Mechanism of Bradykinin-Induced Ca$^{2+}$ Mobilization in Murine Proximal Tubule Epithelial Cells

Manish M. Tiwari, Paul L. Prather, and Philip R. Mayeux

Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas

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ABSTRACT

Despite the recognized physiological role of bradykinin (BK) in the kidney in maintaining glomerular and tubule function and its role in pathological states such as endotoxemia, diabetes, and other diseases, relatively little is known about the mechanisms by which BK can impact kidney function. Furthermore, the signaling of BK receptors in the murine nephron has not been fully characterized. The present studies were undertaken to examine BK-stimulated Ca$^{2+}$ signaling using Fura-2 in the murine proximal tubule epithelial cell line TKPTS. BK produced a concentration-dependent rise in intracellular Ca$^{2+}$ ([Ca$^{2+}$]i) (pEC$_{50}$ = 8.39 ± 0.04). Selective antagonists showed the rise in [Ca$^{2+}$]i was mediated through B2 receptors. The rise in [Ca$^{2+}$]i was rapid and reversible and was maximally stimulated at 1 µM (697 ± 70 nM above basal level of 115 ± 6 nM). Studies with thapsigargin and EGTA showed Ca$^{2+}$ mobilization was dependent on two events: release and influx. Both U73122 [1-6-[[17-β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione] [a phospholipase C (PLC) inhibitor] and genistein (a tyrosine kinase inhibitor) partially inhibited BK-stimulated rise in [Ca$^{2+}$]i. When combined, both agents produced a further decrease, suggesting multiple pathways for PLC activation may be involved. The ability of Ni$^{2+}$ to inhibit influx indicated the activation of a Ca$^{2+}$ release-activated channel (CRAC). Ca$^{2+}$ mobilization did not seem to be affected by cyclic nucleotides or protein kinase C. In summary, the TKPTS murine proximal tubule cell line expresses functional B2 receptors linked to Ca$^{2+}$ mobilization that is dependent on phospholipase C and activation of CRAC.

Kinins are a family of peptides that exert their effects through two receptor subtypes, B1 and B2. Bradykinin (BK) is a nine-amino acid peptide generated by the plasma kallikrein-kinin system of proteinases and is the natural agonist for B2 receptors. Carboxypeptidase M degrades BK to a selective agonist at B1 receptors (Bathon and Proud, 1991). Kallidin is a 10-amino acid peptide produced by the tissue kallikrein-kinin system and is also a natural agonist for B2 receptors and can be converted to BK by plasma aminopeptidase. The efficient catabolism of filtered kinins by the renal tubule (Casarini et al., 1999) suggests that local activation of the kallikrein-kinin system is responsible for the generation of B1 and B2 agonists in the kidney.

The renal kallikrein-kinin system is a paracrine/autocrine system that participates in the regulation of renal hemodynamics and tubular function (Siragy et al., 1996; Wang et al., 2000). The effects of the kinins are thought to be primarily mediated by B2 receptors constitutively expressed throughout the nephron under physiological conditions (Figueroa et al., 1995). The B1 receptor is not considered to be constitutively expressed but can be induced by inflammatory stimuli or by chronic inhibition of angiotensin-converting enzyme (Marin-Castano et al., 2002).

The physiological role of renal B2 receptors has been studied extensively in rats, and the distribution of B2 receptors along the nephron has been well documented in this species.
(Figueroa et al., 1995; Marin-Castano et al., 1996). In contrast, there are much fewer studies in the mouse; however, this is changing because availability of B1 and B2 transgenic mice makes this species especially valuable for physiological and pharmacological studies. For example, mice overexpressing the human B2 receptor display increased renal blood flow, glomerular filtration rate, and diuresis (Wang et al., 2000). Interestingly, B2 receptors seem to be important in blunting renal vascular hypertrophy in angiotensin type 1 receptor-deficient mice (Tsuchida et al., 1999) and reducing renal fibrosis caused by ureteral obstruction (Schanstra et al., 2002). Furthermore, diabetic nephropathy is exacerbated in B2 knockout mice (Kakoki et al., 2004). BK signaling is also implicated in the renal inflammatory response to lipopolysaccharide (Marin-Castano et al., 1998). Despite the recognized physiological role of BK in the kidney in maintaining glomerular and tubule function and its role in pathological states such as endotoxic shock, inflammation, diabetes, and other diseases (Calixto et al., 2000), relatively little is known about the mechanisms by which BK can alter kidney function and injury. Furthermore, the signaling of BK receptors in the murine nephron (Kitamura and Miller, 1994; Mukhin et al., 2003) has not been fully characterized.

