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Kinin-induced Anion-dependent Secretion in Porcine Ileum: Characterization and Involvement of Opioid and Cannabinoid-Sensitive Enteric Neural Circuits

Benedict T. Green[1], Andrew Calvin,

Scott M. O'Grady, and David R. Brown

University of Minnesota, Department of Veterinary PathoBiology, College of Veterinary Medicine (B.T.G., A.C., D.R.B.),

and

Department of Animal Science-Physiology, College of Agriculture, Food and Environmental Sciences (S.M.O.), St. Paul, Minnesota 55108

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Please address all correspondence to:

David R. Brown, Ph.D.

University of Minnesota

Department of Veterinary PathoBiology

1988 Fitch Avenue

St. Paul, MN 55108-6010

Telephone: (612) 624-0713

FAX: (612) 625-0204

E-mail: brown013@umn.edu

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Abbreviations: DALBK, [des-Arg^{9},Leu^{8}]bradykinin; DIDS, 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid; DMSO, dimethylsulfoxide; DPDPE, [D-Pen^{2,5}]enkephalin; HOE-140, D-arginyl-L-arginyl-L-prolyl-*trans*-4-hydroxy-L-prolylglycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-(2α,3β,7αβ)-octahydro-1H-indole-2-carbonyl-L-arginine; HU-210, (6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol)

Abstract

The intestinal secretory actions of the proinflammatory peptide kallidin (lysyl-bradykinin) are mediated partially by enteric neurons. We hypothesized that kallidin produces neurogenic anion secretion through opioid- and cannabinoid-sensitive enteric neural pathways. Changes in short-circuit current (I_{sc}) across sheets of porcine ileal mucosa-submucosa mounted in Ussing chambers were measured in response to kallidin (1 μM) or drugs added to the contraluminal bathing medium. Kallidin transiently increased I_{sc}, an effect reduced after inhibition of neuronal conduction by 0.1 μM saxitoxin, cyclooxygenase inhibition by 10 μM indomethacin, or kinin B_2 receptor blockade by 1 μM HOE-140. Its action was dependent upon extracellular Cl^- or HCO_3^- ions, but was resistant to 10 μM bumetanide or 0.3 mM DIDS, and appeared to involve luminal alkalinization as measured by pH-stat titration. Kallidin-induced I_{sc} elevations were sensitive to saxitoxin in tissues bathed in Cl^--, but not HCO_3^--deficient media. Tissues pretreated with 0.1 μM [D-$Pen^{2,5}$]enkephalin, a selective δ-opioid agonist, displayed reduced I_{sc} responses to kallidin; this effect was prevented by the δ-opioid antagonist naltrindole. At a contraluminal concentration of 1 μM, the cannabinoid receptor agonist HU-210 also attenuated responses to kallidin. Proinflammatory kinins appear to stimulate neurogenic anion secretion in porcine ileum by activating enteric neural circuits expressing inhibitory opioid and possibly cannabinoid receptors.

The intestinal mucosa has evolved a diverse array of innate and acquired mechanisms to protect the vast surface area it encompasses from infection. In the early stages of infection and tissue injury, the proinflammatory peptide kallidin (lysyl-bradykinin) is produced by the kallikrein-catalyzed cleavage of low molecular weight kininogen (Kaplan et al., 2002). Kallikrein-type proteases have been localized in mast cells and goblet cells along the length of the intestinal tract (Hinterleitner and Powell, 1991). Kallidin and its des-lysyl homolog bradykinin act as agonists at kinin $B_1$and B_2 receptors, which are members of the G protein-coupled receptor superfamily (Regoli et al., 2001). The kinin B_2 receptor is constitutively expressed, whereas the B_1 receptor is induced by proinflammatory cytokines released in the course of tissue injury (Couture et al., 2001). These peptides produce pain, vasodilatation and in the digestive tract, evoke active, transepithelial chloride secretion in the small intestine and colon (Gaginella and Kachur, 1989; Cuthbert and Huxley, 1998) or transepithelial bicarbonate secretion in gallbladder and duodenum (Baird and Margolius, 1989; Chen et al., 1997). The actions of kinins on ion transport are attributed to their combined effects on enteric neurons and non-neuronal cells, including intestinal epithelial cells. Moreover, these peptides can induce the formation of arachidonic acid metabolites that in turn act upon both neurons and enterocytes. For example, kinins activate primary afferent nerves in peripheral tissues, including the small intestine, through direct effects on neurons and the formation of eicosanoids such as prostaglandin E_2 and 12-lipoxygenase metabolites (Maubach and Grundy, 1999; Shin et al., 2002).

Natural and synthetic opioids can alleviate diarrhea and produce constipation, actions which have been attributed in part to their intestinal antipropulsive and antisecretory

actions. In most species examined, the latter effect is mediated by inhibitory δ-opioid receptors expressed in submucosal neuronal circuits that are linked to active anion secretion (DeLuca and Coupar, 1996). Indeed, δ-opioid receptor immunoreactivity has been co-localized with immunoreactivity to the cholinergic neural marker choline acetyltransferase in submucosal neurons and nerve fibers of the porcine ileum. Subpopulations of these δ-opioid receptor-positive neurons also express immunoreactivities for the sensory neural markers, calcitonin gene-related peptide and vanilloid VR1 receptor (Poonyachoti et al., 2002). In addition, the selective δ-opioid agonist [D-Pen^{2,5}]enkephalin (DPDPE) inhibits active, neurogenic anion secretion mediated by type 2 proteinase-activated receptors, H_1-histamine receptors, and serotonin receptors in muscle-stripped sheets of porcine ileal mucosa (Green et al., 2000; Poonyachoti and Brown, 2001; Green and Brown, 2002). These studies suggest that submucosal δ-opioid receptors may function to limit neurogenic secretion associated with intestinal inflammation.

In the present investigation, we addressed the hypothesis that kallidin-induced anion secretion, like that evoked by histamine, serotonin or trypsin, is mediated by opioid-sensitive enteric neural circuits in the porcine ileum. Cannabinoids produce antipropulsive actions in the intestine that are similar to opioids, but their ability to alter intestinal secretion has not been clearly defined (Izzo et al., 2001). As immunoreactivity for cannabinoid CB_1 receptors has been detected in the porcine submucosal neurons (Kulkarni-Narla and Brown, 2000), it was of additional interest to compare the effects of the cannabinoid agonist HU-210 with those of the selective δ-opioid agonist DPDPE on kallidin-stimulated neurogenic ion transport in the porcine ileum.

