Action of Bradykinin in the Submucosal Plexus of Guinea Pig Small Intestine

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ABSTRACT

Intracellular recording methods with “sharp” microelectrodes were used to study actions of bradykinin (BK) on electrical behavior of morphologically identified neurons and the identification and localization of BK receptors in the submucosal plexus of guinea pig small intestine. Exposure to BK depolarized the membrane potential and elevated excitability in submucosal neurons with AH-type electrophysiological behavior and Dogiel II multipolar morphology and in neurons with S-type electrophysiological behavior and uniaxonal morphology. BK-evoked depolarizing responses were associated with increased neuronal input resistance in AH-type neurons and decreased input resistance in S-type neurons. The selective B2 BK receptor antagonists HOE-140 (icatabant acetate) and WIN64338 [(S)-4-[2-bis(cyclohexylamino)methyleneamino]-3-(2-naphthalenyl)-1-oxopropylamino]benzyl tributyl phosphonium chloride hydrochloride, but not the selective B1 receptor agonist Kallidin, but not the selective B1 receptor agonist des-arg9-BK mimicked the excitatory action of BK. Western blot analysis and reverse transcription-polymerase chain reaction confirmed the expression of B2 receptor protein and mRNA. Binding studies with a fluorescently labeled BK2 antagonist found expression of B2 receptors on a majority of the ganglion cells. B2 receptors occupied 82% of the neurons that expressed immunoreactivity for neuropeptide Y, 75% of the neurons that expressed vasoactive intestinal peptide, 84% of the neurons that expressed substance P, 71% of the neurons that expressed choline acetyltransferase, and all neurons that expressed calbindin immunoreactivity. The results suggest that the B2 receptor mediates the excitatory action of BK on submucosal plexus neurons. Pathophysiological significance of the excitatory actions on secretomotor neurons might be stimulated mucosal secretion and the secretory diarrhea associated with intestinal inflammatory states.

Chemical signaling is a mode of physiological communication between the enteric nervous system (ENS) and the enteric immune system (Wood, 2002). Neural networks in the myenteric and submucosal plexuses of the ENS interact to form an independent integrative nervous system that controls intestinal motility, secretion, and blood flow and interact to organize the activity of each of the three effector systems into functional patterns of digestive behavior (Wood, 1994a; Gershon, 1998; Wood et al., 1999). In so doing, the ENS functions in interactive concert with the enteric immune system. Populations of immune/inflammatory cells communicate to form an independent integrative nervous system that controls intestinal motility, secretion, and blood flow and interact to organize the activity of each of the three effector systems into functional patterns of digestive behavior (Wood, 1994a; Gershon, 1998; Wood et al., 1999). In so doing, the ENS functions in interactive concert with the enteric immune system. Populations of immune/inflammatory cells (lymphocytes, granulocytes, and mast cells) surround the ENS and expand in number during pathological inflammatory states (e.g., enteric infections, inflammatory bowel disease, and radiation-induced enteritis). Members of the immune/inflammatory cell populations communicate with the ENS in paracrine manner through the release of chemical mediators (Frieling et al., 1994; Wood, 2002; Liu et al., 2003a). Exposure to the inflammatory mediators histamine, mast cell proteases, prostaglandins, leukotrienes, and cytokines modifies both neuronal excitability and neurotransmission (Nemeth et al., 1984; Frieling et al., 1994; Dekkers et al., 1997; Xia et al., 1999; Gao et al., 2002; Liu et al., 2003a,b).

The present study was focused on bradykinin (BK) as one of the putative mediators formed during inflammatory, ischemic, and hemorrhagic states in the bowel (Zeitlin and Smith, 1973; Brown and Roberts, 2001). It was the second phase of a project to understand how BK formation might alter gut motility and secretion through actions in the ENS. The second phase aimed to study the cellular neuropharmacology of BK on neuronal elements of the submucosal plexus with a view toward potential pathological influence on intestinal secretory function. The earlier phase investigated actions, mechanisms of action, and localization of action to

