Bradykinin activates calcium-dependent potassium channels in cultured human airway smooth muscle cells

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Liu B, Freyer AM, Hall IP. Bradykinin activates calcium-dependent potassium channels in cultured human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 292: L898–L907, 2007. First published December 8, 2006; doi:10.1152/ajplung.00461.2005.—Bradykinin (BK) is an inflammatory mediator that can cause bronchoconstriction. In this study, we investigated the membrane currents induced by BK in cultured human airway smooth muscle (ASM) cells. Depolarization of the cells induced outward currents, which were inhibited by tetraethyl-ammonium (TEA) in a concentration-dependent manner with an IC_{50} of 0.33 μM. The currents were increased by elevating intracellular free Ca^{2+} concentration, suggesting they are calcium-activated potassium channels [I_{K(Ca)}]. Preexposure to inhibitor of I_{K(Ca)} of large conductance (BKCa), iberiotoxin, and small conductance (SKCa), apamin, inhibited the increase of outward current induced by BK. The relative contribution of BKCa was greatest in early passage cells. Both nickel and SKF-96365 (10 μM) inhibited the increase of the I_{K(Ca)} induced by BK; however, the t-type Ca^{2+} channel blocker, nifedi-pine, had no effect. Activation of the BK-induced current was inhibited by heparin, indicating dependence on intact heparin 1,4,5-triophosphate (IP_{3})-sensitive intracellular Ca^{2+} stores. BK also increased inositol phosphate accumulation and induced a transient Ca^{2+}-activated chloride current (CACC) and a sustained nonselective cation current (I_{Ca}). In summary, BK activates BKCa, SKCa, CACC, and I_{Ca} via IP_{3}-sensitive stores in human ASM.

Relaxant responses of ASM to agents such as isoprenaline are at least in part dependent on activation of K^{+} channels (26, 29), although other pathways including altered sensitivity of the contractile apparatus to Ca^{2+} may also play a role (7). The exact mechanism whereby ASM tone returns to baseline after exposure to agonists, which raise intracellular Ca^{2+}, remains unclear. As well as sequestration and efflux of Ca^{2+} (20), stimulation of calcium-activated potassium channels [I_{K(Ca)}] by the elevated intracellular Ca^{2+} is thought to contribute to membrane hyperpolarization and thus relaxation (26, 29).

In ASM ex vivo, the major K^{+} current is carried by I_{K(Ca)} of large conductance (BKCa) (29, 50), but ASM is also known to express a number of other K^{+} channels that may contribute to the control of K^{+} efflux (40, 42) including Ca^{2+}-activated K^{+} channels of intermediate or small conductance (SKCa), delayed rectifier K^{+} (K_{IR}) (11, 48), and ATP-sensitive K^{+} (K_{ATP}) (32, 33) channels.

ASM cells are very plastic, and relative channel expression is known to alter once ASM cells are grown in culture (41). Furthermore, ion channel activity is altered in ASM cells from rats with a hyperresponsive phenotype (45), in atrial myocytes grown in an altered extracellular matrix environment (49), in smooth muscle cells exposed to proinflammatory mediators (19), and following exposure to environmental toxins (52). K^{+} channels other than BKCa may thus assume greater importance in ASM cell function in the asthmatic airway, where the matrix environment is altered and inflammatory mediators abound.

Here we show in cultured ASM, where BKCa activity is partly downregulated but some I_{K(Ca)} activity persists, that BK is able to stimulate KCa activity through release of Ca^{2+} from internal stores via inositol 1,4,5-triophosphate (IP_{3})-dependent pathways and Ca^{2+} influx. BK also induces a large calcium-activated chloride channel (CACC), which, in turn, results in a subsequent influx of Ca^{2+} from extracellular sources via a current with properties typical of nonselective ion channel current (I_{Ca}).

METHODS

Collection and dissociation of ASM. Following informed written patient consent and local ethical committee approval (Ethics Committee of City Hospital, Nottingham, United Kingdom), macroscopically normal proximal bronchi of patients undergoing thoracotomy for lung cancer were obtained immediately after operation and transferred in cold physiological salt solution (PSS) (see Solutions and chemicals) to the laboratory within 20 min. Bronchial tissue was washed three times in HEPES-buffered, calcium and magnesium-free HBSS. ASM was carefully stripped from cartilage of the airway and minced finely.
in HBSS supplemented with collagenase A (2 mg/ml) and incubated in the same enzymatic solution for 1 h at 37°C with gentle trituration every 15 min. Cells were collected following filtration through a sterile, 100-μm cell strainer and seeded on a 25-mm plastic cell culture plate at a density of 5–10×10^3 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were grown at 37°C in a humidified incubator under 5% CO₂ and exhibited more than 95% smooth muscle α-actin staining. Cells were also stained for myosin heavy chain. Cells were passaged with 0.05% trypsin and 0.5 mM EDTA. We used cells from four separate donors during passages 1–7.

