The bradykinin B_2 receptor induces multiple cellular responses leading to the proliferation of human renal carcinoma cell lines

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Background: The vasoactive peptide bradykinin (BK) acts as a potent growth factor for normal kidney cells, but there have been few studies on the role of BK in renal cell carcinomas.

Purpose: In this study, we tested the hypothesis that BK also acts as a mitogen in kidney carcinomas, and explored the effects of BK in human renal carcinoma A498 cells.

Methods: The presence of mRNAs for BK B_1 and BK B_2 receptors in A498 cells was demonstrated by reverse transcription–polymerase chain reaction. To study BK signaling pathways, we employed fluorescent measurements of intracellular Ca^{2+}, measured changes in extracellular pH as a reflection of Na^+/H^+ exchange (NHE) with a Cytosensor microphysiometer, and assessed extracellular signal-regulated kinase (ERK) activation by Western blotting.

Results: Exposure to 100 nM of BK resulted in the rapid elevation of intracellular Ca^{2+}, caused a $\geq 30\%$ increase in NHE activity, and a $\geq 300\%$ increase in ERK phosphorylation. All BK signals were blocked by HOE140, a BK B_2 receptor antagonist, but not by a B_1 receptor antagonist. Inhibitor studies suggest that BK-induced ERK activation requires phospholipase C and protein kinase C activities, and is Ca^{2+}/calmodulin-dependent. The amiloride analog 5-(N-methyl-N-isobutyl)-amiloride (MIA) blocked short-term NHE activation and inhibited ERK phosphorylation, suggesting that NHE is critical for ERK activation by BK. BK induced an approximately 40% increase in the proliferation of A498 cells as assessed by bromodeoxyuridine uptake. This effect was blocked by the ERK inhibitor PD98059, and was dependent on NHE activity.

Conclusion: We conclude that BK exerts mitogenic effects in A498 cells via the BK B_2 receptor activation of growth-associated NHE and ERK.

Keywords: A498 cells, G protein-coupled receptors, signal transduction, Na^+/H^+ exchange, extracellular signal-regulated protein kinase

Introduction

Renal cell carcinoma is the most common cancer of the kidney and is one of the most resistant to systemic therapy. Therefore, until recently, surgery was the only medical treatment for patients with limited metastatic disease.¹ During the past 3 years, a better understanding of the molecular biology underlying metastatic renal cell carcinoma has led to the development of new therapeutic techniques. Several new drugs that target the vascular endothelial growth factor, its receptor, and the mammalian target of rapamycin have been shown to provide a clinical benefit in the treatment of advanced or metastatic renal cell carcinoma, but a significant improvement in survival has not yet been convincingly demonstrated across all clinical trials.² Further studies of the specific signaling pathways that lead to the proliferation of renal carcinoma cells will increase the understanding of the biology of this tumor,
and will help to develop new ways for the targeted inhibition of malignant cell proliferation, ultimately leading to more effective therapies.

The endogenous intrarenal kinin hormone bradykinin (BK) plays a significant role as a modulator of renal functions such as electrolyte and water excretion, as well as acting as a vasodilator. In addition to its vasoactive properties, BK plays a role in renal cell growth and proliferation. BK exerts its multiple pathophysiological functions via two known receptors, the bradykinin B₁ (BK B₁) and bradykinin B₂ (BK B₂), receptors, which belong to the superfamily of G protein-coupled receptors (GPCR). We have reported previously that BK is a potent mitogenic factor for a kidney cell line derived from the inner medullary collecting duct of mice (mIMCD-3 cells), and demonstrated that BK-induced cell proliferation was dependent on the activation of the epidermal growth factor receptor (EGFR) tyrosine kinase and the subsequent activation of mitogen- and extracellular signal-regulated kinase (MEK), and on extracellular signal-regulated protein kinase (ERK). There is evidence linking the mitogenic kinin peptides to the carcinogenic process. The histological presence of BK receptors has been demonstrated in clinical specimens of adenocarcinoma, squamous carcinoma, lymphoma, hepatoma, and carcinoid, and in experimental mouse sarcoma 180 and colon adenocarcinoma. In esophageal carcinomas, the expression of BK B₁ and BK B₂ receptors was upregulated in activated mast cells and giant tumor cells. The BK receptors were also highly expressed in small cell and non-small cell carcinomas of the lung. The mitogenic effects of BK have also been reported in primary cultured epithelial breast cancer cells, where BK-stimulated cell proliferation through ERK activation takes place. BK also exerted mitogenic effects in the MCF-7 breast cancer cell line, suggesting that BK has an important role in cancer endorsement and progression. In addition, the BK antagonist CU201 has been reported to inhibit the growth of human lung cancer cell lines. However, only limited studies on the role of BK in renal cell carcinomas have been done. There is one report suggesting that BK, among the other GPCR agonists, causes tyrosine phosphorylation of endogenous EGFR in several kidney cancer cell lines. Another study demonstrated an increased expression of the BK B₁ and BK B₂ receptors in the renal parenchymal tissue adjacent to the clear cell carcinoma. In the present study, we used A498 cells, a transformed cell line established from a clear cell renal cell carcinoma of a 52-year-old patient. A498 human renal cancer cells represent a model used widely for the in vitro study of renal carcinomas. Here we used these cells to explore the possible mitogenic effects and signaling pathways of BK.

