Metabotropic Signal Transduction for Bradykinin in Submucosal Neurons of Guinea Pig Small Intestine

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Received August 27, 2003; accepted December 18, 2003

ABSTRACT

Intracellular recording methods with "sharp" microelectrodes were used to study signal transduction mechanisms underlying the excitatory action of bradykinin (BK) in morphologically identified neurons in the small intestinal submucosal plexus. Exposure to BK evoked slowly activating membrane depolarization and enhanced excitability associated with increased input resistance in AH-type and decreased input resistance in S-type neurons. Preincubation with pertussis toxin did not affect the BK-evoked responses. Pretreatment with the cyclooxygenase inhibitors indomethacin or piroxicam suppressed or abolished the BK-evoked responses. Application of prostaglandin (PG) E2 or PG analogs evoked BK-like depolarizing responses in the submucosal plexus with a potency order of PGE2 > PGE1, > 17-phenyl trinor-PGE2, > PGI2, > sulprostone > PGF2α. Depolarizing responses to bradykinin or PGE2 in S-type neurons were suppressed in the presence of the phospholipase C inhibitor U73122 [(1-6-[[[17β]-3-methoxyestra-1,3,5[10]-tren-17-71)amino]hexyl)-1H-pyrole-2,5-dione]], but not the inactive analog U73343 [(1-6-[[[17β]-3-methoxyestra-1,3,5[10]-tren-17-71)amino]hexyl]-2,5-pyrolinedione)]. The inositol-1,4,5-trisphosphate receptor antagonist 2-aminoethoxy-diphenylborane and the calmodulin inhibitor W-7, but not ryanodine, suppressed both bradykinin- and PGE2-evoked responses. KN-62, an inhibitor of calmodulin kinases, or GF109203X, a specific protein kinase C inhibitor, suppressed both BK- and PGE2-evoked depolarizing responses. Selective protein kinase A inhibitors did not alter BK- or PGE2-evoked depolarizing responses in S neurons. The results suggest that BK stimulates synthesis and release of PGE2, which acts at EP1 receptors to evoke depolarizing responses in submucosal neurons. The postreceptor transduction cascade includes activation of phospholipase C, inositol-1,4,5-trisphosphate production, intraneuronal Ca2+ mobilization, activation of protein kinase C and/or calmodulin kinases, and phosphorylation of cationic channels.

Exposing neurons in the guinea pig small intestinal myenteric or submucosal plexus to bradykinin (BK) in vitro evokes slowly activating depolarization of the membrane potential and enhanced excitability characterized by increased firing frequency during intraneuronal injection of depolarizing current pulses in both AH- and S-type neurons and the appearance of anodal break excitation at the offset of hyperpolarizing current pulses in AH neurons (Hu et al., 2003, 2004). The depolarizing responses are associated with increased input resistance (i.e., decreased conductance) in AH neurons and with decreased input resistance (i.e., increased conductance) in S neurons. Selective BK B2 receptor antagonists, but not BK B1 antagonists, suppress the actions of BK in both myenteric and submucosal plexuses (Hu et al., 2003, 2004). Binding studies with a fluorescently labeled, selective BK B2 receptor antagonist reveal expression of BK B2 receptors on a majority of the ganglion cells in the myenteric and submucosal plexuses. RT-PCR and Western blot analysis confirms the expression of BK B2 receptor mRNA and protein in both plexuses (Hu et al., 2003, 2004).

Inhibition of cyclooxygenase suppresses the excitatory action of BK in the myenteric plexus (Hu et al., 2003). Application of the prostaglandins E2, D2, F2α, or I2 mimics the

ABBRévIATiONS: BK, bradykinin; RT-PCR, reverse transcription-polymerase chain reaction; CaMK, calmodulin kinase; PBS, phosphate-buffered saline; IP3, inositol-1,4,5-trisphosphate; bp, base pair; L-NAME, N-nitro-L-arginine methyl ester; PTX, pertussis toxin; CPA, cyclopiazonic acid; 2-APB, 2-aminoethoxy-diphenylborane; IPSP, inhibitory postsynaptic potential; EP, receptor for prostaglandin E2; EP1, receptor for prostaglandin F2α; PLC, phospholipase C; CaM, calmodulin; PKC, protein kinase C; EPSP, excitatory postsynaptic potential; PKA, protein kinase A; U73122, (1-6-[[[17β]-3-methoxyestra-1,3,5[10]-tren-17-71)amino]hexyl]-1H-pyrole-2,5-dione); W-7, (N-(aminohexyl)-S-chloro-1-naphthalesulfonyl)alanine hydrochloride; KN-62, (1-N,0-bis[5-isouquinolinesulfonyl]-N-methyl-L-tyrosyl]-4-phenylpiperazine; GF109203X, bisindolylmaleimide I; CGP37157, (7-chloro-S-(2-chlorophenyl)-1,5-dihydro-4, 1-benzothiazepin-2(3H)-one, KT5720, (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-12-epoxy-1-H-diindolol[1,2,3-fg: 3',1',kll]pyrrolo[3,4-f][1,6]benzodiazocine-10-carboxylic acid, hexyl ester); WIN64338, (S)-4-[2-bis(cyclohexylamino)methyleneaminio][3-2-naphthalenyl][1- oxopropyl]amino]benzyl tributyl phosphonium chloride hydrochloride; SC19220, (8-chloro-dibenzo[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-acetylhydrazide).
BK-evoked responses in the myenteric plexus. The evidence suggests that bradykinin acts at BK B₂ receptors on myenteric neurons to stimulate the formation of prostaglandins. Once formed and released, the prostaglandins act to elevate the excitability of the same ganglion cells from which they are released and to diffuse and excite neighboring ganglion cells.