ABBREVIATIONS: ENS, enteric nervous system; BK, bradykinin; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; Chat, choline acetyltransferase; NPY, neuropeptide Y; SP, substance P; VIP, vasoactive intestinal peptide; I-V, current-voltage; HOE-140, icatabant acetate; HOE-741, fluorescently labeled HOE-140; WIN64338, [(S)-4-[2-bis(cyclohexylamino)methyleneamino]-3-(2-naphthalenyl)-1-oxopropylamino]benzyl tributyl phosphonium chloride hydrochloride.
specific categories of neurons in the myenteric division of the ENS (Hu et al., 2003). Acquisition of information for each of the two plexuses individually was advised because the functional neurobiology differs for each plexus. Examples of differences include the segregation of motor neurons and specific reflex behaviors between the two plexuses. Excitatory and inhibitory musculomotor neurons are located in the myenteric plexus, and secretomotor neurons are in the submucosal plexus. Reflex circuits for propulsive motility are "hard-wired" into the myenteric plexus (Smith et al., 1990; Kunze and Furness, 1999). Reflex circuitry for the control of intestinal secretion is incorporated in the neural networks of the submucosal plexus (Frieling et al., 1992; Cooke, 2000). Earlier studies in the myenteric plexus found excitatory actions of BK on neurons with AH-type 2 electrophysiological behavior, Dogiel type II multipolar morphology, and immunoreactivity for calbindin and on neurons with S-type electrophysiological behavior, uniaxonal Dogiel type I morphology and immunoreactivity for nitric-oxide synthase (Hu et al., 2003). Calbindin-positive Dogiel type II neurons predominate in the myenteric plexus and are scarce in the submucosal plexus (Brookes 2001a,b). Nitric oxide-positive uniaxonal neurons that express nitric-oxide synthase and project their axon in the aboral direction within the myenteric plexus are identified as inhibitory motor neurons to the musculature (Brookes, 2001a,b). Stimulation of excitability in this neuronal population suppresses contractile activity. A subpopulation of uniaxonal neurons that express immunoreactivity for choline acetyltransferase, neuropeptide Y, and vasoactive intestinal peptide in the submucosal plexus are identified as secretomotor neurons (Bornstein and Furness, 1988). Elevated activity in this neuronal population increases mucosal secretion of H2O and electrolytes, which when exaggerated, leads to neurogenic secretory diarrhea (Cooke, 1994). One of the aims of the present study was to evaluate actions of BK on secretomotor neurons in the submucosal plexus.

Bradykinin B1 and B2 receptors mediate the actions of BK. Both receptors have been defined pharmacologically by use of specific peptide and nonpeptide antagonists. DNA that encodes functional BK B2 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., 1996; Farmer et al., 1998). Bradykinin binds preferentially to the B2 BK receptor and the related kinins generated by cleaving the terminal arginine residue from kallidin and BK (e.g., des-arg10-kallidin, and des-arg9-bradykinin) bind preferentially to the BK B1 receptor. An aim of the present study was to identify the kind of BK receptor expressed by neurons in the submucosal plexus.

Materials and Methods

Adult male Hartley-strain guinea pigs (300–350 g) were stunned by a sharp blow to the head and exsanguinated from the cervical vessels according to protocols approved by The Ohio State University Laboratory Animal Care and Use Committee and United States Department of Agriculture Veterinary Inspectors. Submucosal plexus preparations were obtained from segments of small intestine removed 10 to 20 cm proximal to the ileocecal junction. The segments were microdissected for electrophysiological recording and fluorescence immunohistochemistry as described previously (Frieling et al., 1991).

Electrophysiology. The preparations were placed in 2-ml chambers and superfused with Krebs' solution warmed to 37°C and gassed with 95% O2/5% CO2 (pH 7.3–7.4), at a rate of 10 to 15 ml min−1. The composition of the Krebs solution was 120 mM NaCl, 6 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 1.35 mM NaH2PO4, 14.4 mM NaHCO3, and 11.5 mM glucose. Transmembrane electrical potentials were recorded with conventional "sharp" intracellular microelectrodes filled with 4% biocytin (Sigma-Aldrich, St. Louis, MO) in 2 M KCl or potassium acetate containing 0.05 M Tris buffer (pH 7.4) and having resistances of 80 to 200 MΩ. Methods for electrophysiological recording of electrical behavior in submucosal neurons and procedures for development of the intraneuronal marker biocytin were the same as described previously (Liu et al., 2003a).

