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Actions of bradykinin on electrical and synaptic behavior of neurones in the myenteric plexus of guinea-pig small intestine

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1 Electrophysiologic methods were used to study actions of bradykinin (BK) in neurones of the myenteric plexus of guinea-pig small intestine *in vitro*. Exposure to BK depolarized the membrane potential and elevated excitability in AH- and S-type neurones. Neuronal input resistance associated with the depolarizing responses was either decreased or unchanged in S-type and increased in AH-type neurones.

2 The selective B_2 BK receptor antagonist HOE-140, but not the selective B_1 receptor antagonist des-arg^{10}-HOE-140, suppressed the BK-evoked responses. RT-PCR confirmed the expression of B_2 receptor mRNA, but not B_1 receptor mRNA.

3 Binding of fluorescently- labeled HOE-140 (HOE741) was localized to ganglion cells in whole-mount preparations. BK B_2 receptors were coexpressed with immunoreactivity for calbindin or nitric oxide synthase.

4 Exposure to BK suppressed the amplitude of both fast and slow excitatory postsynaptic potentials. Depolarizing responses evoked by application of serotonin or substance P and nicotinic responses to acetylcholine were not reduced by BK. This suggested that BK action on neurotransmission was presynaptic suppression of neurotransmitter release. Presence of HOE-140 in the bathing solution suppressed or abolished the presynaptic inhibitory action of BK.

5 The cyclooxygenase inhibitor, piroxicam, suppressed both the direct excitatory action of BK and its presynaptic inhibitory action. Application of prostaglandin E_2, D_2, $F_{2\alpha}$ or I_2 mimicked the BK-evoked responses.

6 The results suggest that BK acts at B_2 BK receptors on myenteric neurones to stimulate the formation of prostaglandins. Once formed and released, the prostaglandins act to elevate the excitability of ganglion cells in the myenteric plexus and to suppress the synaptic release of neurotransmitters.

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Abbreviations: ACh, acetylcholine; BK, bradykinin; EPSP, excitatory postsynaptic potential; ENS, enteric nervous system; NOS, Nitric oxide synthase; PBS, phosphate-buffered saline

Introduction

Intestinal functions (e.g. motility, secretion and intramural blood flow) are largely controlled by the enteric nervous system (ENS). The bowel fails to function after loss of the ENS (Wingate, 1993; De Giorgio *et al*., 1996; Krishnamurthy & Shuffler, 1997; Smith *et al*., 1997; Wood, 2000, 2002). Interconnected synaptic networks in the myenteric and submucosal plexuses of the ENS work like a mini-brain in the digestive tract. Firing rates of secretomotor neurones in submucosal ganglia determine rates of mucosal secretion, while musculomotor neurones in the myenteric plexus provide inhibitory and excitatory input to the musculature (Cooke, 1989; Surprenant, 1994; Brookes, 2001). Sensory neurones and interneurones form integrated microcircuits that control firing rates in the pools of motor neurones.

The enteric immune/inflammatory system communicates with the ENS through the release of chemical mediators (Frieling *et al*., 1994a,b). Enteric neuronal microcircuits are 'flooded' by a paracrine overlay of inflammatory mediators during anaphylactic reactions to specific antigens and during intestinal inflammatory states. Exposure to inflammatory mediators including histamine, prostaglandins and cytokines strongly influences ENS function (Nemeth *et al*., 1984; Gaginella & Kachur, 1989; Frieling *et al*., 1994a,b; Dekkers *et al*., 1997; Xia *et al*., 1999).

The present study was focused on bradykinin (BK) as an established inflammatory mediator of documented importance in the small and large intestine. BK is produced from kininogen as a result of tissue injury, anoxia or inflammation. Its action on nociceptive spinal afferent neurones generates pain signals and its action as a stimulant for production of inflammatory cytokines such as tumor necrosis factor, interleukin-1 and interleukin-6 may contribute to inflammatory responses (Baccaglini & Hogan, 1983; Dray *et al*., 1994). Many intestinal cell types express receptors for

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BK including fibroblasts, smooth muscle, neurones and epithelial cells.

BK B_1 and B_2 receptors mediate the actions of BK. Both receptors were defined pharmacologically by use of specific peptide and nonpeptide antagonists. DNA that encodes functional BK B_2 receptors from human, rat, rabbit, mouse and guinea-pig sources have been cloned (McEachern *et al*., 1991; Hess *et al*., 1992; Ma *et al*., 1994; Bachvarov *et al*., 1995; Farmer *et al*., 1998). BK binds preferentially to the B_2 receptor and the related kinins generated by cleaving the terminal arginine reside from kallidin and BK (e.g. des-arg^{10}-kallidin, and des-arg^9-BK) bind preferentially to the B_1 BK receptor.

In the gastrointestinal tract, BK either contracts or relaxes smooth muscle, promotes ion secretion in the mucosa and stimulates the release of acetylcholine (ACh) in the myenteric plexus (Goldstein *et al*., 1983; Yau *et al*., 1986; Kachur *et al*., 1987; Andreeva & Rang, 1993; Zagorodnyuk *et al*., 1998). BK and substance P stimulate extravasation during neurogenic inflammation in the mouse gastrointestinal tract (Figini *et al*., 1997). Ileal secretion and smooth muscle contractility in the guinea-pig are influenced by the B_2 receptor (Kachur *et al*., 1987). BK is also implicated in the elevated sensitivity to rectal distension that occurs during intestinal inflammation in rats (Julia *et al*., 1995).

The evidence for action of BK in the ENS is strong. Nevertheless, there are little specific data on how BK might act at the level of single enteric neurones. The work reported here was oriented to determination of the actions of BK at the single neurone level in the myenteric plexus and to identification of the receptors involved in the action. Some of the results have been published in abstract form (Hu *et al*., 1998).

Methods

Tissue preparation

Adult male Hartley-strain guinea-pigs (300 – 350 g) were stunned by a blow to the head and exsanguinated from the cervical vessels according to the protocols approved by the Ohio State University Laboratory Animal Care and Use Committee and United States Department of Agriculture Veterinary Inspectors. Segments of small intestine 5 – 10 cm in length were removed 10 – 20 cm proximal to the ileocecal junction. Longitudinal-muscle-myenteric plexus preparations were obtained by methods described in detail elsewhere (Wood & Mayer, 1978). The preparations were mounted in a 1.5-ml glass-bottomed recording chamber that was perfused at a rate of 10 – 15 ml min^{-1} with Krebs solution warmed to 37°C and gassed with 95% O_2 – 5% CO_2 to buffer at pH 7.3 – 7.4. The composition of the Krebs solution was (in mM) NaCl, 120.9; KCl, 5.9; $MgCl_2$, 1.2; $NaHCO_3$, 14.4; $CaCl_2$, 2.5; and glucose, 11.5.

