants.

Detection of B2-Receptor mRNA by Reverse Transcription-Polymerase Chain Reaction

Samples of rat testes and kidney (as positive control) were removed and mechanically crushed in liquid nitrogen, and total RNA was extracted using the Qiagen RNeasy Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA from Sertoli cells or peritubular cells in primary culture was obtained using the Qiagen RNeasy Mini Kit. The purity of total RNA was estimated by electrophoresis on a 1.3% agarose gel and by the A260/A280 ratio using a ultraviolet spectrophotometer. Total RNA concentration was determined by absorbance at 260 nm.

Two micrograms of total RNA from each sample were reverse transcribed into cDNAs using 1 μg of oligo dT15-primers (Promega, Mannheim, Germany). Briefly, each reaction tube consisted of 5 μl of 5× reverse transcription (RT) buffer (250 mM Tris [pH 8.3], 375 mM KCl, 15 mM MgCl₂, and 50 mM DTT), 1.25 μl of 10 mM dNTP (10 mM each of; Promega), 25 U of RNasin ribonuclease inhibitor (Promega), 200 U of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Promega), and sterile distilled water to a final volume of 25 μl. This was then incubated at 36°C for 1 h to synthesize the first strand of cDNA. The RT enzyme was inactivated by heating at 70°C for 10 min.

One-fourth (0.6 μl) of this reaction product was used as a template for polymerase chain reaction (PCR) in combination with 1 μl each of the specific bradykinin B2-receptor primers. The following bradykinin B2-receptor primer pair was used: the upstream primer, 5’-CCA TCT TCT CAC CTG CAT TG-3’ (B-R3/98), which spanned intron 2 and corresponded to position 2075–2090 on exon 2 and 2892–2896 on exon 3 according to the rat bradykinin B2 receptor gene sequence published by Wang et al. [25]; and the downstream primer, 5’-CGT CTG GAC CTC GTT GAA CT-3’ (B-R10/99), which derived from position 3615–3596 on exon 3 as previously published by El-Dahr et al. [26]. To compare the expression of bradykinin B2 receptor in different tissues or cell types, bradykinin B2 receptor was coamplified with β-actin, which was also used as an internal control to monitor sample-to-sample variation in the RT and PCR procedures.

Primers for β-actin were as follows: upstream primer, 5’-GGC CAA CCG TGA AAA GAT GAC-3’; and downstream primer, 5’-ATT GCC GAC ATT GAT GAC GAC-3’. Linearity of the assay with respect to the amount of products was verified during preliminary experiments (data not shown) using increasing concentrations of template cDNA. Also, the optimal amplification cycles were determined over a range of 20–40 cycles. Six microliters of RT product and 2 μl of the specific primers (each of 25 μM) were mixed with 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 9.0], 500 mM KCl, and 1% Triton), 3 μl of 25 mM MgCl₂, 1 μl of dATP, dCTP, dGTP, and dTTP (each of 10 mM), 1.25 U of Taq-Polymerase (Promega), and sterile RNase-free distilled water to a final volume of 50 μl. A master mix containing all required reagents except cDNA was used to eliminate intersample variations. After a first-denaturation step at 94°C for 5 min, the following cycling parameters were used for amplification of bradykinin B2 receptor: denaturation at 94°C for 45 sec, annealing at 58°C for 1 min, and extension at 72°C for 40 cycles, with a final extension step at 72°C for 7 min. Amplification of β-actin was performed using the same procedure with the following exceptions: denaturation for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1.5 min for 25 cycles. All PCR experiments were run on a Primus 25/96 PCR Thermal Cycler (MWG, Ebersberg, Germany).

A 9-μl aliquot from each amplified sample tube was resolved onto 2% agarose gel in TBE buffer (90 mM Tris-Borate and 0.2 mM EDTA [pH 8.4]) and visualized by ethidium bromide staining. The expected sizes of the PCR products were 739 and 412 base pairs (bp) for bradykinin B2 receptor and β-actin, respectively. Negative controls were included for each set of RT-PCR by omitting the sample in the RT reaction and the RT product in the PCR step.

The relative optical densities of β2-receptor RT-PCR products were semiquantified by densitometric analysis and normalized with the corresponding RT-PCR products of β-actin using Gelscan Professional imaging software (BioSciTeC GmbH, Frankfurt, Germany).

DNA Sequencing

Representative RT-PCR products of the bradykinin B2-receptor gene were excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s instructions. Purified RT-PCR products were sequenced using B-R3/98 and B-R10/99 primers and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit on an ABI PRISM 310 Genetic Analysis System (PE Applied Biosystems, Weiterstadt, Germany). Sequences were analyzed using the Sequencing Analysis program (PE Applied Biosystems) and compared with the published B2-receptor gene sequence [25] using the Sequence Navigator program (PE Applied Biosystems).

