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Bradykinin increases intracellular calcium levels in rat testis peritubular cells *via* the B_2 receptor subtype

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1 RT–PCR and Western blots were used to detect bradykinin B_2 receptors in testis and isolated peritubular cells of pre-pubertal rats. RT–PCR demonstrated expression of a single transcript, whereas Western blots showed up to three specific bands that were in accordance with the described native, glycosylated and dimeric form of B_2 receptor proteins, respectively.

2 Fura-2-loaded peritubular cells responded with an instantaneous, linear and transient rise in $[Ca^{2+}]_i$ after adding bradykinin. Stimulation of cells with bradykinin concentrations between 1 μM and 1 pM showed a dose dependent increase of $[Ca^{2+}]_i$. The calcium response to bradykinin was diminished after stimulation of peritubular cells in calcium-free buffer. After blocking the SERCA-pumps by thapsigargin and subsequent stimulation with bradykinin, no rise of $[Ca^{2+}]_i$ was appreciated.

3 Multiple stimulation of a single peritubular cell by local perfusion with a brief addition of BK (10 nM) resulted in a fast and immediate response. However, the second and third stimuli had slower rise rates and diminished $[Ca^{2+}]_i$ peaks, showing desensitization of the kinin receptor.

4 Addition of the bradykinin B_1 receptor agonist [des-Arg[9]]-bradykinin (100 nM) to Fura-2-loaded peritubular cells did not change the $[Ca^{2+}]_i$. However, the B_2 receptor antagonist HOE 140 (100 nM) strongly inhibited the bradykinin-induced calcium response.

5 We conclude that the bradykinin-induced increase in $[Ca^{2+}]_i$, in testicular peritubular cells is mediated by the stimulation of kinin receptors of the B_2 subtype.
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Keywords: Kinin B_2 receptor; bradykinin; intracellular calcium; rat; testis; peritubular cells; male reproduction

Abbreviations: BK, bradykinin; B_2R, bradykinin subtype 2 receptor; $[Ca^{2+}]_i$, intracellular calcium ion concentration; HBSS, HEPES-buffered salt solution; HOE 140, DArg-[Hyp[3], Thi[5], DTic[7], Oic[8]]-bradykinin; tKKS, tissue kallikrein kinin system

Introduction

Bradykinin receptors are part of the tissue kallikrein kinin system (tKKS) that triggers a variety of physiological functions. Pharmacologically, two subtypes of bradykinin receptors can be distinguished. The B_2 receptor (B_2R) exhibits high affinity for bradykinin and kallidin (lys-bradykinin), whereas the B_1 receptor (B_1R) shows high affinity for the carboxyl terminally truncated kinins, [des-Arg[9]]-bradykinin and [des-Arg[9]]-kallidin. The B_2R is constitutively expressed in many tissues, whereas the B_1R is almost undetectable but is up-regulated under certain pathological conditions such as inflammation or tissue injury. Both subtypes belong to the G protein-coupled receptor family characterized by seven membrane-spanning helices (Marceau, 1997; Müller-Esterl, 1997; Regoli *et al*., 2001). The signalling pathways of kinin receptors include the activation of G proteins that stimulate the activity of phospholipase C (PLC) resulting in phosphatidylinositol (PI) turnover and a transient increase in intracellular free calcium ion concentration $[Ca^{2+}]_i$ (Lee *et al*., 1993). The rise in $[Ca^{2+}]_i$ increases the levels of nitric oxide (NO) and cGMP. A second signalling cascade includes phospholipase A_2 (PLA_2), which releases the prostaglandin precursor, arachidonic acid (Burch & Axelrod, 1987).

Peritubular cells are contractile myoid cells that are embedded within the lamina propria surrounding the seminiferous tubules of the testis. In a sexually mature adult, each seminiferous tubule has a central lumen lined by an actively replicating germinal epithelium mixed with a population of non-dividing, supporting cells – the Sertoli cells. Peritubular cells produce several factors that are involved in seminiferous tubule contractility and extracellular matrix formation (Holstein *et al*., 1996). It has been suggested that these cells also secrete the peritubular modifying substance (PmodS) which influences the biosynthesis of products from Sertoli cells (Skinner, 1993). In the rat, expression of B_2 receptor mRNA occurs in testis (McEachern *et al*., 1991) and isolated testicular cells (Monsees *et al*., 1999). Recently, we detected B_2R protein in the testis of pre-pubertal rats by immune histochemistry (Monsees *et al*., 2002). However, there is limited knowledge on the presence of kinin receptors in testicular peritubular cells. Thus, the aim of this study was to determine whether or not these cells express a functional B_2 receptor.

