VPAC<sub>1</sub> receptors regulate intestinal secretion and muscle contractility by activating cholinergic neurons in guinea pig jejunum

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Submitted 3 December 2013; accepted in final form 25 February 2014

Fung C, Unterweger P, Parry LJ, Bornstein JC, Foong JP. VPAC<sub>1</sub> receptors regulate intestinal secretion and muscle contractility by activating cholinergic neurons in guinea pig jejunum. Am J Physiol Gastrointest Liver Physiol 306: G748–G758, 2014. First published February 27, 2014; doi:10.1152/ajpgi.00416.2013.—In the gastrointestinal tract, vasoactive intestinal peptide (VIP) is found exclusively within neurons. VIP regulates intestinal motility via neurally mediated and direct actions on smooth muscle and secretion by a direct mucosal action, and via actions on submucosal neurons. VIP acts via VPAC<sub>1</sub> and VPAC<sub>2</sub> receptors; however, the subtype involved in its neural actions is unclear. The neural roles of VIP and VPAC<sub>1</sub> receptors (VPAC<sub>1R</sub>) were investigated in intestinal motility and secretion in guinea pig jejunum. Expression of VIP and VPAC<sub>1</sub> receptors across the jejunum layers was examined using RT-PCR. Submucosal and myenteric neurons expressing VIP receptor subtype VPAC<sub>1</sub> and/or various neurochemical markers were identified immunohistochemically. Isotonic muscle contraction was measured in longitudinal muscle-myenteric plexus preparations. Electrogenic secretion across mucosa-submucosal preparations was measured in Ussing chambers by monitoring secretions. VPAC<sub>1R</sub> expression on the smooth muscle and/or myenteric plexus preparations. VIP induces Cl<sup>-</sup> secretion directly via epithelial VPAC<sub>1R</sub> and indirectly via VPAC<sub>1R</sub> on cholinergic secretomotor neurons. No evidence was obtained for involvement of other neural VIP receptors.

Vasoactive intestinal peptide; VPAC<sub>1</sub> receptor; motility; secretion; enteric

Vasoactive intestinal peptide (VIP) is one of the most abundant neuropeptides in the gut. It is found in many different enteric neural subtypes, including interneurons, inhibitory motor neurons, and secretomotor neurons (18). Although the presence of VIP in these subtypes is conserved across species (20, 27), its functions within the enteric circuitry are unclear. VIP depolarizes many myenteric (38, 49) and submucosal neurons (34, 43) and is found in many axon varicosities within the myenteric (37) and submucosal plexuses (14, 43). However, its role in synaptic transmission and the neuronal subtypes that respond to VIP are unknown. VIP is implicated in pathologies such as bacterial toxin-induced diarrhea (6) and irritable bowel syndrome (39), and a better understanding of its actions may prove beneficial for the development of novel therapeutics.

VIP is known to exert its effects by activating two main G protein-coupled receptor subtypes: VPAC<sub>1</sub> and VPAC<sub>2</sub> (13, 22, 31). In addition, a high-affinity VIP-specific receptor subtype has been identified in guinea pig tenia coli (47, 51). However, the VPAC receptor subtypes expressed by specific enteric neuron subtypes have not been investigated.

Identification of the specific roles of VIP in the gut has been difficult because of the apparent overlapping expression of its receptors on various effector cells and neurons. VIP is clearly important in the control of intestinal motility since VIP knockout mice display disrupted intestinal transit (32). In the myenteric plexus, VIP is contained within inhibitory motor neurons and descending interneurons (18, 41). Muscle contractility studies in guinea pig ileum and colon indicate that VIP directly relaxes circular muscle via activation of VPAC<sub>2</sub> receptors (VPAC<sub>2R</sub>) on the smooth muscle (24, 44) but also stimulates neurogenic contraction in longitudinal muscle preparations (26, 29). Motility studies in rat colon demonstrated involvement of VIP and VPAC<sub>1</sub> receptors (VPAC<sub>1R</sub>) (45). However, VPAC<sub>1R</sub> expression on the smooth muscle and/or myenteric neurons has not been examined. VIP is also a major player in the regulation of water and electrolyte secretion. VIP can directly stimulate the mucosa, which has been shown to express VPAC<sub>1R</sub> (42). In addition, secretion studies have shown that VIP also regulates neurogenic secretory tone, but the receptor subtype involved in this is unclear. Overall, understanding the involvement of VIP in physiology and pathophysiology has been challenging because of the multifaceted role of VIP in motility and secretion. A better understanding of the specific neural VPAC receptor that mediates these effects will provide more insight.