Construction of the Bradykinin B2-Receptor Probe

The following primers, derived from the rat bradykinin B2-receptor gene sequence described by Meachem et al. [28], were used to generate the rat bradykinin B2 receptor probe: B-R5, 5’-TTCACATCACAACC-GC-3’; B-R6, 5’-TTGGTTCATGTTGGTGTCC-3’; and B-R10: 5’-CAGGT-ATCCGCTGATACTC-3’. Messenger RNA was isolated from rat cardiomyocytes and purified using the QuickPrep Micro mRNA purification kit (Pharmacia, Freiburg, Germany) according to the manufacturer’s instructions. Complementary DNA synthesis was performed with the first-strand cDNA synthesis kit (Pharmacia, Freiburg, Germany) and the specific prim
er B2-R6. Approximately 200 ng of mRNA were mixed with 1 μl of di-thiothreitol (200 mM) and 1 μl of B2-R6 primer (50 ng/μl) to a total volume of 10 μl and incubated at 65°C for 10 min. Then, the sample was placed on ice, and 5 μl of the “bulk” solution, including buffer and M-MuLV reverse transcriptase, were added. Reverse transcription was carried out for 1 h at 37°C. For PCR amplification, 1 μl of the RT mixture was used. The total reaction volume of 50 μl contained 50 ng each of the primers B2-R5 and B2-R6, 1.25 U of Taq DNA Polymerase (Boehringer, Mannheim, Germany), 200 μl of dATP, dCTP, dGTP, and dTTP, and 1.5 mM MgCl₂. After 7 min of initial denaturation at 94°C, the samples were amplified for 35 cycles. The amplification profile consisted of denaturation at 94°C for 1 min, primer annealing at 53°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. The PCR products were diluted 10-fold in water, and 1 μl of this dilution was subjected to a second PCR using the primer B2-R5 and the nested primer B2-R10. The amplification profiles of the second PCR were identical to those described for the first PCR.

The 393-bp PCR product was cloned into the HindII site of pGEM3Z (Promega) for sequencing. Sequence analysis showed authentic cDNA for the rat B2-receptor gene (codons 2–132). One base substitution (G → A) was observed at codon 76, which had also been described by Pesquero et al. [33]. To confirm the predicted size (Fig. 1A, lane 2), and the negative control B2-R6. The amplification profiles of the second PCR were identical to those described for the first PCR.

The 393-bp PCR product was cloned into the HindII site of pGEM3Z (Promega) for sequencing. Sequence analysis showed authentic cDNA for the rat B2-receptor gene (codons 2–132). One base substitution (G → A) was observed at codon 76, which had also been described by Pesquero et al. [33]. For further experiments, the PCR product was cloned between the EcoRI and BamHI sites of pGEM3Z.

In Situ Hybridization

Mature rat testis was sectioned to 10-μm thickness on a cryostat, thaw-mounted on coated slides (poly-l-lysine), fixed with 5% paraformaldehyde (freshly prepared, in 0.1 M phosphate buffer [pH 7.4], 30 min, 4°C), washed in PBS, and stored at −70°C until use. Hybridization and labeling were performed as described by Ungeferen et al. [30] using a 393-bp fragment of the bradykinin B2 receptor (see above). The 393-bp labeled sense and antisense RNA probes derived by T7 or T3 polymerase in vitro transcription of the pBS II plasmid containing the Bk receptor fragment were used. For autoradiography, the hybridized sections were dipped into Kodak NTB 2 nuclear emulsion (Integra Biosciences GmbH, Fernwald, Germany) (undiluted, melted at 42°C), dried for 2 h, and exposed at 4°C with desiccant for 3 wk. The autoradiographs were developed with Kodak D 19 (1:1), and the sections stained with hematoxylin and eosin.

Immunostaining of Bradykinin B2 Receptor

Specific antipeptide antibodies to the bradykinin B2 receptor were prepared as described in detail previously [31, 32]. In brief, eight peptide segments were selected from the sequence of the rat B2 receptor by the criteria of hydrophilicity, relative location in the receptor protein, and presence of a cysteine residue for coupling. In this way, peptides covering seven of the eight intra- and extracellular domains of the transmembrane-spanning region were synthesized. Each peptide was covalently coupled to keyhole limpet hemocyanin. The conjugates were used for the generation of eight antisera in rabbits (AS276–AS283) according to established immunization procedures. Specificity and cross-reactivity of the antipeptide antibodies with the rat B2 receptor have been demonstrated in various studies (for overview, see [1]).