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Methods

Isolation and culture of primary peritubular cells

Peritubular cells were isolated from pre-pubertal Sprague–Dawley rats (18–21 days postnatal) as described previously (Wennemuth *et al*., 2000) with some modifications. In brief, testes were rinsed twice in DHM (Dulbecco modified Eagle medium + Ham's F-12 medium; 1 : 1) containing 1 ml of gentamycin solution. After decapsulation, the tissue was minced into small fragments and incubated in DHM containing collagenase (1 mg ml^{-1}) and DNase (20 μg ml^{-1}) 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^{-1} DHM, supplemented with 2 mg ml^{-1} bovine serum albumin) to stop enzymatic cleavage and the tubule fragments again were allowed to settle for 7 min. Then the pellet was resuspended in a solution of collagenase (2 mg ml^{-1}), hyaluronidase (2 mg ml^{-1}), and DNase (20 μg ml^{-1}) in DHM and incubated for 30 min at 37°C. Thereafter, the cell suspension was centrifuged for 45 s at $43 \times g$. The supernatant containing the peritubular cells was removed and diluted with the 1.5 times its volume of 10 mM phosphate buffered saline. Peritubular cells were collected by centrifugation for 10 min at $50 \times g$. The pellet was resuspended in Nutrient Mixture F-10 supplemented with 15% horse serum, 3% foetal calf serum, 2 mM L-glutamine, and 1% penicillin/streptomycin. Peritubular cells were seeded in 250 ml culture flasks and incubated at 37°C. After two passes, the purity of the peritubular cells was >98% as estimated by histochemical staining for α-smooth muscle isoactin (Tung & Fritz, 1990) and morphological examination for germ cells.

Detection of B_2 receptor mRNA by RT–PCR

Samples of rat testes were removed, mechanically crushed in liquid nitrogen, and total RNA were extracted using the Qiagen RNeasy Midi Kit according to the manufacturer's instruction. Additionally total RNA from peritubular cells in primary culture were obtained using the Qiagen RNeasy Mini Kit. RNA from each sample were reverse transcribed into cDNA using oligo dT^{15}-primers. Briefly, each reaction tube consisted of 2 μg of total RNA, 1 μg of oligo dT^{15}-primers, 5 μl of 5 × RT-buffer (250 mM Tris, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 1.25 μl of 10 mM dNTP, 25 U RNAsin ribonuclease inhibitor, 200 U of Moloney murine leukaemia virus reverse transcriptase, and sterile distilled water to a final volume of 25 μl. This was incubated at 36°C for 1 h to synthesize the first strand of cDNA. The RT enzyme was inactivated by heating to 70°C for 10 min.

One-fourth (6 μl) of this reaction product was used as a template for PCR in combination with 1 μM of each of the specific bradykinin B_2 receptor primers, described next. The upstream primer was 5′-CCA TCT CTC CAC CTG CAT TG-3′ (B2R3/98). It spanned intron2 and corresponded to position 2075–2090 on exon2 and 2892–2896 on exon3 according to the rat B_2R gene sequence (Wang *et al*., 1994). The downstream primer was 5′-CGT CTG GAC CTC CTT GAA CT-3′ (B2R10/99). It derived from position 3615–3596 on exon 3 (El-Dahr *et al*., 1997). Primers for β-actin (loading control) were as follows: upstream primer 5′-GGC CAA CCG TGA AAA GAT GAC-3′; downstream primer 5′-ATT GCC GAT AGT GAT GAC CTG-3′ (MacDonald *et al*., 1996). Six μl RT product and 2 μl of each of the 25 μM specific primers described above were mixed with 5 μl of 10 × PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 1% Triton), 3 μl of 25 mM $MgCl_2$, 1 μl of each of the 10 mM dNTP, 1.25 U of Taq-Polymerase, and sterile RNAse-free distilled water to a final volume of 50 μl. After denaturation at 94°C for 5 min, the following cycling parameters were used for amplification of the bradykinin B_2 receptor: denaturation at 94°C for 45 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min. A total of 40 cycles were performed. A final extension step at 72°C for 7 min followed. Amplification of β-actin was performed using the same procedure with the following adjustments: denaturation for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1.5 min. A total of 25 cycles were applied.

A 9 μl aliquot from each amplified sample tube was analysed in a 2% agarose gel in TBE buffer (90 mM Tris-Borate, 0.2 mM EDTA, pH 8.4) and visualized by ethidium bromide staining. The expected sizes of the PCR products were 739 bp and 412 bp for the bradykinin B_2 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.

DNA sequencing

Representative RT–PCR products of the bradykinin B_2 receptor gene were excised from the agarose gel and purified using the QIAquick Gel Extraction Kit according to the manufacturer's instructions. Purified RT–PCR products were sequenced using B2R3/98 and B2R10/99 primers and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit on an automated ABI PRISM 310 Genetic Analysis Sequencer (PE Applied Biosystems, Weiterstadt, Germany). Sequences were analysed using the Sequencing Analysis program (PE Applied Biosystems) and compared with the published B_2R gene sequence (Wang *et al*., 1994) with the Sequence Navigator program (PE Applied Biosystems).

Immunoblotting of bradykinin B_2 receptor

Rat testis were mechanically crushed in liquid nitrogen, dissolved in five times its volume of lysis buffer (1% SDS, 1 mM sodium ortho-vanadate, 10 mM Tris-HCl, pH 7.4) and heated for 15 s at 900 W in a microwave oven. Peritubular cells were washed with cold phosphate-buffered saline (PBS, 10 mM, pH 7.3). Thereafter, lysis buffer at 90°C was added and the cells were scraped from the dish. Protein content from tissue or cell extracts was determined using BioRad's protein assay. Aliquots of the protein (250 μg) or cell (125 μg) extracts were freeze dried and dissolved in sample buffer (125 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.006% bromophenol blue, 1.8% β-mercaptoethanol). These protein extracts were heated for 5 min at 95°C, run in each lane of a 12% polyacrylamide gel and then electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, Applied Biosystems, CA, U.S.A.) using transfer buffer (25 mM Tris-HCl, 190 mM glycine, and 20% methanol).