MATERIALS AND METHODS

Drugs and chemicals. Kallidin, D-arginyl-L-arginyl-L-prolyl-trans-4-hydroxy-L-prolylglycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-(2α,3β,7αβ)-octahydro-1H-indole-2-carbonyl-L-arginine (HOE-140) and [des-Arg^{9},Leu^{8}]bradykinin (DALBK) were obtained from Bachem (Torrance, CA). Atropine, bumetanide, carbamylcholine chloride (carbachol), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), histamine, indomethacin, naltrindole and saxitoxin were obtained from Sigma/RBI Chemical Co. (St. Louis, MO). [D-Pen^{2,5}]enkephalin (DPDPE) was purchased from Peninsula Laboratories (Belmont, CA). (6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol (HU-210) was purchased from Tocris Cookson (Ballwin, MO). HU-210 and indomethacin were solubilized in dimethylsulfoxide (DMSO); this solvent had no effect on baseline or kallidin-stimulated intestinal ion transport. DPDPE was solubilized in 0.01 M acetic acid with 0.1% bovine serum albumin, aliquoted at stock concentrations of 100 μM, and stored until use at –65 °C. All other drugs and reagents were dissolved in distilled water and added to the contraluminal bathing medium unless otherwise noted.

Animals and tissue preparation. Intestinal tissues were obtained from Yorkshire pigs (6-10 weeks of age; 10 - 18 kg body weight) of each sex that were not fasted before sacrifice. Animals were sedated with an intramuscular injection of tiletamine hydrochloride-zolazepam (Telazol®; 8 mg/kg, Fort Dodge Laboratories, Fort Dodge, IA), in combination with xylazine (8 mg/kg). The animals were subsequently euthanized by barbiturate overdose in accordance with approved University of Minnesota IACUC protocols. A midline laparotomy was performed to expose the intestine and a portion of

the ileum, identified by its attachment to the ileo-cecal ligament, was removed and placed in an oxygenated organ preservation solution (composition in mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 0.5; $NaHCO_3$, 25; NaH_2PO_4, 1.0; and D-glucose, 11; pH 7.4).

Ileal segments were stripped of underlying smooth muscle layers and sheets of mucosa-submucosa were mounted between two Lucite half-chambers with a surface area of 2 cm^2. Mucosal sheets were bathed on both sides with a salt solution that approximated the composition of the porcine extracellular fluid (composition in mM: NaCl, 130; KCl, 6; $CaCl_2$, 3; $MgCl_2$, 0.7; $NaHCO_3$, 20; NaH_2PO_4, 0.29; and Na_2HPO_4, 1.3) at pH 7.4 and gassed continuously with 5% CO_2 in O_2 at 39° C (porcine core temperature). In anion substitution experiments, gluconic acid was substituted for chloride ion and HEPES substituted for bicarbonate ion at equimolar concentrations. D-Glucose and mannitol (10 mM) were added to the contraluminal and luminal bathing media, respectively.

Measurement of transepithelial ion transport. Short-circuit current (I_{sc}, in $\mu A/cm^2$) across each mucosa-submucosal sheet was monitored continuously by an automatic voltage clamp apparatus (Model TR100, JWT Engineering, Overland Park, KS, and Model EVC-4000, World Precision Instruments, Sarasota, FL). Experiments were initiated after the basal I_{sc} had stabilized (approximately 25 – 35 min). Tissue conductance (G_t, in mSiemens/cm^2) was calculated by Ohm's law from the current change produced by the periodic delivery of a bipolar 5mV pulse measured throughout each experiment. Data were aquired using a PowerLab data acquisition unit and analyzed with Chart data analysis software (AD Instruments, Grand Junction, CO). Both I_{sc} and G_t were determined immediately before drug administration and at the peak of drug action.

At the end of each experiment, when I_{sc} had returned to baseline, mucosal I_{sc} responses to 10 μM carbachol (contraluminal addition) and 10 mM glucose (luminal addition) were measured in each tissue to assess tissue viability.

Kallidin was added to the contraluminal bathing medium to achieve a final bath concentration of 1 μM. This concentration was chosen because it is in the upper range of the kallidin concentration-effect relationships determined in previous studies *in vitro* with intestinal mucosa preparations (Gaginella and Kachur, 1989). In some experiments, receptor blockers or ion transport inhibitors were added to either the luminal or contraluminal bathing medium 15 min prior to kallidin addition. Some tissues were pretreated contraluminally with 0.1 μM DPDPE or 1 μM HU-210 10 min prior to kallidin addition; in some experiments with DPDPE, saxitoxin (0.1 μM) or the selective δ-opioid antagonist naltrindole (1 μM) was added to the contraluminal bathing medium 5 min prior to DPDPE addition.

pH-Stat titration. Tissue sheets were mounted in Ussing chambers under short-circuit conditions and bathed luminally or contraluminally in physiological salt solution and a salt solution with an equimolar substitution of sodium gluconate for sodium bicarbonate and sodium phosphate on the opposite side. The salt solutions were maintained at 39° C and gassed continuously with 5% CO_2 in O_2 or 100% O_2, respectively. In a second set of experiments, tissues bathed with HCO_3-containing physiological salt solution that was gassed continuously with 5% CO_2 in O_2 and maintained at their individual stable pH baseline (average pH = 7.43-7.44) by continuous titration as measured with a PHM-82 pH meter, model TTT-80 titrator and model ABU-80 autoburette (Radiometer Corp., Copenhagen, Denmark). After the pH baseline had

been established, drugs were added to the contraluminal bathing medium and the bathing medium was titrated with 0.005 N HCl for the HCO_3-substituted salt solution experiments and 0.001 N HCl for the normal physiological salt solution experiments. Changes in luminal or contraluminal pH were monitored and 10 μM carbachol was added to the contraluminal bathing medium at the end of each experiment to determine its action in alkalinizing the bathing medium.

Data analysis. Data are expressed as mean I_{sc} or G_t under baseline conditions, or as mean changes in peak I_{sc} elevations occurring in response to kallidin or other substances. Data from tissues that, at the end of the experimental period, did not respond to carbachol and glucose with elevations in I_{sc} were omitted. In the case that the tissues serving as controls manifested poor responses to glucose and carbachol, data from all tissues obtained from the donor animal were excluded from further analysis. Statistical analyses of data were performed using the PRISM computer software program (version 3.0, GraphPad Software, Inc., San Diego, CA). Comparisons between a control mean and a single treatment mean were made with a two-tailed, paired or unpaired Student's t test when appropriate. Comparisons of a control mean with multiple treatment means were made by one-way analysis of variance followed by Dunnett's test. In all cases, the limit for statistical significance was set at $P < 0.05$.

