Vasoactive intestinal peptide is a local mediator in a gut-brain neural axis activating intestinal gluconeogenesis

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VASOACTIVE INTESTINAL PEPTIDE IS A LOCAL MEDIATOR IN A GUT-BRAIN NEURAL AXIS ACTIVATING INTESTINAL GLUCONEOGENESIS

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ABSTRACT

Intestinal gluconeogenesis (IGN) promotes metabolic benefits through activation of a gut-brain neural axis. However, the local mediator activating gluconeogenic genes in the enterocytes remains unknown. We show that (1) VIP signaling through VPAC1 receptor activates the intestinal glucose-6-phosphatase gene *in vivo*, (2) the activation of IGN by propionate is counteracted by VPAC1 antagonism, and (3) VIP-positive intrinsic neurons in the submucosal plexus are increased under the action of propionate. These data support the role of VIP as a local neuromodulator released by intrinsic enteric neurons and responsible for the induction through a VPAC1 receptor-dependent mechanism in enterocytes.

KEYWORDS

Intestinal gluconeogenesis; vasoactive intestinal peptide; gut-brain axis; glucose-6-phosphatase

KEY MESSAGES

- Vasoactive intestinal peptide is a local neuromodulator released by enteric neurons in response to propionate feeding. This in turn activates intestinal gluconeogenesis, a key process in the regulation of glucose and energy balance.
- Aims/goals: We sought to determine the neural mechanisms by which intestinal glucose production is activated following a propionate-enriched diet.
- We fed rats standard or propionate-enriched diet for two weeks and we evaluated the activation of intestinal gluconeogenesis genes in response to these diets and to VIP signaling (i.e. exogenous VIP and VPAC1 receptor antagonist). Furthermore, we quantified VIP-positive neurons in the enteric nervous system in response to propionate feeding.
- When injected with VIP, rats fed a control diet show an activation of intestinal gluconeogenesis genes. This activation was also present in propionate-fed rats but was reversed by VPAC1 receptor antagonist injection. VIP enteric submucosal neurons were also more numerous in propionate-fed rats.

INTRODUCTION

The gut-brain axis plays a critical role in the regulation of metabolic homeostasis. Intestinal gluconeogenesis (IGN) is a newly described process that promotes a decrease of hunger and an improvement of insulin sensitivity, while decreasing hepatic glucose production(1). The IGN function is described in humans, especially after gastric bypass surgery or during the an-hepatic phase of liver transplantation(2). Intestinal gluconeogenesis is activated when feeding protein(3,4)- or dietary fiber(5)-enriched diets. Specifically, in the case of dietary fiber, microbial-generated propionate binds to free fatty acid receptor 3 (FFAR3) in the nerve endings of the portal vein wall. This leads to the activation of a gut-brain reflex arc, inducing in the end the expression of gluconeogenic genes (glucose-6-phosphatase, G6Pase; phosphoenolpyruate carboxykinase, PEPCK)(5). However, the local enteric relay responsible for the activation of IGN genes in the enterocytes remains to be elucidated. We hypothesized that the release of a local gut neurotransmitter by enteric nervous plexuses in response to a signal from the central nervous system could be involved.

Vasoactive intestinal peptide (VIP) is a small peptide that acts as a neurotransmitter and paracrine neurohormone in non-adrenergic neurons of the enteric nervous system(6). VIP neurons innervate the gut epithelium, and VIP specifically increases cAMP levels in enterocytes through binding to G_s-coupled VPAC1 receptor(7). *In vitro* studies in Caco-2 cells overexpressing the catalytic subunit of G6Pase (G6PC) have shown that transcription of *G6PC* is activated by cAMP-mediated signaling in the intestine(8). Interestingly, isolated intestinal epithelial cells from rat exhibit a high adenylate cyclase sensitivity to VIP(9), and culturing Caco-2 cells overexpressing *G6PC* in the presence of VIP up-regulates the transcription of the gene(8). These data suggest that local release of VIP by enteric neurons may be involved in activation of IGN genes in the gut epithelium.

To test this hypothesis, we fed rats for two weeks with either standard chow or propionate-enriched chow to induce IGN gene expression (see Methods). We investigated the *in vivo* implications of VIP on IGN gene expression and cAMP-mediated signaling in the intestine.

RESULTS AND DISCUSSION

Rats that were fed on standard chow and received a VIP injection had about 2.5-fold higher G6Pase activity in the jejunum compared with rats that received a saline injection. In

line with previous data(5), propionate feeding strongly induced G6Pase activity. It is noteworthy that an injection of VPAC1 antagonist PG 97-269 reversed the induction observed in propionate-fed rats, but had no effect on standard-fed animals (Figure 1A).