Patch-clamp electrophysiology. Conventional whole cell configuration patch-clamp technique was employed to record from cultured human ASM. The cells were placed directly in the cell chamber mounted on the stage of an inverted microscope (Nikon Eclipse 200), allowed to settle, and then washed with PSS at a constant speed (6 ml/min). Pipettes were drawn from borosilicate glass on a pipette puller PIP5 (HEKA, Lambrecht, Germany) and had resistances of 4–7 MΩ when filled with electrolyte. Automatic series resistance compensation was performed routinely and monitored continuously. Recordings were terminated if the access resistance changed by more than 25% during the recording period.

Voltage pulses were delivered through an EPC-9 amplifier. Data were filtered at 1 kHz (–3 dB down) and sampled at 2 kHz using Pulse software (v8.53, HEKA). Cell resting membrane potentials were recorded in current-clamp mode immediately after a stable electrical access had been established. Following this, the cells was depolarized from a holding potential of −60 mV ranging from −100 mV to +100 mV in 10-mV steps for a duration of 400 ms for recording of the outward currents. For recording of inward chloride currents, the cells were voltage-clamped at −60 mV. Reagents containing experimental drugs were delivered through a 1-μm-diameter puffer pipette connected to a pressure injection device controlled electronically (DAD system). The pipette was positioned about 50–60 μm from the cell. The application of the drug into cell was triggered by an offset sent by the amplifier to the command voltage signal. All experiments were carried out at room temperature (20–24°C).

Additional experiments were performed in current clamp mode. Changes in cell membrane potentials were continuously monitored in current clamp mode when stable whole cell recording had been achieved.

[^3H]inositol phosphate accumulation. Medium was aspirated from confluent monolayers of primary human ASM cells grown in a 24-well plate and replaced with 300 μl of inositol-free DMEM containing [^3H]myo-inositol at 2 μCi/ml for 24 h. After this time, the medium was aspirated and replaced with 1 ml of fresh medium without inositol. The cells were then incubated for an additional 24 h. The cells were then washed with ice-cold saline and lysed with 0.1 ml of 1 M NaOH. The lysates were vortexed for 30 s and then centrifuged at 10000 g for 3 min. The supernatant was then transferred to a scintillation vial and 1 ml of scintillation fluid was added. The vials were then counted for [^3H]inositol phosphate accumulation.
medium was again removed and cells washed twice with 1 ml of Hanks’/HEPES buffer. Cells were kept at 37°C while 300 μl of Hanks’/HEPES containing 10 mM of LiCl was added to each well for 15 min and agonist added for the final 10 min as indicated. Reactions were stopped by removing the medium and adding 1 ml of methanol/0.12 M HCl (1:1 vol/vol), which had been stored at -20°C. Samples were stored at -20°C for at least 30 min. An aliquot (800 μl) from each well was neutralized to pH 7 with an appropriate volume (typically 4.8 ml) of buffer (25 mM Tris/0.5 M NaOH/H2O at 0.238/0.025/0.737 vol/vol/vol). [3H]inositol phosphates were separated from free [3H]myo-inositol by anion exchange chromatography on Dowex-Cl columns as per Daykin et al. (8).

**Solutions and chemicals.** PSS consisted of NaCl (135 mM), KCl (5 mM), MgCl2 (1 mM), CaCl2 (1 mM), and HEPES (10 mM) and was pH-adjusted to 7.2 with NaOH. Intracellular solution, consisting of KCl (140 mM), MgCl2 (1 mM), EGTA (0.01 mM), Mg(ATP)2 (2 mM), HEPES (10 mM), pH adjusted to 7.2 with KOH, was used to record the outward currents. For recording of inward currents, KCl was replaced by cesium: CsCl (140 mM), MgCl2 (1 mM), CaCl2 (1 mM), EGTA (3 mM), Mg(ATP)2 (2 mM), HEPES (10 mM), pH adjusted to 7.2 with CsOH, and 10 mM tetraethylammonium (TEA) was included in the bath solution.

Collagenase A, Igepal, BSA, BK, iberiotoxin (Ibt), TEA, nifedipine, SKF-96365, lanthanum, nickel, cobalt, de-Arg10-Hoe 140 and N-adamantaneacetyl-α-Arg-[Hyp3, Thi5,8, D-Phe7]-BK were purchased from Sigma (Poole, Dorset, United Kingdom). Monoclonal mouse anti-smooth muscle α-actin antibody and myosin heavy chain antibody was purchased from Sigma and a goat-anti-mouse immunoglobulin (Ig) G from Dako (Glostrup, Denmark).