RESULTS

Mediators of kallidin action. Baseline I_{sc} and G_t in isolated sheets of ileal mucosa-submucosa averaged 4 ± 3 μA/cm^2 and 23 ± 1 mS/cm^2 (n = 224 tissues from 37 pigs). At a contraluminal concentration of 1 μM, kallidin produced a monophasic rise in I_{sc} that attained a peak elevation of 80 ± 6 μA/cm^2 relative to mean baseline values (n = 97 tissues from 37 pigs). The duration of kallidin action on I_{sc} was 11.2 ± 0.5 min (8 tissues from 4 pigs). The kallidin-induced increase in I_{sc} was 70% of that produced by 10 μM carbachol (ΔI_{sc} produced by carbachol = 114 ± 10 μA/cm^2, n = 65 tissues from 28 pigs). Tissue conductance nearly doubled to 43 ± 3 mS/cm^2 when determined at the time of peak change in I_{sc} produced by kallidin (n = 65 tissues from 28 pigs).

To verify that the action of kallidin was mediated by enteric neurons, mucosal I_{sc} responses to kallidin were examined in tissues pretreated with 0.1 μM saxitoxin, a neuronal Na^+ channel blocker. The neurotoxin significantly increased baseline G_t from 21 ± 5 to 26 ± 6 mS/cm^2 (15 tissues from 7 pigs, $p < 0.05$, paired t test). In the presence of saxitoxin, I_{sc} elevations produced by kallidin decreased by 67% relative to those in toxin-untreated tissues (Fig. 1).

To determine if the activation of muscarinic cholinergic receptors was involved in kallidin action, eight tissues were pretreated with the muscarinic cholinergic antagonist, atropine. At a contraluminal concentration of 0.1 μM, atropine did not significantly alter baseline I_{sc} or G_t, and mucosal I_{sc} responses to kallidin were not significantly altered in

atropine-treated tissues (Fig. 1). In tissues pretreated with atropine, I_{sc} responses to the contraluminal addition of 10 μM carbachol were abolished (data not shown).

Because kallidin action may depend upon the formation of eicosanoids, mucosal responses to kallidin were measured in seven tissues pretreated with indomethacin, a cyclooxygenase inhibitor. At a contraluminal concentration of 10 μM, indomethacin did not significantly alter baseline I_{sc} or G_t. However, it significantly decreased kallidin-induced I_{sc} responses by 68% of peak I_{sc} elevations in untreated tissues (Fig. 1). As DMSO was used to solubilize indomethacin and a few other substances, the effect of this solvent on kallidin-induced I_{sc} elevations was examined as well. At a contraluminal concentration of 0.1% v/v, it did not significantly change baseline I_{sc} or G_t, or alter significantly mucosal responses to 1 μM kallidin (mean ΔI_{sc} after kallidin in DMSO-pretreated tissues = 101 ± 31 $\mu A/cm^2$; P = 0.84 vs. response in DMSO-untreated tissues, Student's t-test; n = 8 tissues from 5 pigs).

The type of kinin receptor mediating mucosal responses to kallidin was determined in tissues pretreated with the kinin B_1 receptor antagonist DALBK or the B_2 receptor antagonist HOE-140. At a contraluminal concentration of 1 μM, HOE-140, but not DALBK, significantly decreased mucosal I_{sc} responses to kallidin (Fig. 2).

Ionic basis for kallidin-evoked short-circuit current. Previous reports have indicated that kinins induce anion secretion across the intestinal epithelium (Gaginella and Kachur, 1989; Cuthbert and Huxley, 1998). Therefore, tissues were pretreated with bumetanide, a blocker of the $Na^+/K^+/Cl^-$ cotransporter which represents one Cl^- entry pathway in intestinal epithelial cells that is of importance in active anion secretion. Bumetanide did not significantly change baseline I_{sc} or G_t after its contraluminal addition

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at 10 μM. At this concentration, it did not significantly decrease the peak I_{sc} elevations occurring in response to kallidin (mean ΔI_{sc} after kallidin in untreated and bumetanide-pretreated tissues = 107 ± 14 and 97 ± 31 μA/ cm^2; respectively; $P > 0.05$, Student's t-test; n = 26 and 5 tissues from 13 and 3 pigs respectively).

Anion substitution experiments were undertaken to assess the dependency of the I_{sc} response to kallidin on extracellular Cl^-- and HCO_3^-. Gluconate was substituted for the Cl^- ions in bathing media to examine the role of HCO_3^- ions in kallidin action; HEPES was substituted for HCO_3^- ions in HCO_3^- -deficient/Cl^--replete media. Removal of either Cl^- and HCO_3^- ions did not significantly change baseline I_{sc} (mean I_{sc} in Cl^--deficient and HCO_3^--deficient conditions = 6 ± 1 and 12 ± 2 μA/cm^2, respectively; n = 33 and 30 tissues from 16 and 10 pigs respectively). Electrical conductance was significantly decreased in tissues bathed in $Cl^—$- or HCO_3^--deficient media compared to those bathed in anion-replete medium (G_t in tissues bathed in Cl^- -deficient and HCO_3^- -deficient media was 15 ± 1 and 24 ± 2 mS/cm^2, respectively; $P < 0.05$ for each anion-deficient condition vs. the anion-replete condition, Dunnett's test, n = 33 and 30 tissues from 16 and 10 pigs respectively).

The peak elevations in I_{sc} produced by kallidin in tissues bathed in HCO_3^--deficient and Cl^--deficient media were significantly decreased relative to those in tissues bathed in anion-replete medium (mean ΔI_{sc} produced by 1 μM kallidin in anion-replete, Cl^--deficient media, and HCO_3^--deficient media = 80 ± 6, 22± 5, and 29 ± 4 μA/cm^2 respectively; $P < 0.05$, Dunnett's test, n = 97, 16 and 22 tissues from 40, 16 and 10 pigs respectively).

