# Pre- and Post-Junctional Bradykinin $B_2$ Receptors Regulate Smooth Muscle Tension to the Pig Intravesical Ureter

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Aims: Neuronal and non-neuronal bradykinin (BK) receptors regulate the contractility of the bladder urine outflow region. The current study investigates the role of BK receptors in the regulation of the smooth muscle contractility of the pig intravesical ureter. Methods: Western blot and immunohistochemistry to show the expression of BK $B_1$ and $B_2$ receptors and myographs for isometric force recordings were used. Results: $B_2$ receptor expression was consistently detected in the intravesical ureter urothelium and smooth muscle layer, where a strong $B_2$ immunoreactivity was observed within nerve fibers among smooth muscle bundles. $B_1$ expression was not detected. On ureteral strips basal tone, BK induced concentration-dependent contractions, which were potently reduced by extracellular $Ca^{2+}$ removal and by $B_2$ receptor and voltage-gated $Ca^{2+}$ (VOC) channel blockade. BK contraction did not change as a consequence of urothelium mechanical removal or cyclooxygenase and Rho-associated protein kinase inhibition. On 9,11-dideoxy-9a,11a-methanoepoxy prostaglandin $F_{2a}$ (U46619)-precontracted samples, under non-adrenergic non-cholinergic (NANC) and nitric oxide (NO)-independent NANC conditions, electrical field stimulation-elicited frequency-dependent relaxations which were reduced by $B_2$ receptor blockade. Kallidin, a $B_1$ receptor agonist, failed to increase preparation basal tension or to induce relaxation on U46619-induced tone. Conclusions: The present results suggest that BK produces contraction of pig intravesical ureter via smooth muscle $B_2$ receptors coupled to extracellular $Ca^{2+}$ entry mainly via VOC (L-type) channels. Facilitatory neuronal $B_2$ receptors modulating NO-dependent or independent NANC inhibitory neurotransmission are also demonstrated. Neurourol. Urodynam. 9999:1–7, 2014. # 2014 Wiley Periodicals, Inc.

Key words: bradykinin receptors; $Ca^{2+}$ signaling; inhibitory neurotransmission modulation; pig intravesical ureter; smooth muscle tension

## INTRODUCTION

Bradykinin (BK), a metabolite of the kallikrein–kinin system, is a potent mediator of inflammation, causing pain, vasodilatation, increased vascular permeability, and smooth muscle contraction.[1,2] BK exerts its action via two receptor subtypes $B_1$ and $B_2$.[3] BK shows a much higher affinity for the $B_2$ receptor, whereas the $B_1$ one exhibits greater selectivity for BK metabolites lacking the C-terminal Arg.[3] BK immunoreactivity (IR) is present in afferent and efferent neurons innervating the bladder and urethra smooth muscle and in the urothelium, where BK exerts a wide range of biological actions including the ability to contract the detrusor smooth muscle, stimulate sensory nerves, and evoke the release of cyclooxygenase (COX) products.[4–7] In the rat bladder, BK contraction is produced independently of phospholipase C involving L-type $Ca^{2+}$ channels, as well as by rho-kinase and phospholipase $A_2$/COX dependent mechanisms.[8] BK also modulates the human urothelial phenotype, accelerating stretch-induced ATP release, release of nerve growth factor, and $TRPV_1$ channel expression. BK-induced changes in urothelial sensory function might contribute to the bladder dysfunction.[9] In the bladder neck, neuronal $B_2$ receptors modulate non-adrenergic non-cholinergic (NANC) inhibitory neurotransmission, whereas urothelial $B_1$ and muscle $B_2$ receptors produce relaxation and contraction, respectively, of smooth muscle.[10] BK receptors have been proposed as therapeutic receptor targets for the stress urinary incontinence, by increasing the outlet resistance.[11] These receptors also play a key role in the pathogenesis of experimental cystitis and kallikrein–kinin system activation has been reported in bladders of patients suffering interstitial cystitis.[12,13] Thus, BK, via $B_2$ receptors, is involved in the genesis of detrusor hyperreflexia during cyclophosphamide-induced cystitis favoring the development of bladder pain and increased urinary frequency.[12,13]

The ureterovesical junction plays an important role in both the active transport of urine bolus from ureter to bladder and the prevention of vesicoureteral reflux during bladder filling.[14] The study of peptide receptors in the distal ureter might be useful to identify therapeutic targets to vesicoureteral reflux. In fact, angiotensin II receptor antagonists have been proposed to prevent renal fibrosis associated with reflux nephropathy.[15] BK receptors play a pivotal role in the regulation of the bladder outflow region tension,[10] no data exist, however, about their possible involvement in distal ureteral tone regulation. Therefore, the current study investigates the role of BK receptors on the pig intravesical ureter smooth muscle contractility.