The present study aimed to compare BK signaling mechanisms in the submucosal plexus with earlier findings reported for the guinea pig small intestinal myenteric plexus (Hu et al., 2003). A second aim was to use neuropharmacological tools to obtain insight into the signal transduction pathways associated with BK B₂ receptor activation in enteric neurons.

Materials and Methods

Adult male Hartley-strain guinea pigs (300–350 g) were stunned by a sharp blow to the head and immediately 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. Segments of small intestine 5 to 10 cm in length were removed 20 cm proximal to the ileocecal junction. Methods for intracellular electrophysiological recording from morphologically identified submucosal neurons and immunohistochemical methods for localization of chemical codes were the same as described previously (Hu et al., 2004). Primary antibodies used in the immunohistochemical studies were mouse anti-CaMKII (1:200) (code C265; Sigma-Al- drich), rabbit anti-CaMKIV (1:500) (code C2851; Sigma-Aldrich), goat anti-choline acetyltransferase (1:100) (code AB 114P; Chemicon International, Temecula, CA), and rabbit anti-vasoactive intestinal peptide (1:100) (code IHC7161; Peninsular Laboratories, Belmont, CA). The preparations were incubated in the primary antibody at room temperature for 24 h followed by 3 × 10-min washes with phosphate-buffered saline (PBS) and then incubated in fluorescein isothiocyanate-labeled donkey anti-rabbit secondary IgG (code 715-165-150) for another 30 min and 3 × 10-min washes with PBS. All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

RT-PCR methods were the same as described previously (Hu et al., 2003). The primers used were as follows: inositol-1,4,5-trisphosphate (IP₃) (5'-CCGTTTCAACGAGCAGAAGGA-3', antisense 5'-CATCGGTGATGTCCTGGTCC-3', IP₃ (24 bp) (BankGen accession nos. NM_009805 and XM-010943), sense 5'-GGACCTGGACCTTGGCAGATGCT-3', antisense 5'-GATCAAGACCGAAGTGGACG-3', IP₃ (54 bp) (BankGen nos. NM_009805 and XM-010943), sense 5'-GGCCAGGGCATGCCAGATCC-3', antisense 5'-ACCGGCATCCCCGACTGTCT-3', IP₃ (65 bp) (BankGen nos. NM_013138 and XM-014015), sense 5'-CTCAAGGAGAAGTGCCTGAACC-3', antisense 5'-AGAAGAAGTGCCTGAAGTCC-3', calmodulin kinase (CaMKII) (396 bp) (BankGen nos. NM-009792 and NM-015981), sense 5'-CATGTCAGGAGAATGCTGAGAA-3', antisense 5'-CTGTGAAGTGCAGGAGATCTG-3', CaMKII (I10) (394 bp) (BankGen nos. NM-007595 and NM-001120), sense 5'-GCTGCCTGTTCCATGTCATC-3', antisense 5'-TAACAGAATGCTGGTAAATCTG-3', and CaMK IV (619 bp) (BankGen nos. J03057 and NM-001744), sense 5'-GAGGTTGTTGCTGATTGGGACGG-3', antisense 5'-CCACCGGGGGAGATGAAGTG-3'.

Pharmacological agents were applied by either addition to the Krebs’ bathing solution or by pressure ejection from micropipettes (10–20-μm tip diameter) positioned within 200 μm of the impaled neuron. Bradykinin, prostaglandin E₂, prostaglandin D₂, indomethacin, piroxicam, prostaglandin F₂α, prostaglandin I₂, Nω-nitro-l-arginine methyl ester (l-NAME), and nitric oxide antagonist phentolamine (PTX), U73122, and tetrodotoxin (TTX) were purchased from Sigma-Aldrich, 2-Aminoethoxydiphenylborane (2-APB), CPG37157, GF109203X, KT5720, WIN56484, and phentolamine were purchased from Tocris Cookson Inc. (Ballwin, MO). KN-62 and myristoylated PKI (14-22) amide were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Data are presented as means ± S.E.M. Concentration-response relationships were constructed using the following least-squares fitting routine. V = Vₘₐₓ[1 + (EC₅₀/Cᵢₖₐₜ)]², where V is the observed membrane depolarization, Vₘₐₓ is the maximal response, C is the corresponding drug concentration, EC₅₀ is the concentration that evokes the half-maximal response, and n₉₅ is the apparent Hill coefficient. All EC₅₀ and IC₅₀ values are given as the mean ± S.E.M. Student’s t test was used for statistical analysis of significance of differences in the means with P < 0.05 accepted as significant.

Results

Exposure to BK evoked BK B₂ receptor-mediated depolarizing responses and enhanced excitability (Hu et al., 2003). Involvement of PTX-sensitive G proteins in the BK B₂-evoked excitatory responses was investigated by incubating submucosal plexus preparations with PTX (2 μg ml⁻¹) for 12 h before recording electrophysiological responses of the neurons to BK (Fig. 1). Incubation in PTX did not alter the responses to BK. Exposure to 100 nM BK evoked depolarizing responses of 15.1 ± 3.6 mV in eight PTX-treated preparations and 16.8 ± 0.6 mV for 12 untreated preparations (P > 0.05; Fig. 1, E–G).