Materials and methods

Reagents and cell culture

Bradykinin, des-Arg⁹ BK, HOE-140, des-Arg¹⁰-HOE-140, probenecid, ethidium bromide, and various salts were purchased from Sigma (St Louis, MO). 5-(N-methyl-N-isobutyl)-amiloride (MIA) was purchased from RBI (Natick, MA). U-73342 (1-[6-[(17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl] amino] hexyl]-1H-pyrole-2,5-dione), U-73343 (1-[6-[(17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl] amino] hexyl]-2,5-pyrrolidinedione), GF109203 (2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide), PD98059 (2’-Amino-3’-methoxyflavone), W-7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide), AG1478 (4-(3-Chloroaniline)-6,7-dimethoxyquinazoline), and BAPTA-AM (1,2-bis (o-Aminophenoxy) ethane N, N,N’,N’-tetraacetic acid tetra (acetoxymethyl) ester) were purchased from Calbiochem (Gibbstown, NJ). The BK B₁ antagonist was purchased from Abcam Inc (Cambridge, MA), and the BK B₂ antibody was purchased from BD Transduction Laboratories™ (Franklin Lakes, NJ). All cell culture media and supplements were purchased from Life Technologies (Carlsbad, CA). Polycarbonate cell culture inserts for microphysiometry were purchased from Corning Costar (Cambridge, MA). Fluo-4 AM was purchased from Molecular Probes (Eugene, OR).

The A498 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Life Technologies) in a cell culture incubator at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the SAT-60 reagent (Tel-Test Inc, Friendswood, TX), and RT-PCR was performed using a one-step RT-PCR kit (Qiagen, Valencia, CA). Both methods were performed following the manufacturers’ instructions. Specific sequences for sense (S) and antisense (AS) primers for BK B₁ and BK B₂ receptors that were published previously were synthesized (IDT Technologies Inc, Coralville, IA). The primers for the BK B₁ receptor were 5′-TTC TTA TTC CAG GTG CAA GCA G-3′ (S) and 5′-CTT TCC TAT GGG ATG AAG ATA T-3′ (AS), yielding a 213-bp fragment. The primers for the BK B₂ receptor were 5′-TGC
TGGTGCTATTTACATTCATGCTGAGTTTGTGAAAGCTGCTCG
CAGTTTGTGAAGTCTGCTCATGACCATGCTGTCACCGTCA
yielding a 235-bp product. The GAPDH primer sets 5’-ACCATG
CATGCTGATCCTGGTACCGTCAATGCTGCTGAC
(S) and 5’-CTCAGCTGATCCTGGTACCGTCAATGCTGCTGAC
(AS), yielding a 432-bp product (IDT Technologies Inc.),
were used as an internal control. The PCR conditions for both
the BK B1 and BK B2 primer sets were cDNA synthesis
at 94°C for 30 minutes and 95°C for 15 minutes (one cycle),
followed by 94°C for 30 seconds, 52.4°C for 30 seconds,
and 72°C for 1 minute during 35 cycles, and 72°C for 10
minutes (one cycle). Amplified products were separated
by electrophoresis on 4%–12% polyacrylamide gel (Life
Technologies) and visualized after staining with ethidium
bromide (1 µg/mL) under UV light using Fluorochem
Image (Alpha Innotech Corp, San Leandro, CA).