We next investigated whether IGN induction was associated with an *in vivo* activation of cAMP-mediated signaling. After VIP injection, the intracellular cAMP content was increased 4-fold compared with saline-injected animals on standard diet. Similar cAMP levels were found in propionate-fed animals. Again, antagonism of VPAC1R abolished this increase (Figure 1B). The increase in cAMP is a key signal triggering the phosphorylation of cAMP responsive element binding protein (CREB). We determined the effect of the aforementioned conditions on the phosphorylation state of CREB. Both VIP injection and propionate feeding increased the levels of phosphorylated CREB, while not affecting the expression of CREB protein. As was the case before, treatment with PG 97-269 reversed the induction only in propionate-fed animals (Figure 1C). Furthermore, propionate feeding effectively increased the expression of phosphorylated CREB specifically in the enterocytes, as assessed by immunohistochemical methods (Figure S1). *G6pc* gene is endowed with CREB binding sites(10). To further document the regulation of this gene in the intestine, we studied the binding of phosphorylated CREB to the *G6pc* promoter by chromatin immunoprecipitation (ChIP). As shown in Figure 1D, the same pattern of activation/no activation was observed when studying the binding of phosphorylated CREB to the *G6pc* promoter.

Since VIP is expressed in enteric neurons, we examined if there was a variation of VIP expression in both enteric nervous plexuses. Using VIP immunofluorescence, we quantified VIP-positive cells in the myenteric and submucosal plexuses, in standard- and propionate-fed rats. In agreement with the observation that VIP is involved in propionate-mediated activation of IGN, propionate feeding effectively increased the ratio of VIP-positive neurons in the submucosal enteric plexus, which is primarily involved in the control of intestinal epithelial functions(6) (Figure 1E and G). No such increase was observed in the myenteric plexus (Figure 1F). However, caution is warranted since the absence of increase in VIP myenteric neurons could have been missed due to a technical issue. Indeed, VIP expression in the myenteric plexus is often studied following colchicine treatment to block its fast anterograde axonal transport, thereby retaining it in the cell body and facilitating labeling. This was not done here.

The purpose of this study was to investigate whether VIP was an enteric relay in the activation of gluconeogenic genes in the intestine in response to portal glucose signals relayed by the central nervous system (here activated by propionate). We show that VIP can induce

G6Pase activity *in vivo*. Moreover, our data strongly suggest that VIP is necessary for propionate-mediated activation of IGN, since blocking of VPAC1 receptors in propionate-fed animals reverses the activation. These elements support the role of VIP as a local neuromodulator released by intrinsic neurons and responsible for the induction of IGN through a VPAC1-dependent mechanism in enterocytes.

VIP neurons are present in submucosal and myenteric plexuses. VIP neurons of the myenteric plexus receive strong vagal inputs(11) while neurons in the submucosal plexus directly regulate enterocyte functions(6). Given the fact that propionate feeding activates neurons of the dorsal motor nucleus of the vagus(5) and that the secretomotor activity of VIP has been linked to VPAC1 activation in either enterocytes or cholinergic enteric neurons of the submucosal plexus(12), it is very likely that activation of IGN may take place through the same pathway.

The involvement of VIP signaling in glucose and energy homeostasis is not restricted to the intestinal area. For example, VIP signaling is needed for normal glucose-mediated insulin release in the endocrine pancreas(13). Interestingly, VIP-null mice have elevated plasma glucose and enhanced taste preference for sweet(14), and streptozotocin-treated diabetic mice have decreased levels of gastrointestinal VIP(15). All these alterations point to a crucial role of gastrointestinal VIP signaling in maintaining whole body glucose homeostasis.

In conclusion, we report here a mechanism by which a nervous signal from the central nervous system activates intestinal glucose production, a function promoting numerous metabolic benefits(1–5). These findings provide a novel understanding of the brain-gut neural relationships. Given the beneficial outcomes deriving from the activation of IGN, this may open novel perspectives in the treatment and prevention of metabolic diseases.

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All protocols were performed in accordance to our local ethics committee at Claude Bernard University Lyon 1.

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AUTHOR CONTRIBUTIONS

FDV and GM were responsible for the study concept; FDV, FP and AGS performed the experiments; FDV, FP, AGS and GM analyzed and interpreted the data; FDV, FP and GM wrote the manuscript.

CONFLICTS OF INTEREST

The authors disclose no conflicts of interest.