Detection and visualization of the B_2R protein was performed using a monoclonal primary antibody and the Amplified Opti 4 CN Detection Kit according to the manufacturer's instructions. In brief, the PVDF membranes underwent the following series of room-temperature incubations, all washing steps lasted 5 min unless otherwise stated: (1) incubation for 5 min in methanol, (2) two washes in PBST (PBS plus 0.1% Tween 20), (3) preincubation for 1 h in PBST containing 3% blocking solution (BioRad), (4) two washes in PBST, (5) incubation with a 1 : 1000 dilution of B_2R antisera in PBST containing 1% bovine serum albumin (BSA) for 1 h, (6) two rinses in PBST, (7) incubation with a 1 : 3000 dilution of the secondary antibody (goat anti-mouse) in PBST containing 1% BSA for 1 h, (8) two rinses in PBST, (9) incubation with amplification reagent (BioRad) for 10 min, (10) four rinses in PBST containing 20% dimethyl sulphoxide of 5 min each, (11) two rinses in PBST, (12) incubation with streptavidin-HRP in PBST (BioRad) containing 1% BSA, (13) two rinses in PBST, (14) incubation with staining solution (BioRad) for 5 – 10 min and (15) four 15 min rinses in water.

Dye loading and photometry

For single cell measurements, cells grown as a monolayer on coverslips were washed three times with HEPES-buffered salt solution (HBSS, pH 7.4; 10 mM HEPES, 135 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM KCl, 0.8% dextrose) before incubation at 32°C for 30 min with Fura-2 AM. Three μl of a 3 mM stock solution of Fura-2 AM (in dimethylsulphoxide) was mixed with 3 μl of Pluronic F-127 (10%) and adjusted with HBSS to a final volume of 1 ml. After incubation with Fura-2 AM for 30 min, cells were washed again twice with HBSS as described above. The intracellular Fura-2 AM was hydrolyzed by incubating the cells an additional 30 min at 32°C with 5% CO_2. For cuvette measurements, cells grown in cell culture flasks were washed three times with HBSS before incubation with trypsin (0.05%) for 30 s. The reaction was stopped with RPMI medium. The cell suspension was placed in a 50 ml Falcon tube and washed twice by centrifugation at $500 \times g$ for 1 min and resuspended in HBSS before it was loaded with Fura-2 AM. Loading was accomplished by resuspending the pellet in 1 ml of a 3 μM Fura-2 AM solution. After 30 min of incubation at 32°C with 5% CO_2, cells were washed again twice by centrifugation at $500 \times g$ for 1 min. Cells were adjusted to a concentration of $1-2 \times 10^6$ cells ml^{-1} with HBSS and were stored at 32°C with 5% CO_2 for 30 – 60 min prior to performing photometric measurements.

The dye-loaded cells were placed into a measuring chamber and examined with a 40× oil objective and a 10× ocular lens in an inverted microscope (Nikon, Diaphot 400, Düsseldorf, Germany) or 2 ml of the cell suspension was placed in a cuvette and fluorescence was monitored by a spectrofluorometer (PTI, Wedel, Germany). Emission intensities were recorded as a ratio (340 – 380 nm^{-1}) at 510 nm with a photomultiplier (PTI, Wedel, Germany). The background-corrected signal (R) was calibrated by applying the standard equation $[Ca^{2+}] = K_d(R\text{-}R_{min})\ (R_{max}\text{-}R)^{-1}$ where R_{min} and R_{max} were determined empirically from cells (Grynkiewicz *et al*., 1985).

Test solutions were added directly to the bath or the cuvette. In some experiments, drugs were applied with a solenoid-controlled, gravity-fed, multibarrelled local perfusion system as previously described (Wennemuth *et al*., 1998, 2000).

Materials

Dulbecco modified Eagle medium: Ham's F-12 medium, Nutrient Mixture F-10, RPMI medium, penicillin/streptomycin solution, horse serum and foetal calf serum were purchased from Gibco BRL (Karlsruhe, Germany). Gentamycin, bovine serum albumin, collagenase, hyaluronidase, DNase, soybean trypsin inhibitor, Pluronic F-127, bradykinin, [des-ArgH]-bradykinin and HOE 140 were obtained from Sigma (Deisenhofen, Germany). L-glutamine originated from Biochrome (Berlin, Germany). Qiagen RNeasy Midi Kit, Qiagen RNeasy Mini Kit and QIAquick Gel Extraction Kit were purchased from Qiagen (Hilden, Germany). Oligo dT^{15}-primers, dNTP Promega, RNAsin ribonuclease inhibitor, Moloney murine leukaemia virus reverse transcriptase and Taq-Polymerase were obtained from Promega (Mannheim, Germany). BioRad protein assay, Amplified Opti 4 CN Detection Kit and secondary antibody (goat antimouse) were purchased from BioRad (Hercules, U.S.A.). The B_2R monoclonal primary antibody (mouse) was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Fura-2 AM was purchased from Molecular Probes (Leiden, Netherlands). RPMI medium originated from Greiner (Frickenhausen, Germany).