Evaluation of VPAC subtypes involved in gut functions has been hampered by lack of reliable antagonists. Nonspecific VIP antagonists have provided some insight, but some inconsistencies in findings between studies have arisen. For instance, the effectiveness of the VIP fragment, VIP(10–28), as a VIP antagonist appears to differ between studies (1, 7, 12, 42), perhaps because of species differences. Yet, despite using the same animal model, another nonspecific VIP antagonist, [4Cl-]D-Phe<sub>6</sub>,Leu<sub>17</sub>]-VIP, was reported to reverse cholera toxin-induced secretion by one group (36) but to be ineffective by another (1). However, the selective VPAC<sub>1R</sub> antagonist PG97–269 has been used successfully to attenuate cholera toxin-evoked hypersecretion and antagonize VIP effects in monolayers of the human colonic cell line T84 in human ileum and in rat jejunum (1).
We employed an antiserum against VPAC1R and the specific VPAC1R antagonist PG97-269 to examine the roles of this receptor in muscle contractility and secretomotor control in guinea pig jejunum. We found that VIP acting via VPAC1R on excitatory motor neurons stimulates ACh-mediated longitudinal muscle contraction. Furthermore, we found that VIP activates VPAC1R on cholinergic secretomotor neurons.

**MATERIALS AND METHODS**

**Identification and quantification of Vipr1 and Vipr2 mRNA**

*Tissue preparation.* Guinea pigs of either sex (180–350 g) were killed by stunning and severing the carotid arteries and spinal cord according to protocols approved by the University of Melbourne Animal Experimentation Ethics Committee. Segments of jejunum (4–5 cm) were removed from the animal and immediately placed in PBS (4°C).

*RNA extraction.* The tissue was segregated into mucosa, submucosa, longitudinal muscle and myenteric plexus, and circular muscle by microdissection. These samples were frozen with liquid nitrogen. Total RNA was extracted as previously described (17).

*RT-PCR.* Expression of VIP receptor 1 gene (*Vipr1*) and VIP receptor 2 gene (*Vipr2*) transcripts (encode VPAC1R and VPAC2R, vasoactive intestinal peptide receptor 1 gene; *Vipr1* and vasoactive intestinal peptide receptor 2 gene; *Vipr2*) in guinea pig jejunum was first assessed using RT-PCR. First-strand cDNA synthesis was performed using 1 μg total RNA in a 20-μl reaction with random hexamers (50 ng/μl) and 200 U SuperScript III (Invitrogen). Guinea pig specific forward and reverse primers for *Vipr1* and *Vipr2* were synthesized by GeneWorks (Adelaide, SA, Australia; Table 1). Each RT-PCR reaction used 1 μl cDNA in a 25-μl reaction containing GoTaq Green Master Mix (Promega, Annandale, SA, Australia), with forward and reverse oligonucleotide primers designed to span an intron (GeneWorks, Hindmarsh, SA, Australia). Guinea pig specific primers for *gapdh* were included as a positive control. PCR reactions were performed using a MyCycler PCR machine (Bio-Rad, West Ryde, NSW, Australia). PCR products were sequenced by the Applied Genetic Diagnostics laboratory (Department of Pathology, The University of Melbourne, Parkville, VIC, Australia).

*Quantitative real-time polymerase chain reaction.* Quantification of *Vipr1* and *Vipr2* gene expression was accomplished using the ΔΔCt method with ribosomal 18S (*Rn18s*) as the endogenous control. Guinea pig specific forward and reverse primers and 6-carboxy fluorescein-labeled TaqMan probes for *Vipr1* and *Vipr2* were designed using RealTimeDesign (Biosearch Technologies, Novato, CA; Table 1). Optimal concentrations for all primers and probes were 10 μM. Quantitative real-time polymerase chain reaction (qPCR) experiments were performed on the Opticon 2 PCR machine (Bio-Rad) in triplicate 20-μl volumes containing 1× SensiMix dT (Quantate, Alexandria, NSW, Australia) and 1–2 μl cDNA. Mean relative gene expression was plotted with the SE.

**Immunohistochemical Localization of VPAC1R**

A jejunal segment (5 cm long) was opened along the mesenteric border, pinned flat, and fixed in 4% paraformaldehyde overnight at 4°C. Tissues were cleared of fixative (3 × 10 min PBS washes) before submucosal plexus and myenteric plexus preparations were dissected and processed for immunohistochemistry as previously described (21). Briefly, preparations were permeabilized with 1% Triton X-100 (ProSciTech, Thuringowa, QLD, Australia) for 30 min and then washed (3 × 10 min; PBS). Primary antibodies were applied and incubated overnight at 4°C. Each submucosal preparation was triple-labeled for Hu (pan-neuronal marker), VPAC1R, and one of three markers of cholinergic neurons: choline acetyltransferase (ChAT, all cholinergic neurons), neuropeptide Y (NPY, secretomotor), or calretinin (secretomotor/vasoconstrictor) (18) (Table 2). Each myenteric preparation was triple-labeled for Hu, VPAC1R, and one of three markers for different functional subsets of neurons: calbindin (intrinsic sensory neurons), calretinin (excitatory longitudinal muscle motor neurons and ascending interneurons), and nitric oxide synthase (NOS; inhibitory motor neurons and descending interneurons) (18) (Table 2). After being washed (3 × 10 min; PBS), preparations were incubated in secondary antibodies (Table 2) for 150 min at room temperature. The preparations were washed again (3 × 10 min; PBS) before mounting in Dakocytofonation fluorescent mounting medium (Carpen-taria).

