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Autonomic Neurotransmitters Modulate Immunoglobulin A Secretion in Porcine Colonic Mucosa

Lisa D. Schmidt¹, Yonghong Xie¹, Mark Lyte², Lucy Vulchanova¹, and David R. Brown^{1,*}

¹ Department of Veterinary and Biomedical Sciences, University of Minnesota, 1988 Fitch Avenue, St. Paul, Minnesota 55108-6010

²Department of Pharmacy Practice, Texas Tech University Health Sciences Center, 3601 Fourth Street, MS 8162, Lubbock, Texas 79430

Abstract

Secretory immunoglobulin A (sIgA) plays a crucial role in mucosal surface defense. We tested the hypothesis that colonic sIgA secretion is under enteric neural control. Immunohistochemistry of the porcine distal colonic mucosa revealed presumptive cholinergic and adrenergic nerve fibers apposed to secretory component (SC)-positive crypt epithelial cells and neighboring IgA⁺ plasmacytes. The cholinomimetic drug carbamylcholine elicited rapid, atropine-sensitive IgA secretion into the luminal fluid bathing mucosal explants mounted in Ussing chambers. The adrenergic receptor agonist norepinephrine also increased IgA secretion, an action inhibited by phentolamine. These effects were independent of agonist-induced anion secretion. In Western blots of luminal fluid, both agonists increased the density of protein bands co-immunoreactive for IgA and SC. Mucosal exposure to enterohemorrhagic *Escherichia coli* did not affect IgA secretion, and carbamylcholine treatment did not affect mucosal adherence of this enteropathogen. Acetylcholine and norepinephrine, acting respectively through muscarinic cholinergic and α -adrenergic receptors in the colonic mucosa, stimulate sIgA secretion and may enhance mucosal defense *in vivo*.

Keywords

mucosal immunity; secretory component; muscarinic cholinergic receptor; α -adrenergic receptor; colonocyte; *Escherichia coli* O157:H7

1. Introduction

Immunoglobulin A (IgA) is the most abundant immunoglobulin present at all mucosal surfaces, where it plays crucial roles in mucosal protection (Woof and Kerr, 2006). It is produced and released as a J chain-linked dimer by resident IgA⁺ plasma cells in mucosal tissues, including the extensive lamina propria of the intestine. Dimeric IgA is recognized by the polymeric Ig receptor (pIgR) expressed by neighboring epithelial cells, subsequently transcytosed through pIgR⁺ cells, and enzymatically cleaved at the mucosal surface to form secretory IgA (sIgA). The higher molecular weight sIgA represents dimeric IgA covalently bound to secretory component (SC), a remnant of the polymeric Ig receptor which prevents sIgA degradation.

*Corresponding author: David R. Brown, Ph.D., Department of Veterinary and Biomedical Sciences, University of Minnesota, 1988 Fitch Avenue, St. Paul, Minnesota 55108-6010, Telephone: (612) 624-0713; FAX: (612) 625-0204; E-mail: brown013@umn.edu

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Due to constitutive pIgR transcytosis in epithelial cells, free SC may also be secreted and appears to possess important protective functions *per se* (Kaetzel, 2005). Factors that regulate the rate and magnitude of sIgA secretion are not completely defined.

Neural stimulation and various neurotransmitter substances appear to alter sIgA released by acinar cells and ductal epithelial cells in lacrimal and salivary glands (Kelleher et al., 1991; Lambert et al., 1994; Proctor and Carpenter, 2002; Teeuw et al., 2004). Experiments with rat salivary glands, for example, indicate that sIgA secretion is regulated by sympathetic and parasympathetic nerves (Carpenter et al., 1998; Proctor et al., 2000). The output of sIgA is also modified by injections of cholinomimetic and sympathomimetic drugs into the salivary gland blood supply (Proctor et al., 2003b) or direct application of adrenergic agonists to salivary gland cells (Carpenter et al., 2004). Although the intestinal tract is a major site for mucosal immunity and is extensively innervated, little is known about the neural regulation of enteric sIgA secretion. The intravenous injection of cholinomimetic drugs or the gut neuropeptides cholecystokinin and substance P have been shown to increase sIgA secretion in isolated loops of the rat small intestine (Wilson et al., 1982; Freier et al., 1987, 1989; McGee et al., 1995). In addition, the luminal output of sIgA from vascularly-perfused segments of the swine ileum is increased by intraarterial substance P and somatostatin (Schmidt et al., 1999).