Measurement of intracellular Ca

A498 cells were plated onto collagen-coated 25 mm cover
slips placed into a single well of a six-well plate. The next
day, the medium was changed to 0.1% fetal bovine serum.
Cells were incubated 24 hours later with the calcium-sensitive
fluorescent probe Fluo-4 AM (2 µM; Molecular Probes) in
a Hank’s balanced salt solution (HBSS)—with a pH of 7.4
and containing 2.5 mM Probenecid and 0.1% bovine serum albumin—for 60 minutes at 37°C. At the end of the incubation,
the cells were washed three times with HBSS and the
cover slip was mounted into a thermo-regulated heating block
at 37°C (Olympus America, Melville, NY), and 1 mL HBSS
was added to the cells. Microscopic images were acquired
using an Olympus Ultra View LCI High Resolution Work-
station (PerkinElmer Life Sciences, Boston, MA) equipped
with a laser-scanning confocal unit (Omnichrome, Chino,
CA), 15-mW krypton three-line laser head, and a UAPON 340
40x 1.35 or PlanAPO 60x 1.4 NA (Olympus America) oil
immersion objective. Fluorescence was excited by using a
488 nm argon laser emission line and collected using a stan-
dard fluorescein isothiocyanate filter set (530 ± 30 nm). An
increase in fluorescence (gray-scale intensity) was considered
an indicator of increased intracellular free calcium and was
recorded and compared between samples.

Microphysiometry

Extracellular acidification rates (ECARs) were measured in
real-time in intact cells placed in an eight-chamber CytosensorTM
microphysiometer (Molecular Devices, Sunnyvale, CA), as
described previously.15,26 A microphysiometer uses a light-
addressable silicon sensor to detect extracellular protons, which
can be derived primarily from Na+/H+ exchange and glycolysis,
and from other metabolic pathways. Rate data transformed by a
personal computer running CytosoftTM (version 2.0; Molecular
Devices) were presented as ECARs in µVolts/s, which roughly
respond to milli-pH units/min. In order to facilitate the com-
parison of data between two channels, values were expressed
as percentage increases of the baseline as determined by com-
puterized analysis of the three data points after the exposure
of the cell monolayers to test substances.

The day prior to experimentation, A498 cells were plated
onto polycarbonate membranes (3 µm pore size, 12 mm size)
at a density of 300,000 cells per insert. The day of the study,
cells were washed with serum-free, bicarbonate-free Ham’s
F-12 medium, placed into the microphysiometer chambers,
and perfused at 37°C with the same medium. The pump
cycle was set to perfuse cells for 60 seconds, followed by a
30-second “pump-off” phase, during which proton efflux was
measured from the 6 seconds through to 28 seconds. Cells
were exposed to the test agents for four cycles (360 seconds).
Valve switches (to add or remove test agents) were per-
formed in the middle of the pump cycle. Data points were
then acquired every 90 seconds. The peak effects during
stimulation were expressed as percentage increases from
average basal ECARs from five rate measurements prior to
the application of the test agent(s).

ERK assays

ERK phosphorylation was assessed using a phosphorylation
state-specific ERK antibody (Cell Signaling, Beverly, MA),
which specifically recognizes threonine202 and tyrosine204,
phosphorylated ERK as described previously.3–11 Cells were
briefly cultured in 12-well plates, serum-starved for 24 hours,
and stimulated with a vehicle or BK. After treatment, cells were
scraped into a Laemmli buffer and subjected to SDS-PAGE
using 4%–20% pre-cast gels (Life Technologies), and then
semi-dry transferred to PVDF membranes (Millipore, Billerica,
MA). The membranes were blocked with a BLOTTO buffer and
incubated with the phospho-ERK antibody (at 1:1000 dilutions),
followed by incubation with goat anti-rabbit alkaline
phosphatase-conjugated IgG (Chemicon International, Inc,
Temecula, CA). Immunoreactive bands were visualized by a
chemiluminescent method (CDP Star™; New England Biolabs,
Ipswich, MA) using pre-flashed Kodak X-AR film, and quanti-
fied using ImageJ software (Image Processing and Analysis
The membranes were stripped using the Re-Blot Plus antibody
stripping solution (Millipore) and reprobed with the control
ERK antibody, which recognizes equally well phosphorylated
and nonphosphorylated ERK, in order to quantify total ERK.
The results are presented as intensities of phospho-ERK bands relative to total ERK bands and expressed as folds of basal phosphorylation (untreated cells).

**Proliferation assays**

A498 cell proliferation was assessed by a BrdU cell proliferation assay (Calbiochem). A498 cells were seeded (10^4 per well) in 96-well microplates and incubated in serum-free minimum essential medium for 36 hours. Next, cells were pre-incubated for 1 hour with 10 µM PD98059 (a MEK inhibitor), with 10 µM MIA (an Na^+/H^+ exchange inhibitor), or a vehicle before the addition of 100 nM BK or 20% fetal bovine serum (positive control) or a vehicle (negative control) for 24 hours. After incubation with a BrdU label for an additional 24 hours at 37°C, the assay was performed according to the manufacturer’s protocol, and the absorbance was measured using a SpectraMax M5/M5® Microplate Reader (Molecular Devices) at dual wavelengths of 450–540 nm.