The TKPTS cell line is an immortal cell line that was grown from proximal tubule explants dissected from the 8Tg(SV40E)Bri7 mouse kidney (Ernest and Bello-Reuss, 1995). This cell line has been used as a model to study drug transport (Ernest and Bello-Reuss, 1995) and proximal tubule cell injury (Arany et al., 2004). Since intracellular Ca2+ is an important second messenger modulating tubular function and tubular injury, the present study was undertaken to use the TKPTS cell line as a model to examine the mechanism of BK-stimulated Ca2+ mobilization in murine proximal tubule epithelial cells.

Materials and Methods

**Chemicals.** Fura-2-acetoxyethyl ester, TMB-8, ionomycin, thapsigargin, BIM, genistein, H89, and KT5823 were purchased from Calbiochem (San Diego, CA). LY294002 was purchased from Axxora (San Diego, CA). BK, the B2 antagonist d-Arg[9-Hyp, d-Phe7]-BK, the B2 antagonist d-Arg[9-Hyp, Thi3, d-Phe7]-BK, the B1 antagonist des-Arg[9-Leu5-BK, the B1 agonist des-Arg9-BK, U73122, U73343, PMA, nifedipine, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Culture of TKPTS Cells.** The TKPTS cell line is mouse proximal tubule cell line developed by Dr. Elsa Bello-Reuss (Ernest and Bello-Reuss, 1995) and was a gift from Dr. Bello-Reuss. These cells were originally grown from proximal tubule explants dissected from the 8Tg(SV40E)Bri6 mouse kidney. Cells were grown at 37°C under 5% CO2 in 50% Dulbecco’s modified Eagle’s medium and 50% Ham’s F-12 medium containing penicillin (100 U/ml) and streptomycin (100 µg/ml) and supplemented with insulin and 5% fetal bovine serum (Di Mari et al., 1999). Experiments were performed on cells from passages 36 to 48.

**Measurement of [Ca2+]i.** Intracellular Ca2+ concentration was measured in stirred suspensions of TKPTS cells harvested using trypsin (0.25%) and EDTA (2.21 mM). Harvested cells were washed and resuspended in a modified Gey’s buffer containing 145.0 mM NaCl, 5.0 mM KCl, 1.0 mM NaHPO4, 0.5 mM MgSO4, 1.0 mM CaCl2, 5.0 mM glucose, 10.0 mM HEPES, and 1.0 mM probenecid, pH 7.4. Cells were loaded with the intracellular fluorescent Ca2+ dye Fura-2-acetoxyethyl ester for 20 min at 35°C. Cells were then washed twice (200g for 2 min) in modified Krebs’ buffer and resuspended in the same buffer to a concentration of approximately 1.5 × 106 cells/ml. Aliquots (1.5 ml) were allowed to warm to 37°C for 3 min before the measurement of Fura-2 fluorescence while inside a 1-cm2 cuvette with continuous stirring. This was achieved using a Hitachi F2000 spectrophotometer (Hitachi Instruments, Danbury, CT) equipped with a thermostatic cell holder and magnetic stirrer. A 1-cm grooved magnetic stir disc was placed into the cuvette, and the suspension of tubules was stirred at an approximate speed of 500 rpm. At the end of this equilibration period, a 30-s baseline reading was made before the addition of the test agent. Test samples were continuously stirred and kept at 37°C, whereas measurements of the 340-380-nm emission ratio were made using an excitation wavelength of 510 nm. The emission was measured at each excitation wavelength every 0.5 s. Maximum fluorescence was determined by the addition of 0.2% Triton X-100 (final concentration). Minimum fluorescence was obtained by the subsequent addition of 10 mM EGTA (final concentration). Intracellular-free calcium [Ca2+]i was calculated using the following formula: [Ca2+]i = KdR / (R - Rmin), where Kd is the dissociation constant for the agonist B2 receptor, R is the ratio 340/380 nm, and Rmin is the minimum fluorescence. Each set of experiments was completed within 30 to 60 min after loading with Fura-2.