## MATERIALS AND METHODS

### Tissue Collection and Dissection

Adult pigs of either sex with no lesions in their urinary tract were selected from the local slaughterhouse. Urinary bladders with attached ureters were removed immediately after the animals were sacrificed, and kept in chilled physiological saline solution (PSS) at 4°C. The adjacent connective and fatty tissues were removed with care, and longitudinal preparations (4–6 mm long and 2–3 mm wide) of the intravesical ureter, which is ureteral segment located inside the bladder wall thickness formed by intramural and submucosal components, were dissected from the bladder.[16]

### Western Blot

Intravesical ureter was homogenized in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 1% SDS, 1 mM sodium vanadate, and 0.01% protease inhibitor cocktail (all from Sigma–Aldrich). Thirty-microgram protein were separated in a 15% polyacrylamide gel (SDS–PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad). All membranes were blocked in 5% non-fat dry milk for 1 h at room temperature. For immunodetection, membranes were incubated overnight at 4°C with rabbit monoclonal anti-$B_1$ or anti-$B_2$ receptor antibodies (1:200, Santa Cruz Biotechnology, Inc., Heidelberg, Germany). Membranes were then washed in 0.05% Tween-20, incubated with a HRP-conjugated anti-rabbit secondary antibody (1:7,000, GE Healthcare) for 1 h at room temperature, and then washed and visualized by chemiluminescence (ECL advance-kit, GE Healthcare).

### Immunohistochemistry

Intravesical ureter segments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), for 2–4 h at 4°C, and subsequently placed in 30% sucrose in PB for cryoprotection. The tissue was embedded and frozen in OCT compound (Tissue-Tek®. Sakura Finetek, Europe B.V.), and stored at −80°C. Transversal sections 5 mm thick were obtained by means of a cryostat and pre-incubated in 10% normal goat serum in PB containing 0.3% Triton-X-100, for 2–3 h. Then, sections were incubated with rabbit anti-$B_1$ or anti-$B_2$ receptor antibodies at 1:50 final concentration, plus a mouse anti-protein gene product 9.5 (anti-PGP 9.5) (Abcam, Cambridge, UK), as neuronal marker, diluted 1:50, during 48 h at 4°C, washed and reacted with the secondary antibodies Alexa Fluor 594 goat–antirabbit (1:200 dilution) to detect $B_1$ or $B_2$ receptors and Alexa Fluor 488 goat–antimouse (1:200 dilution) to detect PGP 9.5, for 2 h at room temperature. The slides were covered with a specific mounting medium with DAPI (Invitrogen, Paisley, UK), which stains all cell nuclei. No immunoreactivity (IR) could be detected in sections incubated in the absence of the primary antiserum.[10]

### Myographs for Isometric Force Recordings

Ureteral strips 4–5 mm long and 2–3 mm wide were suspended horizontally with one end connected to an isometric transducer (Grass FT03C) and the other one to a micrometer screw, in 5 ml organ baths containing PSS at 37°C gassed with carbogen (95% $O_2$ and 5% $CO_2$) to obtain a final pH of 7.4. The signal was continuously recorded on a polygraph (Graphtec MC6621). Passive tension of 2 g was applied to the strips and they were allowed to equilibrate for 60 min. The contractile ability of the strips was determined by exposing them to potassium rich (124 mM) PSS (KPSS).[17] The effect induced by BK receptor agonists was studied on preparation basal tension as well as on 0.1 mM 9,11-dideoxy-9a,11a-methanoepoxyprostaglandin $F_{2a}$ (U46619)-induced contraction. A first control response curve to BK was obtained, the bath solution was then changed every 15 min for a period of 90 min, the preparations were incubated with the specific treatments for 30 min, and then a second cumulative contraction and/or relaxation curve was constructed. The concentration of the agents used was chosen on the basis of previous studies.[10] Mechanical removal of the urothelium was performed by dissection under the microscope and cold light source.

To investigate the role of extracellular $Ca^{2+}$ entry, strips were washed three times and kept for 5 min in $Ca^{2+}$-free PSS containing 0.1 mM EGTA, and then the solution was changed to $Ca^{2+}$ free KPSS without EGTA. Under these conditions, contractions on basal tone elicited by submaximal BK doses in the absence or presence of extracellular $Ca^{2+}$ were obtained.