Five-micrometer paraffin sections of rat testes were deparaffinized in toluene and hydrated through a graded series of ethanol concentrations. Testes had been fixed previously by immersion with freshly prepared methacarn solution (methanol:chloroform:glacial acetic acid, 6:3:1 [v/v/v]). Slides were washed in TBS (50 mM Tris-HCl buffered saline and 150 mM NaCl [pH 7.8]) three times (10 min each). Thereafter, the sections were incubated with 3% hydrogen peroxide for 20 min at room temperature to abolish endogenous peroxidase activity. After three washing steps with TBS (10 min each), nonspecific binding was blocked with 1% Auran BSA-c (Biotrend, Köln, Germany) and 5% preimmune goat serum (DAKO, Hamburg, Germany) in TBS for 30 min at room temperature. Additionally, the sections were incubated for 30 min at room temperature in protein block serum-free solution (DAKO). Sections were then incubated for 20–24 h at room temperature with the primary antibody (polyclonal antipeptide antisera 276–283, diluted 1:300 with TBS containing 1% BSA-c and 5% goat serum). Slides were washed in TBS three times (10 min each) and then blocked again by incubation in TBS containing 1% BSA-c and 5% goat serum for 30 min at room temperature. Thereafter, sections were incubated for 30 min at room temperature with the secondary antibody (EnVision-HRP, goat, ready-to-use solution; DAKO). Slides were washed twice in TBS (10 min each) followed by an additional washing step with Tris-HCl (50 mM Tris-HCl [pH 7.8]) for 10 min. The final reaction product was obtained by incubating the sections for 3 min in Tris-HCl (pH 7.8) containing 0.007% diaminobenzidine tetrahydrochloride (Sigma) and 0.024% H₂O₂. Sections were counterstained with hematoxylin. Negative controls were performed by using preabsorbed primary antibodies and by substituting preimmune serum or buffer for the primary antibody. These sections were immune negative. Rat kidney sections were used as a positive control for the B2 receptor [33].

RESULTS

Integrity and Purity of cDNA

The integrity of cDNA gained by RT-PCR from tissues of testes and kidney was confirmed by amplification of β-actin. This “housekeeping” gene was ubiquitously expressed in all samples, giving a signal at 412 bp, and confirmed the quality of cDNA (Fig. 1). Amplification with bradykinin B2 receptor-specific primers, which bracket an intron, gave a single 739-bp product from cDNA and demonstrated the absence of genomic DNA.

Expression of the Bradykinin B2 Receptor mRNA

Expression of the bradykinin B2 receptor in male rats (immature or mature) was examined by RT-PCR using RNA isolated from testis and kidney, which served as a positive control [34]. In addition, RNA was isolated from purified Sertoli cells and peritubular cells. These cells were isolated from immature rats (age, 18–20 days). A 739-bp PCR product that corresponded to the expected size of bradykinin B2-receptor mRNA was detected in testes of immature and adult rats. Sertoli cells or peritubular cells isolated from immature rats also displayed expression of the B2-receptor mRNA (Fig. 1A). The 739-bp PCR product was subsequently confirmed to be authentic bradykinin B2 receptor by direct nucleotide sequence analysis. The RT-PCR of the positive control (kidney) yielded a product of the predicted size (Fig. 1A, lane 2), and the negative con-
trols (C1, no RNA; C2, no template) displayed no bands, indicating accuracy of reaction conditions. The housekeeping gene, β-actin, served as loading control and was used for normalization of gene expression. Mean values of 4–10 experiments are given in Figure 1B. In the juvenile rat, no significant differences in B2-receptor gene expression were observed between testis and isolated Sertoli cells or peritubular cells. Densitometric analysis also revealed no significant differences in B2-receptor mRNA levels of testes from rats of different ages.

**Cellular Location of the Bradykinin B2-Receptor mRNA**

The cellular distribution of the B2-receptor mRNA in the rat testis was investigated using the in situ hybridization technique. The experiments revealed clear expression of 35S-labeled bradykinin B2-receptor mRNA in the seminiferous epithelium (Fig. 2). Whereas cells on the base of the tubule showed intense labeling, the autoradiographic signals gradually decreased toward the lumen. No bradykinin B2-receptor mRNA could be detected on testicular spermatozoa (Fig. 2, arrows). No significant labeling was seen in the interstitium (Fig. 2, asterisks).