**Analysis of Antagonist Affinity.** The pKd value for d-Arg[9-Hyp, d-Phe7]-BK was determined using nonlinear regression analysis as described by Lew and Angus (1995). The pEC50 values were calculated from the concentration-response curves for BK in the presence of varying concentrations of d-Arg[9-Hyp, d-Phe7]-BK were plotted against the molar concentration of d-Arg[9-Hyp, d-Phe7]-BK and fit to two equations using nonlinear regression:

\[
\text{pEC}_{50} = -\log(b) - \log(c)
\]

\[
\text{pEC}_{50} = -\log\left(b + \frac{1}{c}ight)
\]

where [B] is the concentration of d-Arg[9-Hyp, d-Phe7]-BK, c is the difference between the pKd value for d-Arg[9-Hyp, d-Phe7]-BK and the EC50 value for the BK control curve, and S is the Hill slope. Equation 1 assumes the Hill slope is equal to 1 and therefore the pKd value can be determined. Equation 2 assumes the Hill slope is different from 1 and so only the pA2 value can be determined. The pA2 value is defined as the negative logarithm of the antagonist concentration (in molar) that produced a 2-fold shift in the agonist dose-response curve. The initial value of c was set to 0 in eqs. 1 and 2, and the initial value of S was set to 1 in eq. 2. The F-test was used to determine which equation gave the best fit using the null hypothesis that eq. 1 is the best fit.

**Evaluation of mRNA Levels for BK1 and BK2 Receptors.** All reagents were purchased from Invitrogen (Carlsbad, CA). For RT-PCR, total RNA was extracted from TKPTS cells using TRIzol and treated with DNase I. Total RNA was reverse transcribed using oligo(dT) primer and SuperScript III RNase H-Reverse Transcriptase according to the supplier’s recommendations. Oligonucleotide primers (synthesized by IDT, Coralville, IA) were designed for specific PCR amplification of the murine B1 and B2 receptor. Primers for B1 were 5’-CCG AAG CCT GGC ATC TGC TGT G-3’ (forward) and 5’-CCA GCA ACC TGT AGC GGT CC-3’ (reverse), yielding a 353-bp fragment. Primers for B2 were 5’-TGT CAG CTT GGT CCT CC-3’ (forward) and 5’-GCT CTT GAA CAC CAA CAT GG-3’ (reverse), yielding a 360-bp product. PCR with B1 and B2 primers was performed in 25 µl of PCR buffer in the presence of 0.2 mM dNTP, 1.5 mM MgCl2, 0.5 µM primers, and 0.6 µl of Taq polymerase. PCR conditions for both BK1 and BK2 primer sets were: 94°C for 3 min followed by 55°C for 30 s, 72°C for 30 s, and 94°C for 25 s during 35 cycles. Amplified products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide fluorescence. Human cDNAs for the B1 and B2 receptor were obtained from Guthrie Research Institute (Sayre, PA) and used as positive controls.

**Data Analysis.** All data, including concentration-response data, were analyzed using Prism 4.0 for Mac (GraphPad Software Inc.,
San Diego, CA). Power analysis was used to estimate sample size to have 80% power to detect a 30% change with a significance level of 0.05 (two-tailed). EC_{50} values were determined using nonlinear regression. Data are presented as mean ± S.E.M. unless otherwise noted. Effects of inhibitors were analyzed using analysis of variance followed by the Newman-Keuls post test. When only two groups were compared, an unpaired t test was performed. Each n represents a unique flask or dish of cells from passages 36 to 48.

**Results**

**Effect of Bradykinin on [Ca^{2+}]_i.** [Ca^{2+}]_i was measured in stirred suspensions of TKPTS cells at 37°C. Addition of BK produced a rapid and transient concentration-dependent rise in [Ca^{2+}]_i (Fig. 1). The rise in [Ca^{2+}]_i, typically peaked within 15 s and returned to basal (unstimulated) levels by 100 s.

**BK Receptor Subtype Mediating Changes in [Ca^{2+}]_i.** Total RNA extracted from TKPTS cells was subjected to RT-PCR using specific primers for B1 and B2 mRNA. No product of the expected size was detected using primers for B1. However, primers for B1 did produce a product of the expected size when used against human B1 cDNA (data not shown). Primers for B2 produced a product with the expected size of 360 bp (Fig. 2A). The BK receptor subtype responsible for the rise in [Ca^{2+}]_i was examined directly using selective B1 and B2 inhibitors (Bathon and Proud, 1991). The selective B2 antagonists d-Arg[9][Hyp^{3}, D-Phe^{7}]-BK, and d-Arg[9][Hyp^{3}, Thi^{8}, D-Phe^{7}]-BK have approximately 10^4-fold higher affinity for the B2 receptor than does the B1 agonist des-Arg^{9}-BK or B1 antagonist des-Arg^{9}[Leu^{8}]-BK (Paquet et al., 1999). These agents were tested against a concentration of BK (100 nM) that produced 80 to 90% of maximum response. Both B2 antagonists incubated for 3 min at a concentration of 1 μM blocked the response to BK (100 nM) (Fig. 2B). In contrast, the B1 antagonist des-Arg[9][Leu^{8}]-BK (1 μM) preincubated for 3 min before the addition of BK did not inhibit the response to BK. Furthermore, the B1 agonist des-Arg^{9}-BK (1 μM) failed to elicit a rise in [Ca^{2+}]_i, confirming that B1 receptors are not involved in this response. These data indicate that the BK-stimulated rise in [Ca^{2+}]_i is due to B2 receptor activation.