In electrical field stimulation (EFS) experiments, noradrenergic neurotransmission and muscarinic receptors were blocked by pre-incubation with guanethidine (10 mM) and atropine (1 mM), in the absence or presence of the NO synthase inhibitor $N^G$-nitro-L-arginine (L-NOARG, 100 mM), for 1 h, replacing the solution every 20 min, and these drugs were present throughout the experiment. In U46619-precontracted strips, EFS was performed by delivering rectangular pulses (1 ms duration, 0.5–16 Hz, 20 s trains, with constant current output adjusted to 75 mA), at 4 min intervals, from a Cibertec CS20 stimulator (Barcelona, Spain). These EFS parameters have previously been used to elicit neurogenic relaxations in the intravesical ureter.[18]

### Drugs and Solutions

The following drugs were used: bradykinin (BK), HOE140, indomethacin, Lys-[Des-Arg$^9$]bradykinin (kallidin), R892; 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy]ethyl-1H-imidazole hydrochloride (SKF96365); 9,11-dideoxy-9a,11a-methano epoxyprostaglandin $F_{2a}$ (U46619); and trans-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride (Y27632) from Tocris (UK) and atropine, guanethidine, nifedipine, and $N^G$-nitro-L-arginine (L-NOARG) from Sigma. Indomethacin, nifedipine, and U46619 were dissolved in 96% ethanol. The other drugs were dissolved in distilled water. Preliminary experiments showed that these solvents, at their final concentrations, had no effect on the preparations contractility. The primary BK $B_1$ or $B_2$ receptor antibodies were from Santa Cruz Biotechnology and PGP 9.5 was from Abcam. The secondary antibodies were from Invitrogen.

The composition of PSS was (mM): NaCl 119, KCl 4.7, $KH_2PO_4$ 1.18, $MgSO_4$ 1.17, $CaCl_2$ 1.5, ethylenediaminetetraacetic acid 0.027, and glucose 11. The solution was kept at 378C and continuously gassed with carbogen to maintain pH at 7.4. KPSS was PSS with KCl exchanged for NaCl on an equimolar basis. Stock solutions were prepared daily in distilled water.

### Calculations and Statistics

Contractions to BK receptor agonists are expressed as a percentage of the contraction induced by KPSS (124 mM). Relaxations to EFS are expressed as a reversal percentage of the 0.1 mM U46619-induced tone. For each concentration–response curve (CRC) the concentration required to give half maximal response ($EC_{50}$) was estimated by computerized non-linear regression analysis (GraphPad Prism). The potency and maximal responses to agonists are expressed in terms of $pD_2$ and Emax, respectively. $pD_2$ is defined as the negative logarithm of $EC_{50}$ ($pD_2$ ¼ log $EC_{50}$). Results are expressed as mean SEM of n (number of preparations, 1–2 strips per animal). Statistical significance of the differences was studied by Student's t-test for paired observations and by analysis of variance (ANOVA) and a posteriori Bonferroni method for multiple comparisons. The differences were considered significant with a probability level of $P < 0.05$.

## RESULTS

### Expression of the BK $B_1$ and $B_2$ Receptors

In Western blots of intravesical ureter membranes, antibodies directed against the BK $B_2$ recognized a specific band located at approximately 43 kDa, the expected molecular weight for the $B_2$ receptor, both in smooth muscle and urothelium (Fig. 1A). Moreover, BK receptor subtype antibodies combined with the neuronal marker PGP 9.5 showed $B_2$ receptor expression within nerve fibers distributed in the smooth muscle layer of the intravesical ureter, running parallel to the smooth muscle bundles (n ¼ 5) (Fig. 1B–I). $B_1$ receptor expression was not detected.

### Functional Studies

Ureteral strips were allowed to equilibrate to a passive tension of 1.9 0.1 g (n ¼ 168 preparations from 84 pigs). Under these conditions KPSS produced a contraction above the basal tension of 2.4 0.2 g (n ¼ 168).

### Effect of Urothelium Mechanical Removal and of $B_1$ and $B_2$ Receptor and COX Pathway Blockade on BK Contractions

On preparation basal tone, BK (0.1 nM-30 mM), produced concentration-dependent contractions ($pD_2$ and $E_{max}$ values being 6.3 0.2 and 69.3 3%), which were largely reduced by HOE140 (0.1 mM), a $B_2$ receptor selective antagonist (n ¼ 9) (Fig. 2A and B). These contractions, however, were not modified by urothelium mechanical removal (n ¼ 8) or by pretreatment with R892 (0.1 mM) (n ¼ 7) (Fig. 2C) or with indomethacin (3 mM) (n ¼ 6), a $B_1$ receptor antagonist and a COX inhibitor, respectively. The $B_1$ receptor selective agonist kallidin (0.1 nM–3 mM) failed to increase the smooth muscle basal tension (n ¼ 6) or inhibit the U46619-induced tone (n ¼ 7).