Data analysis

Calibration and kinetic analysis of digital photometric records were performed in Igor Pro Vers. 4.0.2.1 (Wavemetrics, Lake Oswego, OR, U.S.A.). Statistical calculations were performed in Excel (Microsoft, Redmond, WA, U.S.A.).

Results

Expression of the bradykinin B_2 receptor mRNA

A 739-bp PCR product corresponding to the expected size of bradykinin B_2 receptor mRNA was detected in testis and peritubular cells isolated from immature rats (Figure 1). The 739-bp PCR product was subsequently confirmed to be authentic B_2R by direct nucleotide sequence analysis. The negative controls (C1, no RNA; C2, no template) displayed no bands, indicating the accuracy of the reaction conditions. To ensure that equal amounts of RNA were reverse-transcribed and amplified in each reaction tube, β-actin was also amplified in these experiments to yield the expected band at 412 bp.

Immunoblotting of the bradykinin B_2 receptor

Western blots, using a monoclonal antibody raised against the C-terminal peptide 350 – 364 of the bradykinin B_2 receptor, detected two proteins with relative molecular weights of approximately 42,000 and 45,000 in immature testis (Figure 2). Peritubular cells displayed a major band at

approximately 42,000 M_r and two minor bands at approximately 45,000 and 132,000 M_r.

BK induced $[Ca^{2+}]_i$ elevation in peritubular cells

Fura-2-loaded peritubular cells responded with an instantaneous, linear and transient rise in $[Ca^{2+}]_i$ after adding BK. In Figure 3, BK was added to the cuvette at a final concentration of 10 nM. To test the need for extracellular Ca^{2+} we used Ca^{2+}-deficient HBSS (HBSS0). Cells were placed in HBSS0 only a few seconds (<5 s) before stimulation with BK to prevent depletion of internal stores.

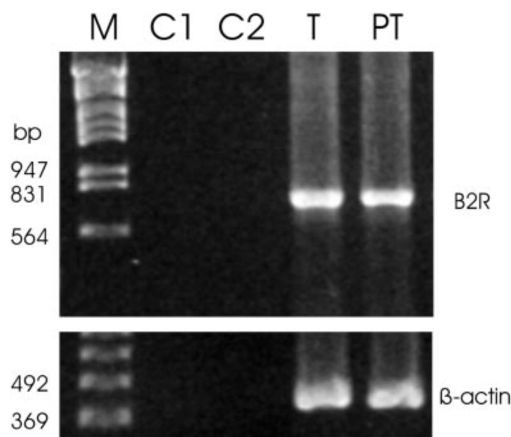

Figure 1 RT–PCR analysis of bradykinin B_2 receptor (B_2R) mRNA expression in testis (T) and isolated peritubular cells (PT) from immature rats (18 days). The expected size of the PCR products is 739 bp for B_2R and 412 bp for β-actin, respectively. Negative controls: C1, no RNA; C2, no template; M, marker.

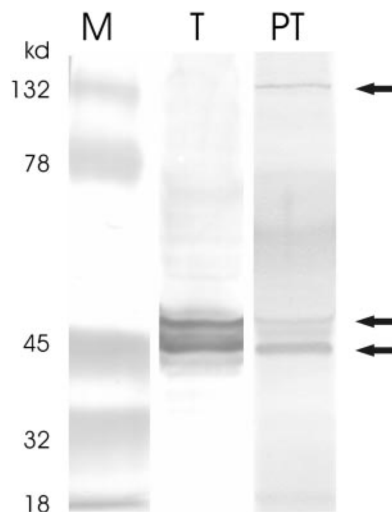

Figure 2 Western blot analysis of the bradykinin B_2 receptor in testis (T) and isolated peritubular cells (PT) from immature rats (18 days). Proteins were separated by electrophoresis using a 12% polyacrylamide gel (SDS–PAGE) and blotted on a PVDF membrane. Detection and visualization of the B_2R protein was performed using a monoclonal B_2R-antibody (mouse, Transduction Laboratories, Lexington, KY, U.S.A.) and the Amplified Opti 4 CN Detection Kit (BioRad, Hercules, U.S.A.). Arrows indicated three B_2R protein bands with relative molecular masses of approximately 42, 45 and 132 kDa. M = molecular weight marker.