In the present study, we tested the hypothesis that sIgA secretion from colonic mucosa explants is modified by the major enteric neurotransmitter substances, acetylcholine and norepinephrine (NE). The porcine distal colon was chosen as an experimental model, despite some interspecies variations, as intestinal immunity in swine is similar in many respects to that in humans (Snoeck et al., 2006). Furthermore, mucosal explants from the porcine colon have been used to investigate the neuroregulation of active epithelial ion transport (Brown and O'Grady, 1997) and the adrenergic modulation of *Escherichia coli* O157:H7 adherence (Green et al., 2004). The main objectives of the present investigation were to establish the presence of presumptive cholinergic and adrenergic nerve fibers in immune effector sites of the porcine colon and characterize the effects of the cholinomimetic drug carbamylcholine (CCh) and NE on lumenally-directed sIgA secretion from isolated sheets of the colonic mucosa.

2. Materials and methods

2.1. Drugs and bacteria

Drugs were purchased from Sigma Chemical Co. (St. Louis, MO). With the exception of indomethacin, which was dissolved in dimethylsulfoxide, drugs were dissolved in distilled water prior to use. In some experiments, colonic mucosa sheets were pretreated with antagonists added to the contraluminal bathing medium 5 min before the contraluminal addition of agonists. Wild-type, *Shiga* toxin-negative enterohemorrhagic *Escherichia coli* nal^R (EHEC; nalidixic acid-resistant) strain 85-170 was obtained from Dr. Mark Stevens (Institute for Animal Health, Compton Laboratory, Berkshire, UK). An additional *Shiga* toxin-negative EHEC strain 700728 (American Type Culture Collection, Manassas, VA) was examined in experiments designed to assess the effects of the cholinergic agonist CCh on mucosal adherence of EHEC. Both EHEC strains were grown to stationary phase in Luria-Bertani broth (Qbiogene, Irvine, CA) in an overnight culture. Bacterial cultures were stored at -80 °C in phosphate-buffered saline (PBS) containing 4% glycerol (Fisher Scientific, Fair Lawn, NJ).

2.2. Animals and tissue preparation

Colonic mucosa explants were isolated from outbred Yorkshire-Landrace pigs of either sex that were 5 to 7 weeks old and weighed between 10 and 18 kg. Animals had continuous access to water and nonmedicated pig feed and were not fasted prior to sacrifice. They were anesthetized with tiletamine hydrochloride-zolazepam (Telazol[®]; 8 mg/kg, i.m. injection; Fort

Dodge Laboratories, Fort Dodge, IA) in combination with xylazine (AnaSed®; 3 mg/kg, Lloyd Laboratories, Shenandoah, IA), and subsequently euthanized with Beuthanasia®-D Special (0.5 ml/kg, i.v. injection; Schering-Plough Animal Health, Union, NJ) in accordance with approved University of Minnesota IACUC protocols. Each explant was stripped of its underlying longitudinal and circular smooth muscle coats and the remaining mucosa with attached submucosa was mounted in Ussing flux chambers (1 cm² flux area for IgA secretion experiments; 2 cm² flux area for Western blot experiments). Explants were bathed on their luminal and contraluminal aspects with a physiological saline solution similar in composition to porcine extracellular fluid (130 mM NaCl, 6 mM KCl, 3 mM CaCl₂, 0.7 mM MgCl₂, 20 mM NaHCO₃, 0.29 mM NaH₂PO₄, and 1.3 mM Na₂HPO₄); D-glucose and mannitol (10 mM) were added to the contraluminal and luminal bathing media, respectively. The buffer solution was maintained at porcine core temperature (39 °C) and aerated with 95% O₂/5% CO₂ by gas lift. In preliminary experiments it was found that the effects of CCh and NE on IgA secretion were nearly halved in tissues held in cold, oxygenated physiological saline solution for > 40 min prior to mounting in Ussing chambers compared to responses in tissues mounted immediately after smooth muscle removal (data not shown). Therefore, mucosal sheets were mounted in chambers within 10 min of tissue preparation.

2.3. Immunohistochemistry

Colonic segments were cut along the mesenteric border, pinned on a Silgard-coated surface mucosal side up, and fixed in modified Zamboni's fixative (4% paraformaldehyde and 0.2% picric acid) for 2 h at room temperature. After extensive washing in PBS, tissues were stored in PBS containing 10% sucrose and 0.05% sodium azide until further use. Slide-mounted cryostat sections (20 µm) were incubated in blocking buffer (PBS containing 0.03% Triton X, 1% bovine serum albumin, 1% normal donkey serum, and 0.01% sodium azide) for 1 h at room temperature, followed by incubation in primary antisera overnight at 4°C. Primary antisera mixtures included goat anti-porcine IgA (1:2000 working dilution; Bethyl Laboratories, Montgomery, TX), mouse anti-porcine secretory component (1:300; Serotec, Raleigh, NC), and one of the following antisera: rabbit anti-protein gene product 9.5 (1:1000; Neuromics Antibodies, Northfield, MN), guinea pig anti-vesicular acetylcholine transporter (1:2000; Fitzgerald Industries International Inc., Concord, MA) or rabbit anti-dopamine beta-hydroxylase (1:500; Immunostar, Hudson, WI). The slides were then rinsed in PBS, incubated in secondary antisera for 1 h at room temperature and coverslipped using DPX mounting resin (Electron Microscopy Sciences, Fort Washington, PA). Controls included omission of primary antibodies and preabsorption of primary antibodies with their cognate peptides or, in the case of IgA and secretory component, preabsorption with varying concentrations (0.1-10%) of normal swine serum. The sequential application of secondary antisera separated by blocking with normal swine serum was necessary to eliminate non-specific staining with the donkey anti-rabbit and anti-guinea pig secondary antisera without interfering with staining with anti-porcine IgA. Images were collected using a confocal laser-scanning microscope (Bio-Rad MRC 1024) and processed using Confocal Assistant 4.1 (freeware) and Adobe Photoshop (version 6.0.1, Adobe Systems, San Jose, CA).