In tissues bathed in Cl^--deficient media, saxitoxin significantly reduced kallidin-induced elevations in I_{sc} (Fig. 3A). In contrast, I_{sc} responses to kallidin in tissues bathed in HCO_3^--deficient media were relatively resistant to the neurotoxin (Fig. 3B). Indomethacin significantly reduced I_{sc} responses to kallidin in tissues bathed in media deficient in either anion (Fig. 3A, B).

Some tissues were treated luminally with 0.3 mM DIDS prior to kallidin addition. Luminal DIDS did not alter kallidin action in tissues bathed in either Cl^--or HCO_3^--deficient media (mean ΔI_{sc} produced by 1 μM kallidin and DIDS pretreated tissues in Cl^--deficient media, and HCO_3^--deficient media = 22 ± 5, 13± 3, and 29 ± 4, 22 ± 5 $\mu A/cm^2$ respectively; $P < 0.05$, Dunnett's test, n = 22, 5, 16 and 3 tissues from 16, 3, 9 and 3 pigs respectively).

pH-stat titration experiments were undertaken to examine further the role of HCO_3^- ions in the kallidin-evoked I_{sc}. Initially, tissues were mounted in Ussing chambers with sodium gluconate substituted physiological salt solution on either the luminal or contraluminal side and normal physiological salt solution on the opposing side. The pH-stat buret was then placed in the reservoir containing the substituted salt solution for the duration of the experiment. During a 10 min period during which baseline alkalinization was measured, the rate of luminal alkalinization was 40 ± 10 nmol base equivalents/min and the baseline pH-stat contraluminal alkalinization was 20 ± 3 nmol base equivalents/min (n = 11 luminal and 8 contraluminal buret tissues from 4 pigs). The contraluminal addition of 1 μM kallidin produced a moderate alkalinization of both the luminal and contraluminal bathing media (luminal alkalinization, 150 ± 27 nmol base equivalents and contraluminal alkalinization, 120 ± 21 nmol base equivalents; n = 11

luminal and 8 contraluminal buret/tissues from 4 pigs). To assess tissue viability 10 μM carbachol was added to the contraluminal side after the addition of kallidin when the tissues had returned to baseline, and the rate of luminal alkalinization was assessed for the duration of the carbachol response of 6.1 ± 0.6 min in 19 tissues from 4 pigs (luminal alkalinization, 250 ± 90 nmol base equivalents and contraluminal alkalinization, 140 ± 75 nmol base equivalents; n = 11 luminal and 8 contraluminal buret/tissues from 4 pigs).

Alkalinization measurements using bicarbonate-containing physiological salt solution bathing both sides of mucosal sheets were undertaken as well. Tissues were maintained at a baseline pH of 7.43 ± 0.02 in luminal buret experiments and a pH of 7.44 ± 0.01 in contraluminal buret experiments. The contraluminal addition of 1 μM kallidin produced a rapid alkalinization of both the luminal and contraluminal bathing media, with the former action being significantly greater than the latter (Fig. 4). The subsequent contraluminal administration of 10 μM carbachol also produced both luminal and contraluminal alkalinization (nmol base equivalents delivered luminally and contraluminally after carbachol = 120 ± 20 and 50 ± 20, respectively; $P > 0.05$, unpaired Students t test, n = 8 and 6 tissues from 7 and 6 pigs respectively). Histamine, in contrast, produced little or no luminal alkalinization after its contraluminal addition at 2 μM (Fig. 4).

Effects of opioid and cannabinoid receptor agonists on kallidin-stimulated ion transport. The δ-opioid agonist DPDPE (0.1 μM, contraluminal addition) did not produce significant changes in baseline I_{sc} or G_t. However, it decreased by 53% the peak I_{sc} elevation produced by kallidin. Tissues pretreated with both saxitoxin and DPDPE appeared to display an additional decrease in I_{sc} responses to kallidin. The selective δ-opioid antagonist naltrindole prevented the inhibitory action of DPDPE (Fig. 5).

At a contraluminal concentration of 1 μM, the non-selective cannabinoid agonist HU-210 did not produce significant changes in baseline I_{sc} or G_t, but significantly decreased subsequent mucosal responses to kallidin (Fig. 5).

DISCUSSION

Consistent with the results of previous investigations on the intestinal secretory actions of kinins (Gaginella and Kachur, 1989), kallidin transiently increased I_{sc} in mucosa-submucosa sheets from porcine ileum after its contraluminal administration. This effect was blunted by saxitoxin and indomethacin, providing evidence that it is mediated in part by enteric neurons and cyclooxygenase metabolites, respectively. The enteric neural circuit(s) mediating kallidin-induced secretion does not appear to contain muscarinic cholinergic receptors because of the insensitivity of kallidin action to atropine. This result is in contrast to that obtained by Diener *et al.* (1988), who reported that atropine decreased significantly mucosal I_{sc} responses to bradykinin in the rat distal colon. However, our previous studies in porcine ileal mucosa which examined the secretory effects of other inflammatory mediators including serotonin (Green and Brown, 2002), proteinases (Green et al., 2000) and histamine (Poonyachoti and Brown, 2001) showed a similar pattern of atropine resistance. These results in combination suggest that proinflammatory mediators stimulate active anion secretion in the porcine intestine through a neural mechanism that does not involve cholinergic secretomotor neurons. Kinin B_2 receptors appear to mediate the effect of kallidin on transepithelial ion transport, as kallidin-induced elevations in I_{sc} were sensitive to the selective B_2 receptor blocker HOE-140, but not to the kinin B_1 antagonist DALBK. This result again is consistent with studies in other intestinal segments and in other mammalian species as well as in

intestinal tissues from B_2 receptor knockout mice, which demonstrate that kinin B_2 receptors mediate the secretory effects of kinins (Gaginella and Kachur, 1989; Cuthbert and Huxley, 1998). The present results show that the porcine ileal mucosa appears to be generally similar to analogous intestinal preparations from other species with respect to the kinin receptor type and the neural and eicosanoid influences contributing to kallidin activity in the intestinal mucosa.