*Cell counts.* Submucosal plexus and myenteric plexus preparations were imaged using a Zeiss Pascal confocal microscope. The proportion of each neuronal subtype (ChAT+, NPY+, calretinin+, calbindin+, NOS−, and/or VPAC1R+) was obtained by examining coexpression with at least 100 Hu+ neurons in each preparation. The mean proportion of each neuronal subtype was determined by averaging the

**Table 1. Guinea pig specific primers/probes used for RT-PCR and qPCR**

<table>
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<tr>
<th>Primer/Probe (Accession No.)</th>
<th>Sequence (5’-3’)</th>
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<th>Annealing Conditions</th>
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<td><em>Vipr1</em> (JN225407)</td>
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<td>TGTTCACAGCCAGGAGGTTG</td>
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<td><em>Vipr2</em> (NM_001173121)</td>
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<tr>
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<td><em>Vipr2</em> (NM_001173121)</td>
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<td>Probe</td>
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*Vipr1*, vasoactive intestinal peptide receptor 1 gene; *Vipr2*, vasoactive intestinal peptide receptor 2 gene.
Table 2. Primary and secondary antisera used for immunohistochemistry

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Product Code and Source</th>
<th>Species</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Product Code and Source</th>
<th>Species</th>
<th>Dilution</th>
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<td>Anti-rabbit CY5</td>
<td>711-065-152; Jackson</td>
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<td>Hu</td>
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<td>Goat</td>
<td>1:500</td>
<td>Anti-sheep 488</td>
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<td>CG1; SWANT</td>
<td>Goat</td>
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<tr>
<td>NPY</td>
<td>Dimension</td>
<td>Sheep</td>
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<tr>
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<td>1:500</td>
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</table>

VPAC1R, vasoactive intestinal peptide receptor 1; ChAT, choline acetyltransferase; NPY, neuropeptide Y; nNOS, neuronal nitric oxide synthase.

proportions obtained from three to five animals. Data are expressed as means ± SE.

In Vitro Measurement of Longitudinal Muscle Contractility

Tissue preparation. The abdominal cavity of the animal was opened, and a segment of jejunum (8–10 cm in length) was excised and placed in physiological saline (Krebs; composition in mM: 118 NaCl, 25 NaHCO3, 11 t-glucose, 4.8 KCl, 2.5 CaCl2, 1.2 MgSO4, and 1.0 NaH2PO4) bubbled with 95% O2-5% CO2 (carbogen).

The segment of jejunum was opened along the mesenteric border and then pinned flat on a Sylgard-lined dish. The mucosa, submucosa, and circular muscle layers were removed by microdissection. The remaining myenteric plexus and longitudinal muscle preparations were cut into longitudinal strips (1.5–2 cm in length) and then suspended in a 6-ml organ bath and attached to isotonic transducers (SDR Technology, Sydney, NSW, Australia) with a resting tension of 0.3 g. Preparations were continuously bathed with carbogenated Krebs maintained at 37°C. Contractile responses were recorded using a Biopac M100A (SDR Technology). Data were analyzed using Acaknowledge 3.2.4 software (SDR Technology).

The guinea pig longitudinal strips were equilibrated for 40 min. Time control experiments involved three repeated additions of VIP (100 nM) separated by washout (complete changes of the bath solution; 3 × 5 min). In separate experiments, various antagonists were added before the second VIP addition and allowed to incubate for 3, 10, or 15 min.

In Vitro Measurement of Short-Circuit Current Using Ussing Chambers

The gut segment was opened along the mesenteric border and pinned flat on a silicone elastomer-lined dish (Sylgard 184; Dow Corning, North Ryde, NSW, Australia). Mucosal-submucosal preparations were produced by removing the smooth muscle layers by microdissection. Each animal provided up to four mucosa-submucosa preparations. Ussing chambers were set up as described previously (16). Briefly, preparations were mounted in Ussing chambers (CHM2; World Precision Instruments, Sarasota, FL). The mucosal and serosal surfaces were independently bathed in continuously circulating carbogenated Krebs (10 ml) maintained at 37°C. The transepithelial voltage potential of the preparation was clamped to zero, and short-circuit current (Isc), a measure of epithelial ion transport (11), was monitored. The tissue was equilibrated for 40 min before data recording. Drugs were added to the serosal half-chamber.

Experimental protocol. Experiments were limited to a duration of 3 h from mounting the preparation to minimize deterioration of the mucosal epithelium. The protocol involved four agonist additions at 20-min intervals. Each addition was separated by a washout (3 changes of fresh Krebs at 5-min intervals) before the agonist-evoked response reached a plateau. The third and fourth time points were chosen for further pharmacological analysis, since agonist-induced responses at these times did not differ significantly from each other.