Focal electrical stimulation of interganglionic fiber tracts in the submucosal plexus evoked characteristic inhibitory postsynaptic potentials (IPSPs) that were abolished by the α-noradrenergic receptor antagonist phentolamine (Fig. 1A). No IPSPs could be evoked in the preparations after incubation in PTX (Fig. 1, B and G). Micropressure application of norepinephrine evoked phentolamine-sensitive IPSP-like responses before incubation in PTX. “Puffs” of norepinephrine did not evoke the IPSP-like responses in the preparations after incubation in PTX. The noradrenergic IPSPs in submucosal neurons are PTX-sensitive and served as a control for effectiveness of PTX to block G protein-coupled responses in the submucosal neurons (Surprenant and North, 1988).

Stimulation of Nitric-Oxide Synthase. We used the nitric-oxide synthase inhibitor l-NAME as a pharmacological tool for testing the hypothesis that BK-evoked synthesis and release of nitric oxide accounted for the excitatory action of BK on the submucosal neurons. The presence of l-NAME (300 μM) in the bathing solution did not suppress the BK-evoked depolarizing responses. Depolarizing responses to 30 nM BK were 12.4 ± 1.2 mV for seven neurons in the presence of l-NAME and were not different from responses of 13.2 ± 0.9 mV in 25 neurons evoked by 30 nM BK in the absence of l-NAME (P > 0.05; Fig. 2D).

Stimulation of Cyclooxygenase. We used the cyclooxygenase inhibitors indometacin and piroxicam for testing the hypothesis that BK-evoked formation of prostaglandins accounts for the excitatory action of BK on the submucosal neurons. The presence of indometacin (60 μM) in the bathing solution reduced the mean amplitude of the BK-evoked depolarizing responses to 26% of the amplitude in the absence of indometacin. The depolarizing responses to 30 nM
BK were $13.8 \pm 0.8$ mV for 13 neurons in the absence of indomethacin and were reduced to $3.6 \pm 0.4$ mV in the presence of indomethacin ($P < 0.001$; Fig. 2D).

Piroxicam (60 μM) in the bathing solution reduced the mean amplitude of the BK-evoked depolarizing responses to 23% of the amplitude in the absence of piroxicam. The depolarizing responses to 30 nM BK were $13.2 \pm 0.9$ mV for 25 neurons in the absence of piroxicam and were reduced to $3.1 \pm 0.6$ mV in the presence of piroxicam ($P < 0.001$; Fig. 2, A and D). Unlike the effects on BK-evoked responses, inhibition of cyclooxygenase by either indomethacin or piroxicam did not change the characteristic slowly activating depolarizing responses to substance P (Fig. 2C).

**Prostaglandin Actions.** Suppression of BK-evoked depolarizing responses by the cyclooxygenase inhibitors suggested that the responses to BK occurred secondary to the release of prostaglandins and their excitatory action on the neurons. We tested this possibility by applying 30 nM PGE$_2$, 30 nM PGD$_2$, 3 μM PGF$_{2\alpha}$, or 3 μM PGL$_2$ to 41 neurons and found that each prostaglandin evoked depolarizing responses associated with action potential discharge that were essentially the same as the responses to BK (Fig. 2B).

Depolarizing responses to PGE$_2$ were concentration-dependent with an EC$_{50}$ value of $5.3 \pm 0.8$ nM ($n = 22$ neurons). Threshold concentration was in the range of 0.3–1.0 nM and the maximal depolarization was $12.0 \pm 0.8$ mV evoked by 100 nM PGE$_2$. The EP$_1$ and EP$_3$ receptor agonists 17-PT-PGE$_2$ and sulprostone mimicked the depolarizing responses and enhanced the excitatory responses evoked by PGE$_2$. Receptors for prostaglandins are named for the natural prostaglandin for which they have the greatest affinity. The receptor type for PGE$_2$ (EP receptor) is subdivided into the subtypes EP$_1$, EP$_2$, EP$_3$, and EP$_4$ receptors based on physiological action and cloning information (Narumiya et al., 1999). The receptor type for PGF$_{2\alpha}$ is the FP receptor and the receptor type for PGL$_2$ is referred to as the IP receptor. The nonspecific EP receptor agonist PGE$_{1\alpha}$, PGL$_{2\alpha}$, an IP receptor agonist and the FP receptor agonist PGF$_{2\alpha}$ each evoked BK-like depolarizing responses. The responses evoked by the prostaglandin analogs were concentration-dependent and the potency order
In A to C were obtained.

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tool to test the hypothesis that stimulation of phospholipase

data for effects of inhibition of prostaglandin formation by indomethacin

substance P in the same neuron were not suppressed by piroxicam. D,

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and coapplication of 10

and the suggestion that the BK-evoked

current do not contribute

min in 10 µM U73122, the mean amplitude of the depolarizing

responses evoked by 100 nM BK was reduced to 47.4 ± 3.1% (P < 0.01) of the amplitude before U73122 in seven neurons (Fig. 3A). The BK-evoked responses for six neurons were abolished after a maximum of 28 min in 10 µM U73122.