Intracellular electrophysiology and morphologic marking

The myenteric plexus was visualized with differential interference contrast optics and epilumination. Ganglia selected for study were immobilized with 100-μM diameter L-shaped stainless-steel wires pressed onto the surface at either side of the ganglion. Recording electrodes were glass micropipettes filled with 4% biocytin (Sigma, St Louis, MO, U.S.A.) in 2 M KCl containing 0.05 M tris (hydroxymethyl)-aminomethane buffer (pH 7.4). Resistances of the electrodes were in the range of 80 – 120 MΩ. The same electrodes were used to inject the neuronal tracer biocytin by the passage of hyperpolarizing current (0.5 – 1.0 nA for 5 – 30 min). WPI M-707 amplifiers (World Precision Instruments, Sarasota, FL, U.S.A.) were used in the bridge circuit mode to record transmembrane potentials and to inject electrical current into the ganglion cell somas. Constant current, rectangular pulses were driven by Grass SD9 stimulators (Grass instrument Division, Astro-Med Inc., W. Warwick, RI, U.S.A.), electrometer output was amplified and observed on an oscilloscope (Tektronics 5113, Tektronics Inc., Beaverton, OR, U.S.A.) and recorded on videotape (A.R.Vetter Co., Rebersburg, PA, U.S.A.) for later analysis. Synaptic potentials were evoked by focal electrical stimulation of interganglionic fiber tracts with electrodes made of 20-μm diameter Teflon-coated platinum wire and connected through a stimulus-isolation unit (Grass SIN5) to a Grass S48 stimulator. Chart records were made on an Astro-Med thermal recorder (Astro-Med Inc., Cleveland, OH, U.S.A.). The amplitude of the spikes, in some of the figures, was blunted by the low frequency response of the recorder. After completion of the electrophysiologic segment of the experiment, the anal end of the preparation was marked and the tissue was washed three times in cold Krebs solution followed by cold fixative in a disposable recording chamber left overnight at 4°C. The fixative contained 2% formaldehyde in a 15% solution of picric acid. The preparations were cleared in three changes of dimethylsulfoxide and three 10-min washes with phosphate-buffered saline (PBS; 0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.0). They were then reacted with avidin coupled to horseradish peroxidase (elite ABC kit, Vector, Burlingame, CA, U.S.A.), carried through a diaminobenzidine color-developing reaction and dehydrated in alcohol. The preparations were mounted in Canada balsam and examined with a Nikon Eclipse E600 microscope.

Imaging the B_2 BK receptor

Whole-mount longitudinal muscle-myenteric plexus preparations were incubated at 37°C in circulating Krebs solution gassed with 95% O_2 and 5% CO_2 for 1 h to allow migration of the receptors to the cellular surfaces. The preparations were then rinsed in ice-cold Krebs solution containing 3 μM nicardipine, 1% bovine serum albumin and the protease inhibitors chymostatin (4 μg ml^{-1}), bacitracin (40 μg ml^{-1}), leupeptin (4 μg ml^{-1}) and captopril (30 μM) followed by incubation for 45 min at 4°C in Krebs solution in the absence or presence of 30 nM fluorescently- labeled selective B_2 receptor antagonist HOE-140 (BODIPY® 576/589, Cat# 741, NEN™ Life Science Products, Boston, MA, U.S.A.). Labeled HOE-140 was able to diffuse into the tissue and bind to the B_2 receptor with the receptor/ligand complex remaining at the cell surface, because internalization did not occur owing to solidification of membrane lipids at 4°C. The preparations were then rinsed in ice-cold PBS and fixed overnight in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0. The tissues were rinsed again in cold PBS. Double labeling with HOE-741 was done with mouse anti-human neuronal protein HuC/HuD (anti-Hu; code A-21271 at 1 : 50; Molecular Probes, Eugene, OR, U.S.A.), sheep

anti-NOS (code AB1529 at 1 : 500, Chemicon, Temecula, CA, U.S.A.) or mouse anticalbindin (code C9848, at 1 : 5000; Sigma, St Louis, MO, U.S.A.) to gain limited insight into the chemical coding for B_2 receptors in the myenteric plexus. Primary antibodies against anti-Hu, nitric oxide synthase (NOS) and calbindin were diluted in PBS containing 10% horse serum and 0.3% Triton X-100. After incubation with the primary antibodies, the preparations were washed (3×10 min) in PBS, transferred to a humid chamber and incubated at room temperature for 30 min in fluorescein isothiocynate-labeled donkey anti-sheep secondary IgG (code 713-095-147, Jackson ImmunoResearch, West Grove, PA, U.S.A.), or donkey anti-mouse secondary IgG (code 705-095-147, Jackson ImmunoResearch, West Grove, PA, U.S.A.) diluted in hypertonic PBS containing 10% normal horse serum, 0.3% Triton X-100 and 0.1% sodium azide. Following a final rinse in PBS, the whole-mounts were cover-slipped with Vectorshield (Vector, Burlingame, CA, U.S.A.). All preparations were examined with an epifluorescence microscope (Nikon Eclipse-600) and analyzed using filter combinations that enabled separate visualization of the different fluorophores. Images were digitized with a SPOT-2 chilled color and B/W camera (Diagnostic Instruments, Sterling Heights, MI, U.S.A.) and analyzed with SPOT III software.

RT-PCR

Total RNA from enzymatically dissociated myenteric ganglia was extracted with Trizol (Life Technology, Gaithersburg, MD, U.S.A.). The tissue was homogenized in Trizol (1 ml 100 mg^{-1} of tissue). Chloroform (10% of the total volume) was added and the sample was then covered, shaken vigorously and placed on ice for 10 min followed by centrifugation for 15 min ($13{,}000 \times g$). The aqueous phase was then removed and the RNA was precipitated with isopropanol (60% of the Trizol volume). The RNA pellet was then washed by resuspension in 70% ethanol. The suspension was centrifuged for 5 min ($10{,}000 \times g$), dried briefly, and then dissolved in diethylpyrocarbonate-treated water. The RNA was then divided into 1 $\mu g\,\mu l^{-1}$ aliquots for further use. Reverse transcription to cDNA was performed by adding the following and incubating at 42°C for 60 min: 2.0 μl of $10 \times$ RT buffer (0.1 M Tris, 0.5 M KCl, pH 8.3), 4.0 μl of 25 mM $MgCl_2$, 2.0 μl Deoxynucleotide Mix (dATP, dCTP, dGTP and dTTP each at 10 mM), 2.0 μl of Oligo-p(dT) Primer (0.8 $\mu g\,\mu l^{-1}$), 50 U of Rnase inhibitor, 20 U of AMV reverse transcriptase (Roche Diagnostics Corp. Indianapolis, IN, U.S.A.), and 1 μl of total RNA. Subsequent enzyme deactivation was accomplished by heating to 99°C for 5 min and then cooling to 4°C for 5 min. The following reaction mixture was added to 5 μl of the cDNA product: 25 μl of PCR master mix (Roche Diagnostics Corp. Indianapolis, IN, U.S.A.), 2.0 μl of primers and 18 μl of sterile water. The primers used were:

1. Upstream: 5′-GGTGTACGCTGCTCCTGAG-3′.
2. Downstream: 5′-TCAGCACAACCAGGAC-3′.
3. Upstream: 5′-TATTCCTGGTGGTGGCTATC-3′.
4. Downstream: 5′-GGAGGCTACCAGTGTGAG-GA-3′.

Primers 1 and 2 were from the mRNA of a guinea-pig lung B_2 receptor clone and were used to amply a 500 bp DNA for the BK B_2 receptor. Primers 3 and 4 were used in the attempt to amply DNA coding for the guinea-pig BK B_1 receptor. Amplification was done with a iCycler™ (BIO-RAD Laboratories, Hercules, CA, U.S.A.). PCR cycles consisted of denaturation for 1 min at 94°C, annealing at 56°C for 90 s, and extension at 72°C for 90 s. A total of 30 cycles was followed by completion of extension for 7 min at 72°C. PCR product (12 μl) was analyzed by electrophoresis on a 2% agarose gel. Sequencing was done with an ABI 373XL DNA sequencer. RT-PCR without AMV reverse transcriptase was used as a negtive control.

Chemicals

Pharmacologic agents were applied either by addition to the bathing solution or by pressure microejection from glass micropipettes (10 – 15 μM tip diameter) with N_2 pulses of controlled duration and pressure. Agents used were: acetylcholine, BK, Lys-BK, des-arg^9-BK, [D-Phe^7]-BK, D-arg-[hyp^3, D-Phe^7]-BK, D-arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, des-arg^9, [Leu^8]-BK, HOE-140, [Des-arg^{10}] HOE-140, forskolin, prostaglandin E_2, prostaglandin D_2, prostaglandin $F_{2\alpha}$ and prostaglandin I_2, piroxicam, substance P and serotonin all from Sigma, St Louis, MO, U.S.A. except for HOE-140, des-arg^{10} HOE-140, acetylcholine, substance P and serotonin each of which were obtained from R.B.I., Natick, MA, U.S.A.