Data Measurement and Statistics

The maximum change in muscle length and maximum change in Isc from baseline (Δls and ΔIsc) evoked by addition of drug was measured. Experiments conducted with tissue from the same animal were matched; control data from adjacent segments of jejunum were not significantly different. Results are presented as mean Δlength ± SE and mean ΔIsc ± SE. Muscle contractility data were analyzed by a one-way ANOVA, and secretion data were analyzed by a two-way ANOVA, followed by a Bonferroni’s posttest where appropriate to determine statistical significance. P < 0.05 was considered significant.

Drugs Used

All drugs were prepared as stock solutions using distilled water. Drugs were directly added to the Krebs bathing the longitudinal muscle preparations or diluted in the reservoir of Krebs supplying the serosal side of the Ussing chamber to achieve the desired final concentration. The maximum volume of each drug added did not exceed 100 μl.

Tetrodotoxin (TTX; Alomone Labs, Jerusalem, Israel), hyoscine hydrobromide and hexamethonium bromide (both from Sigma Aldrich, Castle Hill, NSW, Australia), and VIP (guinea pig; Auspep, Tullamarine, VIC, Australia) were stored at 4°C. [Ac-His5-D-Phe8-Lys3,Arg6-Leu2]VIP(1–7)/growth hormone-releasing factor (8–27) or PG97–269 (Phoenix Pharmaceuticals, Belmont, CA) was stored as aliquots at −20°C. TTX was incubated in the bathing solution for 15 min, hyoscine and hexamethonium were incubated for 10 min, and the VPAC1R antagonist was incubated for 3 min before agonist addition.

RESULTS

Differential Expression of Vipr1 and Vipr2 Across the Gut Layers

Vipr1 and Vipr2 expression was analyzed in separated layers of gut, including the mucosa, submucosal plexus, longitudinal muscle and myenteric plexus, and circular muscle. Both Vipr1 and Vipr2 gene transcripts were detected by RT-PCR in all layers (Fig. 1A). The Vipr1 PCR bands appeared strongest in the mucosa and weakest in the circular muscle. Substantial levels of Vipr1 expression were detected in the submucosal plexus and longitudinal muscle plus myenteric plexus layers. The Vipr2 PCR bands were of similar intensity across all layers. Sequenced Vipr1 and Vipr2 PCR products were analyzed using nucleotide BLAST (NCBI), which confirmed that target gene products were amplified. Our guinea pig Vipr1 sequence shared 85% homology with that of mice and 84% homology with that of rats. Similarly, our guinea pig Vipr2 sequence shares 89 and 87% homology with mouse and rat Vipr2 sequences, respectively. Sequence analysis showed that the PCR products corresponded to an 84-amino acid fragment.
of the VPAC1R and a 72-amino acid fragment of the VPAC2R (Expert Protein Analysis System, ExPASy; Swiss Institute of Bioinformatics). Quantitative analysis of Vipr1 and Vipr2 expression is shown in Fig. 1, B and C. Expression of Vipr1 was significantly higher in the mucosa ($P < 0.001$) than in any other gut layer (Fig. 1B), although substantial levels were also detected in the submucosal plexus. However, the level of expression detected in the longitudinal muscle and myenteric plexus was relatively low. In contrast, Vipr2 expression was highest in the submucosal plexus, which was notably higher than in the mucosa ($P < 0.001$) and longitudinal muscle and myenteric plexus ($P < 0.05$) (Fig. 1C).

Adcyap1r1 [which encodes for the PAC1 receptor (PAC1R)] expression was also examined by RT-PCR and was found in all gut layers, whereas quantitative analysis showed that Adcyap1r1 expression was very low in the mucosa relative to the other layers (data not shown).

Cholinergic Excitatory Motor Neurons and Secretomotor Neurons Express VPAC1R

The expression of VPAC1R within the myenteric and submucosal plexuses was then examined using immunohistochemistry. VPAC1R immunoreactivity was observed in 52 ±
2% of all myenteric neurons (n = 8 animals; Hu+, 5,158 neurons, Fig. 2) and 42 ± ± 4% of all submucosal neurons (n = 5 animals; 2,088 Hu+ neurons, Fig. 3). VPAC₁R staining was specific to neurons, particularly localized to the cytoplasm of their cell bodies and nerve fibers (Figs. 2 and 3). Both strongly and weakly staining neurons were seen. In the submucosal plexus, VPAC₁R+ neurons were observed to be predominantly located in the periphery of the ganglia (not quantified).