**Data and statistical analysis.** Data acquisition and analysis were performed with the Pulse (v8.53) software and Microsoft Excel (Microsoft, Redmond, WA). Results are expressed as the means ± SE of n observations. Statistically significant differences were evaluated by using ANOVA or paired or unpaired two-tailed Student’s t-test as appropriate. A P value of <0.05 was considered to be significant.

**RESULTS**

**Basal electro-physiological properties of human ASM.** The resting membrane potentials of ASM was -36.98 ± 2.89 mV and the membrane capacitance 140.08 ± 6.02 pF (both n = 29). Step depolarizations of ASM from -100 mV to +100 mV from a holding potential of -60 mV led to the activation of slowly activating outward currents. Pharmacological dissection of the currents with 1, 10, and 20 mM TEA reduced mean outward currents at +100 mV by 25.2 ± 1.6% (P < 0.05), 56.2 ± 4.6% (P < 0.05), and 63.4 ± 2.9% (P < 0.01; n = 12, respectively) in a concentration-dependent manner. Steady-state I-V curve analysis demonstrated depolarization-induced outward currents with delayed rectifier properties (Fig. 1).

**BK-induced currents in human ASM.** Application of different concentrations of BK (0.01–10 μM) caused an increase in both inward and outward currents in a dose-dependent manner.
The outward current was dependent on intracellular calcium, as increasing estimated intracellular free calcium from 133 nM 
\( (n = 9) \) to 937 nM \( (n = 12) \) increased the current by 80–90% 
\( (P < 0.05) \) between +20 to +60 mV (data not shown).

To study the inward current further, the main charge carrier in the pipette was changed to cesium, and the cells voltage-clamped at +60 mV. Addition of BK (1 \( \mu \)M) induced a biphasic current: a transient inward current of large amplitude and a sustained current of small amplitude, characteristics suggestive of CACC and \( I_{\text{CAT}} \) respectively (data not shown) (24).

**Role of \( I_{\text{K(Ca)}} \) of large and small conductance in BK-induced currents.** To attempt to define the channels underlying the BK-induced current, we studied early and late passage ASM cells from four different donors. In early passage \( (\text{passages } 2–3) \) cells, preincubation of cells with two concentrations of Ibt (100 nM), an inhibitor of BKCa, inhibited BK-induced currents by 90 ± 15% \( (n = 6; \text{Fig. } 3) \). The magnitude of BK-induced \( I_{\text{K(Ca)}} \) at −90 mV in these early passage cells was 8.4 ± 2.5 pA/pF. The BK-induced current in later passage \( (\text{passages } 6–7) \) cells was reduced in magnitude (4.7 ± 1.4 pA/pF), and Ibt was less effective at blocking \( I_{\text{K(Ca)}} \) in these cells \( (64 ± 12\% \text{ inhibition}, n = 6) \), suggesting that, in early passage cells, the majority of \( I_{\text{K(Ca)}} \) represents residual BKCa, whereas, in the later passage cells, a greater component of \( I_{\text{K(Ca)}} \) is carried by SKCa. In keeping with this observation, apamin (100 nM), which blocks SKCa and had no effect on the basal currents induced by depolarization, partially abrogated currents induced by BK (1 \( \mu \)M) in late passage cells (Fig. 4). Similarly, inward currents induced by histamine, a well-characterized contractile agonist, were also inhibited in later passage cells by apamin. Apamin decreased histamine-induced outward current density at 100 mV by 41% \( (n = 3; P < 0.01) \).

**Effects of Ca channel blockers on BK-induced currents.** As these findings highlight a central role of calcium in depolar-
FIG. 5. Effect of cations and SKF-96365 on BK-induced inward currents. BK (1 μM) applied directly to the cells voltage-clamped at −60 mV for 35 s evoked transient, rapidly inactivating, low-noise inward current (CACC) followed by noisy, sustained, small-amplitude currents (ICAT) at the end of BK application. Treatment of cells with Co2+ (100 μM), Ni2+ (100 μM), La3+ (100 μM), and SKF-96365 (10 μM) all inhibited CACC, whereas Ni2+, La3+, and SKF-96365 inhibited ICAT, while Co2+ had no obvious effect on ICAT. A and E: Co2+; B and F: Ni2+; C and G: La3+; D and H: SKF-96365. ∗P < 0.05; ∗∗P < 0.01 compared with control.