Data analysis

Data are presented as means ± s.e.m. Concentration – response relations were analyzed with the following least-squares fitting routine: $V = V_{max}/[1 + (EC_{50}/C)^{n_H}]$, where V is the observed membrane depolarization, V_{max} is the maximal response, C is the corresponding drug concentration, EC_{50} is the concentration which evokes a half-maximal response, and n_H is the apparent Hill coefficient. Antagonist concentration – inhibition curves were obtained in individual neurones by applying a fixed concentration of BK (100 nM), and progressively increasing the concentration of antagonist. IC_{50} values were calculated by least-squares fitting to $V = V_0/[1 + (IC_{50}/[Ant])^{-n_H}]$, where V and V_0 are peak responses in the presence and absence of antagonist at concentration [Ant]. The graphs in the figures were drawn by averaging results from all experiments and fitting a single concentration – response curve to the pooled data using Sigmaplot software (SPSS Inc., Chicago, IL, U.S.A.). All EC_{50} and IC_{50} values given in the text are the mean ± s.e.m. Student's t-test and paired t-test were used to determine statistical significance with $P < 0.05$ accepted as significant.

Results

Electrophysiology

The neurones were classified as AH- or S-type based on generally accepted criteria (Bornstein *et al*., 1994; Wood, 1994a). Exposure to BK in the superfusion solution (0.01 – 300 nM) evoked slowly activating depolarization of the membrane potential in 71 of 88 AH-type neurones (Figure 1a). Amplitude of the depolarizing responses was concentration-dependent with an EC_{50} of 3.4 ± 0.7 nM, and a threshold of ~1 nM. The peak amplitude of the depolarization

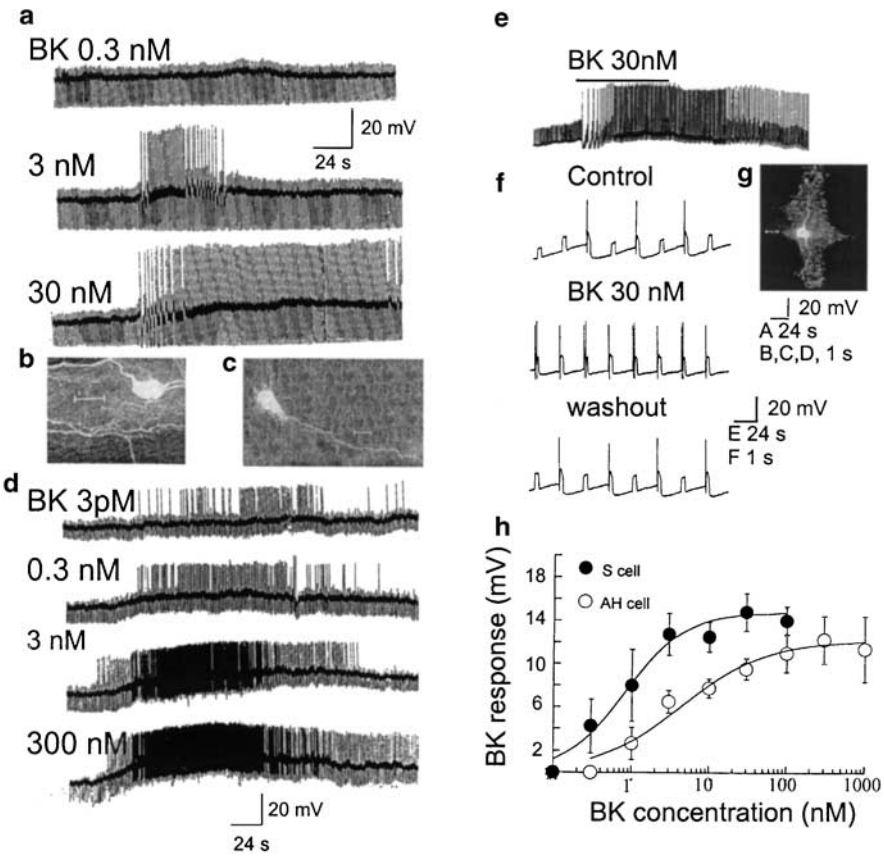

Figure 1 BK-evoked depolarizing responses and enhanced excitability in AH- and S-type neurones in guinea-pig small intestinal myenteric plexus. (a) Action of BK (0.3 – 30 nM) was concentration dependent in an AH-type neurone. Downward deflections are electrotonic potentials produced by intraneuronal injection of constant-current hyperpolarizing pulses. Increased amplitude of electrotonic potentials reflects an increase in the neuronal input resistance. (b) Morphology of the neurone from which the records in (a) were obtained. (c) Morphology of the neurone from which the records in (d) were obtained. (d) Action of BK (3 pM – 300 nM) was concentration dependent in an S-type neurone. Enhanced excitability is reflected by spontaneous spike discharge and anodal-break excitation at the offset of constant-current hyperpolarizing pulses. (e) Depolarizing response to 30 nM BK in an AH-type neurone. (f) Reversible suppression of hyperpolarizing after-potentials by 30 nM BK in the same neurone as (e). (g) Morphology of the neurone from which the records in (e) and (f) were obtained. (h) Concentration – response curves for BK-evoked membrane depolarization in AH- and S-type neurones. Each data point represents four to 13 AH-type neurones and seven S-type neurons. The EC_{50} value was 4.4 ± 0.9 nM for AH-type neurones and 0.8 ± 0.1 nM for S-type neurones. Calibration bars for morphology are 20 μM.

was 12.3 ± 2.2 mV evoked by 300 nM BK in 11 AH neurones (Figure 1a, h).

Enhanced neuronal excitability, as reflected by discharge of action potentials either in response to depolarizing current pulses or by anodal-break excitation at the offset of hyperpolarizing current pulses, was associated with the depolarizing responses in AH neurones (Figures 1a, e, f). Depolarizing responses to BK were associated with increased input resistance in the AH-type neurones (Figures 1a, 2b). Hyperpolarizing after-potentials in AH neurones were suppressed when BK was present in the bathing solution (Figure 1e, f).

Exposure to BK in the superfusion solution (0.01 – 300 nM) evoked slowly activating depolarization of the membrane potential in 22 of 29 S-type neurones (Figure 1d). Amplitude of the depolarizing responses was concentration-dependent with an EC_{50} of 0.7 ± 0.1 nM and a threshold of ~ 0.3 nM (Figure 1h). The peak amplitude of the depolarization was 14.8 ± 7.5 mV evoked by 30 nM BK in five S neurones (Figure 1d, h). Augmented excitability during exposure to BK was evident as increased frequency of spontaneous spike discharge and appearance of anodal-break excitation at the offset of hyperpolarizing current pulses (Figure 1d). There was either no change or a decrease in input resistance associated with the depolarizing responses to BK in S neurones.

Plots of current – voltage relations ($I-V$ curves) for AH and S neurones in the presence and absence of BK reflected the effects on neuronal input resistance (Figure 2a, b). The $I-V$ curves in the absence and presence of BK for the S-type neurone in Figure 2a intersected at ~ -15 mV and was suggestive of a reversal potential near this value. The mean reversal potential estimated from the membrane potentials at which $I-V$ curves intersected was -14.2 ± 3 mV for five S neurones.