**Data analysis**

ERK assays were performed in duplicate and repeated at least three times. Proliferation assays were performed in quadruplicate and repeated at least three times. Data are presented as mean ± standard error of the mean. Statistical evaluations of the data were performed using analyses of variance or Student’s t-tests as appropriate. Differences were considered significant at P < 0.05.

**Results**

**A498 cells express endogenous BK B1 and B2 receptors**

Total RNA extracted from A498 cells was subjected to RT-PCR using specific primers for BK B1 and BK B2 mRNA. As shown in Figure 1A, products with the expected sizes of 213 bp for BK B1 receptors and 335 bp for BK B2 receptors were detected. To confirm the expression of BK receptors at the protein level, we performed Western blotting of A498 lysates with BK B1 and BK B2 receptor antibodies. Western blot analyses showed a major band at 65 kDa that is immunoreactive for BK B2 receptors, as well as a duplet at 40/42 kDa that is immunoreactive for BK B2 (Figure 1B).

**Bradykinin induces elevations in intracellular Ca^{2+} in A498 cells**

Cells grown on collagen-coated cover slips were incubated with the calcium-sensitive probe Fluo-4 AM, and the detection of calcium-dependent fluorescence was performed with a confocal microscope as described. Figure 2A shows representative raw data from a single experiment demonstrating that 100 nM of BK induced a rapid elevation of intracellular Ca^{2+} in A498 cells in the absence of HOE-140 (a BK B2 receptor antagonist). Pre-incubation with 1 µM of HOE-140 completely eliminated the Ca^{2+} signal (Figure 2B), whereas preincubation with the BK B2 receptor antagonist des-Arg^9-HOE140 (1 µM) had no effect (not shown), providing strong evidence that the effect is mediated by BK B2 (and not BK B1) receptors.

**Bradykinin stimulates NHE activity in A498 cells**

Proton microphysiometry was performed on quiescent A498 cell monolayers, as described in the Materials and methods section. ECARs were measured after 100 nM of BK was applied to the cells for four measurement cycles. Figure 3A shows that cells treated with 100 nM BK (open circles) had a rapid increase in extracellular acidification rates that did not occur when cells were exposed to BK after pre-incubation with 5 µM of MIA or NHE-1 and -2 inhibitors (black diamonds). BK-induced proton efflux was blocked by pre-incubation with 1 µM of HOE140, a BK B2 receptor antagonist, while a BK B1 receptor antagonist, des-Arg^9-HOE140, did not change BK-induced ECARs (Figure 3B), supporting the involvement of the BK B2 receptor. Thus, Figure 3 presents evidence that BK B2 receptors in A498 cells activate proton efflux through NHE stimulation.

**Bradykinin stimulates ERK phosphorylation in A498 cells**

A498 cells were stimulated with 100 nM of BK for the indicated periods of time. Figure 4A shows that BK B2 receptors...
in A498 cells activates ERK in a time-dependent manner, with maximal stimulation at 10 minutes. Next, A498 cells were stimulated with the indicated concentrations of BK for 5 minutes. Figure 4B demonstrates that BK in A498 cells activates ERK in a concentration-dependent manner, with maximal activation at 1 µM. In subsequent experiments ERK assays were usually carried out with stimulation by 100 nM BK for 5 minutes, unless otherwise mentioned.

**BK stimulates ERK phosphorylation through a BK B<sub>2</sub> receptor**

A498 cells were pretreated for 30 minutes with the BK B<sub>2</sub> receptor antagonist des-Arg<sup>10</sup>-HOE-140 (1 µM) or the BK B<sub>2</sub> receptor antagonist HOE-140 (1 µM), and then stimulated with 100 nM of BK for 5 minutes. Figure 4C shows that pretreatment with HOE-140 completely prevented the BK-induced activation of ERK, whereas des-Arg<sup>10</sup>-HOE-140 was ineffectual. Thus, BK stimulates ERK activity via the BK B<sub>2</sub> receptor.