In the myenteric plexus almost all calbindin+ intrinsic sensory neurons (18) were VPAC₁R+ (99 ± ± 4%; Fig. 2, A–D; n = 3 animals). A substantial proportion of calretinin+ excitatory motor neurons and ascending interneurons (18) were also VPAC₁R-immunoreactive (61 ± ± 5%; n = 4 animals; Fig. 2, E–H), whereas only 0.88 ± ± 1% of NOS+ neurons expressed VPAC₁R (n = 3 animals; Fig. 2, I–L). Interestingly, in the submucosal plexus, a large proportion of VPAC₁R+ submucosal neurons expressed ChAT: 78 ± ± 4% of VPAC₁R+ neurons were ChAT+ and 61 ± ± 4% of ChAT+ neurons were VPAC₁R+ (n = 5 animals; Fig. 3, A–D). Moreover, the majority of NPY+ secretomotor neurons (18) were VPAC₁R-immunoreactive (78 ± ± 4%; n = 4 animals; Fig. 3, E–H), whereas only 14 ± ± 8% of calretinin+ neurons expressed VPAC₁R (n = 4 animals; Fig. 3, I–L).

**Role of VPAC₁R in Longitudinal Muscle Contraction and Intestinal Secretion**

Given the expression of VPAC₁R on calretinin excitatory motor neurons and cholinergic secretomotor neurons, we examined the effect of VIP on contractions of longitudinal muscle-myenteric plexus preparations and on electrogenic secretion in submucosa-mucosa preparations. Muscle contractility experiments were conducted on tissues from 20 animals (35 preparations). Ussing chamber experiments were conducted on tissues from 60 animals (112 preparations); basal Iₑ was 70 ± ± 4 μA/cm² (n = 112).

**VIP evokes longitudinal smooth muscle contractions by activating VPAC₁Rs on neurons.** Application of VIP (100 nM) evoked reproducible contractions in longitudinal muscle preparations (0.39 ± ± 0.10 mm; n = 8; Fig. 4A). The VIP-evoked contractions were reversibly blocked by incubating the tissue with TTX (voltage-gated Na+ channel blocker; 1 μM) for 15 min before VIP application (P < 0.05; n = 7; Fig. 4B), indicating that VIP was acting on neurons and not directly on the smooth muscle. VIP-evoked contractions were also significantly inhibited in the presence of the VPAC₁R antagonist PG97–269 (1 μM; P < 0.01; n = 6; Fig. 4C), an effect that persisted after washout. Similarly, the muscarinic antagonist hyoscine (10 μM) significantly inhibited the VIP-evoked contractile response (P < 0.01; n = 8; Fig. 4D), and its effects did not wash out. In contrast, the VIP response was unaffected by nicotinic receptor blockade (hexamethonium, 200 μM; n = 6; Fig. 4E).

**VIP-mediated secretion involves activation of cholinergic secretomotor neurons.** Application of VIP (10–300 nM) to the serosal side of the preparation induced a concentration-dependent increase in Iₑ (Fig. 5A). VIP at 50 nM produced a

![Fig. 2. Localizing VPAC₁ receptor (VPAC₁R) in the myenteric plexus of guinea pig jejunum. Images of representative myenteric ganglia, demonstrating neurons stained for Hu (A, E, and I), VPAC₁R (B, F, and J), calbindin (C), calretinin (G), and nitric oxide synthase (NOS; K). Merged images of Hu, VPAC₁R, and calbindin (H) demonstrate colocalization of calbindin with some VPAC₁R+ neurons. In contrast, the merged image of Hu, VPAC₁R, and NOS (L) shows a lack of colocalization of VPAC₁R with NOS. Scale bars = 20 μm. Filled arrows indicate colocalization of VPAC₁R with the neuronal subtype marker, whereas open arrows indicate a lack of colocalization between the markers.](http://ajpgi.physiology.org/DownloadedFrom)
submaximal response; this concentration was used for pharmacological analyses (Fig. 5, B–D). Time controls involved four repeated VIP (50 nM) additions separated by 3 × 5 min washouts to ensure reproducible responses were achieved. The first and second additions evoked responses that were significantly different from each other and were smaller than the \( I_{sc} \) induced by the third and fourth additions (first addition: 64 ± 5 \( \mu A/cm^2 \); second addition: 98 ± 12 \( \mu A/cm^2 \); \( P < 0.05; n = 9 \); Fig. 5B). The third and fourth additions of VIP produced consistent responses (third addition: 123 ± 15 \( \mu A/cm^2 \); fourth addition: 119 ± 19 \( \mu A/cm^2 \); \( n = 9 \)), and thus, in later experiments, the effects of antagonist and washout were examined at these time points, respectively. Because the magnitude of the VIP responses varied between preparations, responses were normalized to the second VIP exposure for each experiment. Data from antagonist experiments were compared with the VIP time controls using two-way ANOVA.