### Effect of Extracellular $Ca^{2+}$ Removal and VOC (L-Type) and Non-VOC Channel and Rho/Rho-Kinase Pathway Blockade on BK Contractions

Extracellular $Ca^{2+}$ removal reduced the response evoked by 3 mM BK (contractions of 61 9% and 3 1% of the KPSS-induced tone, in the presence and absence, respectively, of extracellular $Ca^{2+}$, $P < 0.05$, paired t-test, n ¼ 7) (Fig. 3A and B).

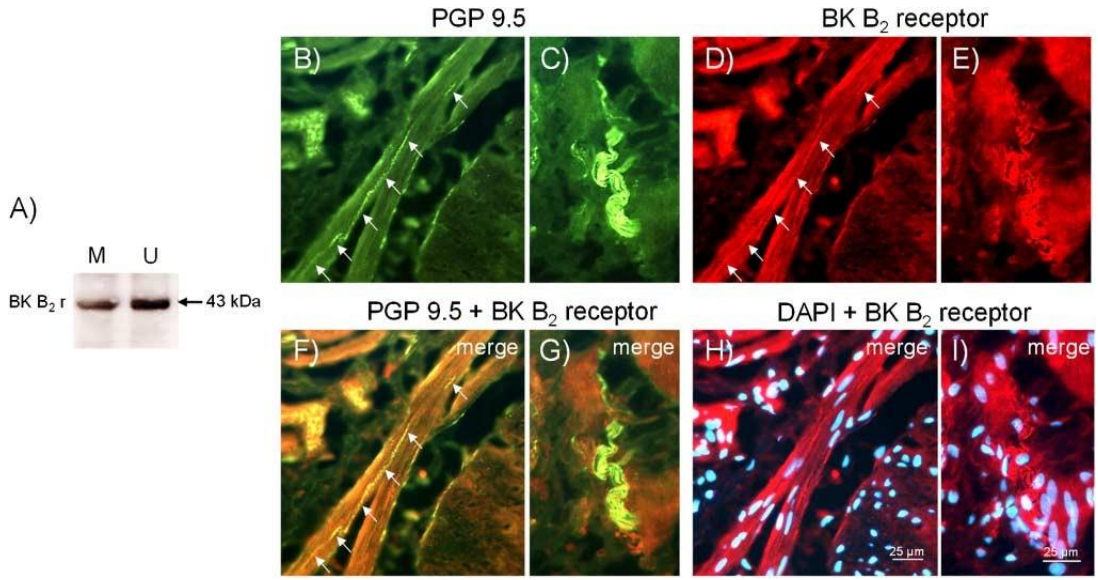


Fig. 1. (A) Western blot of pig intravesical ureter membranes from smooth muscle (M) and urothelium (U) incubated with a bradykinin (BK) $B_2$ receptor antibody, showing a 43 kDa major band corresponding to the expected molecular weight for $B_2$ receptor. (B–I) Intravesical ureter immunohistochemical staining demonstrating the existence of a rich $B_2$ receptor-immunoreactive innervation (n ¼ 5). B, D, F, and H show the same field in all cases. (B) Overall innervation of the intravesical ureter visualized using the general nerve marker PGP 9.5 (green color). (D) $B_2$ receptor immunofluorescence of the intravesical ureter (red color). The $B_2$ immunopositive fibers (D) run parallel to the smooth muscle bundles. (F) Immunofluorescence double labeling for PGP 9.5 and $B_2$ receptor in the smooth muscle showing colocalization in nerve terminals (arrows, yellow color). (H) The cell nuclei were counterstained using DAPI (blue color). (C, E, G, I): High magnification (40 ) for B, D, F, and H, respectively.