2.4. Measurement of mucosal electrical parameters

Baseline short-circuit current (I_{sc} , in microamperes/cm²), a measure of net, active ion transport and therefore tissue viability, was monitored continuously across each mucosal sheet by means of an automatic voltage clamp apparatus (model EVC-4000; World Precision Instruments, Sarasota, FL). The contraluminal side served as reference. A brief 5 mV pulse was delivered at 100 sec intervals for determination of tissue electrical conductance (G_t), which was calculated by Ohm's law. Changes in I_{sc} and G_t were measured immediately before CCh and NE administration and at the peak of drug action with and without their respective antagonists.

2.5. Measurement of immunoglobulin A immunoreactivity

A sample (800 μ l) of the luminal bathing medium was collected at each time point, and replaced with an equivalent volume of physiological saline solution similar in composition to porcine extracellular fluid containing 10 mM mannitol to maintain bath volume (10 ml) and hydrostatic pressure. Samples were taken at time 0, 5, 15 and 30 minutes to assess short-term drug effects and at time 0, 20, 35, 65, and 95 minutes to assess longer-term drug effects. Each sample was centrifuged at 12,000 rpm and 4°C for 5 min, then stored at -20°C. Total IgA was estimated by ELISA with a polyclonal goat anti-porcine IgA antiserum (Bethyl Laboratories), according to the manufacturer's protocol using a modified capture antibody at a 1:500 dilution. Standard curves were generated using porcine reference serum (Bethyl Laboratories).

2.6. Western blots

Luminal and contraluminal bathing media were collected 30 min after fluid replacement and subsequent addition of CCh or NE. Fluid samples were concentrated using Centricon centrifugal filter devices with a molecular weight cutoff of 5 kDa (Millipore, Bedford, MA). Total protein in the samples was determined with the Micro BCA Protein Assay (Pierce Biotechnology, Rockford, IL). Samples were denatured in a non-reducing buffer by heating for 5 min at 70°C. Equivalent amounts of protein (8 ng) were then resolved on 7.5% Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA). In all blots, two lanes were dedicated to molecular weight markers. One of the outermost lanes was assigned to broad range, SDS-PAGE prestained standards (Bio-Rad), whereas the second lane was assigned to broad range, Kaleidoscope prestained standards (Bio-Rad). Proteins were transferred to Immobilon-P (polyvinylidene fluoride (PVDF) microporous) membranes (Millipore) and blocked with casein followed by an avidin-biotin block (Vector Laboratories, Burlingame, CA). Blots were incubated for 30 min at room temperature with monoclonal mouse anti-porcine secretory component or monoclonal mouse anti-porcine IgA (Serotec) diluted 1:300, followed by 30 min incubation in biotinylated horse anti-mouse antibody (1:300 dilution; Vector Laboratories). Immunodetection was then performed using the VECTASTAIN® ABC-AmP kit (Vector Laboratories) followed by the colorimetric development AP conjugate substrate kit (Bio-Rad). Densitometry was performed and analysed using the BioRad Gel Doc 1000 and Bio-Rad "Molecular Analyst" software (ver. 21), respectively. The optical densities of scanned protein bands for CCh- or NE-treated tissues are expressed relative to those obtained from corresponding control tissues from each pig.

2.7. Bacterial treatment

Intestinal mucosa explants were exposed to Shiga toxin-negative enterohemorrhagic *Escherichia coli* O157:H7 strain 85-170. Muscle-stripped colonic mucosa mounted in Ussing chambers were exposed to bacteria on their luminal aspect to achieve an initial bacterial load of $5.9 \pm 0.3 \log_{10}$ colony-forming units (CFU)/ml; bacteria were added at time = 0 min. After 90 min, samples of the bathing media were collected for IgA measurement by ELISA as described in section 2.5.