Presence of an inactive analog of U73122 (10 µM U73343) in the bathing solution for 30 min before application of 100 nM BK did not change BK-evoked depolarizing responses in six neurons (Fig. 3A).

We studied the effects of U73122 on responses to PGE2 in view of the actions of cyclooxygenase inhibitors to suppress BK-evoked responses and the suggestion that the BK-evoked responses reflected the excitatory action of prostaglandins. U73122 (10 µM) in the bathing solution suppressed the amplitude of PGE2-evoked depolarizing responses to 40.6 ± 8.5% (P < 0.01) of the amplitude before application of the drug in five neurons (Fig. 3B). The inactive analog of U73343 (10 µM) did not affect PGE2-evoked responses in any of five neurons (P > 0.05).

Ca2+ Signaling. The depolarizing responses to BK or PGE2 were not altered significantly by removal of Ca2+ from the extracellular bathing medium. Lack of effect of depletion of extraneuronal Ca2+ suggests that opening of Ca2+ channels and inwardly directed Ca2+ current do not contribute significantly to the membrane depolarization evoked by BK or PGE2.

We used CPA to test the hypothesis that mobilization of intraneuronal free Ca2+ is a step in the postreceptor signal transduction mechanism for BK B2 receptor-evoked depolarizing responses. CPA acts selectively to inhibit the Ca2+-ATPase in intracellular membranes (Seidler et al., 1989). Application of 10 µM CPA did not alter the resting membrane potential of the neurons. After a minimum of 4 min in 10 µM CPA, the mean amplitude of the depolarizing responses evoked by 100 nM BK was reduced to 59.5 ± 4.7% (P < 0.01) of the amplitude before CPA in five neurons. These data are not shown in Fig. 3 due to restrictions on space.

One or more of three primary mechanisms might account for mobilization of intracellular Ca2+ in postreceptor signal transduction cascades (Ferris and Snyder, 1992; Meissner, 1994). Release of Ca2+ triggered by IP3 binding to its receptor

based on EC50 values was PGE2 (5.3 ± 0.8 nM) > PGE1 (30.9 ± 4.3 nM) > 17-PT-PGE2 (94.1 ± 22.5 nM) > PG1 (374.1 ± 39.3 nM) > sulprostone (547.7 ± 162.5 nM) > PGF2α (941.0 ± 240.9 nM).

The EP1 receptor antagonist SC19220 was used to investigate possible involvement of the EP1 receptor subtype in the PGE2-evoked depolarizing responses. Depolarizing response to 1 µM 17-PT-PGE2 were 10.2 ± 1.1 mV for six submucosal S-type neurons in the absence of SC19220. Pre- and coapplication of 10 µM SC19220 suppressed the amplitude of the 17-PT-PGE2-evoked depolarizing responses to 5.9 ± 2.5 mV (P < 0.01). Pre- or coapplication of 10 µM SC19220 likewise suppressed the depolarizing responses to BK. The depolarizing responses to 30 nM BK were 12.8 ± 2.1 mV for five neurons before application of SC19220 and 6.4 ± 1.8 mV (P < 0.01) in the presence of 10 µM SC19220.

Phospholipase C. We used U73122 as a pharmacological tool to test the hypothesis that stimulation of phospholipase C (PLC) is a step in the postreceptor signal transduction mechanism for BK B2 receptor-evoked depolarizing responses and elevated excitability in submucosal neurons. U73122 was chosen for its action as a selective inhibitor of PLC (Taylor and Broad, 1998). Exposure of the submucosal preparations to 10 µM U73122 did not alter the resting membrane potential of the neurons. After a minimum of 4

Fig. 2. Formation and action of prostaglandins. A, piroxicam reversibly suppressed BK-evoked responses. B, exposure to prostaglandins D2, E2, F2α, and I2 mimicked the action of BK. C, depolarizing responses to substance P in the same neuron were not suppressed by piroxicam. D, data for effects of inhibition of prostaglandin formation by indomethacin and piroxicam and for inhibition of nitric-oxide synthase by l-NAME (**, P < 0.001). E, morphology of the AH-type neuron from which the records in A to C were obtained.

Fig. 3. Actions of investigative agents that suppress specific steps in postreceptor signal transduction for the depolarizing action of BK and PGE2 in neurons in guinea pig small intestinal submucosal plexus. Rynanidine was used as a tool to inhibit Ca2+-induced Ca2+ release from intraneuronal membranes. 2-APB was used as an intraneuronal IP3 receptor antagonist. U73122 was used as a selective inhibitor of PLC. U73343 is an inactive analog of U73122 and was used as a control. CGP37157 was used as an inhibitor of mitochondrial Na+/Ca2+ exchange. W-7 was used as an inhibitor of Ca2+-CaM regulated enzyme activity. Numbers of neurons are given in parentheses (**, P < 0.001).
on intracellular membranes is one, a second is Ca\(^{2+}\)/H\(^{+}\)-induced Ca\(^{2+}\)/H\(^{+}\) release from intracellular membrane stores, and the third is release from mitochondria by Na\(^{+}\)/Ca\(^{2+}\)/H\(^{+}\) exchange. We used ryanodine as a tool for studying involvement of Ca\(^{2+}\)/H\(^{+}\) mobilization from intraneuronal membranes in the BK B\(_2\) signal transduction cascade because it is a high-affinity ligand for the Ca\(^{2+}\)/H\(^{+}\)-induced Ca\(^{2+}\)/H\(^{+}\) release channel (i.e., the ryanodine receptor). Ryanodine promotes activation of the channel at low concentrations and blocks the channels when present in concentrations greater than 10 \(\mu\)M (Ehrlich et al., 1994).