Reverse Transcriptase-Polymerase Chain Reaction. Total RNA was extracted from submucosal plexus preparations with TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription (1 μg of RNA) was performed by adding the following and incubating at 42°C for 60 min: 2.0 μl of 10× RT buffer (0.1 M Tris, 0.5 M KCl, pH 8.3), 4.0 μl of 25 mM MgCl2, 2.0 μl of deoxynucleotide mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM), 2.0 μl of oligo(dT) primer (0.8 μg/μl), 50 units of RNase inhibitor, 20 units of avian myeloblastosis virus reverse transcriptase (Roche Diagnostics, Indianapolis, IN), and 1 μl of total RNA. Heating to 99°C for 5 min and then cooling to 4°C for 5 min deactivated the enzyme. The following reaction mixture was added to 5 μl of the cDNA product: 25 μl of PCR master mix (Roche Diagnostics), 2.0 μl of primers, and 18 μl of PCR grade sterile water. The primers used were as follows.

1. Upstream: 5'-GGTTCTGTTGGGTGCCTGTATT-3'
2. Downstream: 5'-CAGGAGGACATTGGTGAACAC-3'
3. Upstream: 5'-TATTCCTGGTGGGTGCTGTAC-3'
4. Downstream: 5'-GGAGGCTACCAGTGGAGGA-3'

Primers 1 and 2 were designed from the mRNA of a guinea pig BK B2 receptor (GenBank accession no. NM_007539) and rat BK B2 receptor (GenBank accession no. U66107). Amplification was done with an iCycler (Bio-Rad, Hercules, CA). 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 2% agarose gel. Sequencing was done with an ABI 373XL DNA sequencer after the PCR product was purified with Qiaquick PCR purification kit (QIAGEN, Valencia, CA). RT-PCR without avian myeloblastosis virus reverse transcriptase was used as a negative control.

Western Blot. Submucosal plexus preparations were prepared as described above, washed in cold Kreb's solution, and stored at −70°C. The Complete Mini kit (Roche Diagnostics) was used to obtain samples of total submucosal plexus protein. Samples of total protein (20–100 μg) were loaded onto 11% sodium dodecyl sulfate polyacrylamide gels and blotted onto nitrocellulose filters. The filters were incubated with antiserum in buffer containing 5% nonfat dairy milk for 4 h at room temperature. A rabbit polyclonal antibody specific for the B1 bradykinin receptor (1:1000 code: SC-15048; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or a monoclonal B2 receptor antibody (1:1000 code: 610451; BD Transduction Laboratories, San Diego, CA) was used. The filters were then incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Antibody labeling was detected by chemiluminescence. Color molecular weight standards were run on each gel.