2.8. Measurement of *E. coli* O157:H7 adherence to colonic mucosa

E. coli O157:H7 strain 700728, grown to stationary phase, was added to the luminal bathing medium to achieve an initial bacterial load of $6.1 \pm 0.3 \log_{10}$ CFU/ml. The bacteria were exposed to the colonic mucosa for 90 min. At the end of this time period, tissues were analyzed for bacterial adherence after the method of Knutton *et al.* (1989). Briefly, the exposed tissue was excised from the surrounding tissue, washed 3 times, weighed and placed into 2 ml PBS at pH 7.4. Tissues were then homogenized for 30 seconds at 3000 rpm (Brinkman Polytron PT3000, Littau, Switzerland) prior to serial dilution. Colony forming units were enumerated using a spread-plate technique on Fluorocult agar (Merck KGaA, Darmstadt, Germany) grown

for 18 h at 37°C in a 5% CO₂ atmosphere. Fluorocult agar is a selective medium for *E. coli* O157:H7 (Manafi and Kremsmaier, 2001).

2.9. Data analysis

Data are expressed as mean $\pm$ SE of IgA immunoreactivity (ng/ml), I_{sc} (μ A/cm²) or G_t (milliSiemens/cm²). Statistical analyses of data were performed by using the PRISM computer software program (version 4.0; GraphPad Software, San Diego, CA). In most cases, the amount of immunoreactive IgA present in the luminal medium bathing each mucosal sheet immediately prior to the start of each experiment (time = 0 min) was subtracted from the amount of luminal IgA immunoreactivity measured at subsequent time points. The rate of IgA secretion into the luminal bathing medium was analyzed by linear regression. Comparisons between single control and treatments means were made using a two-tailed Student's *t* test. 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 $P < 0.05$.

3. Results

3.1. Anatomical relationship of enteric nerve fibers to IgA-immunoreactive plasma cells and secretory component-immunoreactive epithelial cells

Nerve fibers immunoreactive for the pan-neuronal marker PGP 9.5 were seen in close proximity to both IgA-positive plasma cells in the lamina propria and neighboring SC-positive epithelial cells in the colonic crypts (Fig. 1A). Presumptive cholinergic and adrenergic nerve fibers in mucosal effector sites of the colon were visualized using antisera directed towards the anti-vesicular acetylcholine transporter and the NE-synthesizing enzyme dopamine- β -hydroxylase, respectively. Cholinergic and adrenergic nerve fibers were observed within several microns of both IgA-immunoreactive plasma cells and crypt epithelial cells immunoreactive for secretory component (Fig. 1B, C).

3.2. Baseline electrical characteristics of colonic mucosal explants and spontaneous IgA secretion

The mean baseline I_{sc} in colonic mucosa explants was $22 \pm 2 \mu$ A/cm² and mean G_t was 25 ± 1 mS/cm² ($n = 183$ colonic tissues from 32 pigs). In control, untreated tissues, IgA immunoreactivity increased spontaneously in the luminal bathing medium over time (Figs. 2 and 3).

3.3. Effects of carbamylcholine (CCh) on luminal secretion of immunoreactive IgA

The cholinergic receptor agonist CCh rapidly increased both I_{sc} (Table 1) and luminal IgA secretion in colonic mucosa explants. Elevations in luminal IgA content were significant within 25 min of CCh addition (Fig. 2, left) and sustained for 60 min (Fig. 2, right). The effects of CCh on active ion transport and luminal IgA secretion were inhibited by the muscarinic cholinergic antagonist atropine (Table 1; Fig. 2). In one set of experiments (Fig. 2, left), atropine decreased early IgA secretion evoked by CCh to levels significantly below those of control tissues.

3.4. Effects of norepinephrine (NE) on luminal secretion of immunoreactive IgA

The adrenergic receptor agonist norepinephrine, like CCh, rapidly increased luminal IgA secretion in colonic explants. Its effects on IgA secretion were manifested within 10 min of its administration (Fig. 3, left) and sustained for 90 min (Fig. 3, right). As reported previously (Traynor et al., 1991), NE transiently increased I_{sc} in colonic mucosa sheets (Table 1). At a relatively low concentration of 0.1 μ M, the α -adrenergic antagonist phentolamine alone did not significantly alter colonic I_{sc} responses to NE; at a 3-fold higher concentration and in

combination with the *beta*-adrenergic antagonist propranolol, it significantly reduced NE-evoked elevations in colonic I_{sc} (Table 1). The effects of NE on luminal IgA secretion were attenuated by phentolamine, but were not further inhibited by propranolol (Fig. 3, left).

3.5. Effects of inhibitors of prostaglandin synthesis, active transport, and axonal conduction on luminal IgA secretion

Neither the cyclooxygenase inhibitor indomethacin nor the Na^+ , K^+ -ATPase inhibitor ouabain at contraluminal bath concentrations of 10 and 50 μM respectively altered luminal IgA secretion over a 60 - 90 min time period (data not shown).

At a contraluminal concentration of 0.1 μM , the axonal conduction blocker saxitoxin had no significant effect on luminal IgA secretion measured over 30 min (data not shown).