We applied a high concentration of ryanodine (10 \(\mu\)M) with 100-nM BK to test the hypothesis that ryanodine release channels are involved in postreceptor signal transduction for the BK B\(_2\) receptor. No significant suppression of BK-evoked responses in the presence of ryanodine was observed in any of 16 neurons (Figs. 3A and 4A). Exposure to 10 \(\mu\)M CGP37157, a drug known to inhibit mitochondrial Na\(^{+}\)/Ca\(^{2+}\)/H\(^{+}\) exchange (Cox and Matlib, 1993), did not alter the action of BK in eight neurons (Fig. 3A).

The selective IP\(_3\) receptor antagonist 2-APB was used to address the question of whether synthesis of IP\(_3\) and its action at intraneuronal IP\(_3\) receptors might be involved in the BK B\(_2\) receptor signal transduction pathway. Application of 100 \(\mu\)M 2-APB did not alter the resting membrane potential of any of seven neurons. 2-APB (100 \(\mu\)M) reduced the amplitude of the depolarizing responses to 100 nM BK to 23.0 \pm 9.3\% (\(P < 0.01\)) of the BK responses before application of 2-APB in seven neurons (Figs. 3A and 4A). The inhibitory action of 2-APB was consistent with involvement of intraneuronal IP\(_3\) receptors in the BK signal transduction cascade. We tested further the hypothesis that synthesis of IP\(_3\) and its binding to intraneuronal IP\(_3\) receptors are steps in the BK B\(_2\) transduction pathway by searching for mRNA transcripts for the receptors with RT-PCR. RT-PCR analysis identified the presence of mRNA transcripts for each of the three types of IP\(_3\) receptors (i.e., IP\(_3\) receptors I, II, and III) in the submucosal plexus preparations (Fig. 4B).

The effects of 2-APB on responses to 100 nM PGE\(_2\) were investigated in view of the suggestion that the BK-evoked responses were secondary to the formation and action of prostaglandins on the neurons. 2-APB (100 \(\mu\)M) in the bathing solution suppressed the amplitude of PGE\(_2\)-evoked depolarizing responses to 26.5 \pm 9.7\% (\(P < 0.01\)) of the amplitude before application of the drug in six neurons (Fig. 3A). The presence of ryanodine (10 \(\mu\)M) in the bathing solution did not affect PGE\(_2\)-evoked responses in five neurons (Fig. 3B).

**Calmodulin Kinases.** Multiple Ca\(^{2+}\)-sensing proteins detect elevation of free cytosolic Ca\(^{2+}\) and become elements in intracellular regulatory pathways for a diversity of cellular responses. Calmodulin (CaM) is one of the important Ca\(^{2+}\) sensing elements. We found CaM immunoreactivity expressed in neurons of the submucosal plexus. The immunoreactivity was concentrated in the cell bodies of most neurons (Fig. 5) with weaker staining in neuronal processes.

We used the membrane permeable calmodulin inhibitor W-7 to test the hypothesis that Ca\(^{2+}\) sensing by CaM is a step in the BK signal transduction cascade. The mechanism of action of W-7 is binding to CaM and inhibition of Ca\(^{2+}\) -CaM regulated enzyme activity (Hidaka et al., 1981). Application of W-7 did not alter the resting membrane potential of the neurons. Pretreatment with W-7 (50 \(\mu\)M) for 15 min reduced responses to 100 nM BK to 5.3 \pm 2.5\% (\(P < 0.001\)) of the amplitude of the depolarizing responses before W-7 in seven neurons.

![Fig. 4. IP\(_3\) receptor and calmodulin, but not ryanodine receptor antagonism, suppressed BK-evoked depolarizing responses in submucosal neurons. A, suppression by IP\(_3\) receptor antagonist by 2-APB and the CaM inhibitor W-7, but not by a high concentration of ryanodine. B, expression of mRNA transcripts for IP\(_3\) receptors I (lane 2), II (lane 3), and III (lane 4) in the submucosal plexus. Lane 1 is 100-bp DNA ladder. C, S-type neuron from which the records in A were obtained.](image-url)
neurons (Figs. 3A and 4A). The responses to BK were abolished in five of the seven neurons. W-7 (50 \mu M) in the bathing solution suppressed the amplitude of depolarizing responses evoked by 300 nM PGE2 to 9.7 ± 5.1% (P < 0.001) of the amplitude before application of the drug in five neurons (Fig. 3B). Washout of W-7 for a minimum of 45 min reversed its inhibitory action on responses to BK or PGE2.

Downstream effects of elevation of cytosolic Ca2+ in intracellular signaling pathways generally include the phosphorylation of proteins and changing of their conformation and activity. CaMKs catalyze the phosphorylation of downstream proteins in signal transduction cascades. CaMKII and CaMKIV are multifunctional kinases known to translate neuronal Ca2+ signals into phosphorylation of channel proteins and intracellular enzymes (Miller and Kennedy, 1986).