Immunohistochemistry. Whole-mounts of the submucosal plexus were incubated in 10% normal horse serum in PBS for 1 h at room temperature before exposure to the primary antiserum diluted in hyper-
tonic PBS containing 10% normal horse serum, 0.3% Triton X-100, and 0.1% sodium azide. The preparations were placed in humidified chambers and processed for indirect single- or double immunofluorescence staining. Incubation for 18 h at room temperature in rabbit or mouse antisera or a mixture of primary antibodies from different species to achieve double labeling. The primary antibodies used were mouse anti-B2 receptor (1:1000) (code 610451; BD Transduction Laboratories), rabbit anti-B2 receptor (1:1000) (code AS276; kindly provided by Prof. Müller-Esterl, Frankfurt, Germany), mouse anti-HuC/HuD neuronal protein (1:2000) (code A21271; Molecular Probes, Eugene, OR), goat anti-choline acetyltransferase (ChAT) (1:100) (code AB 144P; Chemicon International, Temecula, CA); sheep anti-neuropeptide Y (NPY) (1:3000) (code AB1583; Chemicon International), rat anti-substance P (SP) (1:2000) (code MAB356; Chemicon International), rabbit anti-vasoactive intestinal peptide (VIP) (1:100) (code IHC7161; Peninsula Laboratories, Belmont, CA), and goat anti-calretinin (1:5000) (code AB1550; Chemicon International). The preparations were incubated for 24 h in the primary antibody at room temperature followed by 3 × 1-min washes with PBS and then incubated in fluorescein isothiocyanate-labeled donkey anti-rabbit secondary IgG (code 711-095-152), donkey anti-sheep secondary IgG (code 713-095-147), donkey anti-rat secondary IgG (code AP189F) or donkey anti-goat secondary IgG (code 705-095-147), and Cy3-labeled donkey anti-rabbit secondary IgG (code 711-165-152) or donkey anti-mouse secondary IgG (code 715-165-150) for another 30 min at 37°C followed by 3 × 10-min washes with PBS. All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The preparations were cover-slipped in Vectorshield (Vector Laboratories, Burlingame, CA). Fluorescent labeling was examined with a Nikon Eclipse E-600 fluorescent microscope that was equipped with appropriate filters and a SPOT-2 chilled color and BW digital camera (Diagnostic Instruments, Sterling Heights, MI).

Drugs and Chemicals. Pharmacological agents were applied either by addition to the bathing solution or by micropressure ejection from micropipettes (10–20-μm tip diameter) positioned within 200 μm of the impaled neuron. Tetrodotoxin, N-methyl-d-glucamine, choline chloride, BK, des-arg⁹-BK, des-arg⁹-leu⁶-BK, indomethacin, calbindin, and picroxican were obtained from Sigma-Aldrich. HOE-140 and des-arg⁹-HOE-140 were purchased from Sigma/RBI (Natick, MA). WIN64338 was purchased from Tocris Cookson Inc. (Ballwin, MO). HOE-741 was obtained from PerkinElmer Life Sciences (Boston, MA). For experiments involving low Na⁺ Krebs’ solution, Na⁺ was substituted with equal molar N-methyl-d-glucamine or choline chloride. The substitution reduced the Na⁺ concentration of the bathing solution from 146.2 to 26.2 mM.

Data Analysis. Data are presented as means ± S.E.M. Concentration-response curves were constructed using the following least-squares fitting routine. 

\[ V = V_{	ext{max}}/[1 + (EC_{50}/C)^{n_{H}}] \]

where \( V \) is the observed membrane depolarization, \( V_{\text{max}} \) is the maximal response, \( C \) is the corresponding drug concentration, \( EC_{50} \) is the concentration that evoked a half-maximal response, \( n_{H} \) is the apparent Hill coefficient. Antagonist concentration-inhibition curves were obtained in individual cells by using a fixed concentration of BK (30 nM), and progressively increasing the concentration of antagonist. \( IC_{50} \) values were calculated by least-squares fitting to the following equation. 

\[ V = V_{\text{max}}/[1 + (IC_{50}/[Ant])^{n_{H}}] \]

where \( V \) is the observed percentage of inhibition and \( V_{\text{max}} \) is peak percentage of inhibition, \( [Ant] \) is the corresponding antagonist’s concentration, \( IC_{50} \) is the antagonist concentration that produced half-maximal inhibition of BK-evoked responses, and \( n_{H} \) is the apparent Hill coefficient. The graphs in the figures were drawn by averaging results from all experiments and fitting a single concentration-response curve to the pooled data with SigmaPlot software (SPSS Science Inc., Chicago, IL). All \( EC_{50} \) and \( IC_{50} \) values are the mean ± S.E.M.

Results

Identification of the Submucosal B₂ Receptor. An RT-PCR product with 500 nucleotide base pairs was amplified by a pair of specific primers for the guinea pig BK B₂ receptor (Fig. 1A, lane 2). No RT-PCR product was amplified for the B₁ receptor (Fig. 1A, lane 3). The positive control (Fig. 1B, lane 3) was obtained from a rat pituitary protein extract. The difference in molecular mass of 3 kDa between the rat and guinea pig BK B₂ receptor accounts for differences seen on Western blots (SWISS-PROT: O70526 for guinea pig and P25023 for rat). Expression of B₁ receptor protein was not detected in submucosal plexus extracts (Fig. 1B, lane 1).