The addition of BK showed a diminished maximal $[Ca^{2+}]_i$ response with a mean value of 124.23 nM ($\pm$22.55 SE; $n=7$; Figure 4A). Subsequent addition of 2 mM Ca^{2+} led to a high increase in $[Ca^{2+}]_i$ (Figure 4B). Pretreatment of cells with 5 μM thapsigargin showed a transient rise in $[Ca^{2+}]_i$, but subsequent stimulation with BK did not increase $[Ca^{2+}]_i$ (Figure 4C). Stimulation of cells with BK concentrations between 1 μM and 1 pM showed a dose dependent rise of $[Ca^{2+}]_i$. Figure 5 depicts the averaged $\Delta[Ca^{2+}]_i$ signals as a function of BK – where $\Delta[Ca^{2+}]_i$ is defined as the maximal Ca^{2+} response to BK minus the resting $[Ca^{2+}]_i$. We used local perfusion to stimulated single peritubular cells with BK multiple times. Between the stimuli, cells were perfused with HBSS. Figure 6A shows a typical example of the outcome of eight independent experiments. The cell was stimulated three times with a brief (12–40 s) perfusion of BK (10 nM). All stimuli showed a fast and immediate response. The second and third stimuli had slower rise rates and diminished $[Ca^{2+}]_i$ peaks, pointing to a receptor desensitization process.

To evaluate what subtype of kinin receptors are required for peritubular cells to respond to BK, we used a specific agonist of the bradykinin B_1 receptor (B_1R) and a specific antagonist of the bradykinin B_2 receptor, respectively. Stimulation of B_1R, by addition of [des-Arg^9]-BK (100 nM) to the cuvette containing Fura-2-loaded peritubular cells, showed no influence on the $[Ca^{2+}]_i$ (Figure 6B). Also a 1 μM concentration of [des-Arg^9]-BK did not evoke a rise in calcium levels (not shown). Application of BK (10 nM) after the addition of [des-Arg^9]-BK did not show any difference when compared to stimulation with BK alone. The average value of the $[Ca^{2+}]_i$ peak after BK stimulation was 606.95 nM ($\pm$39.55 s.e.; $n=6$). Preincubation (2 min) with the specific inhibitor of B_2R, HOE 140 (100 nM), did not change the $[Ca^{2+}]_i$ level (Figure 6C). However, subsequent stimulation with BK (10 nM) resulted in a dramatic decrease of the $[Ca^{2+}]_i$ peak (17.74 nM$\pm$6.13 s.e.; $n=9$) when compared to the uninhibited reaction.

Discussion

The major finding of the present study is the observation that the bradykinin-induced increase in $[Ca^{2+}]_i$ in testicular

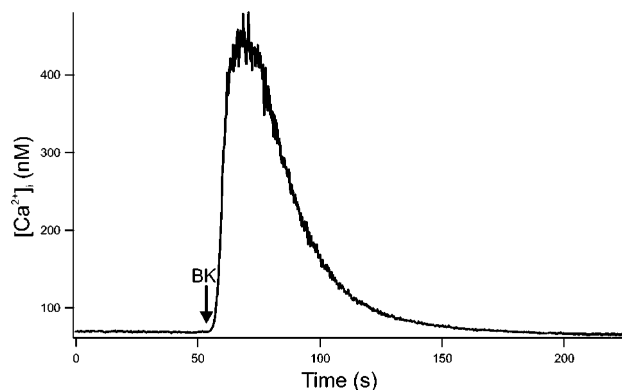

Figure 3 Effect of BK on $[Ca^{2+}]_i$ in Fura-2-loaded peritubular cells. Original recording of peritubular cells stimulated with BK in a final concentration of 10 nM. 200,000 cells ml^{-1} were stimulated in a cuvette-based system. The arrow marks the point of BK addition to the cuvette. Subsequently the cells responded with a transient rise in $[Ca^{2+}]_i$. The picture shows one typical experiment out of seven.