Depletion of intracellular stores inhibits both inward and outward currents induced by BK. As these data suggested a role for sarco-endoplasmic reticulum Ca2+-ATPase (SERCA)-regulated Ca2+ stores in BK-induced CACC and ICAT, we investigated the effect of thapsigargin, a selective inhibitor of SERCA of intracellular organelles. Cells were dialyzed with electrode solution containing thapsigargin (2 μM), and SKF-96365 (10 μM) inhibited the BK-induced amplification by 10.13% (P > 0.05; n = 6), 26.08% and 34.54% (both P < 0.05; n = 6), respectively. To confirm that the inhibition of the inward currents translated into inhibition of the outward current, we studied the effect of the Ca2+ channel blockers on the K+ current. Co2+ (100 μM), Ni2+ (100 μM), and SKF-96365 (10 μM) inhibited the BK-induced amplification by 10.13% (P > 0.05; n = 6), 26.08% and 34.54% (both P < 0.05; n = 6), respectively (Fig. 6). Nifedipine, a voltage-gated calcium channel antagonist, did not alter BK-induced inward or outward currents significantly (Fig. 7).

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Role of IP3 and ryanodine receptors in BK-induced inward and outward currents. To confirm the importance of internal calcium stores in BK-induced calcium signaling, we studied the effect of 10 mg/ml heparin (which inhibits IP3 receptor-mediated Ca\(^{2+}\) release) and 50 \(\mu\)M ruthenium red (a ryanodine receptor antagonist) on BK-induced currents. Figure 9 shows typical examples of these experiments. CACC in the heparin group (\(n = 8\)) was decreased by 88% (\(P < 0.001\)) compared with control (\(n = 12\)). \(I_{\text{CAT}}\) by 27% (\(P > 0.05\)), and K\(^+\) efflux by 82.04% (\(P < 0.01; n = 7\)), while the negative control de-N-heparin (\(n = 8\)) had no significant effect. Furthermore, 10 \(\mu\)M 2-aminoethoxydiphenyl borate (\(n = 10\)) decreased BK-induced Ca\(^{2+}\) influx by 78% (\(P < 0.001\)) and K\(^+\) efflux by 48% (\(P < 0.01\)). Ruthenium red (\(n = 7\)) had no effect on CACC, had variable effects on the K\(^+\) currents, and increased \(I_{\text{CAT}}\) in cultured ASM (results not shown).

BK induces inositol phosphate production. We sought support for the hypothesis that internal calcium stores were central to BK-mediated signaling by measuring inositol phosphate formation in response to BK. BK (1 \(\mu\)M) maximally induced a 33-fold increase in total \([3\text{H}]\)inositol phosphate accumulation (EC\(_{50}\) = 8.95 nM, 95% confidence intervals 4.58–17.47 nM, \(n = 4\)) in human ASM cells.

Activation of both inward and outward currents by BK is mediated through B\(_2\) receptors. To verify that the BK effect was specific, we used the B\(_1\) antagonist, de-Arg\(^{10}\)-Hoe 140, and the B\(_2\) receptor antagonist, \(N^w\)- adamantaneacetyl-d-Arg-[Hyp\(^3\), Thi\(^{5,8}\), d-Phe\(^7\)]-BK in an attempt to inhibit BK-induced currents. \(N^w\)- adamantaneacetyl-d-Arg-[Hyp\(^3\), Thi\(^{5,8}\), d-Phe\(^7\)]-BK inhibited the BK-induced outward potassium currents in a concentration-dependent manner, whereas de-Arg\(^{10}\)-Hoe 140 produced no significant effect on BK-induced SKCa, whereas Co\(^{2+}\) had no obvious effects.

DISCUSSION

Asthma is a common disease characterized by airway inflammation, hyperresponsiveness, and airway remodeling. In asthmatic patients, increased BK and its related kinins have been detected (35). Kinins are produced endogenously in many tissues and are implicated in both physiological and pathophysiological conditions. In the airways, BK exerts multiple effects, including bronchoconstriction, vasodilatation, plasma extravasation, and cough, by acting directly on ASM or indirectly due to the release of other inflammatory mediators. It has long been recognized that asthmatic individuals are more sensitive to the bronchoconstrictor properties of BK than nonasthmatic individuals (12). More recent studies have determined that inflammation upregulates BK receptor expression in ASM cells (38, 53) and potentiates calcium signaling in response to BK (1, 2). However, less is known about the relaxant mechanisms that follow the bronchoconstrictor response. As...
channels are thought to be important in the regulation of
both basal smooth muscle cell tone and agonist-induced relax-
ation (5, 15, 22, 25, 29, 46), we decided to investigate the effect
of BK on K^+ channel activity.