3.6. Western blot analysis of tissue bathing fluids

IgA-immunoreactive protein bands obtained from luminal fluid samples were co-immunoreactive for SC, confirming the identity of these >203 kDa bands as secretory IgA. In addition, free SC was resolved in a lower molecular weight band of approximately 80 kDa (Fig. 4A, B). Bands co-immunoreactive for IgA and SC obtained in luminal fluid samples from tissues treated with either 10 μM CCh or NE were more intense as measured by densitometry than bands obtained from bathing medium of untreated explants (Fig. 4A-C). Furthermore, the amount of total SC-immunoreactive material (i.e. free SC and SC complexed to secretory IgA) in the luminal medium bathing tissues treated with CCh or NE was significantly greater than that recovered from the contraluminal medium ($P < 0.05$, paired t test, $n = 1$ tissue pair from each of 3 pigs per treatment condition).

3.7. Effects of *E. coli* O157:H7 on luminal secretion of immunoreactive IgA and action of CCh on bacterial adherence

Mucosal exposure to *E. coli* O157:H7 nal^R strain 85-170 for 90 min did not significantly alter the amount of IgA present in the luminal medium bathing colonic explants relative to untreated control tissues (1753 ± 508 and 1195 ± 330 ng/ml, respectively; $n = 1$ tissue pair from each of 6 pigs; $P > 0.05$, paired t test).

Carbamylcholine, at a contraluminal concentration of 10 μM , did not alter the number of *E. coli* O157:H7 strain 700728 colonies adhering to colonic mucosal sheets exposed to the microorganism for 90 min (3.68 ± 0.06 log CFU/g in untreated control tissues and 3.74 ± 0.06 log CFU/g in CCh-treated tissues; $P > 0.05$, paired t test, $n =$ one tissue pair from each of 7 pigs).

4. Discussion

In agreement with the present results, previous immunohistochemical studies found that IgA-immunoreactive plasma cells are localized to lamina propria surrounding the crypt regions of the porcine small and large intestinal mucosae (Atkins et al., 1971) and that SC-immunoreactive epithelial cells in the crypts of the porcine small intestine are in close apposition to these plasma cells (Allen and Porter, 1973). Similar observations have been made in the intestines of other mammalian species, including the human colon (Poger and Lamm, 1974). The localization of IgA⁺ plasmacytes to the colonic lamina propria appears to be mediated by chemokine-receptor interactions involving the mucosa-associated epithelial chemokine CCL28 with its cognate receptor CCR10 (Brandtzaeg and Johansen, 2005). Although presumptive cholinergic and adrenergic nerve fibers have been localized previously in the intestinal lamina propria (Schafer et al., 1998; Green et al., 2004), their anatomical relationship to the cellular components of the mucosal IgA system has not hitherto been

examined. These nerve fibers lie in close proximity to both antibody-secreting cells and SC-immunoreactive epithelial cells and it is not possible, at a morphological level, to distinguish their precise cellular target. Although IgA is the predominant immunoglobulin secreted by the porcine intestinal mucosa, significant amounts of IgM are likely to be secreted as well (Hansen et al., 2006). The relationship of IgM-expressing plasma cells to nerve fibers and SC-immunoreactive epithelial cells was not examined in this study, but would be predicted to be similar to that observed with IgA-positive plasmacytes.

Colonic mucosal explants spontaneously secreted IgA-immunoreactive material into the luminal bathing media. Western blot analyses of the IgA-immunoreactive material recovered from luminal fluid samples demonstrated the co-migration of electrophoretic bands immunoreactive for both IgA and SC, confirming the identity of this material as sIgA. In comparison, contraluminal fluid samples contained smaller amounts of IgA-immunoreactive material that was not immunoreactive for SC; this material likely represents dimeric IgA that did not undergo epithelial transcytosis and binding to SC. Protein bands containing free SC resolved at a molecular weight of approximately 80 kDa, which is in accordance with previously published Western blot assays (Proctor et al., 2000). Basal IgA secretion has been noted previously in vascularly-perfused, isolated segments and mucosal explants of the porcine small intestine (Schmidt et al., 1999; Hansen et al., 2006) as well as intact rat salivary glands (Proctor et al., 2003a). This is probably due to the continuous secretion of polymeric IgA from plasma cells, and ongoing transcytosis of the polymeric Ig receptor in crypt epithelial cells. The results of experiments with indomethacin and ouabain indicate that spontaneous, lumenally-directed sIgA secretion is not dependent upon endogenous prostanoid formation or active ion transport. Moreover, it was not altered in tissues treated with the neuronal sodium channel blocker saxitoxin, a finding suggesting that it is not driven by basal activity in the enteric nervous system.