Increased slopes of $I-V$ curves for AH-type neurones in the presence of BK reflected the increased input resistance that accompanied the depolarizing responses. $I-V$ curves for AH neurones in the presence and absence of BK intersected at membrane potentials between – 80 and – 90 mV with a mean of -87.0 ± 4.0 mV for seven AH neurones (Figure 2b). These values are near the estimated equilibrium potential for K^+ in enteric neurones and may reflect decreased K^+ conductance

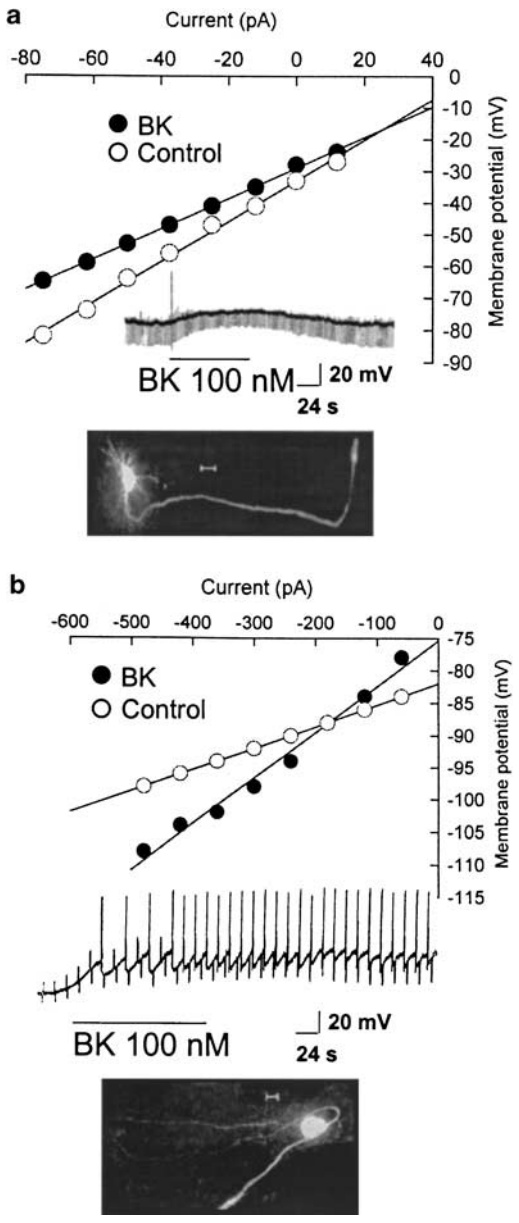

Figure 2 Current – voltage relations ($I-V$ curves) for AH- and S-type myenteric neurones in the absence and presence of BK. (a) $I-V$ curves for an S-type neurone in the absence (○) and presence (●) of 100 nM BK. Decreased slope in the presence of BK reflected decreased input resistance. The $I-V$ curves intersected at −15 mV. The insets show a BK-evoked response and morphology for the S-type neurone from which the $I-V$ curves were obtained. (b) $I-V$ curves for an AH-type neurone in the absence (○) and presence (●) of 100 nM BK. Increased slope in the presence of BK reflected increased input resistance. The $I-V$ curves intersected at − 88 mV. The insets show a BK-evoked response and morphology for the AH-type neurone from which the $I-V$ curves were obtained.

during the depolarizing responses to BK in AH neurones (North, 1973).

The excitatory effects of BK in AH and S neurones were reversed after 5 – 7 min of washout from the superfusion chambers and tubing. No apparent desensitization was observed with repeated exposures at intervals of 10 – 15 min.

The BK-evoked responses persisted with 300 nM tetrodotoxin present in the bathing solution. Likewise, reduction of Ca^{2+} to 0.5 mM and elevation of Mg^{2+} to 12.5 mM in the bathing solution did not suppress the amplitude of BK-evoked depolarizing responses. Since voltage-activated Na^+ channels underlie action potential generation in ENS axones, tetrodotoxin was expected to block the channels and thereby the axonal release of neurotransmitters (Wood, 1994b). Elevated Mg^{2+} and reduced Ca^{2+} prevent Ca^{2+} entry into axone terminals and thereby suppress the release of neurotransmitters. Failure of tetrodotoxin or reduced Ca^{2+} and elevated Mg^{2+} to alter the excitatory responses to BK is indicative of a direct action at receptors on the neurone rather than an indirect action because of excitation of neighboring neurones that provide synaptic input to the recorded neurone.

BK B_2 receptor pharmacology

The actions of the related kinins, Lys-BK (kallidin) and des-Arg^9-BK on myenteric neurones, were investigated as part of the study. Kallidin (10 nM – 3 μM) evoked responses that were essentially the same as those evoked by BK. Nevertheless, higher concentrations were required to evoke responses of the same magnitude as BK. The EC_{50} for Lys-BK was 35.5 ± 9.0 nM for five AH neurones. Exposure to des-Arg^9-BK evoked no responses in any of seven neurones when applied in concentrations as high as 10 μM. The agonist potency order was BK > Kallidin > des-arg^9-BK.

Receptor-specific antagonists were added to the bathing solution before and with the application of BK to help identify the BK receptor type/s responsible for the excitatory responses to BK. The selective B_2 receptor antagonist, HOE-140, concentration dependently suppressed the responses evoked by 100 nM BK with an IC_{50} of 14.5 ± 3.4 nM for five AH neurones (Figure 3). HOE-140 (100 nM) suppressed 89.3 ± 5.9% of the depolarizing response evoked by 100 nM BK. HOE-140 (300 nM) abolished responses evoked by 10 nM BK in three S neurones. Full recovery required a 30 – 45 min washout period. The B_2 BK receptor antagonists D-Arg, [Hyp^3, Thi^{5,8}, D-phe^7]-BK also suppressed BK-evoked responses in seven neurones. On the other hand, the B_1 BK receptor antagonist des-arg^{10}-HOE-140 (300 nM – 3 μM, $n=5$ neurones) or des-arg^9, [leu^8]-BK (300 nM – 3 μM, $n=7$ neurones) had no effect on BK-evoked depolarizing responses in AH or S neurones.

BK B_2 receptor expression

RT-PCR was used to search for and identify BK receptor mRNA transcripts. Amplification of cDNA from total myenteric plexus RNA with a pair of specific primers for the guinea-pig BK B_2 receptor resulted in a single 500 bp RT-PCR product that corresponded to the BK B_2 receptor (Figure 4a). The 500 bp RT-PCR product was sequenced and found to be identical to the guinea-pig lung BK B_2 receptor (Farmer *et al*., 1998). Evidence for expression of BK B_1 receptor mRNA was not found.

A second approach in the study of expression of the B_2 receptor used the BODIPY 576/589 fluorescently labeled B_2 receptor antagonist HOE-140 (HOE741) in a concentration of 30 nM to bind and label B_2 receptors in live whole-mount myenteric plexus preparations. Fluorescence microscopy showed that binding of HOE741 was localized to ganglion cells in the plexus (Figure 4b, c). HOE741 fluorescence was concentrated at the periphery of the ganglion cells