BODIPY 576/579 is a commercially available fluorescent probe that can be bound to a variety of compounds, including the selective BK B₂ receptor antagonist HOE-140. HOE-741 is the BODIPY 576/579 tagged form of HOE-140. We used 30 nM HOE-741 as a tool for localizing BK B₂ receptor expression with specific primers for the B₁ receptor is in lane 3. B, Western blot analysis with a polyclonal antibody to the bradykinin B₁ receptor found no evidence for expression of the B₁ receptor (lane 1). Analysis with a monoclonal bradykinin B₂ receptor antibody generated a 42-kDa band. Lane 3 is a positive B₂ receptor control obtained for rat pituitary gland. The known difference in molecular mass of 3 kDa between the rat and guinea pig BK B₂ receptor accounts for differences seen on Western blots (SWISS-PROT: O70526 for guinea pig and P25023 for rat). Expression of BK B₂ receptor protein was not detected in submucosal plexus extracts (Fig. 1B, lane 1).
lective B2 antagonist HOE-140 before exposure to HOE-741 blocked binding of HOE-741 to the B2 receptor. B, preincubation with the intestinal submucosal plexus. A, localization of HOE-741 binding to neurons were labeled with HOE-741. Colocalization of binding with calretinin immunoreactivity was not active neurons were labeled by HOE-741 and I, immunoreactivity for VIP colocalized with HOE-741 fluorescence.

Most of the neurons (74.6%; 1894/2540) with immunoreactivity for VIP also expressed the B2 receptor (Fig. 2, G–I). A large number of SP-immunoreactive neurons (83.6%; 424/507) and all calbindin-immunoreactive neurons were labeled by HOE-741 and 70.5% (2403/3409) of the ChAT-immunoreactive neurons were labeled with HOE-741. Colocalization of HOE-741 binding with calretinin immunoreactivity was not found (data not shown).

Indirect immunofluorescence with a mouse anti-B2 receptor antibody (code 610451; BD Transduction Laboratories) was used to study the distribution pattern of the BK B2 receptor and as a control for the results with HOE-741. The distribution pattern for the B2 receptor with the anti-B2 receptor antibody was similar to that found with HOE-741 (Fig. 3). All NPY neurons (n = 2135) expressed BK B2 receptor immunoreactivity (Fig. 3, A–C). NPY-immunoreactive neurons are known to be a subset of cholinergic neurons in the guinea pig submucosal plexus (Brookes, 2001a,b). Double labeling of BK B2 receptor immunoreactivity with ChAT and VIP showed localization of the B2 receptor with both ChAT- (data not shown) and VIP-positive neurons (Fig. 3, D–F). Ninety-nine percent of neurons in the guinea pig submucosal ganglia express ChAT or VIP (Brookes, 2001a,b). Our finding that the BK B2 receptor is colocalized with both ChAT and VIP might suggest that 99% of the neuronal population in the submucosal plexus express the B2 receptor.

As a separate control, we also used a rabbit polyclonal B2 receptor antibody (code AS276) that was characterized by Reyes-Cruz et al. (2000). The same immunostaining pattern for distribution of the B2 receptor was obtained with this antibody as with the mouse antibody described above (data not shown).