The kallidin-induced elevation in I_{sc} across the porcine ileal epithelium bathed in media containing Cl^- and HCO_3^- ions was not sensitive to the loop diuretic bumetanide, which blocks Cl^- loading in enterocytes through the $Na^+/K^+/Cl^-$ co-transporter. However, the related loop diuretics furosemide and piretanide have been shown to decrease the actions of kallidin in the mouse and rat intestine (Cuthbert and Margolius, 1982; Cuthbert, 2001). Sensitivity of mucosal I_{sc} responses to loop diuretics has been taken as presumptive, albeit indirect evidence that active transport of the chloride anion is responsible for the transient I_{sc} elevations induced by kallidin (Cuthbert and Huxley, 1998). Anion substitution experiments were undertaken to examine in further detail the contributions of the major extracellular anions, Cl^- and HCO_3^-, to the kallidin-evoked change in I_{sc}. Kallidin action was reduced by similar magnitudes in tissues bathed in media deficient in either Cl^- or HCO_3^- ions, indicating that it is dependent on both extracellular anions. From the results of these experiments, we propose that kallidin stimulates bumetanide-insensitive, anion-dependent secretion in porcine ileum.

Saxitoxin decreased I_{sc} responses to kallidin in tissues bathed in Cl^--deficient media, but had no significant effect on responses to kallidin in tissues bathed in HCO_3^--free media. Serotonin-evoked changes in I_{sc} in porcine ileal mucosa sheets bathed in anion-

deficient media exhibit a similar pattern of saxitoxin sensitivity (Green and Brown, 2002). The results suggest that neurogenic ion transport induced by kallidin is dependent on extracellular HCO_3^- ions. Indomethacin markedly attenuated mucosal I_{sc} responses to kallidin in tissues bathed in media deficient in either Cl^- or HCO_3^- ions, and we hypothesize that both the neurogenic and non-neurogenic components of kallidin action are dependent on the formation of cyclooxygenase metabolites.

Because the neurogenic actions of kallidin appeared to involve electrogenic HCO_3^- secretion, we examined the effects of DIDS at a relatively high luminal concentration on mucosal responses to the peptide. DIDS has been shown to inhibit epithelial HCO_3^- transport mediated by both Na^+-coupled HCO_3^- transporters and Cl^-/HCO_3^- exchangers (Boron, 2001). As DIDS did not significantly alter mucosal I_{sc} responses to kallidin, it is possible that neither mode of HCO_3^- transport contributes to the effects of the peptide. Alternatively, DIDS-resistant HCO_3 transport mechanisms have been reported to exist (Tsuruoka and Schwartz, 1999; Luo et al., 2001). To further clarify a role for HCO_3^- secretion in the action of kallidin, the ability of this peptide to alkalinize the luminal medium bathing mucosal sheets was examined through pH stat titration experiments. Tissues bathed either luminally or contraluminally in HCO_3-free media and gassed with 100% O_2 sustained a baseline rate of alkalinization into the opposite medium, but kallidin did not evoke an increase in alkalinization rate. On the other hand, kallidin produced a rapid increase in luminal alkalinization and a smaller increase in contraluminal alkalinization in tissues bathed on both sides with HCO_3-containing media and gassed with carbogen. On the basis of these data, we speculate that kallidin stimulates a transcellular anion transport pathway across the ileal epithelium that delivers HCO_3^- ions

into the luminal bathing medium in excess of the basal paracellular HCO_3^- flux. Carbachol but not histamine evoked luminal alkalinization as well. We have previously reported that carbachol increases luminal alkalinization in porcine ileum (Chandan et al., 1991). Kinins have been found to evoke active HCO_3^- secretion in a few other gastrointestinal preparations *in vitro*. For example, luminal addition of bradykinin to the guinea pig gallbladder mucosa produced increases in I_{sc} that were abolished in HCO_3^--deficient media (Baird and Margolius, 1989). Moreover, bradykinin increases luminal alkalinization in rat duodenum through an action on kinin B_2 receptors (Chen et al., 1997). The present results suggest that kallidin stimulates HCO_3^--dependent, electrogenic ion transport in the porcine ileum as well.

Enteric δ-like opioid receptors appear to modulate electrogenic ion transport evoked by transmural stimulation of submucosal neurons in the porcine ileum (Poonyachoti et al., 2001). Moreover, I_{sc} elevations produced by either trypsin, histamine, serotonin or an immediate hypersensitivity reaction to a food allergen in the porcine ileal mucosa are attenuated by the selective δ-opioid agonist DPDPE (Green et al., 2000; Poonyachoti and Brown, 2001; Green and Brown, 2002). We tested the hypothesis that δ-opioid receptors are expressed in a common enteric neuronal circuit that mediates secretory responses to inflammatory mediators, including kallidin. In support of this hypothesis, DPDPE markedly attenuated mucosal I_{sc} responses to kallidin, and its effects were prevented by the selective δ-opioid antagonist, naltrindole. When tissues were pretreated with both saxitoxin and DPDPE, there appeared to be a small additional decrease in kallidin action. This may be due to a minor action of kallidin on additional enteric neural pathways that do not express δ-opioid receptors. In previous studies, the combination of the neurotoxin

and DPDPE was without any additional effect on mucosal I_{sc} responses to histamine or serotonin (Poonyachoti and Brown, 2001; Green and Brown, 2002). Based on this result and our previous data, we postulate that the indomethacin-sensitive portion of HCO_3^--dependent, neurogenic ion transport stimulated by kallidin is mediated by an enteric neural circuit that contains inhibitory δ-opioid receptors. The potent cannabinoid agonist HU-210, like DPDPE, decreased mucosal I_{sc} responses to kallidin. Unlike DPDPE however, HU-210 does not alter I_{sc} elevations in sheets of porcine ileal mucosa that are evoked by serotonin or transmural electrical stimulation (Green and Brown, 2002; S. Poonyachoti, H. Albasan and D.R. Brown, unpublished results). Thus, cannabinoids may act on a more circumscribed enteric neural pathway that contains cannabinoid CB_1 and kinin B_2 receptors, and possibly δ-opioid receptors as well. Indeed, some myenteric neurons from porcine ileum maintained in primary culture manifest immunoreactivity for both δ-opioid and cannabinoid CB_1 receptors (Kulkarni-Narla and Brown, 2001). Cannabinoid receptor agonists have been found previously to decrease mucosal ion transport in rat intestine evoked by electrical stimulation *in vitro* (Tyler et al., 2000) and intestinal fluid accumulation in intact rats (Izzo et al., 1999). A role for cannabinoids and cannabinoid receptors in the antisecretory actions of HU-210 must await confirmation with future studies employing cannabinoid antagonists and additional selective agonists.