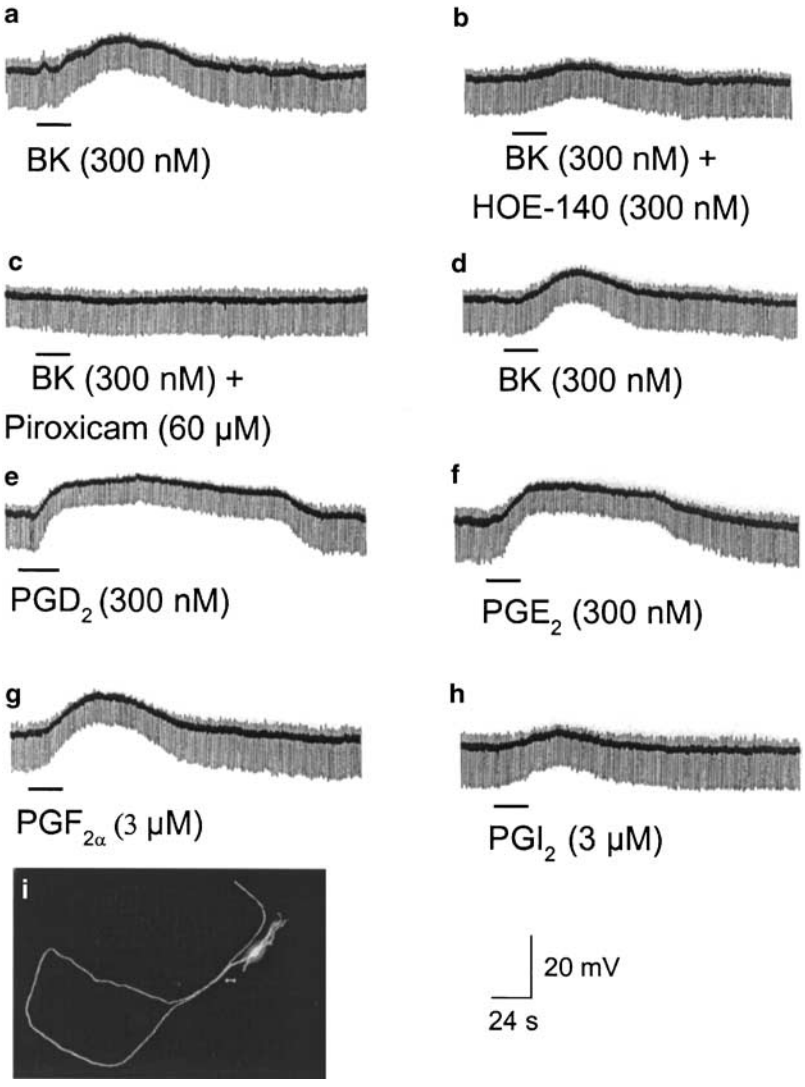

Figure 3 The BK B_2 receptor subtype and the release of prostaglandins-mediated BK-evoked depolarizing responses. (a) Depolarizing response to 300 nM BK in an S-type myenteric neurone. (b) The selective B_2 receptor antagonist HOE-140 (300 nM) suppressed the response to BK. (c) The cyclooxygenase inhibitor piroxicam (60 μM) abolished the response to BK. (e – h) Depolarizing responses to PGD_2, PGE_2, $PGF_{2\alpha}$ or PGI_2 mimicked the action of BK. All records are from the same neurone. Downward deflections are electrotonic potentials evoked by intraneuronal injection of constant-current hyperpolarizing pulses. Decreased amplitude of the electrotonic potentials reflects decreased input resistance. (i) Morphology of the neurone from which the records were obtained. Calibration bar 20 μM.

suggesting that binding was occurring at receptors on the neuronal surface. As a control for the specificity of HOE741 labeling, HOE-140 (300 nM) was applied for 40 min prior to the addition of HOE741 to the bathing medium. Pretreatment with HOE-140 quenched the fluorescence normally seen with HOE741 alone and suggested that localization of HOE741 fluorescence reflected specific labeling of the BK B_2 receptor.

Anti-Hu antibody was used in combination with HOE741 to determine the proportion of the total neuronal population that expressed the B_2 receptor. Anti-Hu immunoreactivity marks all neurones in enteric ganglia and is therefore a useful histologic tool for obtaining accurate counts of all ganglion cells in each individual ganglion (Lin *et al*., 2002). Double labeling with HOE741 and the anti-Hu antibody suggested that all of the myenteric neurones for the most part expressed the B_2 receptor (Figure 4d, f). Validity of this suggestion was reinforced by findings with double labeling of HOE741 together with the neuronal markers calbindin or neuronal NOS. Calbindin is a marker for ganglion cells with AH-type electrophysiology and Dogiel Type II morphology in the guinea-pig small intestine (Furness *et al*., 1990; Brookes, 2001). Uniaxonal myenteric neurones with axons projecting in the anal direction and expressing NOS immunoreactivity are inhibitory musculomotor neurones (Furness & Anderson, 1994; Brookes, 2001). All of the myenteric neurones that expressed immunoreactivity for either calbindin or NOS also expressed the BK B_2 receptor as determined by colocalization with HOE741 binding (Figure 4g, l). The histoanatomical results overall are in agreement with the electrophysiologic findings that exposure to BK-evoked excitatory responses in most of the neurones studied.

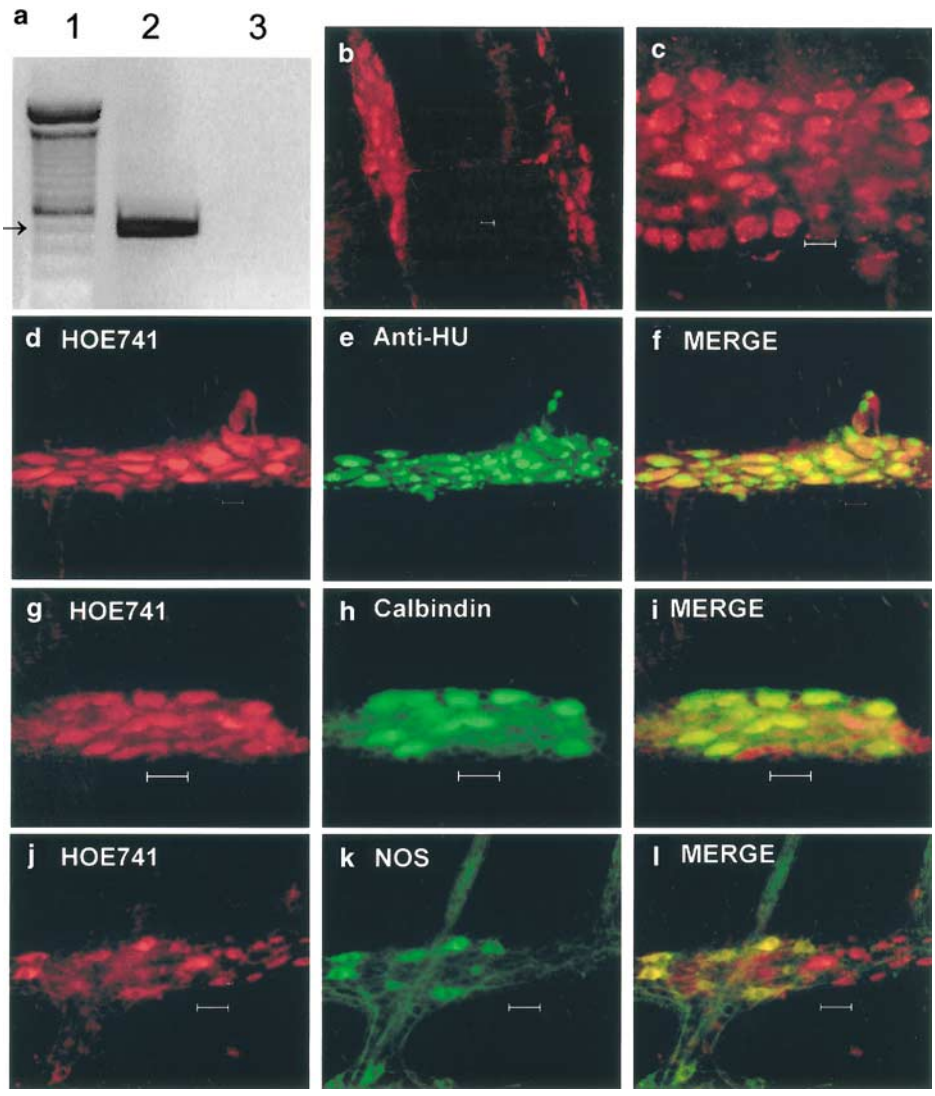

Figure 4 Expression of the BK B2 receptor in guinea-pig small intestinal myenteric plexus. (a) RT-PCR identification of BK B_2 receptor mRNA expression. Lane 1 is a DNA marker. The arrow indicates 500 bp. Lane 2 is the RT-PCR product obtained with specific primers for the B_2 receptor. Lane 3 is the negative RT-PCR result when specific primers for the B_1 receptor were used. (b) Low-power image of binding for HOE741 in a longitudinal muscle-myenteric plexus preparation. (c) Higher magnification image of HOE741 binding. (d – f) Double staining with anti-Hu antibody and HOE741 showed that nearly all myenteric ganglion cells expressed the B_2 receptor. (g – i) Double staining for calbindin and HOE741 revealed calbindin immunoreactivity colocalized with HOE741 binding. (j – l) Double staining for NOS and HOE741. NOS immunoreactivity was colocalized with HOE741. Calibration bars 20 μM.