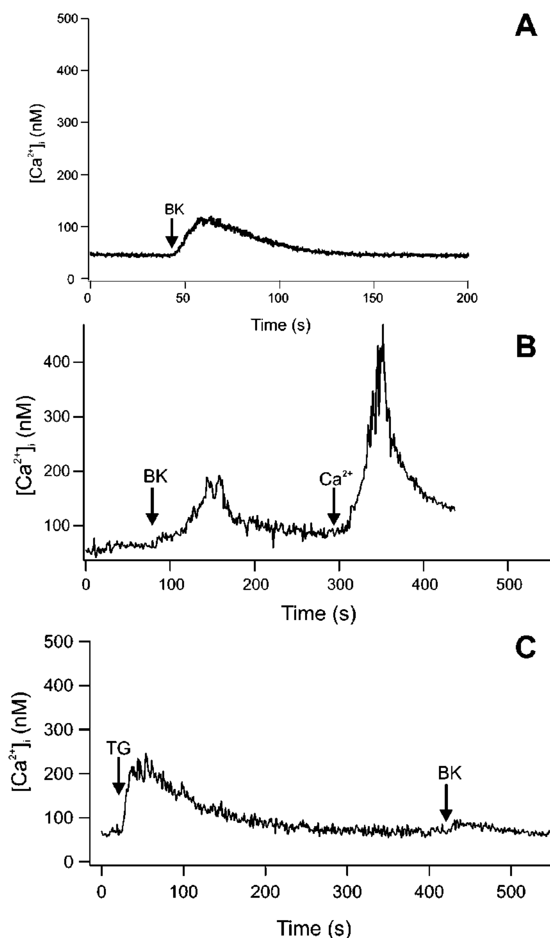

Figure 4 (A) Increase of $[Ca^{2+}]_i$ in peritubular cells by stimulation with BK at a final concentration of 10 nM in a nominally Ca^{2+}-free external HBSS. The stimulus was followed by a diminished calcium response compared to the stimulation in the presence of external calcium. (B) Addition of Ca^{2+} (2 mM) after BK stimulation in nominally Ca^{2+}-free external HBSS led to an instantaneous and linear rise of $[Ca^{2+}]_i$ (C) The SERCA-pumps of the endoplasmic reticulum were blocked by 5 μM thapsigargin. After a transient rise in $[Ca^{2+}]_i$ cells were stimulated with BK (10 nM). No substantial rise in $[Ca^{2+}]_i$ was detectable. All figures show one typical experiment out of at least seven.

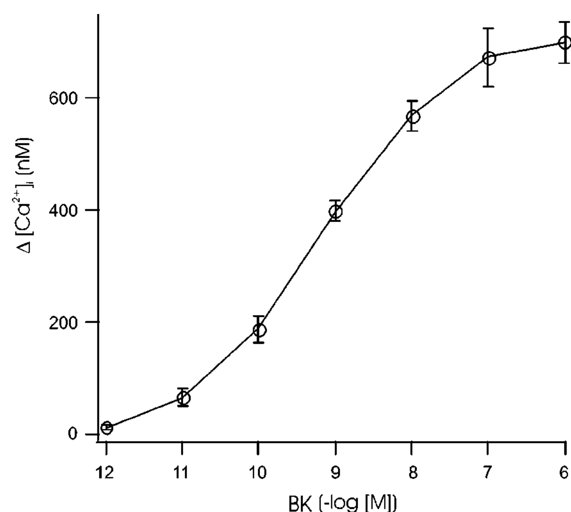

Figure 5 Response of Fura-2-loaded peritubular cells to increasing concentrations of BK. BK concentrations of 1 μM to 1 pM were used to stimulate the cells in a cuvette-based system and monitored by a photomultiplier. The basal $[Ca^{2+}]_i$ was subtracted from the maximal Ca^{2+} responses ($\Delta[Ca^{2+}]_i$). At least five experiments were averaged for each BK concentration and are graphed with their standard errors.

peritubular cells is mediated *via* B_2 receptors. Local production of kinins occurs on the surface of the target cell by the action of the protease tissue kallikrein on the inactive precursor low molecular weight kininogen (Hess, 1997). The main source for kininogens are liver and kidney tissue. However, at least in rodents, small amounts of low molecular weight kininogen are also expressed in other tissues including the testis (Takano *et al*., 1997), but their precise cellular location is still unknown. Tissue kallikrein is also expressed in rat testis (Clements, 1997). Using immune histochemistry we located tissue kallikrein on the acrosomal cap of round and elongated spermatids of pubertal and mature rats (Monsees *et al*., 1999) and – to a lesser extent – on peritubular cells of pre-pubertal rats (unpublished observation). Thus, formation of kinins in rodent testis, especially in the seminiferous tubule, is likely. The liberated kinins act on the B_2 receptor or are rapidly inactivated by specific peptidases which are called kininases and are also located on the plasma membrane. Kininases are present in Sertoli cells of the rat testis (Monsees *et al*.,1996a, b; 1998). Recently we also detected activities of several kininases in peritubular cells isolated from pre-pubertal rat testis (unpublished observations).

The present study demonstrates for the fist time a functional bradykinin B_2 receptor in peritubular myoid cells of the pre-pubertal rat testis. Previously, McEachern *et al*. (1991) showed expression of B_2 receptor mRNA in different tissues of the rat, including kidney, vas deferens and testis. By immune histochemistry, we recently located the B_2 receptor in different testicular cells, including peritubular and endothelial cells, pachytene spermatocytes and spermatids (Monsees *et al*., 2002). Here, we showed expression of the B_2 receptor gene by RT – PCR and identified three B_2 receptor proteins using immune blot technique. The first protein band consistent with cDNA data of rat B_2R, i.e. an unmodified protein of 366 residual amino acid with an M_r of 41,696 (McEachern *et al*., 1991). The two proteins with higher molecular weights may correspond to glycosylated structures of the bradykinin B_2 receptor, that likely form oligomers after activation (Abd Alla *et al*., 1996; Blaukat *et al*., 1999).

Bradykinin was found to increase intracellular calcium levels, $[Ca^{2+}]_i$, in different cell types, including fibroblasts, tracheal, epithelial and adrenal cells (Farmer *et al*., 1991; Houchi *et al*., 1993; Issandou & Rozengurt, 1990; Ricciardolo *et al*., 1998). The present study demonstrated, that bradykinin also stimulated $[Ca^{2+}]_i$ in testicular myoid peritubular cells and that bradykinin B_2 receptors are involved in this mechanism. This conclusion is supported by several lines of evidence. On the molecular level, we showed expression of the B_2R mRNA using primers (Monsees *et al*., 2002) and B_2R using a monoclonal antibody specific for the B_2-subtype of the kinin receptor (Ju *et al*., 1998). On the pharmacologic level, we observed that an increase in $[Ca^{2+}]_i$ is induced by physiological concentrations of BK, which had a high affinity