Cultured human ASM are a widely used and validated model
for the study of ASM cell physiology and pharmacology as
they retain many of their ex vivo characteristics but show
important differences to ASM from other species (14). This

![Diagram](image-url)

Fig. 7. A–F: The L-type Ca\(^{2+}\) channel antagonist, nifedipine (Nif), does not inhibit BK-induced potassium currents. Steady-state I-V curves of outward currents were induced by applying depolarizing pulses from −100 to +100 mV (hp = −60 mV) in 10-mV increments for a duration of 400 ms. Nifedipine (1 μM) had no effects on outward currents induced by BK (1 μM).

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Fig. 8. Predepletion of intracellular stores blocks the BK-induced CACC and SKCa. Intracellular electrode solution containing thapsigargin (Thaps; 2 μM) was used
to dialyze the cells, which were voltage-clamped at −60 mV. BK (1 μM) was delivered through a puffer pipette directly to the cells. CACC induced by BK were
continuously recorded. SKCa were induced by depolarizing the cells from −100 to +100 mV from an hp of −60 mV. A: control; B: following application of Thaps
for 5 min prior to BK; C: summary of effects of BK on CACC; D: effect of Thaps on BK induced SKCa. **P < 0.01 compared with BK only.
Present study thus extends observations previously made in animal ASM cells. Initial characterization of cultured human ASM cells at baseline confirmed the presence of a Ca\(^{2+}\)-dependent K\(^{+}\) current with delayed rectifier properties (Fig. 1) in keeping with earlier findings in ferret cells (11, 42).

Early passage cells retained some BKCa activity, which has been demonstrated in acutely dissociated cells (Fig. 3; Refs. 23, 28, 41, 42), although the magnitude of this current was reduced compared with those previously described in acutely dissociated cells, and in later passage cells, BKCa declined further, probably because of progressive loss of expression of this channel in culture (41). BK increased K\(^{+}\) efflux in a concentration-dependent manner (Fig. 2). In early passage cells, the majority of this increase was Ibt sensitive, and in later passage cells, an element of BK-induced BKCa remained; however, in these later passage cells, apamin also reduced BK-induced current, suggesting a contribution from BK activation of SKCa, which is known to be expressed in both fresh and cultured ASM cells (41). The progressive reduction in BK-mediated activation of BKCa (47) is probably because BKCa channel expression is gradually lost following prolonged cell culture. The mechanism for the transition from predominant K\(^{+}\) channels of large conductance identified in acutely dissociated cells to that of small conductance in later passage cultured human ASM (41) is not very clear. ASM isolated from bronchial tissues undergo morphological and physiological transformation in vitro, that is from a contractile phenotype to a synthetic one, which is thought to mirror some of the in vivo changes during airway remodeling (13). This raises the interesting possibility that during remodeling and ASM phenotype transformation BKCa may be downregulated, leaving SKCa as a major mechanism in regulating and counteracting effects of raised cytosolic Ca\(^{2+}\) concentrations.

The type of calcium signal that activates \(I_{K(Ca)}\) appears to depend on the cell type (43). The signaling pathway underlying BK-induced currents in ASM cells is still uncertain. Several studies have shown that BK is a Gq/G11-coupled receptor agonist with the potential to couple to phosphatidylinositol-specific phospholipase C (PI-PLC) (45), phosphatidylocholine phospholipase C (PC-PLC) (37), or phospholipase D (36). Stimulation of PI-PLC will promote the production of the second messenger, IP3, which releases Ca\(^{2+}\) from intracellular stores to the cytoplasm, and 1,2-diacylglycerol, which activates PKC. In addition to this Ca\(^{2+}\) signaling pathway, Hyvelin and colleagues have described functional ryanodine/caffeine-sensitive Ca\(^{2+}\)-release channels in ASM cells, which appear to modulate BK-induced Ca\(^{2+}\) signaling (18a). In the current study, we used heparin, an antagonist of IP3 receptors on the intracellular stores, and ruthenium red, a relatively specific antagonist of ryanodine receptors (RYR), and found that heparin
abolished the BK-induced currents, while ruthenium red had little effect (Fig. 8). We deduced, therefore, that in this system, BK-activated currents were mediated through B2 receptors rather than B1 receptors, although we did not directly examine the possibility that atypical or B3 receptors are involved in BK-inducedCa2+ and K+ signaling.

In conclusion, we have shown that BK stimulates a biphasic Ca2+ response: release from IP3-sensitive stores and influx through store-operated cation channels, which then activates a Ca2+-activated K+ efflux through Ca2+-activated K+ channels involving both BKCa and SKCa.

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