We found CaMKIV immunoreactivity in most of the neurons in submucosal ganglia, with condensed fluorescence apparent in the nuclear regions and weak fluorescence in the surrounding cytoplasm (Fig. 5C). The number of neurons expressing CaMKII immunoreactivity was variable from ganglion to ganglion (Fig. 5, B and E). Unlike CaMKIV, immunoreactivity for CaMKII was localized to the cytoplasm. Strong immunoreactivity for CaMKII was localized exclusively to submucosal ganglion cells that expressed immunoreactivity for vasoactive intestinal peptide (VIP) and believed to be secretomotor neurons based on the presence of slow noradrenergic synaptic inputs (Fig. 5, D–F). Much weaker fluorescence was seen in neurons containing choline acetyltransferase (data not shown).

Immunohistochemical evidence for expression of CaMKs in the submucosal plexus was reinforced by results obtained with RT-PCR. RT-PCR identified mRNA transcripts for CaMKIIα, CaMKIIβ, and CaMKIV in the submucosal plexus (Fig. 6B).

We used KN-62 as the pharmacological tool for study of involvement of CaMKs in the intraneuronal signal transduction pathway for the depolarizing responses to BK. KN-62 selectively inhibits CaMKs by binding directly to the calmodulin binding site on the enzyme (Tokumitsu et al., 1990). Application of 3 \mu M KN-62 alone did not alter the resting membrane potential of the neurons. After a minimum of 30 min in 3 \mu M KN-62, the mean amplitude of the depolarizing responses evoked by 100 nM BK was reduced to 43.4 ± 5.7% (P < 0.01) of the amplitude before KN-62 in 14 neurons (Figs. 6A and 7A). Washout of KN-62 for a minimum of 45 min reversed its inhibitory action on responses to BK.

The effects of KN-62 on responses to 100 nM PGE2 were determined in view of the evidence presented above that BK-evoked responses might be secondary to stimulation of cyclooxygenase and release and action of prostaglandins. The presence of KN-62 (3 \mu M) in the bathing solution suppressed
the amplitude of PGE₂-evoked depolarizing responses to 35.9 ± 8.4% (P < 0.01) of the responses before addition of KN-62 to the tissue bath in five neurons (Fig. 7B).

Protein Kinase C. The results obtained with the variety of pharmacological tools described above suggest that activation of protein kinase C (PKC) might be a parallel path in post-receptor transduction for the BK₂ receptor. We used GF109203X, which is a potent (IC₅₀ of 10 nM) and widely used inhibitor of the multiple subspecies of PKC (Toullec et al., 1991) to test for PKC involvement. Application of GF109203X did not alter the resting membrane potential. Pretreatment with 1 μM GF109203X for 30 min reduced responses to 100 nM BK to 34.9 ± 5.3% (P < 0.01) of the amplitude of the depolarizing responses before GF109203X in 13 neurons (Figs. 6A and 7A). Washout required a minimum of 45 min for full recovery from GF109203X inhibition.

We investigated the actions of both GF109203X and KN-62 on each of 13 submucosal S-type neurons. KN-62 (3 μM) abolished the depolarizing responses to 100 nM BK in three of the neurons, whereas GF109203X did not alter the responses in the same neurons. GF109203X (1 μM) abolished responses to 100 nM BK in two neurons, whereas KN-62 had no effect in the same neurons. Simultaneous application of GF109203X and KN-62 abolished the BK-evoked responses in 8 of the 13 neurons.

Based on the evidence reported above that the actions of BK reflect the formation prostaglandins and their excitatory effects on the neurons, we applied PGE₂ in the presence of GF109203X. GF109203X (1 μM) in the bathing solution suppressed the amplitude of PGE₂-evoked depolarizing responses to 38.4 ± 8.5% (P < 0.01) of the responses before treatment with GF109203X in five neurons (Fig. 7B).

Protein Kinase A. Putative neurotransmitters and paracrine messengers that evoke responses such as slow excitatory postsynaptic potentials (slow EPSPs) in enteric neurons (e.g., histamine, substance P, and 5-hydroxytryptamine) act to stimulate formation of cAMP in enteric ganglia (Baidan et al., 1992; Xia et al., 1994, 1996). Based on the involvement of cAMP as a second messenger in signal transduction for other slow EPSP mimetics, we used inhibitors of protein kinase A (PKA) to address the question of whether the adenylate cyclase/cAMP/PKA transduction pathway might be involved in generation of the slow EPSP-like depolarizing responses to BK B₂ receptor activation in submucosal neurons. Myristoylated PKI (14-22) amide (300 nM), a highly specific cell-permeable inhibitor of cAMP-dependent protein kinase (Harris et al., 1997), and KT5720, which was characterized by Barajas-Lopez (1993) as an effective inhibitor of PKA in the submucosal plexus, were used to investigate a possible role for PKA in bradykinin- and PGE₂-evoked responses.

No suppression of BK- or PGE₂-evoked responses occurred in eight preparations that were incubated for 30 min in 300 nM myristoylated PKI (14-22) (Figs. 6A and 7B) or 3 μM KT5720 (Fig. 7A). Both PKI (14-22) and KT5720 were effective in blocking the activity of PKA because PKA-mediated responses that were evoked by forskolin in AH-type enteric neurons (Nemeth et al., 1984) were suppressed or abolished by pretreatment with myristoylated PKI (14-22) or KT5720 (data not shown).