The response to VIP was usually biphasic; an initial phase of the response was present in 95% of the preparations examined. However, because the peak of the initial phase was often subtle, only the second phase was quantified. TTX (1 \( \mu M \)) abolished the initial phase in all preparations examined and reversibly depressed the second phase (\( P < 0.05; n = 7 \); Fig. 5C). The TTX-resistant VIP response was about 80% of the corresponding VIP time control. Hyoscine (10 \( \mu M \)) abolished the first phase in every preparation that displayed a biphasic response and irreversibly depressed the second phase (\( P < 0.01; n = 5 \); Fig. 5D) to about 70% of the corresponding time control. Furthermore, there was no involvement of fast nicotinic synaptic transmission, since the VIP response in the presence of hexamethonium (200 \( \mu M \)) was not significantly different from that of the paired time control (39 ± 8 and 37 ± 8 \( \mu A/cm^2 \) respectively; \( n = 9 \)).

VIP evokes a small neurogenic secretory component involving VPAC1R. PG97–269 (1 \( \mu M \)) was used to examine the role of VPAC1R in the VIP-evoked \( I_{sc} \). This VPAC1R selective antagonist was applied 3 min before the addition of VIP, since this duration of incubation was previously reported to be most effective (15). The antagonist blocked the initial phase of the VIP response in 33% of preparations examined and reversibly inhibited the second phase (\( n = 6; P < 0.001 \); Fig. 5C) to about 37% of the corresponding time control.

PG97–269 and TTX were applied together to determine whether there is a VPAC1R-independent neural response to...
VIP. The first phase of the response was abolished by the VPAC1R antagonist and TTX combined in 83% of preparations examined. The combination of both antagonists reversibly depressed the second phase of the response (n = 110056) compared with time controls (n = 9; P < 0.01, Fig. 5C). Their combined effect was no greater than that of PG97–269 alone, but it exceeded that of TTX alone. Hence, no VPAC1R-independent neural effect could be detected.

**Neurally mediated basal secretion involves VPAC1R.** Baseline I_sc of the time controls did not change significantly over the course of the experiment. TTX significantly depressed basal secretion by 63% (P < 0.01; paired t-test; n = 11). Hyoscine reduced the baseline by 15% (P < 0.05; n = 10), and PG97–269 reduced the baseline by 12% (P < 0.05; n = 16). However, hexamethonium did not affect basal secretion (n = 9). Application of PG97–269 together with hyoscine also significantly depressed basal I_sc by 15% (P < 0.05; n = 5) but to a similar extent as hyoscine and PG97–269 alone.

**DISCUSSION**

Our results demonstrate the involvement of neural VPAC1R in both motility and secretion. We have localized VPAC1R to cholinergic motor neurons innervating longitudinal muscle and cholinergic secretomotor neurons. Furthermore, we showed that this receptor subtype is involved in neurally mediated longitudinal muscle contraction and neurogenic secretion with no evidence for involvement of noncholinergic neurons in either response. VIP-mediated effects involve activation of cholinergic neurons that is independent of nicotinic neurotransmission. Our data also confirm that a major action of VIP is to directly activate secretion via VPAC1R on the mucosal epithelium. Collectively, it appears that VPAC1Rs are involved at the output end of the motor pathways of the myenteric and submucosal plexuses.

**Exogenous VIP Activates VPAC1R to Evoke Longitudinal Muscle Contraction**

VIP was previously associated with smooth muscle relaxation via a direct effect on smooth muscle (24, 44). In this study, we show that VIP evokes neurogenic longitudinal muscle contraction, since the VIP-evoked contraction was abolished in the presence of TTX. This complements previous reports in guinea pig ileum and distal colon.
We are the first to demonstrate that VPAC1R is the receptor subtype primarily involved in this response, since it was almost abolished by the VPAC1R antagonist PG97–269. Hence, there is little evidence for the involvement of any other receptor subtypes, namely VPAC2R or the VIP-specific receptor. We have localized VPAC1R immunohistochemically to calretinin/H11001 myenteric neurons, many of which are excitatory motor neurons innervating the longitudinal smooth muscle (18). VPAC1R immunoreactivity was also localized to calbindin/H11001 neurons (myenteric intrinsic sensory neurons), consistent with VIP depolarizing myenteric afterhyperpolarizing neurons (38), although the role of VPAC1R on these neurons is less clear. Calretinin is also contained within cholinergic ascending interneurons. However, there is little evidence for the involvement of either the ascending interneurons or the intrinsic sensory neurons in VIP-induced contraction, since blocking fast nicotinic transmission with hexamethonium did not alter the response. There was virtually no colocalization of VPAC1R with NOS (which labels inhibitory motor neurons), so this receptor subtype is unlikely to be involved in neurally mediated smooth muscle relaxation. This is also consistent with the notion that VIP directly relaxes circular smooth muscle (24, 44). VIP-evoked contraction appears to be exclusively mediated via ACh, since the response was blocked by hyoscine. These results also suggest that VIP has no direct effect on the longitudinal muscle. If VPAC1R is indeed only localized to myenteric neurons, perhaps a lack of VPAC1R expression in the longitudinal muscle led to the low level of \textit{Vipr1} expression detected in the longitudinal muscle and myenteric plexus samples by qPCR. Collectively, the data indicate that VIP directly activates excitatory motor neurons via VPAC1R to evoke ACh release, thereby activating muscarinic acetylcholine receptors on the longitudinal muscle to cause contraction. We propose that the most likely source of VIP input is from descending interneurons since it has been previously shown that VIP-immunoreactive varicosities closely oppose the cell bodies of calretinin\textsuperscript{+} myenteric neurons in guinea pig jejunum (37).