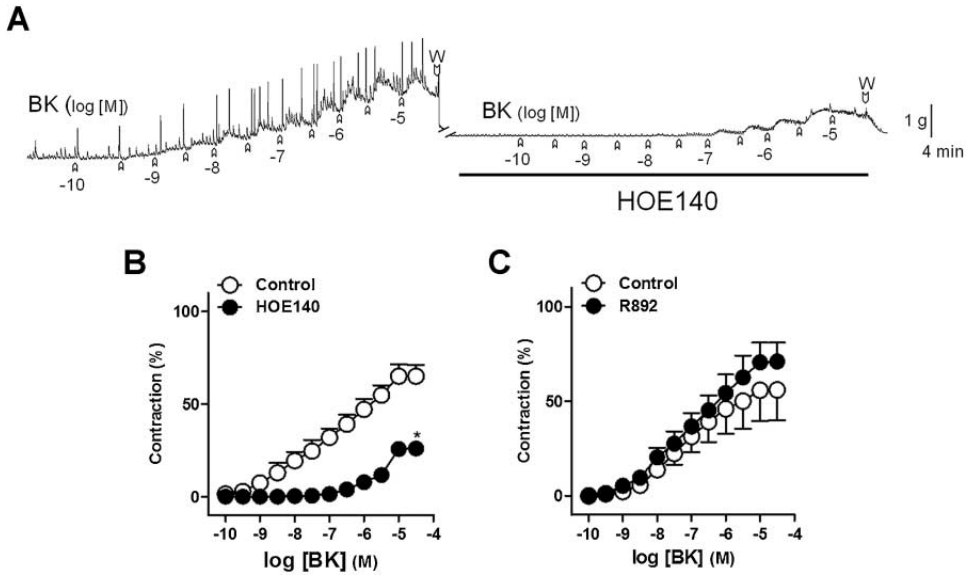


Fig. 2. (A) Isometric force recordings showing the contractions evoked by bradykinin (BK, 0.1 nM—30 mM) on pig intravesical ureter strips basal tension, in the presence or absence of HOE140 (0.1 mM), a BK $B_2$ receptor selective antagonist. Vertical bar shows tension in g and horizontal bar time in min. W: wash out (B, C) Log concentration–response contraction curves to BK in the absence (open circles) or in the presence (closed circles) of HOE140 (0.1 mM) (B) or the $B_1$ receptor selective antagonist, R892 (0.1 mM) (C). Results are expressed as a percentage of the KPSS-induced contraction, and represent mean SEM. of 7–8 preparations from four pigs. $P < 0.05$, versus control (paired t-test).

Nifedipine (1 mM, n ¼ 6) and SKF96365 (10 mM, n ¼ 6), inhibitors of VOC (L-type) and non-VOC channels, respectively, potently reduced BK contractions (Fig. 3D). Pretreatment with nifedipine plus SKF96365 evoked a similar inhibition to that produced by nifedipine alone (n ¼ 8) (Fig. 3C and D). Y27632, a selective inhibitor of the Rho-associated protein kinase, did not change the BK contractions (n ¼ 6).

### Effect of $B_1$ and $B_2$ Receptor Blockade on NANC Neurogenic Relaxations

To investigate the role of BK receptors in NANC inhibitory neurotransmission, intravesical ureter strips were pretreated with guanethidine and atropine, noradrenergic neurotransmission, and muscarinic receptor blockers, respectively, and contracted with the thromboxane analogue U46619 (0.1 mM).