Proinflammatory kinins appear to stimulate neurogenic HCO_3^- secretion in porcine ileum by activating enteric neural circuits expressing inhibitory opioid and possibly cannabinoid receptors. The active secretion of HCO_3^- ions in the duodenum and colon is an important contributor to luminal pH in these intestinal segments (Seidler et al., 2000), and HCO_3^- transport induced by kallidin or other inflammatory mediators may have a

similar role in the ileum. Bicarbonate transport is also linked to absorption of short-chain fatty acids (SCFA), which appear to alleviate intestinal inflammation; chronic ileitis is associated with a reduction in SCFA/HCO_3^- exchange (Manokas et al., 2000). Bicarbonate concentrations in the intestinal lumen may influence cytoplasmic pH regulation in phagocytic cells extruded from the epithelium and thus indirectly modulate their defensive functions (Grinstein et al., 1991). Luminal HCO_3^- ions also stimulate the expression of bacterial gene products mediating attachment and effacement of enterohemorrhagic *Escherichia coli* on epithelial cells (Kenny et al., 1997; Abe et al., 2002). By acting through the enteric nervous system to alter bicarbonate secretion induced by kallidin and other inflammatory mediators, opioids and possibly cannabinoids could alter interactions between the intestinal mucosa and enteropathogenic microorganisms.

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Footnotes

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Address reprint requests to: Dr. David R. Brown, University of Minnesota, Department of Veterinary PathoBiology (Pharmacology section), 1988 Fitch Avenue, St. Paul, Minnesota 55105-6010. E-mail: brown013@umn.edu.

[1]Present address of B.T.G. is United States Department of Agriculture, Agricultural Research Service, Clay Center, Nebraska 68933-0166.

Figure Legends

Fig. 1. Effects of inhibitors on mucosal I_{sc} responses to kallidin. Tissues were pretreated by contraluminal addition of 0.1 μM saxitoxin or atropine, or 10 μM indomethacin prior to contraluminal addition of 1 μM kallidin. Control tissues were not treated with inhibitors prior to kallidin addition. Bars represent the mean ± SE of peak change in I_{sc} produced by kallidin measured in 26 tissues from 13 pigs that served as controls, 14 tissues from 7 pigs pretreated with saxitoxin, 9 tissues from 6 pigs pretreated with atropine, and 7 tissues from 6 pigs pretreated with indomethacin. *$P < 0.05$ vs. control mean, Dunnett's test.

Fig. 2. The effect of kinin B_1 and B_2 receptor blockers on mucosal I_{sc} responses to kallidin. Tissues were pretreated by contraluminal addition of 1 μM [des-Arg^9, Leu^8]bradykinin (DALBK) or HOE-140 prior to contraluminal addition of 1 μM kallidin. Control tissues were not treated with blockers prior to kallidin addition. Bars represent the mean ± SE peak change in I_{sc} produced by kallidin measured in 6 tissues from 3 pigs that served as controls, 5 tissues from 3 pigs pretreated with DALBK, and 6 tissues from 3 pigs pretreated with HOE-140. *$P < 0.05$ vs. control mean, Dunnett's test.

Fig. 3. Dependence of neurogenic and prostanoid components of kallidin-induced I_{sc} elevations on extracellular anions. Responses to 1 μM kallidin (contraluminal addition) were measured in mucosal sheets bathed (top) in Cl^--deficient media or (bottom) in HCO_3^--deficient media and either untreated (control) or pretreated with contraluminally-administered saxitoxin (STX, 0.1 μM) or indomethacin (INDO, 10 μM). Bars represent the mean ± SE peak change in I_{sc} produced by kallidin under chloride-free conditions in 22 tissues from 16 pigs that served as controls, 14 tissues from 6 pigs pretreated with saxitoxin, and 14 tissues from 7 pigs pretreated with indomethacin. Under bicarbonate-free conditions, the peak change in I_{sc} produced by kallidin was measured in 16 tissues from 9 pigs that served as controls, 4 tissues from 4 pigs pretreated with saxitoxin, and 4 tissues from 3 pigs pretreated with indomethacin. *$P < 0.05$ vs. control mean, Dunnett's test.

Fig. 4. Luminal alkalinization produced by kallidin in comparison that produced by with histamine. The chart records shown are representative tracings of luminal buret pH-stat experiments from tissues treated contraluminally with either 1 μM kallidin (top) or 2 μM histamine (bottom). The kallidin tracing is representative of 8 tissues from 7 pigs. The histamine tracing is representative of one tissue from each of two pigs; in these two tissues, histamine increased I_{sc} by 52 and 111 $\mu A/cm^2$. The inset histogram under the kallidin chart record depicts the mean nanomoles ± S.E. of hydrochloric acid delivered into the luminal or contraluminal bathing medium that were necessary to clamp the pH to 7.43 or 7.44 respectively, in tissues treated with kallidin integrated over the period encompassing the peak I_{sc} response to kallidin. *$P < 0.05$, luminal vs. contraluminal

means determined in experiments using 8 tissues from 7 pigs and 6 tissues from 6 pigs respectively; Student's unpaired t-test.

Fig. 5. Inhibitory actions of the δ-opioid agonist [D-Pen^{2,5}]enkephalin (DPDPE) and the non-selective cannabinoid receptor agonist HU-210 on kallidin-induced I_{sc} elevations in porcine ileal mucosa. Responses to kallidin, added to the contraluminal bathing medium at a concentration of 1 μM, were measured in mucosal sheets untreated (control) or pretreated with 0.1 μM DPDPE (contraluminal addition). In some cases, naltrindole (Naltrindole/DPDPE) or saxitoxin (Saxitoxin/DPDPE) was present in the contraluminal medium at a concentration of 1 or 0.1 μM respectively prior to DPDPE addition. Some tissues were pretreated contraluminally with 1 μM HU-210 prior to kallidin administration. Control tissues were not treated with drugs prior to kallidin addition. Bars represent the mean ± SE peak change in I_{sc} produced by kallidin measured in 58 tissues from 25 pigs that served as controls, 19 tissues from 9 pigs pretreated with DPDPE, 7 tissues from 3 pigs pretreated with naltrindole and DPDPE, 7 tissues from 3 pigs pretreated with saxitoxin and DPDPE, and 15 tissues from 6 pigs pretreated with HU-210. *$P < 0.05$ vs. control condition, Dunnett's test.