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ABBREVIATIONS

ChIP: Chromatin immunoprecipitation; CREB: cAMP responsive element binding protein; FFAR3: Free fatty acid receptor 3; G6Pase: Glucose-6-phosphatase; G6PC: Glucose-6-phosphatase catalytic subunit; IGN: Intestinal gluconeogenesis; PEPCK: Phosphoenolpyruate carboxykinase; VIP: Vasoactive Intestinal Peptide

FIGURE LEGENDS

Figure 1. VIP signaling mediates induction of intestinal gluconeogenesis. After two weeks on a standard or propionate-enriched diet, 3h-fasted rats were injected with VIP, VPAC1 antagonist PG 97-269 or only a saline solution.

A. Glucose-6-phosphatase activity in the jejunum of the rats. **B.** Intracellular cAMP in the jejunum of the rats. **C.** Effect of VIP signaling on CREB phosphorylation. Total CREB and pCREB proteins were assessed with western blots on intestinal lysates. **D.** ChIP assay of CREB occupancy over the rat *G6pc* promoter.

Data are mean ± SEM of N = 6 rats per group. ***, $P < 0.001$ vs. standard + saline, one-way ANOVA followed by Dunnett's post-hoc test.

E to G. Increase in VIP-positive neurons in myenteric and submucosal plexuses by propionate feeding. **E and F.** Box plot showing 5 to 95 percentiles. Points below and above the whiskers are drawn as individual dots. VIP-positive cells were counted in 25-32 enteric plexuses of N = 3 rats per group. **, $P < 0.01$, Mann-Whitney's sum of rank test. **G.** Representative jejunal sections of VIP-positive and hematoxylin-stained cells of rats fed a standard or a propionate-enriched diet. Enteric plexuses are lined in white, VIP-positive and negative neurons are tagged respectively with short and full arrows. SMP: submucosal plexus; MEP: myenteric plexus; CML: circular muscle layer; LML: longitudinal muscle layer. Scale bar: 20 μm.

SUPPORTING INFORMATION

Figure S1. Representative sections of phosphorylated CREB-labeled intestinal cells (black arrows in the epithelium) of standard- and propionate-fed rats. Scale bar: 20 μm.

MATERIALS AND METHODS

Animals and housing

Adult male Sprague-Dawley rats (Charles River, France), aged 6 to 8 weeks and weighing 260-280 g at the time of their arrival, were housed in a climate-controlled room (22 ± 2°C) subjected to a 12-hour light/dark cycle with lights on at 7:00 AM, with free access to food and water. Standard diet was SAFE A04 (Augis, France). Sodium propionate (Sigma-Aldrich) was incorporated into the diet at 5% weight/weight. After two weeks of diet, animals were fasted for 3 hours and given an injection of either 0.9% NaCl (saline), or 100 μg/kg body weight VIP (Bachem) or PG 97-269 (Bachem).

Intestine sampling and G6Pase activity

Rats were anesthetized 3 hours after drug injection with a single intraperitoneal injection of sodium pentobarbital (100 mg/kg) and the intestine was sampled as described previously(16). 10 μm-thick sections of the jejunum were used in histology experiments.

Frozen intestine samples were grinded to powder with a stainless steel mortar bathing in liquid nitrogen. The powder was further homogenized in 10 mM HEPES and 0.25 M sucrose, pH 7.4 (in a 9:1 volume ratio) and sonicated. G6Pase activity and non-specific phosphatase

activity were assessed at maximum velocity in duplicates for each homogenate after addition of respectively 20 mM glucose-6-phosphate or β-glycerophosphate (Sigma-Aldrich). After 10 minutes at 30°C, the reaction was arrested by addition of excess 0.11 M ascorbic acid in 10% trichloroacetic acid (Sigma-Aldrich). After centrifugation for 10 minutes at 2,000 g, the supernatant was collected. The released inorganic phosphate was assayed by spectrophotometry at 700 nm by complexation with molybdate ions (excess molybdate ions were precipitated by sodium meta-arsenite). Specific G6Pase activity was computed as the G6Pase activity minus the non-specific phosphatase activity in each sample. Protein concentrations were assayed by Bradford's method and activity was expressed in U/g of protein.

Western Blot

Tissue was grinded in 0.1 M HCl (CREB and pCREB). After denaturing electrophoresis, western blot was performed using rabbit anti-phospho CREB (1113-1, Epitomics, 1:500). For CREB blotting, pCREB membrane was stripped and western blot was performed using rabbit anti-CREB (1496-1, Epitomics, 1:1,000). Secondary antibody was goat anti-rabbit IgG, HRP-conjugated (170-5046, Bio-Rad, 1:10,000).

cAMP assay

cAMP in the intestine was quantified using a direct cAMP enzyme immunoassay kit according to the manufacturer's instructions (ADI-900-066, Enzo Life Sciences).