Electrophysiology. Submucosal neurons were classified as AH- or S-type based on generally accepted criteria (Bornstein et al., 1994; Wood, 1994a). Application of BK (0.3–300 nM) by addition to the superfusion solution for 50 s evoked a concentration-dependent depolarization of the membrane potential coincident with enhanced excitability in 23 of 26 AH-type neurons and 59 of 71 S-type neurons (Fig. 4A). Depolarizing responses in AH-type neurons were associated with an increase in neuronal input resistance and with decreased input resistance in S-type neurons. The electrophysiological results were consistent with the histological finding that most submucosal ganglion cells express the BK receptor. The depolarizing responses began after a 5- to 10-s delay and peaked in 2.5 to 3 min. Enhanced excitability was reflected by an increased number of action potentials evoked by intraneuronal injection of constant current depolarizing pulses, anodal break excitation at the offset of hyperpolarizing current pulses, and the occurrence of spontaneous spike discharge. Suppression of the characteristic hyperpolarizing after-potentials in AH-type neurons was another action of BK (data not shown). The effects of BK were fully reversible after a 10- to 20-min washout period. Time for recovery during washout was directly related to the concentration. No apparent desensitization was observed with repeated exposures at 10- to 20-min intervals. Bradykinin-evoked responses persisted when 300 nM tetrodotoxin was coapplied with BK in seven S-type neurons. Likewise, reduction of Ca2+ to 0.5 mM and elevation of Mg2+ to 12.5 mM in the bathing solution did not suppress the amplitude of BK-evoked depolarizing responses. Tetrodotoxin blocks generation of action potentials in ENS axons and the axonal release of neurotransmitters (Wood, 1994b). Elevated Mg2+ and reduced Ca2+ prevent Ca2+ entry into axon terminals and thereby suppress the release of neurotransmitters. Failure of tetrodotoxin or reduced Ca2+ and elevated Mg2+ to alter the excitatory responses to BK was indicative of a direct action at receptors on the ganglion cell rather than an indirect action due to exci-
tation of neighboring neurons that provided synaptic input to the recorded ganglion cell.

**Concentration-Response Relations (S-Type Neurons).** Concentration-response relations for BK-evoked depolarizing responses were determined only for S-type neurons due to scarcity of AH-type neurons in the guinea pig submucosal plexus.

Threshold concentration for a measurable effect of BK on the membrane potential was 0.1 to 3 nM. The EC$_{50}$ was 2.7 ± 0.4 nM for seven S-type neurons and the maximal depolarizing response was 18.2 ± 2.5 mV (n = 9 S-type neurons) evoked by 300 nM BK (Fig. 4, A and C). The actions of the selective BK B$_2$ receptor antagonists des-arg$^9$-leu$^8$-BK and des-arg$^{10}$-HOE-140 were compared with the actions of the selective BK B$_1$ receptor antagonists HOE-140 and a nonpeptide B$_2$ receptor antagonist, WIN64338. HOE-140 (1–300 nM) concentration dependently suppressed the depolarizing action of 30 nM BK with an IC$_{50}$ value of 31.0 ± 6.6 nM for eight S-type neurons (Fig. 5, A–A$_7$). The threshold concentration for HOE-140 was 1 to 3 nM. The action of HOE-140 was long-lasting, requiring up to 40 min of washout for full recovery. WIN6438 (0.1–3 μM) also suppressed BK-evoked depolarization in concentration-dependent manner with an IC$_{50}$ value of 0.7 ± 0.1 μM in seven S-type neurons. The IC$_{50}$ value for WIN6438 was 20-fold higher than for HOE-140 (Fig. 5E). Neither 300-nM des-arg$^9$-leu$^8$-BK in five ganglion cells nor 300 nM des-arg$^{10}$-HOE-140 in seven ganglion cells suppressed BK-evoked responses. The selective BK B$_2$ receptor agonist kallidin (10 nM–3 μM) acted to depolarize the membrane potential with an EC$_{50}$ value of 298.9 ± 61.5 nM for five S-type neurons. On the other hand, the selective B$_1$ receptor agonist des-Arg$^8$-BK was ineffective in concentrations of 1 to 3 μM in nine neurons (Fig. 5C).

**Ionic Mechanisms.** Depolarizing responses evoked by BK (0.3–300 nM) were associated with increased neuronal input resistance in 22 of 75 neurons and with decreased input resistance in 39 of 75 neurons. Input resistance was unchanged in 14 of 75 neurons. The input resistance was decreased or unchanged in S-type neurons with uniaxonal morphology and increased in AH-type neurons with multipolar morphology.

Figure 6A shows the current-voltage (I-V) relationship for an S-type neuron in which the depolarizing responses to BK were associated with decreased input resistance. The I-V curves obtained in the absence and presence of BK intersected at 8 mV, which is the estimated reversal potential for the depolarizing response. The mean extrapolated reversal potential for the BK-evoked depolarizing responses associated with decreased input resistance was −10.8 ± 8.4 mV (range −28 to +10 mV) for five S-type neurons.