**pK_{B} Determination.** Full concentration-response curves for BK were generated in the presence of increasing concentrations of d-Arg[9][Hyp^{3}, D-Phe^{7}]-BK (Fig. 3A). The concentration-response curve for BK reached maximum response at 1 μM and yielded a pEC_{50} value of 8.39 ± 0.04 (4 nM). The average peak change in [Ca^{2+}]_i at 1 μM BK (697 ± 70 nM) and 10 μM BK (841 ± 62 nM) was not different (P = 0.206; n = 6), indicating maximum response was achieved by 1 μM.

Increasing concentrations of the selective B2 antagonist d-Arg[9][Hyp^{3}, D-Phe^{7}]-BK produced rightward parallel shifts in the concentration-response curve for BK (no differences in the Hill slopes; P = 0.655) and inhibition was surmountable. Equation 1 gave the best fit (F = 25.01; p = 0.1256; do not reject the null hypothesis), and the pK_{B} value was determined to be 8.45 ± 0.15 (3.5 nM) (Fig. 3B).

**Effect of Pertussis Toxin.** To test the involvement of G_{i}/G_{o} G proteins, TKPTS cells were exposed to pertussis toxin in complete growth media at a concentration of 200 ng/ml for 18 h to inactivate G_{i}/G_{o} (Tomura et al., 1997). Treatment with pertussis toxin did not alter basal [Ca^{2+}]_i (Table 1) nor did it inhibit 100 nM BK-induced rise in [Ca^{2+}]_i. Interestingly, the peak rise was instead slightly but significantly elevated (589 ± 40 nM for control versus 713 ± 30 nM after pertussis toxin treatment; P < 0.05; n = 6). The ability of the pertussis toxin treatment to inhibit G_{i}/G_{o} was confirmed by the loss of Ca^{2+} mobilization upon addition of 100 nM adenosine, an agonist at A1 receptors coupled to G_{i}/G_{o} (Tomura et al., 1997). Adenosine (100 nM) raised [Ca^{2+}]_i 98 ± 9 nM above basal. In cells treated with pertussis toxin, the response to adenosine was 1 ± 1 nM (P < 0.001; n = 6).
These data show that the rise in [Ca\(^{2+}\)]\(_i\), elicited by BK is not due to stimulation of B2 receptors coupled to G\(_i/G_o\).

Source of the Rise in Intracellular [Ca\(^{2+}\)]\(_i\). To examine the source of the rise in [Ca\(^{2+}\)]\(_i\), elicited by BK, we compared responses in the absence and presence of three Ca\(^{2+}\) inhibitors (Fig. 4). All experiments were carried out in the presence of 1 mM CaCl\(_2\). TMB-8 (Smith and Iden, 1979) was used to inhibit intracellular Ca\(^{2+}\) release, thapsigargin (Thastrup et al., 1990) was used to deplete intracellular Ca\(^{2+}\) stores, and EGTA was used to eliminate extracellular Ca\(^{2+}\) influx. Preincubation with TMB-8 (100 \(\mu M\)) or thapsigargin (5 \(\mu M\)) for 10 min before the addition of BK (100 \(nM\)) completely blocked the rise in [Ca\(^{2+}\)]\(_i\). The inhibitory effect of thapsigargin was not due to its dimethyl sulfoxide vehicle (0.1%) because vehicle alone did not affect the response to BK (458 ± 60 versus 474 ± 32 nM with vehicle; \(n = 4\)). Thapsigargin did slightly increase basal [Ca\(^{2+}\)]\(_i\), whereas TMB-8 or EGTA did not alter basal [Ca\(^{2+}\)]\(_i\) (Table 1). EGTA (10 mM) added 30 s before the addition of BK decreased the rise in [Ca\(^{2+}\)]\(_i\) by approximately 50%. These data suggest that the rise in [Ca\(^{2+}\)]\(_i\), elicited by BK is due to both intracellular release and influx. The data obtained with TMB-8 and thapsigargin, two dissimilar inhibitors of intracellular release, indicate that intracellular release of Ca\(^{2+}\) is the trigger for extracellular influx.

