Received: 10 May 2017 | Accepted: 20 June 2017

DOI: 10.1111/nmo.13162

ORIGINAL ARTICLE

WILEY Neurogastroenterology & Motility NGM

Synergy between 5-HT$_4$ receptor stimulation and phosphodiesterase 4 inhibition in facilitating acetylcholine release in human large intestinal circular muscle

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Abstract

Background: Gastroprokinetic properties of 5-HT$_4$ receptor agonists, such as prucalopride, are attributed to activation of 5-HT$_4$ receptors on cholinergic nerves innervating smooth muscle in the gastrointestinal smooth muscle layer, increasing acetylcholine release and muscle contraction. In porcine stomach and colon, phosphodiesterase (PDE) 4 has been shown to control the signaling pathway of these 5-HT$_4$ receptors. The aim of this study was to investigate the PDE-mediated control of these 5-HT$_4$ receptors in human large intestine.

Methods: Circular smooth muscle strips were prepared from human large intestine; after incubation with [^{3}H]-choline, electrically induced tritium outflow was determined as a measure for acetylcholine release. The influence of PDE inhibition on the facilitating effect of prucalopride on electrically induced acetylcholine release was studied.

Key Results: The non-selective PDE inhibitor IBMX enhanced the facilitating effect of prucalopride on electrically induced acetylcholine release. The selective inhibitors vinpocetine (PDE1), EHNA (PDE2) and cilostamide (PDE3) did not influence, while rolipram and roflumilast (PDE4) enhanced the prucalopride-induced facilitation to the same extent as IBMX.

Conclusions & Inferences: In human large intestinal circular muscle, the intracellular pathway of 5-HT$_4$ receptors facilitating cholinergic neurotransmission to large intestinal circular smooth muscle is controlled by PDE4. If the synergy between 5-HT$_4$ receptor agonism and PDE4 inhibition is confirmed in a functional assay with electrically induced cholinergic contractions of human large intestinal circular smooth muscle strips, combination of a selective 5-HT$_4$ receptor agonist with a selective PDE4 inhibitor might enhance the in vivo prokinetic effect of the 5-HT$_4$ receptor agonist in the large intestine.

KEYWORDS

5-HT$_4$ receptor, acetylcholine release, human large intestine, phosphodiesterase, prucalopride

Abbreviations: 5-HT$_4$ receptor, 5-hydroxytryptamine 4 receptor; cilo, cilostamide; EFS, electrical field stimulation; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; GI, gastrointestinal; K_i, inhibitory constant; IBMX, 3-isobutyl-1-methylxanthine; ns, not significant; pru, prucalopride; roflu, roflumilast; roli, rolipram; S_1, first application of EFS at the 13th minute (sample 5); S_2, second application of EFS at the 73rd minute (sample 25); S_2/S_1, ratio of the tritium release by S_2 compared to the release by S_1; vinpo, vinpocetine.

1 | INTRODUCTION

5-HT_4 receptor agonists are used for gastrointestinal (GI) hypomotility disorders such as gastroparesis and constipation.[1] Their GI prokinetic effects are attributed to activation of 5-HT_4 receptors on cholinergic neurons innervating the smooth muscle layer.[2] These receptors, facilitating acetylcholine release and GI contraction, were first shown in the GI tract of the guinea-pig[3,4] and later of several species as rat,[5] dog,[6,7] pig,[8,9] and man.[6,10-12] 5-HT_4 receptors are also present in porcine and human atria.[13] The highly selective 5-HT_4 receptor agonist prucalopride, which is marketed for chronic idiopathic constipation, only induces a weak and transient inotropic effect in porcine and human atrial tissue.[14,15] 5-HT_4 receptors are G_s protein-coupled receptors, linked to adenylyl cyclase and signaling via generation of the second messenger cAMP. The weak response to 5-HT_4 receptor agonism in cardiac tissue is due to the pronounced control of the cAMP signal by phosphodiesterases (PDEs), degrading cAMP and limiting the effect of 5-HT_4 receptor agonists[15]. This regulation is mediated by PDE3 plus PDE4 in porcine heart[16,17] and by PDE3 in human heart.[16,18]

The facilitation of enteric cholinergic neurotransmission by prucalopride in the porcine and human GI tract is pronounced and sustained.[6,19-21] Still, this effect was shown to be enhanced by the selective PDE4 inhibitor rolipram in porcine stomach and colon.[9,22] We recently confirmed this[23] with the selective PDE4 inhibitor roflumilast, marketed for chronic obstructive pulmonary disease,[24] and the highly selective 5-HT_4 receptor agonist velusetrag,[25] that is actually under clinical development. These results demonstrate that the intracellular pathway of the 5-HT_4 receptors on the myenteric cholinergic neurons in the porcine GI tract is controlled by PDE4. If confirmed in humans, combination of a 5-HT_4 receptor agonist with a selective PDE4 inhibitor might thus enhance the gastroprokinetic effect of the 5-HT_4 receptor agonist without potentiation of its cardiac effects, which are controlled by PDE3.