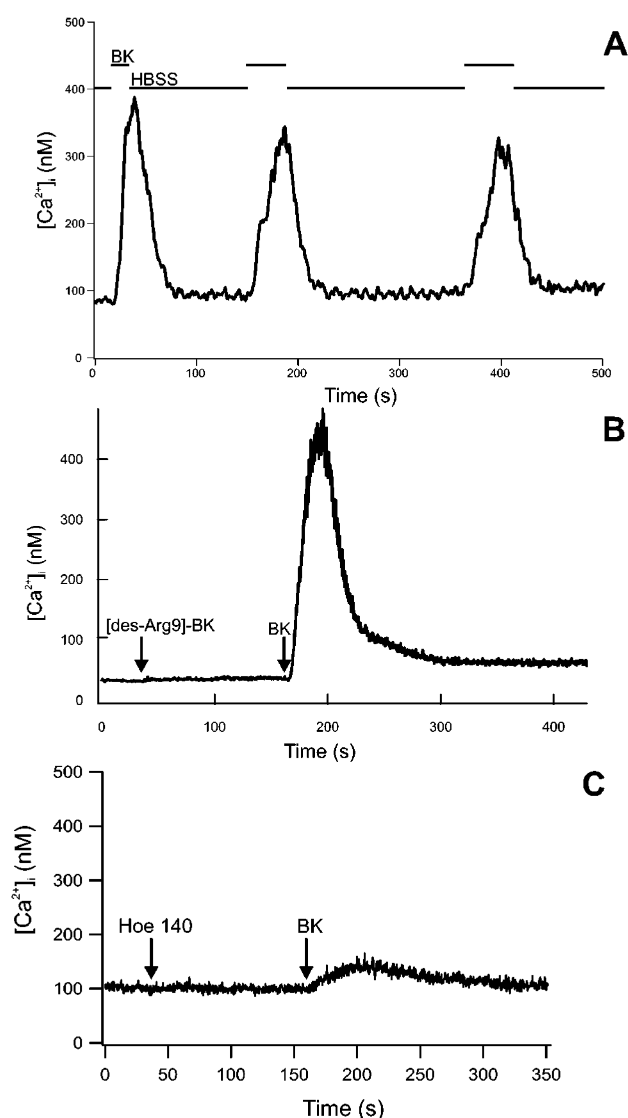

Figure 6 (A) Local perfusion of peritubular cells with BK. Fluorescence was recorded from a single Fura-2-loaded peritubular cell perfused with HBSS. The lines above the recording indicate the change between HBSS and BK perfusion. The recording shows three brief stimulations with 10 nM BK. BK was applied with a local, solenoid-controlled perfusion device with an exchange time between different buffers of <1 s. All three stimulations led to an increase in $[Ca^{2+}]_i$. (B) $[Ca^{2+}]_i$ response to BK following stimulation of bradykinin B_1 receptor (B_1R). In cuvette experiments, the specific B_1R agonist [des-Arg⁹]-BK had no effect on $[Ca^{2+}]_i$ when added to Fura-2-loaded peritubular cells 2 min prior to stimulation with BK. No inhibition of the $[Ca^{2+}]_i$ response was noticed after adding BK (10 nM). (C) Using the same protocol described in the legend to Figure 6B with the specific B_2R antagonist HOE 140 instead of [des-Arg⁹]-BK showed a strong inhibition of the $[Ca^{2+}]_i$ response after BK stimulation. HOE 140 itself did not alter the $[Ca^{2+}]_i$.

to the B_2-subtype kinin receptor (Hall & Morton, 1997). In contrast, even μM concentrations of [des-Arg⁹]-bradykinin, a typical B_1-subtype receptor agonist (Hall & Morton, 1997), were totally inactive. Furthermore, the selective B_2 receptor antagonist HOE 140 (Hall & Morton, 1997) strongly inhibited the increase in $[Ca^{2+}]_i$ induced by bradykinin. These findings indicate that the rise in $[Ca^{2+}]_i$ in testicular peritubular cells is mediated by the stimulation of kinin receptors of the B_2 subtype. The pharmacological profile of the agonists and antagonists used in this study have been studied in detail previously (Regoli & Barabé, 1980; Regoli *et al*., 1998; 2001). Based on the results of different bioassays, rank orders of potency were described for agonists and antagonists of the B_1R and B_2R respectively. The selective and specific B_2R antagonist HOE 140 blocks with high affinity ($IC_{50}=6\ 10^{-10}$ M) the effect of agonists on the B_2R but shows almost no antagonistic properties ($IC_{50}>10^{-5}$ M) on the B_1R. Similarly, [des-Arg⁹]-bradykinin binds with high affinity ($EC_{50}=5\ 10^{-8}$ M) to the B_1R but is almost inactive ($EC_{50}>10^{-5}$ M) towards the B_2R. Corresponding EC_{50} values for bradykinin are $>10^{-5}$ M and $3\ 10^{-9}$ M, respectively (Regoli *et al*., 2001).