Mechanism of Intracellular Ca\(^{2+}\) Release. The release of intracellular Ca\(^{2+}\) stores suggested that BK activates a signaling pathway involving phospholipase C. To explore this pathway, TKPTS cells were preincubated for 5 min with the selective Gq-coupled PLC-\(\beta\) inhibitor U73122 (20 \(\mu M\)) or inactive analog U73343 (Smith et al., 1990). U73122 inhibited the rise in [Ca\(^{2+}\)]\(_i\), elicited by BK (100 \(nM\)) by approximately 50% (Fig. 5), indicating that activation of PLC-\(\beta\) is involved in this signaling pathway. U73343 was ineffective. Intracellular Ca\(^{2+}\) can also be released by the action of PI3K (Ching et al., 2001). To address this potential pathway, the

![Figure 3](https://jpet.aspetjournals.org/doi/10.1124/jpet.2017.801)

**Fig. 3.** Concentration-response curves for BK-induced elevation in [Ca\(^{2+}\)]\(_i\). A, BK produced a concentration-dependent rise in [Ca\(^{2+}\)]\(_i\), with a pEC\(_{50}\) = 8.39 ± 0.04 nM. The selective B2 receptor antagonist d-Arg\(_1\)-Hyp\(_3\), D-Phe\(_7\)-BK produced rightward parallel shifts in the concentration-response curves for BK. Each data point is the mean ± S.E.M. from four separate concentration-response curves. B, determination of increasing concentrations of D-Arg\(_0\)-[Hyp\(_3\), D-Phe\(_7\)]-BK were determined using the concentration-response curves from A. These values were plotted using the equation pEC\(_{50}\) = \(-\log(B) + 10^{\text{pK} B} - \log c\) as described in the text. Using nonlinear regression to solve for pK\(_B\) yielded a pK\(_B\) value of 8.45 ± 0.15.

![Figure 4](https://jpet.aspetjournals.org/doi/10.1124/jpet.2017.801)

**Fig. 4.** Effect of calcium inhibitors on the response to BK. TMB-8 (100 \(\mu M\)) was used to inhibit intracellular Ca\(^{2+}\) release, thapsigargin (5 \(\mu M\)) was used to deplete intracellular Ca\(^{2+}\) stores, and EGTA (10 mM) was used to chelate extracellular Ca\(^{2+}\). TMB-8 or thapsigargin was added 5 min before BK. EGTA was added 30 s before BK. Both TMB-8 and thapsigargin completely inhibited the BK response. EGTA produced a partial but significant inhibition. Data are mean ± S.E.M. (\(n = 4\)). **, P < 0.01 compared with 10-min control; †, P < 0.05 compared with 30-min control.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effects of test agents on basal [Ca(^{2+})](_i).</th>
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<tbody>
<tr>
<td>Agent</td>
<td>Basal [Ca(^{2+})](_i)</td>
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<tr>
<td>---------</td>
<td>----------------</td>
</tr>
<tr>
<td>10-min Control</td>
<td>114 ± 5</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>TMB-8</td>
<td>135 ± 9</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>184 ± 10**</td>
</tr>
<tr>
<td>LGTA</td>
<td>115 ± 13</td>
</tr>
<tr>
<td>LY294002</td>
<td>88 ± 20</td>
</tr>
<tr>
<td>U73343</td>
<td>100 ± 18</td>
</tr>
<tr>
<td>U73122</td>
<td>136 ± 6</td>
</tr>
<tr>
<td>Genistein</td>
<td>113 ± 15</td>
</tr>
<tr>
<td>NiCl(_2)</td>
<td>134 ± 7</td>
</tr>
<tr>
<td>GdCl(_3)</td>
<td>98 ± 11</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>132 ± 7</td>
</tr>
<tr>
<td>Dibutyryl-cAMP</td>
<td>108 ± 16</td>
</tr>
<tr>
<td>8-Br-cGMP</td>
<td>116 ± 21</td>
</tr>
<tr>
<td>30-min Control</td>
<td>180 ± 16**</td>
</tr>
<tr>
<td>FMA</td>
<td>127 ± 9</td>
</tr>
<tr>
<td>BIM</td>
<td>155 ± 18</td>
</tr>
<tr>
<td>H89</td>
<td>140 ± 7</td>
</tr>
<tr>
<td>KTs823</td>
<td>216 ± 18†</td>
</tr>
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</table>
Ca²⁺ combinations resulted in a further decrease in BK-induced influx (Xu et al., 1995; Utz et al., 1999). None of these agents altered basal [Ca²⁺]i (Table 1). Only Ni²⁺ produced a concentration-dependent rise in [Ca²⁺]i, elicited by BK (Table 1). These findings suggest that blocking intracellular release prevents extracellular Ca²⁺ release. To address whether U73122 and genistein inhibit intracellular release, the combinations of EGTA + U73122 or EGTA + genistein were studied. These combinations resulted in a further decrease in BK-induced Ca²⁺ mobilization compared with EGTA alone (Fig. 5). We also studied the addition of U73122 + genistein and found that Ca²⁺ mobilization was inhibited by approximately 90%, indicating their effects were additive. None of the agents tested altered basal [Ca²⁺]i (Table 1). These findings suggest that blocking intracellular release prevents extracellular influx.