Discussion

Most neurons with S-type electrophysiological behavior and uniaxonal morphology and those with AH-type electrophysiological behavior and multipolar Dogiel type II morphology in the submucosal plexus express the BK B₂ receptor (Hu et al., 2004). BK acts at the B₂ receptor to evoke slowly activating depolarizing responses associated with augmented excitability in both S- and AH-type neurons. Increased cationic conductance underlies the BK-evoked depolarizing responses in S-type neurons and decreased K⁺ conductance is responsible for the depolarizing response in AH-type neurons (Hu et al., 2004).

BK B₂ receptor activation in sympathetic ganglion cells, like in enteric neurons, enhances neuronal excitability. Nevertheless, the ionic mechanism in sympathetic neurons differs from our results for enteric neurons (Hu et al., 2003, 2004). The excitatory effects of BK B₂ receptor activation in sympathetic ganglion cells result from inhibition of M-type membrane current after stimulation of PLC and release of Ca²⁺ from IP₃-sensitive intraneuronal Ca²⁺ stores (Cruzblanca et al., 1998; Delmas et al., 2002).

The present study of signal transduction mechanisms for the B₂ receptor focused on S-type neurons because they are the predominant neuronal type in the guinea pig small intestinal submucosal plexus; AH-type neurons make up less than 10% of the total submucosal neuronal population. Moreover, slow EPSP-like responses, which are associated with decreased conductance in AH-type enteric neurons, are mediated by a cAMP-PKA postreceptor transduction mechanism (Wood and Kirchgessner, 2004). Less is known about transduction cascades for slow EPSP-like responses associated with opening of nonselective cation channels and increased conductance in S-type neurons. BK, mast cell proteases and other immune/inflammatory mediators, which might act in paracrine manner in vivo, evoke slow EPSP-like responses that are associated with increased membrane conductance (Gao et al., 2002). The cAMP-PKA pathway seems not to be involved in generation of the slow EPSP-like responses that are associated with increased ionic conductance in S-type neurons because, unlike AH-type neurons, direct stimulation of adenylate cyclase by forskolin has no depolarizing action in S-type neurons (Nemeth et al., 1984). More-
over, neither inhibition of Gs protein-coupling to adenylate cyclase by PTX (see below) nor inhibition of PKA altered the depolarizing action of BK on S-type neurons in the presently reported study.

**Pertussis Toxin.** G proteins couple receptors for multiple signal substances to adenylate cyclase and/or phospholipase C to initiate postreceptor cascades of reactions that culminate in the slow EPSP in AH neurons (Wood and Kirchgessner, 2004). Bertrand and Galligan (1995) reported that G protein coupling for the slow EPSP-like action of the neurokinin-3 agonist senktide did not involve a pertussis toxin-sensitive G protein in AH-type enteric neurons. On the other hand, Pan et al. (1997) offered evidence that pertussis toxin sensitive Gαi coupled the 5-hydroxytryptamine₁₁ receptor to the signaling cascade for the slow EPSP-like responses to serotonin. We found that the slow EPSP-like action of BK was undisturbed by preincubation in PTX, which leads to the conclusion that postreceptor coupling for BK does not involve a PTX-sensitive G protein. As a control for efficacy of PTX to suppress G protein-linked responses, we examined the effects of preincubation in PTX on electrically evoked noradrenergic IPSPs in the same submucosal neurons. Preincubation in PTX suppressed the IPSPs that are known to be mediated by an adenylate cyclase/cAMP transduction pathway (Surprenant and North, 1988).

**Prostaglandins.** Bradykinin acts at intramural BK B₂ receptors to stimulate firing in primary sensory afferents that supply the rat small intestine. Blockade of cyclooxygenase suppresses the excitatory action of BK on the afferents (Maubach and Grundy, 1999). Our findings were much like those of Maubach and Grundy (1999) who concluded that BK acted directly at B₂ receptors on the afferent terminals with the responses to BK being dependent on the presence of prostaglandins, particularly PGE₂.

Bradykinin is known also to act at BK B₂ receptors in the kidney (Maeda et al., 1996) and in mesenteric vascular beds (Peredo et al., 1997) to stimulate formation of eicosanoids and nitric oxide. Stimulation of phospholipase A₂ and formation of arachidonic acid in the signal transduction pathway for BK accounts for the prostaglandin production in these preparations. Prostaglandins, produced in the guinea pig colon in vitro, act to excite submucosal secretomotor neurons and thereby stimulate mucosal secretion (Frielings et al., 1994a,b). Inhibition of prostaglandin formation by indomethacin or piroxicam suppresses the neurally mediated secretory responses that are evoked by BK B₂ receptor activation in guinea pig, rabbit, and rat intestine (Musch et al., 1983; Diener et al., 1988). Consideration of these actions of BK at the integrated organ level of organization was the motivation for testing the hypothesis that inhibition of nitric oxide and/or prostaglandin formation would suppress the excitatory action of BK on neurons in the submucosal plexus.