In isolated sheets of porcine colonic mucosa, CCh produces a rapid, but transient (approximately 10 min duration) increase in I_{sc} , an effect attributed previously to active anion secretion (Traynor et al., 1991). In this tissue preparation, CCh was found to additionally produce a sustained increase in lumenally-directed IgA secretion. Biochemical analyses of the luminal and contraluminal fluids bathing mucosal sheets indicated that CCh increased sIgA and free SC immunoreactivity in the luminal bathing medium, but decreased IgA content in the contraluminal bath. Based on this finding, CCh appears to induce vectorial secretion of both free SC and sIgA towards the mucosal surface. The effects of CCh on I_{sc} and IgA secretion were inhibited by atropine, indicating that they are both mediated by muscarinic cholinergic receptors. Changes in anion transport induced by CCh are directly mediated by drug interactions with colonocytes (Traynor et al., 1991), and it is probable that an epithelial site of action can be ascribed to the effect of CCh on IgA secretion as well. The present results are in general agreement with those of Wilson et al. (1982), who reported that several cholinergic agonists, including pilocarpine, muscarine and bethanechol increase IgA output from perfused loops of the rat intestine by an atropine-sensitive mechanism of action. Moreover, they support previous findings that atropine decreases basal IgA secretion from perfused rat intestinal loops (Wilson et al., 1982; Freier et al., 1987). In a more complex, vascularly-perfused porcine ileum preparation however, electrical stimulation of extrinsic nerves innervating the intestine increases sIgA secretion only after atropine and phentolamine pretreatment; this effect is inhibited by hexamethonium, a blocker of ganglionic neurotransmission (Schmidt et al., 1999). This result suggests that there may exist important inhibitory reflex circuits containing muscarinic cholinergic and α -adrenergic receptors which modulate the enteric neural control of sIgA output. Additional investigations will be necessary to establish how basal sIgA secretion onto the mucosal surface *in vivo* is modulated by the release of acetylcholine from enteric nerves.

Like CCh, NE increased I_{sc} and IgA secretion in colonic mucosa explants. NE similarly induced vectorial secretion of both free SC and sIgA into the luminal bathing medium. Its transient effects on I_{sc} in mucosal sheets from the porcine distal colon are of similar duration to those produced by CCh, are due to increased net anion secretion and are mediated in part through α 1-adrenoceptors (Traynor et al., 1991). In the present study, NE-induced increases in I_{sc} were inhibited after combined α - and β -adrenoceptor blockade by phentolamine and propranolol, respectively. However, sustained, lumenally-directed IgA secretion evoked by NE was inhibited only by phentolamine and therefore is mediated by α -adrenoceptors. In comparison to CCh given at an equimolar concentration, the actions of NE on IgA secretion appeared to occur with a marginally faster onset and slower offset. Based on its longer time course, different sensitivity to adrenoceptor antagonists, and resistance to ouabain, IgA secretion evoked by NE or CCh is probably not linked to the shorter-lived effects of these drugs on active ion transport. Preliminary findings by Hopkins et al. (1997) are in agreement with the hypothesis that IgA transcytosis is mechanistically independent of active transepithelial anion transport. Under physiological conditions in vivo, the early effects of CCh and NE on net anion secretion would produce a transepithelial osmotic gradient favoring lumenally-directed water movement, which in turn might provide a hydraulic force capable of facilitating the movement of lumenally-directed sIgA from the colonic crypts to the surface epithelium.

Intestinal infection by *E. coli* O157:H7 is associated with the production of sIgA directed against lipopolysaccharide and other microbial surface antigens (Siddons and Chapman, 1993). We tested the hypothesis that acute *E. coli* O157:H7 exposure to the colonic mucosa, a major target for infection by this microorganism, activates local neural reflexes linked to sIgA secretion. Previous studies in our laboratory demonstrate that significant adherence of *E. coli* O157:H7 to the porcine colonic mucosa occurs within 90 min of luminal inoculation (Green et al., 2004). Interactions between *E. coli* O157:H7 and the colonic mucosa over this time interval did not elicit a significant change in lumenally-directed sIgA output. Thus, *E. coli* O157:H7 adherence to the colonic mucosa does not appear to activate local submucosal nerves linked to sIgA secretion. However, neurogenic sIgA secretion might be increased gradually after longer periods of bacterial exposure, by invasive rather than adherent enteropathogens, or by enteroenteric neural reflex activity evoked by infections occurring in distant intestinal segments. These alternate hypotheses remain to be tested in future investigations. Both SC and innate (i.e. antigen-nonspecific) sIgA have important roles in mucosal defense against bacterial infection (Perrier et al., 2006; Wijburg et al., 2006). Although CCh increased sIgA content in the luminal bathing fluid over a 90 min time frame, it did not alter the number of *E. coli* O157:H7 adhering to the colonic mucosa. This negative result might be due to the ineffectiveness of free SC or innate sIgA to impair bacterial adherence. However, it might also be attributable to the experimental design in which tissue bathing media are continuously mixed by gas lift. This mixing action could potentially diminish the formation of a mucus layer on mucosal sheets and reduce the local amounts of sIgA or free SC present at the mucosal surface.