**Immunohistochemical Location of the Bradykinin B2-Receptor in the Testis**

To determine the cellular location of the bradykinin B2-receptor protein in the rat testis, sections were immunostained for the B2 receptor using a set of polyclonal antibodies that detect different epitopes of this receptor [31, 32]. In sexually mature rats (age, 53 days) (Fig. 3, A and B), positive immune reaction occurred on germ cells of the adluminal compartment of the seminiferous tubule, especially on pachytene spermatocytes (triangles) and spermatids (black arrows). Somatic cells near the base of the tubule, such as peritubular cells (green arrows), Sertoli cells, or spermatagonia, showed no staining. Interstitial Leydig cells (blue arrows) displayed some faint staining. In immature rats (age, 18 days) (Fig. 3, C and D), bradykinin B2-receptor protein was detected in pachytene spermatocytes, peritubular cells, and Leydig cells. Sertoli cells and spermatagonia did not show positive staining (Fig. 3C). The immune reaction was specific, because incubation with preadsorbed B2-receptor antibody (Fig. 3, B and D) or preimmune serum (data not shown) produced no staining. Moreover, in all testicular sections, endothelial cells of major blood vessels also showed a positive reaction (Fig. 3C, red arrow). These cells are known to express the bradykinin B2-receptor protein [35] and, therefore, served as an internal positive control. In the external positive control, the kidney, B2 receptors were specifically detected in the limb of Henle, the collecting tubules, and the connecting piece between the distal tubule and collecting tubules. These areas are known to express the bradykinin B2 receptor [27, 32]. The corresponding kidney negative controls did not show specific staining in these areas (data not shown).

In rats with fully developed spermatogenesis (age, ≥53 days), expression of the bradykinin B2-receptor protein was dependent on the stage of the seminiferous cycle (Fig. 4). Only faint or no significant staining was observed during the early stages of spermatogenesis. At stages II and III (Fig. 4A, left), some slight immune reaction occurred in elongated spermatids (black arrowheads) and, possibly, also in the adluminal compartment of Sertoli cells but not on round spermatids (white arrows) and pachytene spermatocytes (white triangles). No significant staining was observed at stages III and IV (Fig. 4B, white symbols). Clear expression of the bradykinin B2 receptor was detected earliest on cell membranes and cytoplasm of pachytene spermatocytes (black triangles) and round or early elongated spermatids (black arrows) at stage VIII (Fig. 4C). With progress of spermatogenesis (stage XI) (Fig. 4A, right), positive staining was observed on pachytene spermatocytes from stages VIII to XIII and on elongated spermatids from stages IX to XII. No significant immune reaction was seen at stage XIV (data not shown). The overview slide (Fig. 4D) displayed tubules with intense (black arrows) and low immune reaction (white arrows), pointing to a clear stage-dependent expression of the B2-receptor protein.

**DISCUSSION**

We investigated the expression of the bradykinin B2-receptor mRNA and protein in the rat testis. Our findings demonstrated, to our knowledge for the first time, that the B2 receptor is located in peritubular cells, pachytene spermatocytes, and spermatids of the seminiferous tubule. Presence of the B2-receptor mRNA in different tissues of the rat, including testis, vas deferens, and kidney, was also shown by McEachern et al. [28] using Northern blot analysis.

Semi-quantitative RT-PCR did not show significant differences in B2-receptor mRNA levels in testes isolated from rats of different ages. However, using immunohistochemistry with a set of polyclonal antibodies that detect different epitopes of this receptor, we found hints of development-dependent changes in the location of the B2-receptor protein. In juvenile rats (age, 18 days), the B2-receptor protein was detected in peritubular cells and pachytene spermatocytes, which are the highest-developed germ cells at this age. Leydig cells in the interstitium also showed a positive immune reaction. However, we did not observe specific
staining in peritubular cells in the older rat (age, 53 days). Also, immune reaction in Leydig cells appeared to be less intense compared to that in the juvenile rat. This may point to a possible involvement of the tKKS during testicular maturation and is currently under further investigation.

Division and differentiation of germ cells is a complex process that follows a precise pattern. A stage or cell association is a defined grouping of germ cell types at particular phases of development in cross-sectioned tubules. All germ cells pass through several stages, the number of which differs depending on the species (e.g., six in the human, 14 in the rat). The succession of all stages along time is called the spermatogenic cycle. In rats with fully developed spermatogenesis (age, 53 days), the B$_2$-receptor protein was stage-dependently expressed in pachytene spermatocytes and in round and elongated spermatids. The first prominent expression of the B$_2$-receptor protein was noticed at stage VIII of the cycle. Here, elongated step 19 spermatids are ready to be released into the tubular lumen. At the following stage (stage IX), the mature spermatids have left the seminiferous epithelium, and the next wave of round spermatids begins to differentiate into elongated germ cells. Highest immune reactivity was observed at middle to late stages of the spermatogenic cycle. In contrast, low (elongated spermatids) or no significant (pachytene spermatocytes and round spermatids) staining was detected at early and very late stages. This does not necessarily mean that the bradykinin receptor is absent at these stages. Round spermatids especially should express the receptor, because this protein is present earlier in their predecessors, the pachytene spermatocytes, and also again later in elongated spermatids. We therefore suggest a weak expression of the bradykinin-receptor protein in round spermatids, which may be too low to be detected by our antibody.