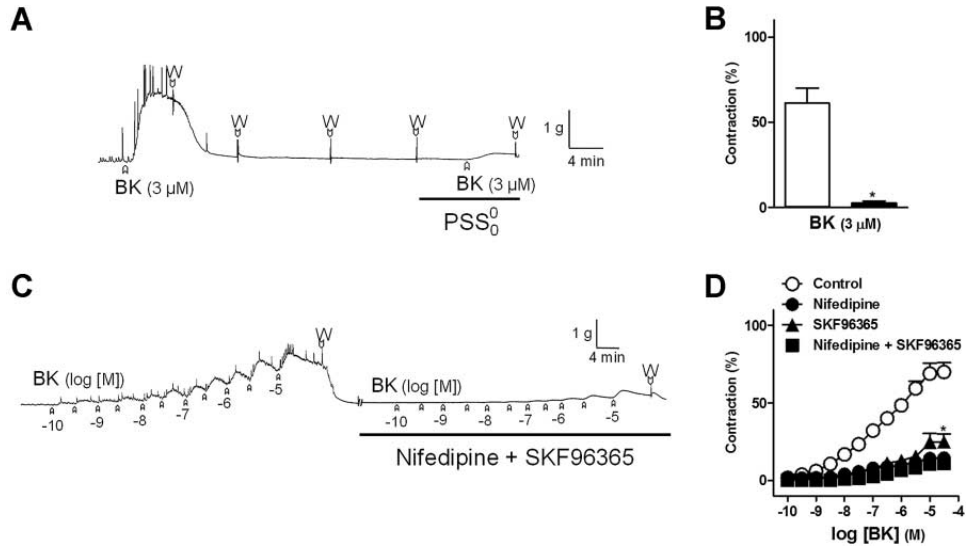


Fig. 3. (A) Isometric force recordings showing the contractions to bradykinin (BK, 3 mM) on pig intravesical ureter strips basal tension, in normal physiological saline solution (PSS) and in $Ca^{2+}$-free PSS ($PSS^o_o$). (B) Diagram showing the contraction produced by 3 mM BK in normal PSS (open bar) and in $PSS^o_o$ (closed bar). (C) Isometric force recordings showing the contractions evoked by BK (0.1 nM–30 mM) on ureter intravesical strips basal tension, in the absence or presence of nifedipine (1 mM) plus SKF96365 (10 mM), blockers of, respectively, L-type VOC and non-VOC channels. Vertical bar shows tension in g and horizontal bar time in min. W: wash out. (D) Log concentration–response contraction curves to BK in the absence (open circles) or presence (closed symbols) of nifedipine (1 mM, closed circles), SKF96365 (10 mM, closed triangles) or nifedipine plus SKF96365 (closed squares). Results are expressed as a percentage of the KPSS-induced contraction, and represent mean SEM. of 6–8 preparations from 3 to 4 pigs. $P < 0.05$, versus control (paired t-test).

Under these conditions, EFS (0.5–16 Hz) evoked frequency-dependent relaxations (maximal responses of 83 5% at 16 Hz, n ¼ 14 from nine pigs), which were reduced by HOE140 (0.1 mM, n ¼ 8), a $B_2$ receptor antagonist, in the absence (Fig. 4A and B) or presence (Fig. 4C) of the NO synthase inhibitor L-NOARG. Intravesical ureter neurogenic relaxations were unchanged by $B_1$ receptor blockade (Fig. 4D, n ¼ 6).

## DISCUSSION

Our results provide morphological and functional evidence for an involvement of neuronal and smooth muscle $B_2$ BK receptors in the regulation of muscle tension to the pig intravesical ureter. BK produces contraction via activation of smooth muscle $B_2$ receptors essentially coupled to extracellular $Ca^{2Þ}$ entry via L-type VOC channels. In addition, neuronal $B_2$ receptors facilitating the NO-dependent and independent NANC inhibitory neurotransmission are also involved. This conclusion is supported by the following observations: (i) The presence of the $B_2$ receptor in the smooth muscle and urothelium, as well as $B_2$ receptor-IR detected within nerve fibers widely distributed in the muscle layer of the intravesical ureter. (ii) BK-induced contraction was profoundly reduced as a consequence of extracellular $Ca^{2Þ}$ removal and by $B_2$ receptor and L-type VOC channel blockade. (iii) The NANC neurogenic relaxation elicited by EFS, in the absence or presence of NO synthase inhibitors, was reduced by $B_2$ receptor selective blockade.

The autonomic nervous system plays an essential role in the maintenance of ureteral motor activity. Thus, noradrenaline[16] and acetylcholine[15] stimulate intravesical ureter phasic activity and basal tone through adrenergic and muscarinic, respectively, receptors. Nitric oxide (NO) and unknown nature mediator/s are involved in the non-adrenergic non-cholinergic (NANC) inhibitory neurotransmission of the pig intravesical ureter.[18] Peptides as substance P or neurokinin A evoke a direct contraction through smooth muscle $NK_2$ receptors,[20] whereas vasoactive intestinal peptide (VIP) or pituitary adenylate cyclase-activating polypeptide 38 (PACAP 38) relax the intravesical ureter through smooth muscle VIP/PACAP receptors.[21]

In the current study, BK evoked a potent contraction on ureteral strip basal tension, which was not changed as a consequence of urothelium mechanical removal, thus suggesting the involvement of smooth muscle receptors in such response. This was also suggested by Western blot and immunostaining of intravesical ureter samples showing a $B_2$ receptor expression throughout the smooth muscle layer, which agrees with the $B_2$ receptor-IR described in the pig bladder neck.[10] BK is an endogenous kinin receptor agonist that displays selectivity for $B_2$ over $B_1$ receptors.[3] This together with the facts that the $B_1$ receptor selective agonist kallidin failed to modify the preparations basal tone and that the $B_1$ receptor expression was not detected, suggested the mediation of smooth muscle $B_2$ receptors in intravesical ureter BK-elicited contractions. The involvement of this BK receptor is also supported by the inhibition produced by HOE140, a $B_2$ receptor selective antagonist, on BK contractile responses. This antagonist shifted the BK CRC to the right in a non-competitive manner, which may be explained on the basis of the presence of neuronal $B_2$ receptors above mentioned or alternatively by the