In summary, the current findings indicate that, as in the salivary gland, sIgA is rapidly secreted in mucosal explants from porcine distal colon in response to both cholinergic and adrenergic receptor agonists, but remains unaltered by acute exposure to an enteroadherent microbial pathogen. The enteric neurotransmitters acetylcholine and NE appear to act on muscarinic cholinergic and α -adrenergic receptors expressed by crypt epithelial cells to promote transcytosis of the polymeric Ig receptor and enhance vectorial secretion of both sIgA and free SC towards the mucosal surface. In its early stages, this action appears to be temporally coincident with, but mechanistically independent of active ion transport. The precise receptor types and associated signal transduction mechanisms mediating sIgA secretion by these and possibly other enteric neurotransmitters at this mucosal immune effector site remain to be characterized. Both CCh and NE stimulate colonic mucin secretion (Smith and Butler,

1974;Neutra et al., 1984Barcelo et al., 2001); both IgA and SC bind to intestinal mucus and may function in a cooperative fashion with mucus in inhibiting bacterial adherence to the mucosal surface (Bouvet et al., 1993;Phalipon et al., 2003). The functional interrelationships between IgA and mucin secretion as well as other mucosal defense processes which are subject to enteric neuromodulation clearly warrant investigation.

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Fig 1.

Proximity of neural elements to IgA-expressing plasma cells and secretory component (SC)-expressing epithelial cells in porcine distal colon. Confocal micrographs are representative of observations in a minimum of three transverse tissue sections from each of five pigs. **(A)**. Nerve fibers immunoreactive for the neural marker PGP 9.5 (red) are in close proximity to both IgA-immunoreactive plasma cells (green) and epithelial cells immunoreactive for SC (blue) in the colonic crypts. **(B and C)**. Higher magnification show that nerve fibers immunoreactive for the vesicular acetylcholine transporter (red, B) and dopamine *beta*-hydroxylase (red, C) are closely apposed to (arrowheads) B lymphocytes immunoreactive for IgA (green) and epithelial cells immunoreactive for SC (blue)., B lymphocytes immunoreactive for IgA (green) and epithelial cells immunoreactive for SC (blue). Abbreviations: Cr, colonic crypt; L, intestinal lumen; SM, submucosa. Scale bars: A, 100 μ m; B and C, 15 μ m.

Fig 2.

Carbamylcholine (CCh) increases luminal accumulation of IgA immunoreactivity through interactions with muscarinic cholinergic receptors in colon. Tissues were treated with either CCh (10 μ M) in the absence or presence of the muscarinic cholinergic antagonist atropine (0.1 μ M) added to the contraluminal bathing medium; tissues untreated with drugs served as controls in each experiment. **(Left)** Short-term effects of CCh on immunoreactive IgA secretion into the luminal fluid. Luminal fluid samples were taken at time = 0 min prior to the contraluminal addition of drugs. Immediately after collection of the initial luminal fluid sample, some tissues were pretreated with atropine. CCh was added to the contraluminal bathing medium 5 min after the start of each experiment (i.e. time = 5 min). Mean IgA immunoreactivity at 0 min was 477 ± 72 , 443 ± 82 and 390 ± 78 ng/ml from control, CCh-treated, and atropine-/CCh-treated tissues, respectively. Points represent the mean ($\pm$ S.E.) luminal IgA immunoreactivity in six control, CCh-treated, and atropine-/CCh-treated tissues from six pigs. Line slopes were significantly different ($F = 11.3$, $P = 0.04$) as determined by linear regression analysis. **(Right)** Longer-term effects of CCh on luminal IgA secretion. Luminal fluid samples were taken at time = 0 min prior to the contraluminal addition of drugs. Immediately after collection of the initial luminal fluid sample, some tissues were pretreated with atropine. CCh was added to the contraluminal bathing medium 5 min after the start of each experiment (i.e. time = 5 min). Mean IgA immunoreactivity at 0 min was 432 ± 53 , 354 ± 41 and 336 ± 55 ng/ml from control, CCh-treated, and atropine-/CCh-treated tissues, respectively. Points represent the mean ($\pm$ S.E.) luminal IgA immunoreactivity in 6 - 11, 6 - 12 and 6 - 12 control, CCh-treated, and atropine-/CCh-treated tissues from seven pigs, respectively. The line elevation for CCh alone was significantly different from control ($F = 76.0$, $P = 0.0004$) as determined by linear regression analysis. * $P < 0.05$ and ** $P < 0.01$ vs. control condition at identical time points, Dunnett t test.

Fig 3.