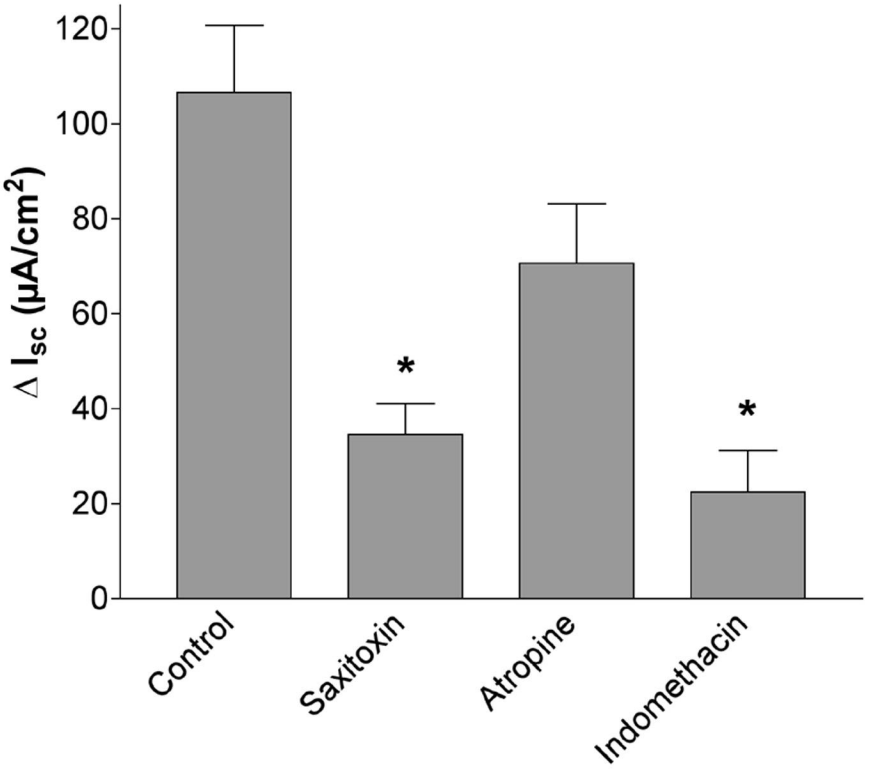


FIG. 1/ JPET #47829

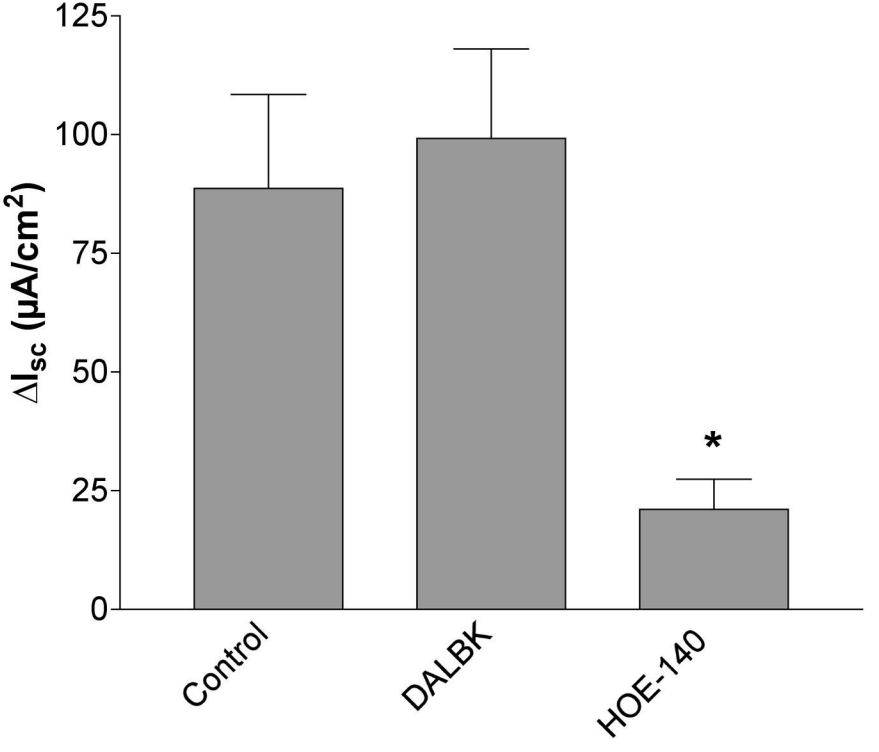
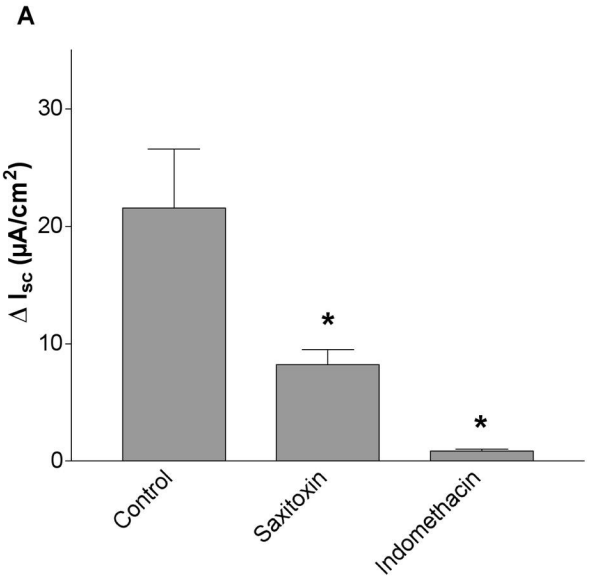