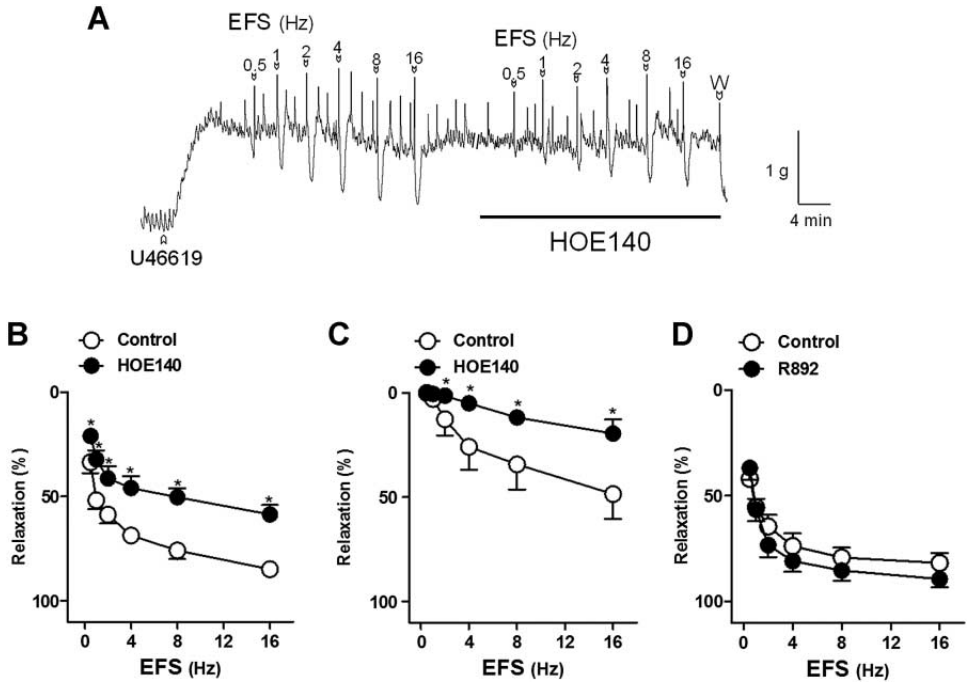


Fig. 4. (A) Isometric force recordings showing the relaxations evoked by electrical field stimulation (EFS, 1 ms duration, 0.5–16 Hz, 20 s trains) in the absence or presence of the $B_2$ receptor selective antagonist HOE140 (0.1 mM), on 1 mM U46619-precontracted pig intravesical ureter strips treated with guanethidine (10 mM) and atropine (1 mM), blockers of noradrenergic neurotransmission, and muscarinic receptors, respectively. Vertical bar shows tension in g and horizontal bar time in min. W: wash out. (B–D) Frequency–response relaxation curves to EFS, in control conditions (open circles) or in the presence (closed symbols) of HOE140 (0.1 mM), alone (B) or in the presence (C) of the nitric oxide synthase inhibitor L-NOARG (100 mM), or the $B_1$ receptor selective antagonist R892 (0.1 mM) (D). Results are expressed as a reversal percentage of the U46619-induced contraction and represent mean SEM of 6–8 preparations from four pigs. $P < 0.05$, versus control (paired t-test).

contribution of a heterogeneous BK receptor population in the BK-elicited contraction. The lack of effect of kallidin on basal tension and of R892, a $B_1$ receptor selective antagonist, on BK contractions ruled out a possible role for $B_1$ receptors. The fact that the BK contraction was not modified as consequence of urothelium removal suggested a lack of contractile effect mediated via an urothelial $B_2$ receptor subtype, which agrees with those found in bladder neck.[10] $B_2$ receptors have previously been reported to promote proliferation of human prostate stromal cells via activation of ERK-1/2 pathways.[22] In the current study, since urothelial $B_2$ receptors are not involved in the regulation of the smooth muscle tension, their role might possibly be ascribed to proliferative actions.

During the filling phase, the bladder shows local contractions produced by a basal myogenic mechanical activity that may be increased by the local release of COX-derived prostanoids and these spontaneous contractions are able to generate activity in afferent nerves that may contribute to detrusor overactivity.[23] In our study, however, indomethacin, a COX inhibitor, failed to modify BK-elicited responses, thus indicating that COX pathway-derived prostanoids are not to be involved in the BK contractions.