The aim of the present study was therefore to investigate whether the signal transduction pathway of 5-HT_4 receptors in myenteric cholinergic neurons innervating human large intestinal circular muscle is controlled by PDEs, and if so whether PDE4 is the isozyme involved. This was done by measuring the influence of PDE inhibitors on the facilitating effect of prucalopride on electrically induced acetylcholine release.

Key points

- In porcine stomach and large intestine, phosphodiesterase (PDE) 4 has been shown to control the signaling pathway of 5-HT_4 receptors on cholinergic nerves innervating the smooth muscle layer; this was now investigated in the human large intestine.
- In human large intestinal circular smooth muscle strips, the facilitating effect of the 5-HT_4 receptor agonist prucalopride on electrically induced acetylcholine release was enhanced by non-selective PDE inhibition with IBMX. The selective PDE4 inhibitors rolipram and roflumilast mimicked the effect of IBMX, while inhibitors of PDE1 (vinpocetine), PDE2 (EHNA), and PDE3 (cilostamide) did not.
- The signaling pathway of 5-HT_4 receptors facilitating cholinergic neurotransmission toward human large intestinal circular smooth muscle is thus controlled by PDE4. If the synergy between 5-HT_4 receptor agonism and PDE4 inhibition is confirmed in a functional assay with electrically induced cholinergic contractions, combination of a 5-HT_4 receptor agonist with a selective PDE4 inhibitor might also in vivo enhance the prokinetic effect of the 5-HT_4 receptor agonist.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue preparation

The study was approved by the Ethical Committee of Ghent University Hospital. All patients (n=59), including 17 women/42 men with a mean age of 66±2 years, provided written informed consent and underwent resection of the large intestine because of cancer.

A specimen from the macroscopic healthy segment of the resected large intestinal tissue (3 caecum, 5 ascending, 9 transverse, 7 descending, 20 sigmoid colon, and 15 rectum) was placed in oxygenated (95% O_2+5% CO_2) ice-chilled Krebs-Henseleit solution (composition in mmol L^{-1}: NaCl 118.0, KCl 4.69, $MgSO_4$ 1.18, KH_2PO_4 1.18, $CaCl_2$ 2.51, glucose 11.1, and $NaHCO_3$ 25.0) and transported to the laboratory. Adipose tissue, adhering mesentery and mucosa were removed by blunt dissection and the tissue was stored overnight in Krebs-Henseleit solution at 4 °C. Within 24 hours after surgery, four full thickness intertaenial smooth muscle strips of approximately 1 to 1.5 cm in length and 0.3 cm in width were cut in the direction of the circular smooth muscle from each human large intestinal specimen to study acetylcholine release.

2.2 | Measuring acetylcholine release

Circular smooth muscle strips were mounted vertically between two platinum wire electrodes (40 × 0.5 mm, 4 mm apart) under a load of 2 g in organ baths (2 mL) filled with oxygenated Krebs-Henseleit solution from now on containing choline (1.5 μmol L^{-1}), ascorbic acid (57 μmol L^{-1}), and guanethidine (4 μmol L^{-1}) at 37 °C. During 1 hour of equilibration, the tissues were superfused (2 mL min^{-1}) with Krebs-Henseleit solution using a peristaltic pump (Minipuls 3; Gilson S.A.S., Villiers le Bel, France). The last 20 minutes, continuous electrical field stimulation (EFS; 1 millisecond monophasic square wave pulses, 40 V, 0.5 Hz) was applied by means of a stimulator (Grass S88; Grass Technologies, West Warwick, RI, USA) with a constant voltage unit.