We did not observe a clear, specific immune reaction in juvenile or mature Sertoli cells by histochemical staining. Only during early stages of the seminiferous cycles did Sertoli cell cytoplasm in the adluminal compartment possibly show some faint staining. This may be related to restricted
sensitivity of the immunohistochemistry procedure compared to RT-PCR, which did detect the B₂-receptor mRNA in purified Sertoli cells isolated from prepubertal rats (age, 18–21 days). Previously, we displayed the occurrence of B₂-receptor mRNA in these cells by Northern hybridization technique [10]. We also detected B₂-receptor protein in isolated juvenile Sertoli cells by Western blot analysis using a very sensitive detection system (unpublished results). However, our Sertoli cell cultures are not entirely pure; they contain some peritubular cells and germ cells. Therefore, we cannot exclude the possibility that mRNA or protein signals in Sertoli cell cultures are caused by these contaminating cells.

Bradykinin is liberated from low molecular weight kininogen by the specific action of tissue kallikrein. We have located this protease on round and elongated spermatids in the adult rat testis [10]. Kininogens are mainly expressed in the liver and kidney. Low molecular weight kininogen is also expressed in other tissues, including the testis [36]. Thus, bradykinin likely is liberated within the murine adult testes. In contrast, we did not detect tissue kallikrein in juvenile or prepubertal rat testes. Specific immunostaining for tissue kallikrein appeared first in 28-day-old rats during the first spermatogenic cycle in tubules containing round spermatids [37]. Therefore, the earliest in vivo formation of bradykinin within the rat seminiferous tubule may start at this time of puberty.

Our data suggest a potential function of the tKKS as a local testicular factor in the seminiferous tubule. The tKKS can stimulate cell proliferation under several physiological and pathological conditions. In vitro, bradykinin enhances the proliferation of fibroblasts, keratinocytes, arterial smooth muscle cells, endothelial cells, and a variety of human tumor cell lines [38]. Thus, the tKKS may also be involved in triggering the proliferation of juvenile peritubular cells and Sertoli cells. Regarding germ cells, tissue kallikrein has been reported to increase the number of spermatocytes in the rat [20]. Atanassova et al. [22] observed a significant stimulation of rat prespermatogonial cell proliferation in organ cultures of immature rat testis after exposure to bradykinin. Using specific B₁/B₂-receptor agonists and antagonists, they further showed that this cellular response is probably mediated via B₂ receptors. Our results concerning occurrence of the B₂ receptor within the germ cell epithelium would fit into this hypothesis. Bradykinin was further shown to induce cell growth and differentiation [38, 39]. Both processes are also essential in spermatogenesis. Pachytene spermatocytes grow rapidly in size, whereas round spermatids highly differentiate into elongated spermatids. We showed that these germ cells express bradykinin B₂ receptors, which points to a possible involvement of the tKKS in spermatogenesis. Small peptides such as oxytocin are regulators of peritubular myoid cell contraction [40]. Thus, bradykinin may also be involved in trig-
gering the contractility of these cells. This view is supported by the fact that cGMP or prostaglandins, which are involved in the second-messenger cascade of oxytocin as well as bradykinin receptors, also stimulate peritubular cell contractions [41–43]. Our recent experiments (unpublished results) showed that a physiological concentration of bradykinin induced an instantaneous and transient rise in intracellular calcium ion concentration in juvenile peritubular cells. These data gave evidence for a functional bradykinin B3 receptor in this cell type (unpublished results).

On the other hand, gene-targeted mice lacking the B3 receptor were fertile and had normal litter size, but their testis morphology and sperm function were not investigated [44]. This implies that the tKKS may not be essential for spermatogenesis, because its action can be compensated for by appropriate backup systems. However, specific expression of the B3 receptor during certain stages of the spermatogenic cycle stresses a potential function of the tKKS in the local regulation of spermatogenesis.

In conclusion, the tKKS may act via bradykinin B3 receptors as a local modulator of spermatogenesis and seminiferous tubule cell functions in the rat testis.

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