Presynaptic inhibition

Single or repetitive focal stimulation applied to interganglionic fiber tracts in the myenteric plexus evokes slow nonadrenergic, noncholinergic excitatory postsynaptic potentials (EPSPs) in the ganglion cells (Katayama & North, 1978; Wood & Mayer, 1978). Exposure to BK in a concentration range of 0.3 – 100 nM reversibly suppressed the amplitude of stimulus-evoked slow EPSPs in 18 of 22 AH-type neurones and nine of 17 S-type neurones. Inhibition of the slow EPSPs was concentration-dependent with an IC_{50} of 9.2 ± 2.1 nM and threshold concentrations of 0.3 – 1 nM for pooled data from 10 AH and five S neurones (Figures 5 and 6). Both the depolarizing responses to BK and suppression of the slow EPSPs were prevented when 300 nM HOE-140 or 60 μM piroxicam was present in the bathing solution. This effect of cyclooxygenase inhibition suggests that activation of BK B_2 receptors on myenteric ganglion cells leads to the formation and release of prostaglandins, which in turn act to suppress slow synaptic excitation. Findings that application of prostaglandins D_2 (100 nM), E_2 (100 nM), $F_{2\alpha}$ (3 μM) or I_2 (3 μM) suppressed stimulus-evoked slow EPSPs in eight AH-type neurones are consistent with this suggestion (Figure 6).

Substance P and serotonin both mimic stimulus-evoked slow EPSPs when experimentally applied to AH-type myenteric neurones in the guinea-pig small intestine (Katayama & North, 1978; Wood & Mayer, 1979). We applied either 30 nM substance P or 300 nM serotonin to five neurones and found slow EPSP-like responses that were the same as described in the earlier reports. The presence of 100 nM BK did not change

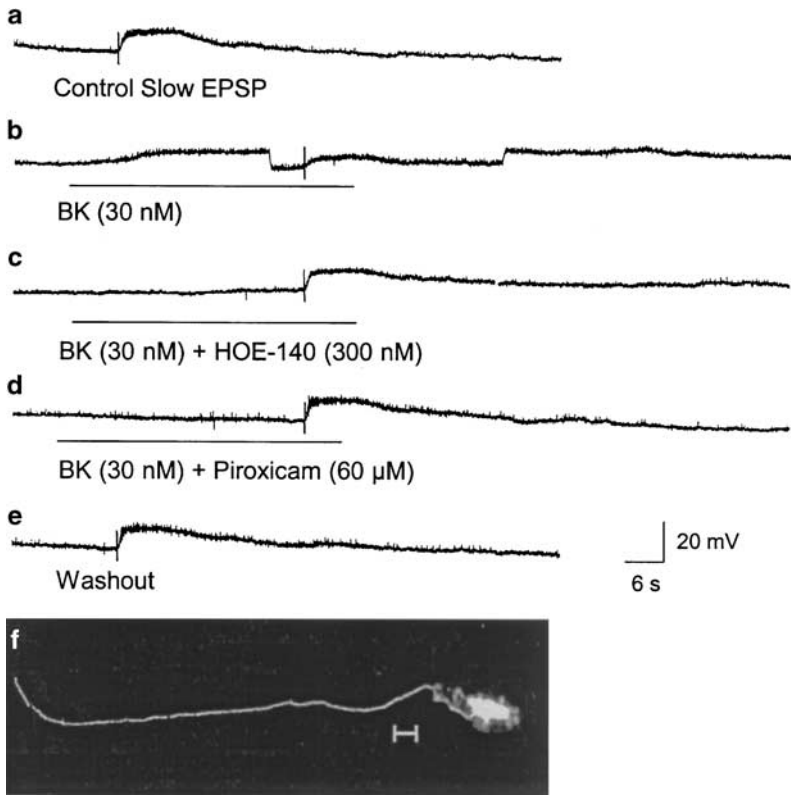

Figure 5 The BK B_2 receptor subtype and the release of prostaglandins-mediated suppression of slow EPSPs by BK in an S-type myenteric neurone. (a) Control, slow EPSP evoked by repetitive stimulation (six pulses; 20 Hz) of an interganglionic connective. (b) Bradykinin (30 nM) evoked slowly activating membrane depolarization. The slow EPSP was suppressed in the presence of BK as reflected by reduced amplitude when the membrane potential was current-clamped at the resting potential of -62 mV. (c) The selective BK B_2 receptor antagonist HOE-140 (300 nM) prevented both BK-evoked depolarizing responses and suppression of the slow EPSP. (d) The cyclooxygenase inhibitor piroxicam (60 μM) prevented both BK-evoked depolarizing responses and suppression of the slow EPSP. (e) Inhibitory action of piroxicam was reversed after washout. (f) Morphology of the S-type neurone from which the results were obtained.

the responses to substance P or serotonin in any of the five neurones (data not shown). Suppression of stimulus-evoked slow EPSPs by BK together with findings that it did not suppress responses to the two slow EPSP mimetics is consistent with a presynaptic inhibitory action to suppress the release of neurotransmitters for the slow EPSP. Suppression of the BK action on the slow EPSPs by piroxicam and inhibition of the slow EPSPs by prostaglandins implicates BK B_2 receptor stimulation of synthesis and release of prostaglandins as the presynaptic inhibitory mechanism.

Focal electrical stimulation of interganglionic fiber tracts evokes nicotinic fast EPSPs in S-type neurones of the guinea-pig small intestinal myenteric plexus (Nishi & North, 1973; Hirst *et al*., 1974). Fast EPSPs, that were suppressed or abolished by 100 μM hexamethonium and similar to those in earlier reports, were also recorded in the present study. Application of 100 nM BK in the bathing solution inhibited the fast EPSPs by $35.2 \pm 4.2\%$ in seven of 17 neurones (Figure 7). Pretreatment with 300 nM HOE-140 or 60-μM piroxicam, blocked the inhibitory action of BK on the fast EPSPs. Application of ACh by micropressure ejection evoked hexamethonium-sensitive depolarizing responses in the same neurones in which BK suppressed the fast EPSPs. Application of 300 nM BK in the bathing medium did not suppress the depolarizing responses to ACh. Suppression of nicotinic fast EPSPs by BK and failure of BK to suppress nicotinic responses to ACh suggest that the mechanism of action of BK was presynaptic inhibition of release of ACh from presynaptic nerve terminals. Suppression of BK action on the fast EPSPs by piroxicam implicates BK B_2 receptor stimulation of synthesis and release of prostaglandins as the presynaptic inhibitory mechanism.