**Bradykinin-induced ERK phosphorylation requires phospholipase C (PLC) and protein kinase C (PKC) activities and is Ca<sup>2+</sup>- and calmodulin-dependent**

Because PKC-dependent and Ca<sup>2+</sup>/calmodulin (CaM)-dependent mechanisms of BK-induced ERK activation have been described previously in vascular smooth muscle cells (VSMC), we next tested if the BK B<sub>2</sub> receptor employs the same pathway in our cell model. PLC inhibitors decreased BK-induced ERK activation, suggesting that PLC is involved in this process (Figure 5A). PKC inhibitors and PKC depletion by the prolonged treatment with phorbol 12-myristate 13-acetate (PMA) also effectively blocked BK-induced ERK activation, supporting a role for PKC (Figure 5B). Next, A498 cells
induced ERK activation but did not alter BK-induced signals, the inhibition of EGFR kinases effectively blocked EGF-BK or 10 ng/mL EGF for 5 minutes. Cells were pretreated for 30 minutes with a vehicle or with 1 µM HOE140 to block ERK activation and CaM are important for BK-induced ERK activation. Because it has been reported that BK causes tyrosine kinase activity does not depend on EGFR tyrosine kinase activity. Because it has been reported that BK causes tyrosine phosphorylation of endogenous EGFR in several kidney cancer cell lines, and because in our previous work we demonstrated that BK-induced ERK activation in kidney cells was dependent on EGFR transactivation, in the next series of experiments we studied the role of EGFR in BK-induced ERK activation in A498 cells. Cells were pretreated for 30 minutes with a vehicle or with 1 µM of AG1478 (an EGFR kinase inhibitor) before stimulation with 100 nM BK or 10 ng/mL EGF for 5 minutes. Figure 5D shows that the inhibition of EGFR kinases effectively blocked EGF-induced ERK activation but did not alter BK-induced signals, indicating that EGFR is not involved in BK-induced ERK phosphorylation in A498 cells.

Bradykinin activates ERK through a pathway that requires NHE activity

Because BK B₂ receptor activated both NHE (Figure 3) and ERK (Figure 4) in A498 cells, in the next series of experiments we investigated the possible relationship between these two pathways. Cells were pretreated with either 10 µM PD98059 to block ERK activation or 5 µM MIA for 30 minutes prior to stimulation with 100 nM BK. NHE activity was assessed by microphysiometry, and ERK phosphorylation by Western blotting with phosphorylation state-specific antibodies. While the pretreatment of cells with 5 µM MIA as expected completely blocked BK-induced ECARs, preincubation with 10 µM of PD98059 did not change BK-induced ECARs, suggesting that ERK does not play a regulatory role in the activation of NHE in A498 cells (Figure 6A). At the same time, pretreatment with PD98059 completely abolished BK-induced ERK phosphorylation (Figure 6B), confirming that ERK phosphorylation in A498 cells proceeds through MEK. Interestingly, pretreatment with MIA—which blocks NHE activity in A498 cells (Figure 6A)—attenuated ERK phosphorylation induced by BK, suggesting that NHE activity is required for ERK activation (Figure 6B).

Bradykinin stimulates the proliferation of A498 cells

The mitogenic effect of BK was evaluated by measuring the proliferation of A498 cells by a BrdU cell proliferation assay. BK caused a significant increase in BrdU-positive cells (48% ± 8% over control values; P < 0.05, n = 4). MEK inhibitors completely blocked this increase without changing the basal proliferation rate. NHE inhibitors decreased both the basal level of proliferation and BK-induced BrdU incorporation (Figure 6C). These findings demonstrate that BK stimulates the proliferation of A498 cells, and implicate roles for ERK and NHE in BK mitogenic activity.

Discussion

In this study we found the presence of BK B₁ and BK B₂ receptor mRNAs in renal carcinoma A498 cells, and demonstrated the presence of these receptors at the protein level by Western blotting (Figure 1). In order to investigate whether these receptors are functional, we tested three signaling pathways known to be stimulated by BK in other cell types. The BK B₁ receptor usually couples to the GTP-binding protein G_{q} and stimulates phospholipase C activity,
which leads to the generation of inositol triphosphate and to
the subsequent mobilization of intracellular Ca\(^{2+}\) from the
endoplasmic reticulum.\(^{28}\) We demonstrated that BK causes
an elevation of intracellular Ca\(^{2+}\) in A498 cells, which
was blocked by the BK B\(_2\) receptor antagonist HOE-140, but
not by the BK B\(_1\) receptor antagonist des-Arg\(^1\)-HOE-
140, indicating a role for the BK B\(_2\) receptor (Figure 2).
Similar BK-induced intracellular Ca\(^{2+}\) mobilization has
been described in several kidney cell lines, including rat
mesangial cells,\(^{29}\) the mIMCD-3 murine inner medullary
collecting duct cell line,\(^{25}\) Kirsten murine sarcoma-virus
transformed rat kidney KNRK cells,\(^{30}\) the mouse proximal
tubule epithelial cell line,\(^{31}\) and in the human embryonic
kidney (HEK293) cell line.\(^{26}\)