Mode of Extracellular Ca²⁺ Influx. To address the mechanism of influx, we examined the effects of the L-type Ca²⁺ channel inhibitor nifedipine, the nonselective cation channel blocker Gd³⁺, and the CRAC channel inhibitor Ni²⁺ (Xu et al., 1995; Utz et al., 1999). None of these agents altered basal [Ca²⁺]i (Table 1). Only Ni²⁺ reduced the Ca²⁺ response (Fig. 6), suggesting activation of a CRAC channel. Representative Ca²⁺ responses shown in Fig. 7 illustrate the consequence of influx to the Ca²⁺ transient. The response to 3 nM BK is shown for comparison because this concentration produced a similar maximal rise in [Ca²⁺]i as 100 nM BK in the presence of influx inhibitors (Table 2). U73122, genistein, Ni²⁺, and EGTA each caused the Ca²⁺ transient to return to basal levels more quickly, indicating that influx prolongs the Ca²⁺ signal (Table 2).

Role of Cyclic Nucleotides and Protein Kinases. Cyclic nucleotides and activation of protein kinases can affect Ca²⁺ mobilization in epithelial and smooth muscle cells depending on the agonist. To explore this, we examined the effects of PMA (1 μM; an activator of PKC), dibutyryl-cAMP (100 μM), 8-Br-cGMp (100 μM), BIM (1 μM; an inhibitor of PKC), H89 (10 μM; an inhibitor of PKA), and KT5823 (10 μM; an inhibitor of PKG) on the BK response. Concentrations used for these agents were based on the literature (Kitamura and Miller, 1994; Yang and Yingst, 1998; Utz et al., 1999). A 30-min incubation with PMA did not affect the BK response (Fig. 8) but inhibited the response to adenosine (100 nM) by 90 ± 7% (n = 6). KT5823 significantly reduced the response to BK (Fig. 8). However, addition of KT5823 caused Ca²⁺ mobilization itself before addition of BK and kept basal levels elevated (Table 1). None of the other agents affected the response to BK.

Discussion

Important interactions between the kallikrein-kinin and renin-angiotensin systems (Schmaier, 2003) and the roles BK may play in renal pathology (Tsuchida et al., 1999; Kakoki et al., 2004) emphasize the importance of understanding BK signaling in the neprhon. The goal of the present study was to use the TKPTS cell line as a model to examine the mechanism of BK-stimulated Ca²⁺ mobilization, since Ca²⁺ is an important regulator of tubule function, such as ion transport and Na,K-ATPase (Cheng et al., 1999).

We have shown that a murine proximal tubule epithelial cell line TKPTS constitutively expresses functional B2 receptors coupled to intracellular calcium mobilization. Functional B1 receptors do not seem to be constitutively expressed. BK produced a concentration-dependent rise in [Ca²⁺]i, with a pEC₅₀ value of 8.39 ± 0.04 (4 nM). This is the first reported EC₅₀ value for BK in the mouse proximal tubule epithelial cell and is very similar to EC₅₀ values or estimates reported for BK signaling in rabbit proximal tubules (Abolian and Nord, 1988), in the murine inner medullary collecting duct cell line mIMCD-3 (Mukhin et al., 2001, 2003), and in cultured rat mesangial cells (Bascands et al., 1991). The B2 antagonists d-Arg⁹[Hyp³, d-Phe⁷]-BK and d-Arg⁹[Hyp³,Thi⁸,⁹]-BK...
Mechanism of BK-Induced Ca\textsuperscript{2+} Mobilization in TKPTS Cells

**Fig. 7.** Representative responses to BK in the presence of release and influx inhibitors. Shown are the responses to 100 nM BK (heavy line) in the presence of U73122 (10 μM), genistein (50 μM), NiCl\textsubscript{2} (Ni, 5 mM), and EGTA (10 mM). All conditions were carried out in the presence of 1 mM CaCl\textsubscript{2}. The addition of BK is indicated by the arrow. For comparison, the response to 3 nM BK is superimposed (thin line). At 3 nM BK, the peak response was similar to that for 100 nM BK in the presence of inhibitor (Table 2). Shown are responses superimposed from a representative experiment repeated four times.