Prostaglandins

Piroxicam, a cyclooxygenase inhibitor, was used to test the possibility that the excitatory actions of BK were secondary to the release of prostaglandins. Piroxicam (60 μM), when co-applied with 100 nM BK, suppressed BK-evoked depolarizing responses from 9.3 ± 1.3 to 0.8 ± 0.5 mV for seven neurones ($P < 0.001$, Student's paired t-test). Figure 3 illustrates how 100 nM BK evoked membrane depolarization associated with decreased input resistance in an S-type neurone with uniaxonal morphology. Pretreatment with HOE-140 (300 nM) partially suppressed the BK-evoked response and addition of 60 μM piroxicam in the bathing solution reversibly abolished the response. In the same neurone, exposure to prostaglandins D_2 (100 nM), E_2 (100 nM), $F_{2\alpha}$ (3 μM) or I_2 (3 μM) evoked depolarizing responses that mimicked the responses to BK. Results of this nature were obtained from an additional 11 neurones. These results suggest that activation of BK B_2 receptors on myenteric ganglion cells leads to the formation

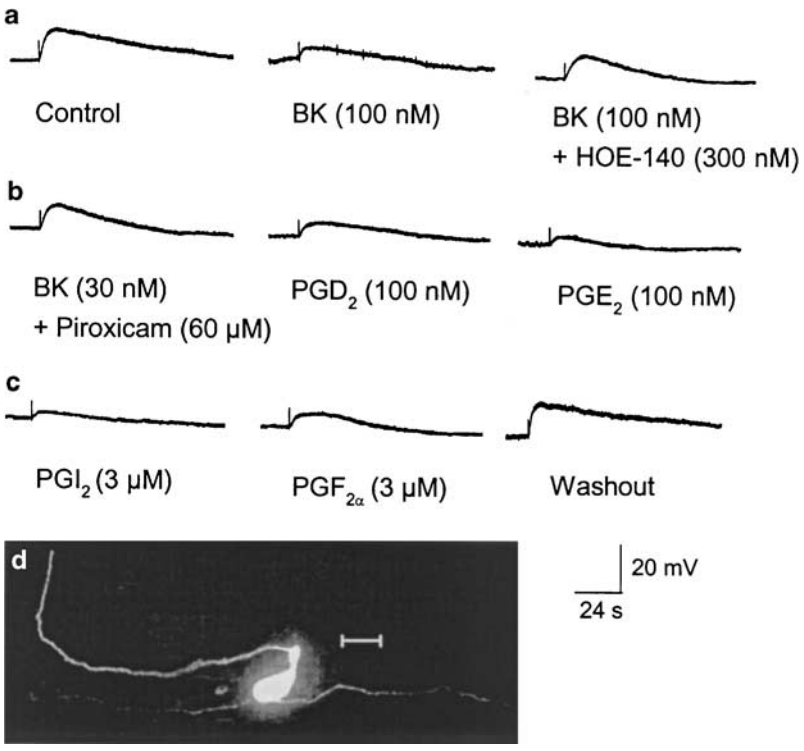

Figure 6 The BK B_2 receptor subtype and the release of prostaglandins-mediated suppression of slow EPSPs by BK in an AH-type myenteric neurone. (a) Control slow EPSP evoked by repetitive stimulation (six pulses; 20 Hz) of an interganglionic connective. BK (100 nM for 1.5 – 2 min) suppressed the slow EPSP. Addition of the selective BK B_2 receptor antagonist HOE 140 reversed the inhibitory action of BK on the slow EPSP. (b) Pretreatment with the cyclooxygenase inhibitor piroxicam (60 μM) offset the inhibitory action of 30 nM BK on the slow EPSP. Application of the prostaglandins PGD_2 (100 nM) and PGE_2 (100 nM) suppressed the slow EPSP. (c) The prostaglandins PGI_2 (3 μM) and $PGF_{2\alpha}$ (3 μM) also suppressed the slow EPSP. The slow EPSP returned to near normal amplitude after washout.

and release of prostaglandins, which in turn act to evoke excitatory responses in the neurones.

Discussion

The work reported here was based on evidence that BK is released during inflammatory and ischemic conditions in the intestine, and after release might act as a paracrine mediator in the ENS and thereby alter secretory and motility functions at the organ level. We found that excitation of ganglion cells in the myenteric plexus was a primary action of bradykinin. Several lines of evidence suggested that the BK B_2 receptor was the exclusive mediator of the neuronal excitatory responses. No evidence for involvement of the BK B_1 receptor was obtained. The evidence for BK B_2 involvement included blockade of BK-evoked responses by the selective BK B_2 receptor antagonist HOE-140, lack of effect of selective BK B_1 receptor antagonists, selective binding of a fluorescently-labeled B_2 receptor antagonist to myenteric ganglion cells, and the presence of the mRNA transcript for the B_2 but not the B_1 receptor.

Both the electrophysiologic results and the fluorescent histochemistry suggested that the majority of neuronal cell bodies in the ganglia expressed the B_2 receptor. The receptor was associated with neurones that had multipolar Dogiel Type II morphology, expression of immunoreactivity for the calcium-binding protein calbindin and AH-type electrophysiologic behavior. Neurones with uniaxonal Dogiel Type I morphology, expression of immunoreactivity for NOS and S-type electrophysiologic behavior also expressed the BK B_2 receptor.

Excitation of large numbers of AH- and S-type myenteric neurones by BK has global implications for function/malfunction at the organ level. On the one hand, very little is known about integrative processes at the microcircuit level of organization to predict the outcome in terms of total organ performance. On the other hand, some predictions can be made. For example, it is clear that myenteric ganglion cells that express immunoreactivity for NOS and have their single axone projecting in the aboral direction are inhibitory musculomotor neurones (Brookes, 2001). Elevation of excitability in this population of neurones would be expected to suppress contractile activity of the intestinal musculature. Likewise, ganglion cells with Dogiel Type II morphology, calbindin expression and AH-type electrophysiology are believed to be interneurones synaptically connected one with another to form positive feed-forward networks that project excitatory input to populations of motor neurones and synchronize the firing of the neurones in a motor pool (Wood, 1994b; Thomas *et al*., 1999). Excitation of AH-type interneurones would therefore be expected to work in concert with direct excitation of individual motor neurons to augment the output from pools of motor neurones to their effectors (e.g.,inhibitory and excitatory musculomotor neurons). Emergent behavior at the neural network and whole-organ levels of