Because BK B\(_2\) receptors have also been shown to
activate sodium-proton exchanges in mIMCD-3 cells,\(^{25}\)
HEK293 cells,\(^{26}\) KNRK cells,\(^{30}\) and renal tubular epithelial
cells,\(^{32}\) we tested potential roles for BK receptors in the regu-
lation of sodium-proton exchanges in A498 cells. A498 cells
treated with 100 nM BK demonstrated a rapid increase in
ECARs as measured by microphysiometry (Figure 3), which
was blocked by preincubation with 5 \(\mu\)M MIA, an inhibitor
of NHE-1 and NHE-2. This response to BK was blocked by
the BK B\(_2\) receptor antagonist HOE-140, but not by the BK
B\(_1\) receptor antagonist des-Arg\(^1\)-HOE-140, supporting a role
for the BK B\(_2\) receptor.

The activation of ERK by BK in kidney cells has been dem-
onstrated in cultured mesangial cells,\(^{3,6}\) rabbit cortical collecting

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**Figure 5** Effects of phospholipase C (PLC), protein kinase C (PKC), Ca\(^{2+}\), and calmodulin (CaM) inhibitors on BK-induced ERK phosphorylation.

**Notes:** (A) Cells were preincubated with a vehicle, 10 \(\mu\)M U73122 (a PLC inhibitor), or 10 \(\mu\)M U73343 (negative control) for 30 minutes before stimulation with 100 nM BK for 5 minutes. (B) Cells were pretreated with either 2 \(\mu\)M GF109203X (a PKC inhibitor) for 30 minutes or with 160 nM PMA for 20 hours, followed by either BK 100 nM or PMA 1 \(\mu\)M stimulation for 5 minutes. (C) A498 cells were pretreated for 30 minutes with a vehicle, 10 \(\mu\)M of BAPTA (an intracellular Ca\(^{2+}\) chelator), or 50 \(\mu\)M of W7 (a CaM inhibitor), and then stimulated for 5 minutes with 100 nM BK. (D) BK-induced ERK phosphorylation does not depend on EGFR tyrosine kinase activity: cells were pretreated for 30 minutes with a vehicle, with 1 \(\mu\)M of AG1478 (an EGFR kinase inhibitor) before stimulation with 100 nM BK, or with 10 ng/mL EGF for 5 minutes. ERK phosphorylation was detected by immunoblotting with an anti-phospho-ERK antibody. Bars represent intensities of phospho-ERK bands relative to total ERK expressed as a fold of basal of control (cells treated with a vehicle). Insets show representative phospho-ERK blots. The same blots were stripped and reprobed with antibodies for total ERK to assure the equal loading of a protein sample on a gel (total ERK). Experiments were performed three times in duplicate. Data are presented as mean \(\pm\) SEM. Statistical probability in figures is expressed as \(*P<0.05, \text{and as } **P<0.01 \text{versus vehicle-treated samples.}

**Abbreviations:** BK, bradykinin; ERK, extracellular signal-regulated kinase; PMA, phorbol 12-myristate 13-acetate; SEM, standard error of the mean.
Bradykinin

B₂ receptor

Gq/11

PLC

PKC

Ca²⁺/CaM

NHE

Proliferation

Figure 7 Scheme of proposed signaling pathway used by bradykinin to stimulate proliferation in A498 cells. This scheme is described in the Discussion section.

Notes: BK stimulates ERK through the B₂ receptor-Gq/11 pathway, which includes the activation of phospholipase C (PLC) and protein kinase C (PKC). PLC-dependent Ca²⁺/CaM mobilization, and calmodulin (CaM) activity. BK B₂ receptors stimulate NHE1 activity, which is necessary for the ERK-dependent proliferation of A498 cells. The dashed line indicates a putative pathway used by BK to activate NHE upstream of ERK.

Abbreviations: BK, bradykinin; ERK, extracellular acidification rate; NHE, Na⁺/H⁺ exchange; CaM, calmodulin; PLC, phospholipase C; PKC, protein kinase C; Ca²⁺/CaM, calcium- and calmodulin-dependent mechanisms of ERK activation in VSMC and COS-7 cells, transiently transfected with BK B₂ receptor.