Chromatin immunoprecipitation (ChIP)

ChIP was performed using Active Motif ChIP-IT® Express Enzymatic kit (53009), according to the manufacturer's instructions. Briefly, 50 to 100 mg of frozen intestine was fixed in 1% formaldehyde in PBS-protein inhibitory cocktail. After chromatin harvesting and fragmentation, an input was prepared as a load control. After removing cross-links, DNA was purified by the phenol/chloroform method and quantified using a NanoDrop®. DNA integrity was verified by agarose gel electrophoresis. For immunoprecipitation, 10 μg of chromatin were used with a rabbit anti-phospho CREB antibody (9191, Cell Signaling, 1:50). A GFP

antibody (Abcam Ab6556) was used as a negative control. Q-PCR was performed using Fast-start SYBR Green PCR reagents (Roche) with the following sequences on *G6pc* promoter:

- Forward GTTTGCTATTTTACGTAAATCACCCT
- Reverse CCTCTGCTATCAGTCTGTGCCTTGC.

VIP immunofluorescence

Jejunum sections were cleared of paraffin, rehydrated and incubated for 35 minutes at 95°C in citrate buffer for antigen retrieval. Sections were incubated in a blocking solution (PBS with 0.3% Triton X-100 and 5% bovine serum albumin - 5% goat serum) for 2 hours and further incubated with the primary antibody (rabbit anti-Neurofilament L, Millipore AB9568, 1:1,000) overnight at 4°C. After several PBS rinses, sections were incubated with the secondary antibody (Alexa 633 goat anti-rabbit IgG, Molecular Probes A-21071, 1:2,000) for 2 hours at room temperature. After several rinses in PBS, a new step of blocking was performed for two hours. Sections were then incubated with rabbit anti-VIP antibody (Abcam Ab78536, 1:1,000) overnight at 4°C, rinsed with PBS and incubated with Alexa 488 goat anti-rabbit IgG (Molecular Probes A-11008, 1:2,000) for two hours. Following rinses in PBS, sections were counterstained with 10 μg/mL Hoechst 33342 (Molecular Probes) and mounted using ProLong Gold® mounting medium (Molecular Probes). Specific staining was absent in the control experiments with omission of primary antibodies and no cross-reactivity was found between antibodies.

Images were obtained using a Carl Zeiss LSM 780 confocal microscope with 40x oil-immersion objectives. All photographs were imported to ImageJ (http://imagej.nih.gov/ij), adjusted for output and threshold to include all information-containing pixels, and adjusted for brightness and contrast to best demonstrate visible fluorescence labeling. No additional photographic alterations were performed. Colocalization of the two markers was quantified using Pearson's coefficient and scatter plot tools available with the software. Cells were counted in the submucosal and myenteric plexus and the proportion of VIP+ cells was obtained by examining co-expression with neurofilament L.