**TABLE 2**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Peak [Ca\textsuperscript{2+}]</th>
<th>Time to Return to Basal [Ca\textsuperscript{2+}]</th>
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</thead>
<tbody>
<tr>
<td>BK (3 nM)</td>
<td>302 ± 8</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>BK (100 nM) + U73122</td>
<td>256 ± 60</td>
<td>46 ± 6***</td>
</tr>
<tr>
<td>BK (100 nM) + genistein</td>
<td>231 ± 27</td>
<td>62 ± 5***</td>
</tr>
<tr>
<td>BK (100 nM) + NiCl\textsubscript{2}</td>
<td>313 ± 30</td>
<td>55 ± 12***</td>
</tr>
<tr>
<td>BK (100 nM) + EGTA</td>
<td>229 ± 32</td>
<td>34 ± 4***</td>
</tr>
</tbody>
</table>

***P < 0.001 compared with BK (3 nM).

**Fig. 8.** Effect of cyclic nucleotides and protein kinases. The response to 100 nM BK was compared after preincubation for 10 min with dibutyryl-cAMP (100 μM) or 8-Br-cGMP (100 μM), or for 30 min with PMA (1 μM, an activator of PKC), BIM (1 μM, an inhibitor of PKC), H89 (10 μM, an inhibitor of PKA), or KT5823 (10 μM, an inhibitor of PKG). Data are mean ± S.E.M. (n = 6–9). *, P < 0.05 compared with BK alone.

D-Phε\textsuperscript{7}-BK both blocked the response to BK, indicating the rise in [Ca\textsuperscript{2+}], elicited by BK was mediated through a B2 receptor. This was confirmed by the inability of the selective B1 antagonist des-Arg\textsuperscript{9}[Leu\textsuperscript{5}]-BK to block the response to BK and by the inability of the selective B1 agonist des-Arg\textsuperscript{9}-BK to alter [Ca\textsuperscript{2+}]. D-Arg\textsuperscript{9}[Hyp\textsuperscript{3}, D-Phε\textsuperscript{7}]-BK (also known as NPC 567) acted as a high-affinity competitive antagonist with a pK\textsubscript{A} value similar to its pK\textsubscript{D} value reported for contraction of human umbilical vein and rat uterus (Pauquet et al., 1999).

The B2 receptor subtype belongs to the G protein-coupled receptor family (Bathon and Proud, 1991). We found that BK caused a rapid increase in [Ca\textsuperscript{2+}], that is consistent with activation of PLC by Gq. Response was blocked by TMB-8 or thapsigargin or by the combination of genistein plus U73122. Thapsigargin depletes intracellular Ca\textsuperscript{2+} stores, whereas TMB-8 inhibits inositol 1,4,5-trisphosphate-mediated Ca\textsuperscript{2+} release from the endoplasmic reticulum, perhaps in part through inhibition of inositol 1,4,5-trisphosphate generation (Smith and Iden, 1979). U73122 is an inhibitor of PLC that is more effective against PLC-β than PLC-γ (Thompson et al., 1991). Genistein is a tyrosine kinase inhibitor that can inhibit phosphorylation and activation of PLC-γ and the IP\textsubscript{3} receptor (Jayaraman et al., 1996; Tolloczko et al., 2000). When tested alone, U73122 and genistein only partially inhibited BK-induced rise in [Ca\textsuperscript{2+}]. In combination, the inhibitory effects of U73122 and genistein were additive, suggesting two parallel pathways for PLC activation: PLC-β activated by the α-subunit of Gq and activation of PLC-γ by tyrosine kinase (Venema et al., 1998). Additional studies are needed to fully understand the role of tyrosine kinase in BK signaling in murine tubule epithelial cells. Although the B2 receptor is not known to couple to or possess intrinsic tyrosine kinase activity, G protein-coupled receptor-induced transactivation of growth factor receptor tyrosine kinase can occur (Mukhin et al., 2003). Another possibility is that B2 receptor activation also activates cytosolic/soluble tyrosine kinases to alter Ca\textsuperscript{2+} mobilization (Tolloczko et al., 2000). PI3K can also cause Ca\textsuperscript{2+} mobilization from intracellular stores as well as influx through the actions of phosphatidyl inositol 3,4,5-trisphosphate (Ching et al., 2001), but this pathway does not seem to be involved since the PI3K inhibitor LY294002 was ineffective.