In our experiments, the inhibition of PLC or PKC altered ERK activation by BK, suggesting that these proteins are involved in the signal transduction pathway (Figures 5A and B). We also demonstrated that Ca²⁺ and CaM are important for the BK stimulation of ERK in A498 cells (Figure 5C). Therefore, mapping studies with chemical inhibitors of candidate molecules revealed that BK-induced ERK activation in A498 cells was dependent on PLC, PKC, and Ca²⁺/CaM activities (Figure 7).

Figure 6 NHE activity is required for bradykinin-induced ERK activation.

Notes: (A) BK-induced NHE activity does not require ERK activation. Cells were pretreated with either 10 µM of an MEK inhibitor (PD98059) or with 5 µM of an NHE inhibitor (MIA) for 30 minutes prior to the application of 100 nM of BK for four measurement cycles, and ECAR was assessed by proton microphysiometry as described in the Materials and methods section. Experiments were performed at least three times. The data are means ± SEM. (B) BK-induced ERK phosphorylation is NHE-dependent. Cells were pretreated with 10 µM PD98059 or with 5 µM MIA for 30 minutes prior to stimulation with 100 nM BK for 5 minutes. Bars represent the intensities of phospho-ERK bands relative to the total ERK expressed as a fold of control (cells treated with a vehicle). Experiments were performed three times in duplicate. Data are presented as means ± SEM. (C) Bradykinin stimulates the proliferation of A498 cells. A498 cells were preincubated for 1 hour with a vehicle, or with 10 µM PD98059 or 5 µM MIA before the addition of 100 nM BK or 20% fetal bovine serum (positive control) or a vehicle (negative control) for 24 hours. After incubation with a BrdU label for an additional 24 hours at 37°C, the BrdU cell proliferation assay was performed according to the manufacturer’s protocol. Experiments were performed at least three times in triplicate. Data are presented as means ± SEM. Statistical probability in figures is expressed as *P < 0.05, and as **P < 0.01 versus vehicle-treated samples.

Abbreviations: BK, bradykinin; ECAR, extracellular acidification rate; ERK, extracellular signal-regulated kinase; MEK, mitogen- and extracellular signal-regulated kinase; MIA, 5-(N-methyl-N-isobutyl)-amiloride; NHE, Na⁺/H⁺ exchange; SEM, standard error of the mean.
In our previous work we established that the BK B₂ receptor stimulates early mitogenic signals associated with the activation of ERK in kidney mIMCD-3 cells, and demonstrated that BK-induced cell proliferation was dependent on EGFR activation. We hypothesized that BK uses a similar pathway to activate ERK in A498 cells. It appears, however, that the inhibition of EGFR tyrosine kinase activity did not alter ERK activation by BK, suggesting that EGFR transactivation is not necessary in this signal transduction pathway (Figure 5D). Interestingly, in A431 epidermoid carcinoma cells, and in head and neck squamous cell carcinomas, BK-induced ERK activation also did not require EGFR tyrosine kinase activity. Thus, the pathway of the B₂ receptor-induced ERK activation in renal carcinoma A498 cells was different from the one described for the kidney mIMCD-3 cells, and similar to the one reported in tumor cell lines.

Our data also support the presence of mRNA for the BK B₁ receptor, but do not support a role for BK B₁ receptors in the stimulation of the tested pathways. Therefore, the functions of the BK B₁ receptor in A498 cells remain unclear. One possible function of the BK B₁ receptor in A498 cells could be cross-talk between BK B₁ and BK B₂ receptors. In that regard, the formation of a complex relationship between BK B₁ and BK B₂ receptors has been described in prostate cancer PC3 cells.