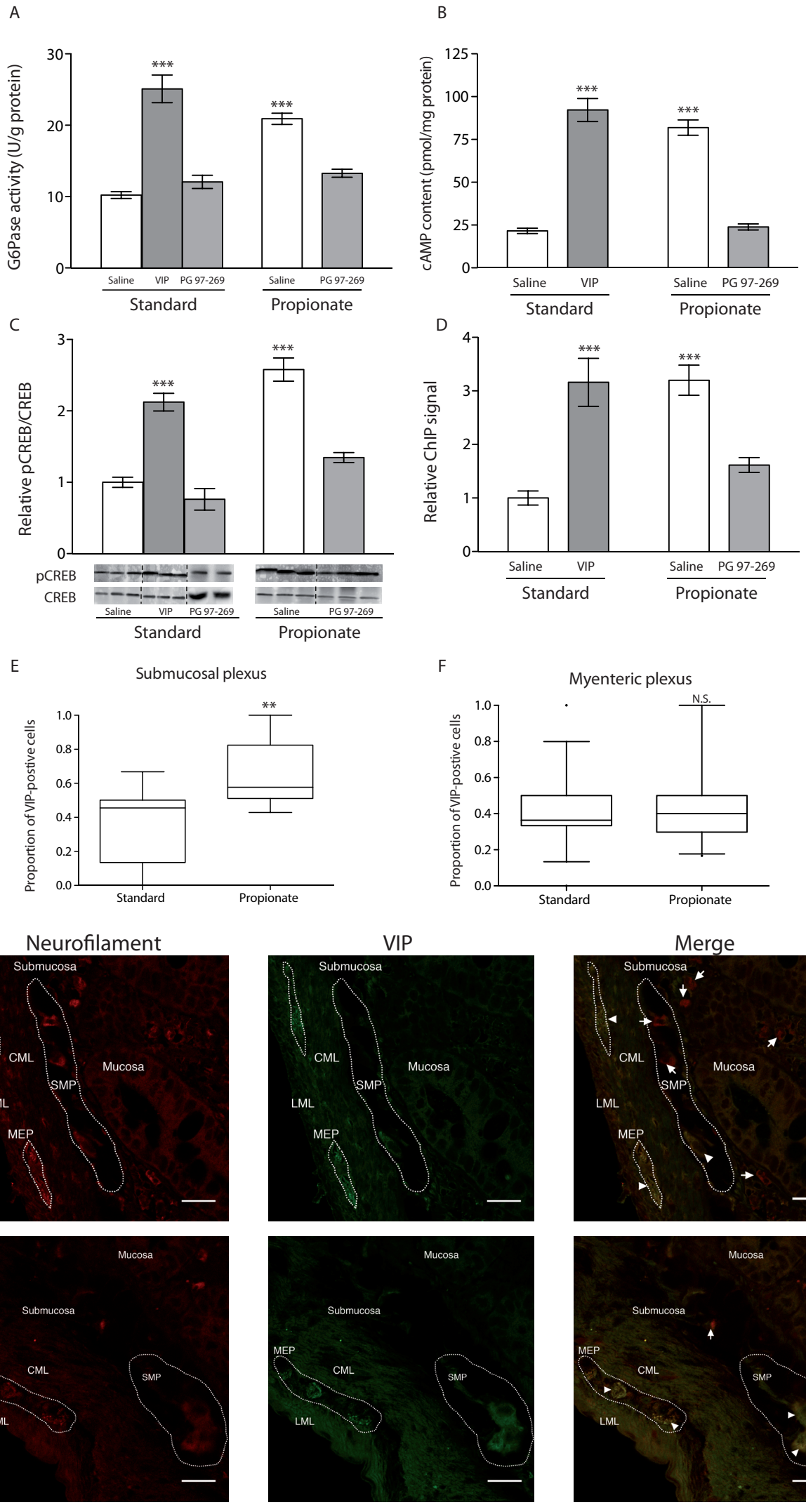
pCREB immunohistochemistry

Jejunum sections were cleared of paraffin, rehydrated and incubated for 35 minutes at 95°C in citrate buffer for antigen retrieval. After 30 minutes of incubation with a peroxidase blocking solution (Dako kit K0690), sections were incubated in PBS with 0.3% Triton X-100 with 5% bovine serum albumin and 5% goat serum for 2 hours and further incubated with the primary antibody (rabbit anti-pCREB, Epitomics 1113-1, 1:250) overnight at 4°C. After several PBS rinses, sections were incubated with the secondary antibody (biotinylated goat anti-rabbit IgG, Vector BA-1000, 1:300) for 2 hours at room temperature. After several rinses in PBS, staining was performed using streptavidin-HRP and a chromogen reagent for 30 seconds (Dako kit K0690). Specific staining was absent in the control experiment with omission of primary antibodies.

Images were acquired using a light microscope (Nikon Eclipse E400) and Histolab software (Microvision Instruments).

Statistical analysis

GraphPad Prism® was used for statistical analysis. Groups were compared using one-way ANOVA followed by Dunnett's post-hoc test. The non-parametric Mann-Whitney's test was used for cell counting. $P < 0.05$ was considered as statistically significant.


A
G6Pase activity (U/g protein)
30
20
10
0
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Saline
VIP
PG 97-269
Saline
PG 97-269
Standard
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B
cAMP content (pmol/mg protein)
125
100
75
50
25
0
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***
Saline
VIP
Saline
PG 97-269
Standard
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C
Relative pCREB/CREB
3
2
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pCREB
CREB
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Relative ChIP signal
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Saline
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Saline
PG 97-269
Standard
Propionate
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Submucosal plexus
Proportion of VIP-postive cells
1.0
0.8
0.6
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Standard
Propionate
F
Myenteric plexus
Proportion of VIP-postive cells
1.0
0.8
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N.S.
Standard
Propionate
G
Neurofilament
VIP
Merge
Standard
Propionate
Submucosa
CML
Mucosa
SMP
LML
MEP