FIG. 2/ JPET #47829



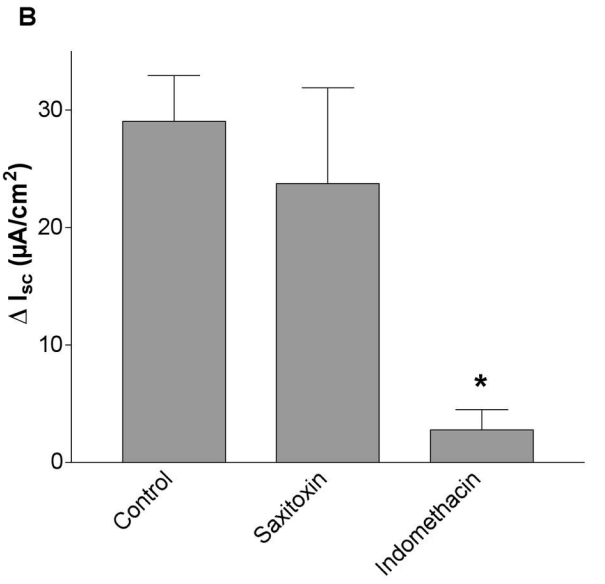
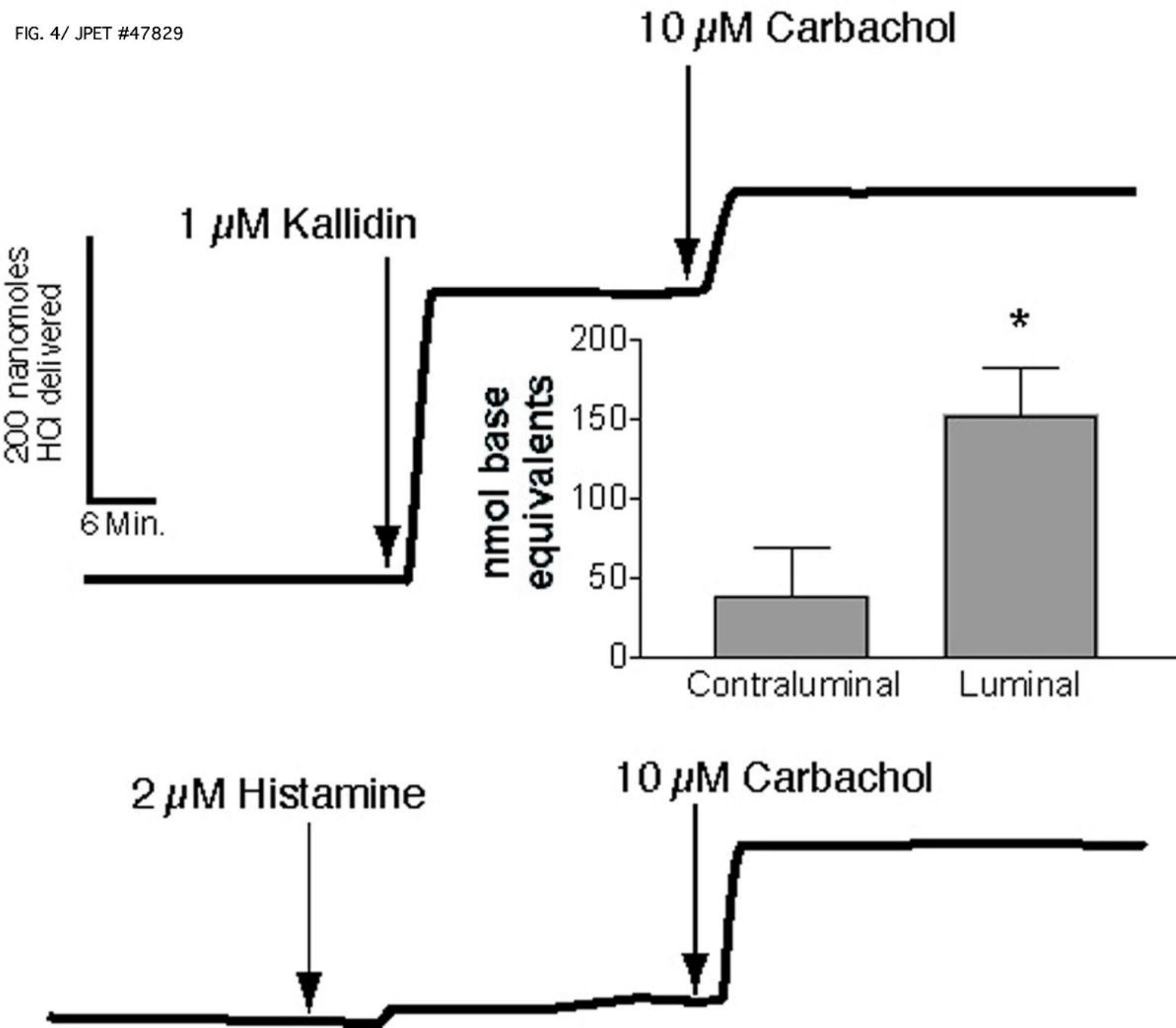


FIG. 3/ JPET #47829


10 μM Carbachol
1 μM Kallidin
200 nanomoles HQ delivered
6 Min.
nmol base equivalents
200
150
100
50
0
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Contraluminal
Luminal
2 μM Histamine
10 μM Carbachol

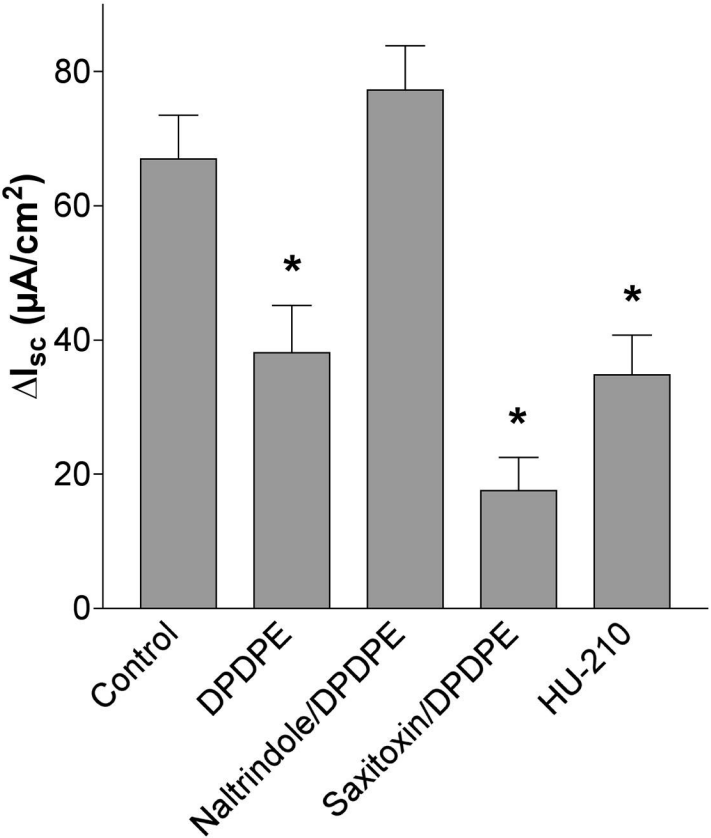

FIG. 5/ JPET #47829