Our data clearly showed that the specific B_1R agonist [des-Arg⁹]-bradykinin did not influence $[Ca^{2+}]_i$ in isolated peritubular cells. In many tissues, B_1 receptors co-exist and mediate similar effects, including transient increases in $[Ca^{2+}]_i$ (Marceau, 1997). As this and our previous study (Monsees *et al*., 2002) investigated only the expression and transcription of the B_2R, the expression and involvement of the bradykinin subtype 1 receptor in peritubular cell or testis physiology was not quantified. The B_1R is generally absent from healthy tissues and animals but can be induced under pathological conditions such as injury or inflammation. The agonists for B_1R are the carboxyl truncated kinins, [des-Arg⁹]-kallidin and [des-Arg⁹]-bradykinin. Thus, B_1R upregulation by injury may extend the tissue response to kinin metabolites. In some pathological systems such as chronic inflammation, the B_1R-mediated response becomes more evident with time as the effect mediated by B_2R fades (Marceau, 1997).

In the present study we used peritubular cells which were isolated from pre-pubertal rats (18 days). At this age the tight junctions of the Sertoli cell barrier are not developed which simplifies tissue dissociation. Also, spermatogonia and pachytene spermatocytes are the only germ cells present, which makes it easier to get a pure preparation of peritubular cells. Preliminary data may point to possible development-dependent expression of the B_2R (Monsees *et al*., 2002). Although semiquantitative RT–PCR analysis did not show significant differences in testicular B_2R mRNA levels, immune histochemistry demonstrated variations in the location of the B_2R protein in rats of different ages. Positive immune staining was observed in peritubular cells of testis slices from pre-pubertal (18 days) but not from sexually mature rats (53 days), which paralleled our results of the immune reaction for tissue kallikrein. However, further studies are needed to clarify development-dependent expression and the influence of androgens on the B_2R expression in testicular cells.

In various cell types, both the influx of Ca^{2+} and the mobilization of sequestered intracellular Ca^{2+} have been demonstrated (Houchi *et al*., 1993; Yang *et al*., 1994). In our study, removal of external Ca^{2+} by use of a calcium-free buffer decreased the $[Ca^{2+}]_i$ response of peritubular cells to bradykinin exposure. This indicates the possible release of Ca^{2+} from internal stores. The addition of Ca^{2+} after stimulating cells with BK in a Ca^{2+}-free medium led to an $[Ca^{2+}]_i$ increase. In another experiment the endoplasmic reticulum, a major internal Ca^{2+} store, was emptied by thapsigargin treatment. Subsequently, no $[Ca^{2+}]_i$ rise was detectable after BK stimulation. This finding is an indicator

that in the presence of external Ca^{2+}, the initial rise in $[Ca^{2+}]_i$ might lead to an opening of store operated channels (I_{crac}) localized in the plasma membrane. Repeated or continuous exposure to kinins alters the response of bradykinin receptors to agonists in a way that the cellular response is dampened or completely switched off. This phenomenon is called adaptation or desensitization and occurs in all biological systems (Böhm *et al*., 1997; Yang *et al*., 1994). We observed adaptation of the kinin receptor in peritubular cells of the rat testis. The desensitization process may result from receptor endocytosis preceded by agonist-induced dimerization and phosphorylation of the B_2 receptor (Abd Alla *et al*., 1999; Blaukat *et al*., 1996; Pizard *et al*., 1999). Internalization of the B_2 receptor seems to be a prerequisite for resensitization and receptor recycling to the plasma membrane (Lamb *et al*., 2001; Zhang *et al*., 1997).

Little is known about calcium responses induced by hormones in peritubular cells of the testis. Endothelin-1 (ET-1), produced by Sertoli cells, stimulates increases in $[Ca^{2+}]_i$ in peritubular cells *via* ET_A-subtype receptors (Santiemma *et al*., 1996). Recently, we showed that the macrophage migration inhibitory factor (MIF) induced a Ca^{2+} response in peritubular cells (Wennemuth *et al*., 2000). Small peptides such as oxytocin are regulators of peritubular myoid cell contraction (Niemi & Kormano, 1965). Thus, bradykinin may also be involved in triggering 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 (Burch & Axelrod, 1987; Buhrley & Ellis, 1975; Farr & Ellis, 1980).

Bradykinin is known to induce cell growth and differentiation (Walsh & Fan, 1997), which are essential processes in testicular and peritubular cell development. Within the germ cell epithelium, bradykinin B_2 receptors seem to be involved in the local regulation of spermatogenesis. In organ cultures of immature rat testis, a significant stimulation of prespermatogonial cell proliferation after exposure to bradykinin was reported (Atanassova *et al*., 1998). We recently demonstrated that certain germ cells express the B_2R protein, which pointed to a possible involvement of the tissue kallikrein kinin system in regulating growth of pachytene spermatocytes and differentiation of round spermatids into highly elongated spermatozoa (Monsees *et al*., 2002).

The results of this study confirm that the bradykinin-induced increase in $[Ca^{2+}]_i$ in testicular peritubular cells is mediated by stimulation of bradykinin B_2 receptors. Thus, the tKKS may act as a local modulator of peritubular cell function in the rat testis.

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