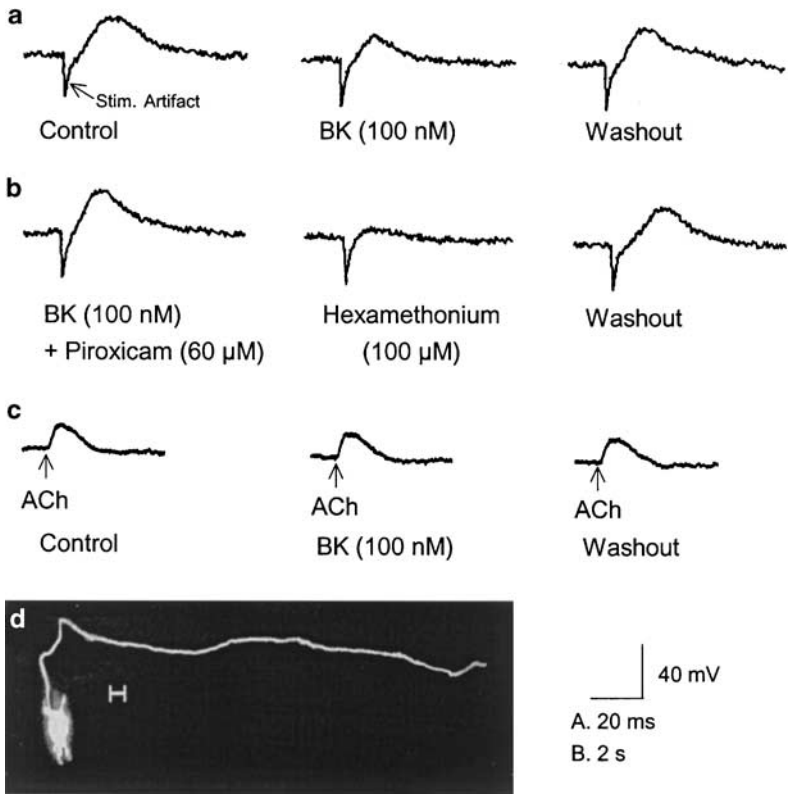

Figure 7 The BK B_2 receptor subtype and the release of prostaglandins-mediated suppression of fast nicotinic EPSPs by BK in an S-type myenteric neurone. (a) BK (100 nM for 1.5 – 2 min) suppressed the fast EPSP and pretreatment with 300 nM HOE-140 offset the action of BK. (b) Pretreatment with the cyclooxygenase inhibitor piroxicam (60 μM) offset the inhibitory action of 100 nM BK on the fast EPSP. Application of the nicotinic receptor antagonist hexamethonium (100 μM) reversibly suppressed the fast EPSP in the presence of piroxicam. Downward deflections in (a,b) are stimulus artifacts. (c) Micropressure pulses (20 ms) of ACh evoked depolarizing responses that mimicked the nicotinic properties of the fast EPSP. BK (100 nM) did not suppress the ACh-evoked responses.

organization that results from actions at the level of single neurons in the networks cannot be predicted with full confidence. Nevertheless, changes undoubtedly occur within the neural microcircuits that influence the functional state of the whole organ. BK levels are known to be elevated in intestinal inflammatory states (Zeitlin & Smith, 1973) and this raises the question of how much of the altered behavior of the inflamed bowel reflects neuronal actions of BK.

Full resolution of the ionic mechanisms of the excitatory actions of BK at the single neuronal level will require detailed studies of the underlying ionic currents that are beyond the scope of the present study. Nevertheless, results from the present study suggest that the ionic mechanism of action differs for AH- and S-type myenteric neurones. Increased input resistance associated with BK depolarizing action in AH neurones suggests that the ionic mechanism of depolarizing action includes reduction of a resting K^+ conductance that is known to be present in AH neurones (Grafe *et al*., 1980). Suppression of hyperpolarizing after-potentials by BK in AH neurones suggests that the ionic mechanism of action includes suppression of the Ca^{2+}-gated K^+ conductance known to exist in AH neurones (North, 1973). Decreased input resistance associated with BK depolarizing action in some S-type myenteric neurones suggests that opening of cationic channels is probably involved in the ionic mechanism of action. Absence of any change in input resistance during BK-evoked depolarizing responses in some other S-type neurones may reflect interactive opening and closure of different species of cationic channels.

Aside from its depolarizing action, the presence of BK suppressed both fast and slow EPSPs. Persistence of nicotinic depolarizing responses to exogenously applied ACh, while BK suppressed nicotinic fast EPSPs, suggests that the action of BK on fast EPSPs was presynaptic inhibition of ACh release. Serotonin, substance P and ACh are putative neurotransmitters for slow EPSPs in guinea-pig small intestinal myenteric plexus (Katayama & North, 1978; Wood & Mayer, 1979). The presence of atropine throughout our study suggested that the slow EPSPs were not muscarinic cholinergic. Persistence of slow EPSP-like depolarizing responses to exogenously applied serotonin or substance P, while BK suppressed slow EPSPs, suggests that the action of BK on slow excitatory neurotransmission was presynaptic inhibition of the release of neurotransmitter/s for the slow EPSP.

Suppression of fast and slow EPSPs by BK was reduced or abolished by the presence of the selective B_2 receptor antagonist HOE-140 in the bathing solution. Action of HOE-140 together with failure of selective B_1 receptor antagonists to suppress BK-evoked inhibition of synaptic transmission suggests that the presynaptic inhibitory action of BK was mediated by the B_2 receptor. Taken together, the evidence suggests that the BK B_2 receptor is expressed by

ganglion cells in the myenteric plexus. Our failure to find evidence for expression of the B_1 receptor is consistent with the conclusions of others that the B_2 receptor is the only functional BK receptor found in the guinea-pig (Kachur *et al*., 1987; Pruneau *et al*., 1995; Seabrook *et al*., 1997).

Neither the excitatory action of BK at the cell bodies of myenteric neurones nor the inhibitory action on neurotransmission occurred after blockade of synthesis of prostaglandins. Moreover, exogenous application of prostaglandins E_2, D_2, $F_{2\alpha}$ or I_2 in our study and the action of prostaglandins E_2 and D_2 reported by others (Dekkers *et al*., 1997; Frieling *et al*., 1994c) mimicked both the excitatory actions of BK at the ganglion cell soma and the BK action to suppress neurotransmission. These findings suggest that both actions of BK were mediated by the release of prostaglandins. Others who studied the actions of BK in the intestinal tract reached similar conclusions (Yau *et al*., 1986; Griesbacher, 1992; Rangachari *et al*., 1993; Gelperin *et al*., 1994). Gelperin *et al*. (1994) concluded that neuronal release of prostaglandins mediated the action of BK to elevate cytosolic Ca^{2+} in cultured rat myenteric neurones. Our evidence suggests that this is the case also for the longitudinal-muscle-myenteric plexus preparation from the guinea-pig small intestine. We found, with binding of the fluorescently tagged B_2 receptor antagonist HOE741, that the B_2 receptor was expressed exclusively by neurones in longitudinal-muscle-myenteric plexus preparations. The combined evidence suggests that BK acts at the BK B_2 receptor subtype on myenteric neurones to stimulate the formation and release of prostaglandins from the neurones. Once released, the prostaglandins may feedback onto the same neurone and may also diffuse in the extracellular milieu to influence the behavior of neighboring neurones; the primary influences on neuronal behavior being elevation of neuronal excitability and presynaptic inhibition of release of neurotransmitters.

Measurements by Goldstein *et al*. (1983) and Yau *et al*. (1986) of BK-evoked release of ACh from intestinal segments in organ baths *in vitro* might appear to contradict our finding that exposure to BK suppressed cholinergic neurotransmission. The fact that neurotransmitter release occurs at two sites in the ENS explains the apparent contradiction. One site is at neuronal – neuronal synapses in the integrative microcircuits. The second site is from long varicose motor axons at neuro-effector junctions including neuro-muscular junctions, neuro-epithelial junctions and neuro-vascular junctions. Release at the neuro-effector junctions is *en passant* from multiple varicosities along each motor axon. The amounts of neurotransmitter released at neuro-effector junctions by neuronal excitatory agents or experimental transmural electrical stimulation, and therefore that can be collected as 'spill-over' from segments of intestine into bathing solutions *in vitro,* are undoubtedly several orders of magnitude greater than the component of release that originates at neuronal – neuronal synapses. Our results show that the BK_2 receptor is expressed by most enteric neurons including motor neurons. The results show also that motor neurons are stimulated by BK to fire at high frequencies and this activity is expected to be transformed into release of neurotransmitters at their junctions with the effector systems during application of bradykinin. Stimulated firing of cholinergic motor neurons can account for the massive release of neurotransmitter that must occur from intestinal segments in order to be detected in the organ bath. A logical interpretation is that BK-evoked release of prostaglandins does not lead to suppression of neurotransmitter release from the multiple axonal varicosities that form the neuro-effector junctions, while at the same time BK exposure does lead to suppression of neurotransmission at the synaptic connections within the microcircuits. Earlier findings that prostaglandins do not suppress muscular contractile or chloride secretory responses to transmural electrical stimulation of intestinal segments *in vitro* indicates absence or reduced activity at neuroeffector junctions; whereas, results in the present manuscript clearly demonstrate that neurotransmission at neuron-to-neuron synapses in the integrative neural networks is suppressed by prostaglandins.

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