Fig. 5. Effects of various antagonists on the second phase of VIP-induced change (Δ) in short-circuit current (I\textsubscript{sc}). A: addition of VIP (10–300 nM) to the serosal side of the jejunum preparation induced a concentration-dependent increase in I\textsubscript{sc}, with 50 nM producing a submaximal response. B: repeated additions of VIP (50 nM), where each addition was separated by 3 × 5 min wash with normal Krebs. The first two additions (solid black bars) evoked responses that were significantly different from each other and were significantly smaller than the 3rd and 4th additions of VIP (striped bars; n = 9). Because the 3rd and 4th VIP additions produced consistent responses, these time points were used to examine the effects of antagonist and washout. C: the VIP response was significantly reduced in the presence of PG97–269 (1 μM; n = 6), TTX (1 μM; n = 7), and the combination of both antagonists (n = 6), but TTX did not further reduce the response in the presence of PG97–269. D: the VIP-evoked response was significantly reduced in the presence of hyoscine (10 μM; n = 5) compared with the VIP time control (n = 9).
Exogenous VIP Activates VPAC\(_1\)R on Cholinergic Secretomotor Neurons

Our PCR data showed expression of Vipr1 mRNA in submucosal plexus, and VPAC\(_1\)R immunoreactivity was identified in cholinergic submucosal neurons, notably by most NPY secretomotor neurons. From our secretion studies, the biphasic VIP response suggests that VIP evokes both cholinergic and noncholinergic responses (8, 10). The first phase was abolished by hyoscine and TTX, indicating that it results from activation of cholinergic neurons. Furthermore, hyoscine reduced the second phase, and, similarly, TTX also reduced the second phase, albeit only by a small amount. These results are consistent with the finding of VPAC\(_1\)R on cholinergic neurons and collectively indicate that VIP stimulates neurons in a cholinergic secretomotor pathway. However, like VIP-evoked longitudinal muscle contractions, VIP-evoked secretion was also unaltered by blockade of nicotinic fast synaptic transmission. This suggests that VIP directly excites cholinergic secretomotor neurons. Although Vipr2 expression was highest in the submucosa, we found no evidence for involvement of VPAC\(_1\)R or the VIP-specific receptor. Rather, the neural component of the VIP response appears to involve VPAC\(_1\)R, since TTX in combination with the VPAC\(_1\)R antagonist did not further reduce the response. Considering that Vipr2 transcripts were expressed in the submucosa, an effective antiserum to localize VPAC\(_2\)R and a specific VPAC\(_2\) antagonist would be invaluable in providing insight into the role of these receptors. We and several others have shown that submucosal neurons in guinea pig are divided into two major populations (ChAT\(^{+}\) and VIP\(^{+}\)) (4, 19, 46), and we have found that submucosal neurons that did not label for ChAT are VPAC\(_{2}\)R \(^{+}\) (data not shown). Thus, by inference, because 20% of VPAC\(_{2}\)R \(^{+}\) neurons were noncholinergic, these neurons must also be VIP\(^{+}\). Nonetheless, there was no evidence of VIP acting on VIP secretomotor neurons.

Our results also show that TTX and the VPAC\(_1\)R antagonist separately and in combination reduced basal secretion, supporting previous reports of neurogenic secretory tone (9, 15, 28) and that VIP neurons are tonically active (9).

Signaling Pathways Involving VIP, PACAP, and NO

VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) are closely related in the VIP family of peptides. PACAP activates PACAP-selective PAC1R and displays similar affinity for the VIP receptors as VIP (48). PACAP, like VIP, is involved in regulating motility and secretion (25, 30). It was shown in the guinea pig ileum that PACAP is expressed by neuronal fibers in the myenteric plexus and smooth muscle (40) and by inhibitory motor neurons innervating circular muscle (18). This is consistent with our finding in guinea pig small intestine that Adcyap1r1 is expressed in the myenteric plexus and smooth muscle layers. Portbury and others (1995) reported that the submucous neurons did not express PACAP in their cell bodies and that there were PACAP-immunoreactive fibers in submucosal ganglia but not the mucosa of guinea pig small and large intestines (40). In accordance with this, we observed Adcyap1r1 expression in the submucous plexus, and the level of mRNA detected in the mucosa was the lowest compared with the other gut layers (40). This suggests that PAC1R is not likely to play a major role in secretory function at the level of the mucosal epithelium.