In the bladder, receptor-mediated contraction is largely dependent on extracellular $Ca^{2+}$ entry via dihydropyridine-sensitive L-type $Ca^{2+}$ channels.[24] In the current investigation, the potent reduction of the BK-induced contraction obtained in a $Ca^{2+}$-free PSS, or by pre-treatment with nifedipine, a L-type VOC channel blocker, suggests that the BK contractions occur through $Ca^{2+}$ influx via L-type $Ca^{2+}$ channel-dependent mechanisms. In addition, a non-L-type $Ca^{2+}$ entry mechanism also contributes to the BK-elicited contraction. This mechanism could include voltage-gated channels, such as T- or P/Q-type channels, and/or voltage-independent store- and receptor-operated channels.[10] In the intravesical ureter, the inhibition produced by the $Ca^{2+}$ entry inhibitor SKF96365, indicates that other $Ca^{2+}$ channels different from the L-type $Ca^{2+}$ may be also involved in BK-induced $Ca^{2+}$ entry. The slight contraction exhibited by submaximal BK concentrations in a $Ca^{2+}$-free medium suggests that a consistent intracellular $Ca^{2+}$ mobilization is not likely to be involved. Bladder contractility is partly independent of changes in intracellular $Ca^{2+}$ concentration via Rho/Rho-kinase pathway and myosin phosphatase inhibition.[25] In the current study, Y27632, a selective inhibitor of the Rho-associated protein kinase, failed to modify the BK contraction, which seems to rule out the possible involvement of a Rho/Rho-kinase pathway in such effect.

In the pig intravesical ureter, both NO-dependent and independent NANC inhibitory neurotransmission has been demonstrated.[18] Thus, neuronal-released NO relaxes smooth muscle through a guanylyl cyclase-dependent mechanism involving $K_{ATP}$ channel activation.[17] In this structure, neuronal peptidergic receptors have been identified modulating the release of inhibitory transmitters. Thus, facilitatory $PAC_1$ receptors located at capsaicin-sensitive primary afferents and coupled to NO release, and inhibitory VPAC receptors at motor neurons are involved in the relaxations to PACAP 38 and VIP, respectively.[21] For this reason, we investigated the possible modulatory role exerted by kinin receptors on NANC inhibitory neurotransmission. In the present study, $B_2$ receptor IR was also observed within nerve fibers among smooth muscle bundles, and the $B_2$ receptor antagonist HOE140 reduced both NO-dependent and independent neurogenic relaxations. These results suggest the presence of a neuronal $B_2$ receptor population stimulating the release of NO and other/s inhibitory neurotransmitters in the intravesical ureter.

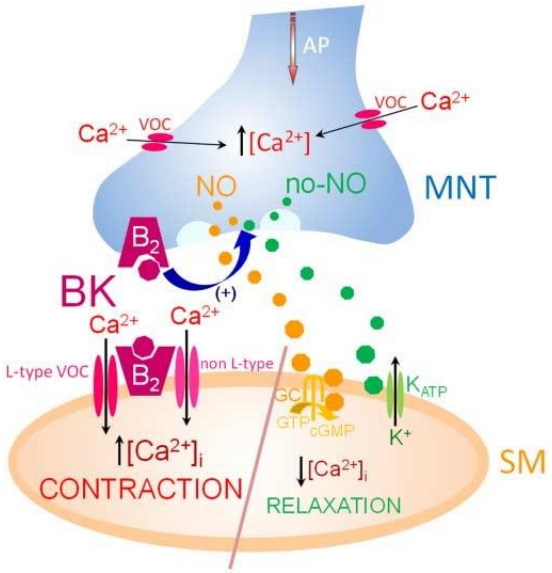


Fig. 5. Proposed mechanism for pre- and post-junctional bradykinin (BK) receptors in the regulation of pig intravesical ureter tension. Arrival of action potentials (AP) at the motor nerve terminal (MNT) evokes membrane depolarization and activation of voltage-gated $Ca^{2+}$ (VOC) channels with the subsequent $Ca^{2+}$ influx, thus favoring NO and unknown nature mediator/s synthesis and release from nerves. BK induces contraction of the intravesical ureter via smooth muscle (SM) $B_2$ receptor activation mainly coupled to extracellular $Ca^{2+}$ entry via VOC (L-type) and non-L type $Ca^{2+}$ channels. In addition, pre-junctional facilitatory $B_2$ receptors stimulate the neuronal NO and unknown neurotransmitter/s release, which produce smooth muscle relaxation through a guanylyl cyclase-dependent mechanism involving $K_{ATP}$ channel activation.

BK receptors have been proposed as therapeutic targets for the stress urinary incontinence.[11] The fact that pre- and post-junctional $B_2$ receptors regulate the intravesical ureter smooth muscle tension raises the possibility these receptors might also be considered as pharmacological targets for treatment of vesicoureteral reflux.

## CONCLUSIONS

Present results suggest that BK elicits contraction of the pig intravesical ureter through smooth muscle $B_2$ receptors essentially coupled to extracellular $Ca^{2+}$ entry mainly via VOC (L-type) channels. Neuronal facilitatory $B_2$ receptors modulating NO-dependent or -independent inhibitory neurotransmission have also been shown (Fig. 5).


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