In addition, the response to BK was not affected by pertussis toxin, an inactivator of G\textsubscript{i}/G\textsubscript{o} G proteins, whereas pertussis toxin completely blocked the Ca\textsuperscript{2+} response to adenosine, a known activator of G\textsubscript{i}/G\textsubscript{o} (Tomura et al., 1997). In Chinese hamster ovary cells transfected with a novel human BK receptor coupled to Gq, pertussis toxin did not inhibit Ca\textsuperscript{2+} mobilization either, whereas U73122 did inhibit (Boels and Schaller, 2003). Together, our data suggest that the pertussis
toxin-insensitive B2 receptor couples to activation of PLC-β and PLC-γ, possibly through Gq. Although the α-subunit of Gq activates PLC leading to release of Ca2⁺ stores, the βγ-subunits released from Gq could affect Ca2⁺ levels by activating protein kinases or Ca2⁺ channels. The CRAC pathway is an influx pathway activated by the release of intracellular Ca2⁺ (Hoth and Penner, 1992). Experiments with EGTA showed that a component of the Ca2⁺ transient was due to extracellular influx. The ability of TMB-8 or thapsigargin to completely block the Ca2⁺ transient suggests that the CRAC channel was responsible for Ca2⁺ influx. Furthermore, both U73122 and genistein diminished Ca2⁺ influx. Thus, the Ca2⁺ transient produced by BK is a result of two linked events. The initial rapid rise in [Ca2⁺]i is due to release from intracellular stores. This then triggers extracellular influx. Ni2⁺, in millimolar concentrations, is known to inhibit many types of release-activated channels, including a CRAC (Utz et al., 1999). Neither Gd³⁺, a nonselective cation channel blocker, nor nifedipine, an L-type calcium channel blocker, inhibited extracellular influx. The ability of Ni2⁺ to inhibit influx suggests that influx occurs through activation of CRAC (Utz et al., 1999).

Cyclic nucleotides and protein kinases can modulate Ca2⁺ mobilization in certain cell types. In smooth muscle cells, phenylephrine-induced Ca2⁺ mobilization is inhibited by U73122, genistein, H89, and BIM, and a nonselective inhibitor of protein kinases (Utz et al., 1999). In tracheal epithelial cells, BK-induced Ca2⁺ mobilization and IP₃ generation is reduced by preactivation of PKC (Yang et al., 1998). BK-stimulated Ca2⁺ mobilization in TKPTS cells seems to be regulated differently. Using the same or similar inhibitors and concentrations, our studies did not suggest a role for PKC in regulating BK-induced Ca2⁺ mobilization. Neither PMA nor BIM altered the response to BK.

Kitamura and Miller (1994) reported in their simian virus 40-transformed mouse proximal tubule cell line that BK-induced Ca2⁺ influx was regulated (inhibited) by dibutyryl-cAMP. In their system, cells were incubated for at least 45 min in Ca2⁺-free buffer before the addition of BK. These conditions could artificially alter Ca2⁺ stores, making interpretation difficult. Our results also differ with those reported in MDCK cells. Jan et al. (1998) reported that PKA, PKC, and cGMP can modulate BK-induced Ca2⁺ mobilization in MDCK cells. In TKPTS cells dibutyryl-cAMP, 8-Br-cGMP, or H89 had no effect, although the possibility that different concentrations and/or different incubation times might affect the response to BK cannot be ruled out. The only effect we observed in TKPTS cells was an inhibitory effect of the PKG inhibitor KT5823. However, KT5823 itself mobilized Ca2⁺ raising the possibility that BK-releasable Ca2⁺ stores may have been altered. Additional studies are required to fully establish the role of cGMP and PKG in TKPTS cells. Interestingly, unlike in TKPTS cells, BK produces a biphasic concentration-response curve in MDCK cells. These differences in the regulation of BK signaling between these two cell lines may be due to the fact that the MDCK cell line is derived from the canine kidney and is considered to be of distal tubule or cortical collecting duct origin.

In summary, results from this study indicate that murine proximal tubule epithelial cells express functional and constitutive B2 receptors that couple to pertussis toxin-insensitive Ca2⁺ mobilization, most likely mediated by coupling to Gq. BK-induced Ca2⁺ mobilization occurs through activation of PLC-β and possibly PLC-γ. Intracellular release is responsible for influx resulting in prolongation of the Ca2⁺ signal. Furthermore, the Ca2⁺ signal does not seem to be regulated by the downstream protein kinases PKC or KA. These data show that the TKPTS cell line can be used as a model to study BK signaling and its impact on proximal tubule function.

References


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Address correspondence to: Dr. Philip R. Mayeux, Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, 4301 West Markham St., #611, Little Rock, AR 72205. E-mail: prmayeux@uams.edu