There are strong interactions between VIP, PACAP, and NOS in neuronal reflex pathways. In rat studies, VIP and PACAP release is regulated by nitric oxide (NO) and vice versa in the control of smooth muscle tone, whereas VIP and NO show synergistic effects in regulating secretion (3, 35). In the guinea pig ileum, VIP and PACAP are colocalized with NOS in inhibitory motor neurons, which innervate circular muscle, whereas VIP and NOS are colocalized in inhibitory motor neurons, which supply the longitudinal muscle (18). Furthermore, VIP- and NOS-immunoreactive interneurons from the myenteric plexus provide inputs to VIP and cholinergic submucosal neurons (33). Interestingly, NO suppresses some slow excitatory postsynaptic potentials and enhances inhibitory postsynaptic potentials, yet increases the overall excitability of VIP secretomotor neurons to depolarization. Thus, NO may be acting to fine tune the numerous inputs to VIP neurons (5). Hence, it is possible that NO may modulate the activity of VIP interneurons, which provide inputs to VPAC\(_1\)R \(^{+}\) excitatory motor neurons (18, 37) and secretomotor neurons (33, 43).

VPAC\(_1\)R is the Major Receptor Subtype in the Mucosa

The Vipr1 transcript was most strongly expressed in the mucosa, with substantial levels in the submucosa and much lower expression in the muscle. Expression of Vipr2, which encodes the other major VIP receptor subtype VPAC\(_2\), was significantly lower in the mucosa. This is consistent with VPAC\(_2\)R being the major receptor subtype mediating VIP-evoked secretion (1, 6). Accordingly, we found that VIP response was largely inhibited by blocking VPAC\(_1\)R. Furthermore, the VIP response was inhibited by the VPAC\(_1\)R antagonist to a larger extent than by TTX; this together with the PCR data indicates that VIP-evoked secretion is mainly mediated via VPAC\(_1\)R by a direct action on the mucosa. Although, even in the presence of the VPAC\(_1\)R antagonist, there was a residual response to VIP suggesting involvement of another VIP receptor subtype, possibly VPAC\(_2\) as we found Vipr2 expression in the mucosa. However, this was not further investigated because of the lack of a specific VPAC\(_2\) receptor antagonist (23). The residual response may also be attributed to the efficacy of the antagonist at the concentration (1 \(\mu\)M) used or the duration of incubation before applying VIP. These were selected because a previous secretion study (15) reported this concentration and timing to be most effective. The same study reported that PG97–269 (1 \(\mu\)M) suppressed or blocked 0.5 \(\mu\)M VIP responses, but, despite the lower concentration of VIP (50 nM) used in our study, PG97–269 did not block the response. The reason for this discrepancy is unclear. Nonetheless, a higher concentration of antagonist or longer incubation might be more effective (1).

Enhanced Secretory Responses with Repeated Agonist Exposure

Responses to VIP increased with repeated exposures before stabilizing. VIP may potentiate the cholinergic component via intracellular secondary messenger systems. Agents that increase cAMP levels, including VIP, enhance the secretory response to calcium-dependent secretagogues (50), and the
VIP receptors excite submucosal and myenteric nerves

Calciu-dependent cholinergic component of the secretory response to electrical stimulation (10) in guinea pig distal colon and ileum, respectively. There is also evidence of this in human T84 epithelial cell monolayers, where cholera toxin and forskolin (both raise cAMP levels) significantly increase the response to exogenous ACh (2). However, details of the intracellular pathways and how the two systems may interact synergistically remain unknown.

In conclusion, we have shown that cholinergic excitatory motor neurons innervating longitudinal smooth muscle and cholinergic secretomotor neurons express VPAC1 and VPAC2 and are involved in mediating neurogenic contractile and secretory responses to exogenous VIP, respectively. However, the neural pathways involved are independent of nicotinic transmission. Identification of the physiological circuitry that leads to release of endogenous VIP on these two classes of cholinergic neurons will be the next step in understanding the place of VPAC1 receptors in the enteric neural circuitry controlling motility and secretion.

ACKNOWLEDGMENTS
We thank Maria Jelinic and Dr. Yu May Soh for assistance with the PCR studies.

GRANTS
The study was supported by a grant from the National Health and Medical Research Council Australia (Grant No. APP1006453). C. Fung is supported by an Australian Postgraduate Award.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: C.F. and P.U. performed experiments; C.F. and P.U. analyzed data; C.F.; L.J.P., J.C.B., and J.P.P.F. interpreted results of experiments; C.F. and J.P.P.F. prepared figures; C.F. and J.P.P.F. drafted manuscript; C.F., P.U., L.J.P., J.C.B., and J.P.P.F. approved final version of manuscript; C.F. and J.P.P.F. prepared figures; C.F. and J.P.P.F. drafted manuscript; C.F., L.J.P., J.C.B., and J.P.P.F. interpreted results of experiments.

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