Application of BK by pressure microejection (“puffs”) evoked slowly activating and prolonged depolarizing responses that were similar to responses evoked by bath application (n = 10 neurons). No rapidly activating depolarizing responses similar to nicotinic responses to acetylcholine were ever observed. The prolonged depolarization in response to puffs of BK was associated with the firing of action potentials (n = 7 neurons). The input resistance decreased in most of the neurons tested (6/10) with pressure microejection of 100 nM BK. The amplitude of the BK-evoked depolarizing responses that were associated with decreased input resistance increased when the membrane potential was current-clamped in the hyperpolarizing direction and decreased when the membrane potential was shifted in the depolarizing direction (Fig. 6, C and D).

Electrophysiological behavior of this nature suggested that BK activates a cationic conductance in the S-type neurons. We tested this hypothesis by examining BK-evoked depolarizing responses in bathing solutions in which the concentration of Na$^+$ was reduced by 82% by substitution of NaCl with N-methyl-D-glucamine or choline chloride. The membrane
potential was current-clamped at its original level before application of BK in the studies with reduced Na\(^+\) solution. In low Na\(^+\) solution, the amplitude of depolarizing responses evoked by 100 nM BK were reduced by 50.7 \(\pm\) 5.8\% in seven S-type neurons. BK evoked no spike discharge in low Na\(^+\) solution (data not shown).

Suppression of Ca\(^{2+}\) entry in bathing solutions containing 16 mM Mg\(^{2+}\) and with Ca\(^{2+}\) reduced from 2.5 mM to 1 mM did not significantly reduce the amplitude of the depolarizing responses to BK in S-type neurons. This suggested that the depolarizing responses to BK in S-type neurons might not be dependent on influx of extracellular Ca\(^{2+}\).

Exposure to BK evoked membrane depolarization associated with increased input resistance in 22 AH-type neurons. An I-V relationship for an AH-type neuron in the presence and absence of BK is shown in Fig. 6B.

I-V curves obtained in the absence and presence of 30 nM BK intersected at \(-88\) mV. The mean reversal potential for the depolarizing responses that were associated with increased input resistance was \(-92.0 \pm 4.5\) mV (range \(-85\) to \(-105\) mV) for five AH-type neurons. These values are near the estimated equilibrium potential for K\(^+\) in enteric neurons and may reflect decreased K\(^+\) conductance during the depolarizing responses to BK in AH-type neurons (North, 1973).

**Discussion**

Bradykinin is formed in inflammatory, ischemic and hemorrhagic conditions in the gut (Brown and Roberts, 2001). It is a peptide cleaved from \(\alpha_2\) globulins termed kininogens. Specific proteases called kallikreins release bradykinin from the kininogens (Bhoola et al., 1992). The present studies tested the suggestion that after intramural formation in the intestine, BK might act on the ENS as a paracrine mediator to alter neural control of secretory and motility functions at the organ level.

Excitation of ganglion cells in the submucosal plexus was found to be 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 B1 receptor was obtained. The evidence for BK B2 involvement included blockade of BK-evoked responses by the selective BK B2 receptor antagonist HOE-140, lack of effect of selective BK B1 receptor antagonists, selective binding of fluorescently labeled HOE-741 to submucosal ganglion cells, and expression of the mRNA transcript and receptor protein for the B2, but not the B1 receptor in the submucosal plexus. The evidence is consistent with earlier published reports that the B2 receptor is the sole receptor for BK in the guinea pig (Kachur et al., 1987; Pruneau et al., 1995). A similar conclusion was reached for the mouse where knockout of the B2 receptor eliminated all responses to BK in both in vivo and in vitro preparations (Borkowski et al., 1995; Seabrook et al., 1997).

The B2 receptor, unlike the B1 receptor, is constitutively expressed in most tissues; B2 receptors are not constitutively expressed. Bradykinin B1 receptors are up-regulated by inflammation and cytokines (Dray and Perkins, 1993). This leaves open the untested possibility that enteric neurons might be found to express the B1 receptor if examined in intestinal inflammatory states.