Another interesting fact is that the NHE1 inhibitor MIA decreased BK-induced levels of ERK phosphorylation in A498 cells (Figure 6B), suggesting that NHE1 may act upstream of ERK in the BK-induced signaling pathway in renal carcinoma cells. Because NHE and ERK have both been implicated as key mediators of growth signals, the relationships between these pathways are very important although not completely understood. It has been well established that NHE1 could be regulated by ERK, although our group and others were unable to demonstrate any role of ERK in the regulation of NHE1 in several cell types. In addition, there have been several reports suggesting that NHE1 might play a role in regulating ERK activation. For example, in human colon cancer epithelial cells, NHE1 inhibition suppressed ERK activation and led to decreased production of interleukin-8 in response to inflammatory signals. It also has been shown that an antagonist of NHE1 inhibits the activation of ERK under osmotic shrinkage in Ehrlich–Lettre ascites cells and glucose-induced ERK activation in a high-glucose model of cardiomyocyte hypertrophy. Therefore, such NHE1-dependent regulation of ERK in most cases has been described in cells stimulated by mechanical stretching, osmotic shrinkage, hypertrophy, and inflammatory mediators. Little is known about GPCR-induced NHE1-dependent ERK regulation. One report suggests that NHE1 is not a regulator for LPA-induced ERK activation in C6 glioma cells, though, our group showed that NHE1 activity plays a necessary role in the activation of ERK by angiotensin II AT₁, and serotonin 5-HT₂A receptors in VSMC. The results of the current study demonstrate that BK B₂ receptor-induced ERK activation in A498 cells depends on NHE activity, suggesting that the critical role of NHE1 in GPCR-induced ERK activation is not restricted to one specific cell type or receptor. Figure 7 depicts one possible scheme, which can account for our findings. Based on previous studies from our group and others, which described BK-induced NHE1 activation in renal cell lines, we hypothesize that BK-induced pathways that lead to NHE1 activation in A498 cells may involve PLC and elevated intracellular Ca²⁺ and CaM; however, detailed mapping studies of this pathway were outside of the scope of this study.

In the next series of experiments we evaluated the physiological significance of BK in A498 cells and assessed BK-induced proliferation by a BrdU cell proliferation assay. The results presented in Figure 6C show that BK exerts mitogenic effects in A498 cells, and that BK-induced proliferation is mediated by ERK. Another interesting observation is that the NHE1 inhibitor MIA decreased BK-induced levels of proliferation in A498 cells (Figure 6C), suggesting that NHE1 may be upstream of ERK in the signaling pathway, which leads to the increased proliferation of renal carcinoma cells.

Although the role of NHE1 has been well established in the myocardial remodeling and heart failure process, its role in renal function is less known. Mice with a spontaneous point mutation that results in truncation between the eleventh and twelfth NHE1 transmembrane domains and a loss of NHE1 function do not present a visible renal phenotype, consistent with the concept that NHE1 “housekeeping” activity under normal conditions is not required. Previous studies from our laboratory demonstrated that the activation of the BK B₂ receptor leads to ERK-dependent proliferation in normal renal (mIMCD-3) cells, and also described BK-induced activation of NHE1 in the same cells through the pathway, which involved PLC, elevated intracellular Ca²⁺, CaM, and Janus kinase 2. However, pretreatment with NHE1 inhibitors did not change the levels of BK-induced ERK phosphorylation in mIMCD-3 cells, suggesting that NHE1 activity does not play a necessary role in the activation of ERK by BK in this model. Thus, an observation that NHE1 acts upstream of ERK in the signaling pathway, which leads to the increased proliferation of A498 cells, suggests that this role of NHE1 is specific to A498 renal cancer cells.
Previously, NHE1-mediated intracellular alkalization has been proposed to play a role in cancer cell growth because it has been shown that an increased pH of tumor cells is associated with increased in vivo tumor growth, DNA synthesis, and cell-cycle progression, suggesting that an overexpression of NHE1 contributes to the transformed phenotype of multiple cancer cells. The cellular alkalization of tumor cells induced by hyperactivation of NHE1 has been shown to be directly related to increased protein synthesis and tumor cell growth. It has been suggested that the mechanism of NHE1-mediated tumor cell growth and metastasis does not depend on its ion-transporting activities, but rather employs NHE1 as a scaffolding protein to directly regulate cytoskeletal dynamics. Further it has been shown that NHE1 antisense genes suppress cell growth, induce cell apoptosis, and partially reverse the malignant phenotypes of human gastric carcinoma cells.

Similarly, the silencing of the NHE1 gene by siRNA interference and/or the inhibition of NHE1 activity by amiloride analogs effectively blocked the invasiveness of human hepatocellular carcinoma cells. The results of the current study demonstrate that NHE also plays a role in the proliferation of A498 cells, suggesting that the inhibition of NHE1 might result in an antiproliferative effect.

**Conclusion**

In summary, we demonstrated that BK exerts mitogenic effects in A498 cells via an endogenously expressed BK receptor, which is functionally coupled to PLC-dependent Ca2+ mobilization, NHE activation, and ERK phosphorylation. Our results demonstrate that NHE1 is involved in BK-induced ERK activation and the proliferation of A498 cells, suggesting that NHE1 may be a potential target for chemotherapeutics for the treatment of renal carcinoma.

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**Disclosure**

The authors report no conflicts of interest in this work.

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