We found no evidence for inhibition of nitric oxide formation. On the other hand, the present study in the submucosal plexus and an earlier one in the guinea pig small intestinal myenteric plexus (Hu et al., 2003) found that the depolarizing responses to BK were suppressed or abolished by cyclooxygenase inhibitors. This was reminiscent of the excitatory action of BK on intestinal sensory afferents reported by Maubach and Grundy (1999) and suggested that bradykinin might act at BK B₂ receptors on submucosal neurons to stimulate the formation of prostaglandins. Once formed and released, the prostaglandins feedback to elevate the excitability of the same ganglion cell and to act in paracrine manner to excite neighboring neurons in the same or adjacent ganglia (Hu et al., 2003). The suggestion of an auto feedback mechanism is reinforced by our finding that exposure of submucosal neurons to PGE₂ and other prostaglandins mimicked the actions of BK to depolarize the membrane potential and elevate neuronal excitability. Our findings in this respect are reminiscent of BK action to stimulate enteric neuronal cyclooxygenase in myenteric neuronal cultures. BK B₂ receptor activation in the cultures released prostaglandins that fed back to mobilize free cytoplasmic Ca²⁺ in the neurons (Gelperin et al., 1994).

Evidence from our study with agents that inhibit intraneuronal Ca²⁺ signaling cascades and that of Gelperin et al. (1994) suggests that mobilization of intraneuronal Ca²⁺ follows BK B₂ receptor activation. Most of the elevation of intraneuronal Ca²⁺ in the submucosal neurons reflects release from intraneuronal stores. BK-evoked depolarizing responses in our study were not reduced by depletion of Ca²⁺ in the bathing solution, which suggested that there was little or no dependence on transmembrane influx of extracellular Ca²⁺.

**Signal Transduction in Submucosal Neurons.** Our results are consistent with the hypothesis that the postreceptor signal transduction steps illustrated in Fig. 8 underlie the neuronal excitatory responses evoked by stimulation of both the BK B₂ receptor and PGE₂ receptor in S-type uniaxonal neurons (primarily secretomotor neurons) in the submucosal plexus. Suppression of BK-evoked responses by inhibition of PLC, by an IP₃ receptor antagonist and by inhibition of Ca²⁺.

![Fig. 8. Overview of postulated metabotropic signal transduction cascade for excitatory actions of BK and prostaglandins on S-type uniaxonal neurons in the submucosal plexus of guinea pig small intestine.](image-url)
ATPase in intraneuronal membranes supports stimulation of PLC as an early step in the transduction process. RT-PCR analysis supplemented conclusions from studies with IP3 receptor antagonists by confirming expression of mRNA transcripts for IP3 receptors in the submucosal plexus.

Involvement of a transduction step with Ca2+ sensing by CaM is supported by immunohistochemical evidence for CaM expression in submucosal neurons and by suppression of BK- and PGE2-evoked responses by an agent that inhibits Ca2+-CaM regulated enzyme activity. RT-PCR analysis revealed expression of mRNA transcripts for CaMKIIα, CaMKIIβ, and CaMKIV in the submucosal plexus, and submucosal neurons also expressed immunoreactivity for CaMKII and CaMKIV. This, together with the finding of suppression of BK- and PGE2-evoked responses by a pharmacological agent that selectively inhibits CaMks, is evidence for CaMks as an intermediate transduction step. Suppression of both BK- and PGE2-evoked excitatory responses during exposure to an agent known to be an effective inhibitor of the enzymatic activity of PKC adds evidence in favor of PKC as a step in the transduction cascade.

When tested in tandem on the same neurons, we found that exposure to either a CaM inhibitor (KN-62) or a PKC inhibitor (GF109203X) suppressed BK-evoked responses in most neurons (i.e., 8 of 13). This is consistent with parallel activation of PKC in BK and CaM in the transduction process. Nevertheless, exclusive operation of the CaMk pathway was suggested when CaMk inhibition suppressed the depolarizing responses to BK in three of the 13 neurons, whereas inhibition of PKC did not alter the responses. On the other hand, PKC inhibition abolished responses to BK in two neurons, whereas inhibition of CaMk was without effect and suggested exclusive operation of the PKC path in these two neurons. Findings for the entire group of 13 neurons do not allow an unequivocal conclusion as to whether one of the steps leading to activation of PKC is exclusively by Ca2+ release from intracellular stores, exclusively by second messenger function of diacylglycerol, or by the parallel occurrence of intraneuronal Ca2+ mobilization and formation of diacylglycerol.

**Secretomotor Neurons.** Electrophysiological and synaptic behavior and morphology and chemical coding of the BK- and PGE2-responsive submucosal neurons identified most as secretomotor neurons. Secretomotor neurons are the final step in the enteric nervous system to the intestinal secretory glands (i.e., crypts of Lieberkühn). They transmit the signals for autonomic minute-to-minute regulation of mucosal secretion and liquidity of the intestinal contents in concert with submucosal vasodilation and increased blood flow in support of stimulated secretion (Andriansitohaina and Surprenant, 1992; Cooke, 2000). Enhanced mucosal secretion, after elevation of excitability in secretomotor neurons, increases the liquidity of the luminal contents and might lead to neurogenic secretory diarrhea. Suppression of excitability in secretomotor neurons (e.g., by opiates, clonidine, or somatostatin analogs) has the opposite effect of producing drier stools and constipation (Wood and Galligan, 2004). Gagninella and Kachur (1989) called attention to observations that BK levels are elevated in diarrheal states associated with intestinal inflammation. Based on the knowledge that BK stimulates prostaglandin synthesis and intestinal secretion they suggested that the primary importance of BK might be a pathophysiologial mediator in the gut.

**References**


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