Results from both electrophysiological and histological studies suggested that the majority of neuronal cell bodies in the ganglia expressed the B2 receptor. Bradykinin selectively stimulated excitability in neurons that expressed immunoreactivity for VIP and/or ChAT. VIPergic and cholinergic neurons with S-type electrophysiological behavior and uniaxonal morphology in the submucosal plexus are known to function as secretomotor neurons (Brookes, 2001a,b). Enhanced excitability in the population of secretomotor neurons elevates intestinal mucosal secretion of electrolytes, H2O, and mucus and can underlie the neurogenic secretory diarrhea often associated with inflammatory states and ischemia-reperfusion injury (Cooke, 2000).

Exposure to BK evoked depolarizing responses in submucosal neurons with S-type electrophysiological behavior and uniaxonal morphology, and the depolarization was associated with decreased neuronal input resistance. A reversal potential near −10 mV for the depolarizing responses suggested that the underlying ionic mechanism for the responses in the S-type neurons might be opening of nonselective cationic conductance channels. Findings that the amplitude of the BK-evoked responses was reduced in bathing solution with reduced Na+ and remained unaffected in solutions with reduced Ca2+ and elevated Mg2+ were consistent with elevated Na+ (and perhaps K+) conductance being the ionic mechanism for the depolarizing responses. The responses to BK in S-type neurons were similar to reported actions for substance P, serotonin, and acetylcholine in the submucosal plexus, which were interpreted by Shen and Surprenant (1993) to be due to an increase in a cation-selective ("mainly sodium") conductance.

AH-type neurons were not a primary focus for the present study because their numbers in the submucosal plexus were less than 10% of the neuronal population. Nevertheless, both direct and indirect immunofluorescence studies confirmed the expression of BK B2 receptors by submucosal neurons with a combination of AH-type electrophysiological behavior, multipolar morphology, and double labeling with calbindin, which is a chemical code for AH-type neurons in the guinea pig ENS. Bradykinin evoked depolarizing responses in the AH-type neurons and the depolarization was associated with increased neuronal input resistance and reduction in amplitude of the characteristic long-lasting hyperpolarizing afterpotentials in these neurons. The mean reversal potential for the depolarizing responses to BK in AH-type neurons was −92 mV and was characteristic of the depolarizing responses reported for the action of other excitatory mediators (e.g.,

![Fig. 6](https://example.com/fig6.png)
histamine, substance P, and serotonin) on AH-type neurons (Katayama and North, 1978; Wood and Mayer, 1979; Nemeth et al., 1984). The reversal potential of –92 mV in AH-type neurons was close to the estimated K⁺ equilibrium potential and suggested of decreased K⁺ conductance as the ionic mechanism underlying the depolarizing responses. Suppression of the hyperpolarizing after-potentials in AH-type neurons by BK was also characteristic for the action reported for other excitatory mediators, including substance P, histamine, and serotonin (Katayama and North, 1978; Wood and Mayer, 1979; Nemeth et al., 1984).

Bradykinin seems not to be an excitatory neurotransmitter in the submucosal plexus because there is no evidence that BK is stored and released by the ganglion cells. On the other hand, BK-containing neurons were reported to be present in the central nervous system (Correa et al., 1979). Our evidence overall suggests that BK behaves solely as a paracrine mediator in the ENS with excitatory actions on both S- and AH-type neurons in the submucosal plexus. The S-type neurons are primarily secretomotor neurons that receive excitatory synaptic input from their AH-type neighbors (Cooke, 2000). Enhanced excitability in both populations of submucosal neurons by BK is a mechanistic explanation for reports of stimulated mucosal secretion during exposure to BK (Kachur et al., 1987; Gaginella and Kachur, 1989). The pathophysiological significance of the neuronal actions of BK can therefore be related to the secretory diarrhea associated with intestinal inflammatory states in which the BK levels increase and the BK B₂ receptor is overexpressed (Stadnicki et al., 1998).

References

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