Norepinephrine (NE) increases luminal accumulation of IgA immunoreactivity through interactions with *alpha*-adrenergic receptors in colon. Tissues were treated with either NE (10 μ M) in the absence or presence of the *alpha*-adrenergic antagonist phentolamine (PTL) added to the contraluminal bathing medium. In some experiments, the *beta*-adrenergic antagonist propranolol (PPL) was also added. Tissues untreated with drugs served as controls in each experiment. **(Left)** Short-term effects of NE on immunoreactive IgA secretion into the luminal fluid. Luminal fluid samples were taken at time = 0 min prior to the contraluminal addition of drugs. Immediately after collection of the initial luminal fluid sample, some tissues were pretreated with adrenergic receptor antagonists. NE was added to the contraluminal bathing medium 5 min after the start of each experiment (i.e. time = 5 min). Mean IgA immunoreactivity at 0 min was 294 ± 85 , 369 ± 99 , 222 ± 62 and 241 ± 44 ng/ml from control, NE-treated, PTL-/NE-treated and PPL-/PTL-/NE-treated tissues, respectively. Points represent the mean ($\pm$ S.E.) luminal IgA immunoreactivity in five control, NE-treated, PTL (0.3 μ M)-/NE-treated and PPL (0.3 μ M)-/PTL-/NE-treated tissues from five pigs. Line slopes were significantly different ($F = 41.3$, $Dfn = 3$, $Dfd = 4$, $P = 0.002$) as determined by linear regression analysis. **(Right).** Longer-term effects of NE on luminal IgA secretion. Luminal fluid samples were taken at time = 0 min prior to the contraluminal addition of drugs. Immediately after collection of the initial luminal fluid sample, some tissues were pretreated with adrenergic receptor antagonists. NE was added to the contraluminal bathing medium 5 min after the start of each experiment (i.e. time = 5 min). Mean IgA immunoreactivity at 0 min was 1014 ± 154 , 972 ± 120 and 840 ± 112 ng/ml from control, NE-treated, and PTL (0.1 μ M)-/NE-treated tissues, respectively. Points represent the mean ($\pm$ S.E.) luminal IgA immunoreactivity in nine control, NE-treated, and PTL-/NE-treated tissues from six pigs. Line elevations were significantly different ($F = 20.8$, $Dfn = 2$, $Dfd = 5$, $P < 0.004$) as determined by linear regression analysis. * $P < 0.05$ and ** $P < 0.01$ vs. control condition at identical time point, Dunnett t test.

Fig 4.

Effects of carbamylcholine (CCh) and norepinephrine (NE) on vectorial secretion of IgA from mucosal explants. (**A**, **B**). Levels of IgA- and secretory component (SC)-immunoreactive proteins in bathing media from tissues treated with CCh (panel A) or NE (panel B). Lanes 1 - 4 show SC-immunoreactive electrophoretic bands at 80 and >203 kDa detected with monoclonal mouse anti-swine SC and lanes 5 - 8 display IgA-immunoreactive bands at >203 kDa detected with monoclonal mouse anti-swine IgA. In panels A and B respectively, the effects of 10 μ M CCh or NE on contraluminal protein secretion are seen in lanes 1 and 5; the effects of these drugs on luminal protein secretion are seen in lanes in 2 and 6. Immunoreactive proteins recovered in contraluminal bathing medium from control tissues are visualized in lanes

3 and 7, and those recovered from the luminal bathing medium appear in lanes 4 and 8 baseline luminal secretion in unstimulated tissues. The location of pre-stained molecular weight markers are annotated on the left side of each electrophoretogram. Arrow heads and arrows indicate IgA and SC, respectively. The Western blots were produced under non-reducing conditions. (C). Quantification of the effects of CCh and NE on the release of secretory IgA (i.e. bands co-immunoreactive for both IgA and SC) into the tissue bathing media. Results represent pooled data from three separate experiments, expressed as band intensities relative to those obtained from samples of media bathing untreated, control tissues. Error bars indicate standard error of the mean.

Table 1Peak changes in short-circuit current (I_{sc}) in drug-treated mucosal explants from porcine colon

Drug (final concentration in contraluminal bathing medium)	Peak ΔI_{sc} ($\mu A/cm^2$)	n tissues/N pigs
Carbamylcholine (10 μM)	84 $\pm$ 9	18/13
Carbamylcholine (10 μM) + atropine (0.1 μM)	22 $\pm$ 7 ^{****}	19/13
Norepinephrine (10 μM)	144 $\pm$ 16	18/9
Norepinephrine (10 μM) + phentolamine (0.1 μM)	135 $\pm$ 15	17/9
Norepinephrine (10 μM)	140 $\pm$ 28	6/6
Norepinephrine (10 μM) + phentolamine (0.3 μM)	89 $\pm$ 27 [‡]	6/6
Norepinephrine (10 μM) + phentolamine (0.3 μM) + propranolol (0.3 μM)	67 $\pm$ 14 ^{‡‡}	6/6

**** P < 0.0001 vs. effect of carbamylcholine in the absence of atropine, unpaired t test.

[‡]P < 0.05 and ^{‡‡}P < 0.01 vs. norepinephrine in the absence of antagonists, Dunnett t test.