After superfusion was stopped, the cholinergic transmitter stores were labeled by incubating the tissues with [^{3}H]-choline (5 μCi mL^{-1})

under continuous EFS (1 millisecond, 40 V, 2 Hz) for 30 minutes. EFS and incubation were stopped and loosely bound radioactivity was washed-out by superfusing (2 mL min^{-1}) the tissues for 90 minutes with Krebs-Henseleit solution from now on also containing hemicholinium-3 (10 μmol L^{-1}), physostigmine (10 μmol L^{-1}), and atropine (1 μmol L^{-1}) to prevent re-uptake of choline, hydrolysis of acetylcholine and auto-inhibition of acetylcholine release respectively.

After ending superfusion for wash-out, the organ baths were filled with 1 mL Krebs-Henseleit solution. The organ bath content was collected and replaced every 3 minutes for a total of 35 samples. EFS (1 millisecond, 15 V, 4 Hz) was applied twice for 2 minutes (S_1 and S_2): S_1 started at the 13th (sample 5) and S_2 at the 73rd (sample 25) minute after finishing the wash-out period. Drugs were added before S_2 and remained present until the end of the experiment: PDE inhibitors or their solvent from the 37th minute (sample 13; 36 minutes before S_2) and prucalopride from the 58th minute (sample 20; 15 minutes before S_2). Tissue strips were blotted and weighed at the end of sample collection (28.75±1.48 mg for the 184 strips included in the results).

0.5 mL of each sample was mixed with 2 mL scintillator containing solution Ultima Gold (Perkin Elmer, Waltham, MA, USA). Total tritium outflow was measured as disintegrations per minute (dpm) by liquid scintillation counting (Packard Tri-Carb 2100 TR; Packard Instrument Company, Downers Grove, IL, USA) with external standardization to correct for counting efficiency. Total tritium outflow, containing [^{3}H]-acetylcholine, [^{3}H]-phosphorylcholine and [^{3}H]-choline, can be considered as a marker for acetylcholine release as we previously showed by separation of the three radioactive compounds that EFS predominantly increases [^{3}H]-acetylcholine release and that EFS-induced changes in total tritium outflow parallel those in [^{3}H]-acetylcholine.[12]

2.3 | Experimental protocols and design

In four separate series, the influence of 10 μmol L^{-1} IBMX (non-selective PDE inhibitor) on the effect of 0.03 μmol L^{-1} prucalopride and the influence of 10 μmol L^{-1} IBMX, 1 μmol L^{-1} rolipram or 0.3 μmol L^{-1} roflumilast (both selective PDE4 inhibitors) on the effect of 0.01 μmol L^{-1} prucalopride was studied. Four parallel strips (same patient) were used for each series: either PDE inhibitor alone, prucalopride alone, or prucalopride in the presence of the PDE inhibitor was added before S_2; the fourth control strip did not receive active compounds.

The influence of 100 μmol L^{-1} vinpocetine (PDE1 inhibitor), 30 μmol L^{-1} EHNA (PDE2 inhibitor) and 1 μmol L^{-1} cilostamide (PDE3 inhibitor) on the effect of 0.01 μmol L^{-1} prucalopride was studied in a fifth series with four parallel strips: one with administration of prucalopride alone, and three where prucalopride was added after previous administration of vinpocetine, EHNA or cilostamide.

In two final series the solvent of IBMX, ethanol, and of vinpocetine, cilostamide, rolipram and roflumilast, DMSO, was tested in the highest concentration used upon administration of the PDE inhibitors: 0.05% for ethanol and 1% for DMSO. Their possible influence on the effect of prucalopride was tested by four parallel strips receiving the solvent alone, prucalopride alone, prucalopride in the presence of the solvent and a control not receiving active compounds.

The four experimental conditions per series were rotated over the four organ baths from experiment to experiment.

2.4 | Data and statistical analysis

EFS induced a clear-cut increase in tritium outflow, by S_1 in samples 5 up to 7 and by S_2 in samples 25 up to 27. The EFS-induced increase in tritium outflow was determined by subtracting the corresponding basal tritium outflow, which was calculated by fitting a regression line through the values of four samples just before stimulation and the values of the four samples starting from the sixth sample after stimulation. S_2/S_1 ratio was then calculated: the sum of the tritium outflow above baseline of samples 25 to 27 as ratio of the sum of the tritium outflow above baseline of samples 5 to 7.

Strips were excluded from analysis when: the S_1- and/or S_2-induced release peak showed an aberrant pattern eg double peak (24 out of 236 strips), the basal outflow was unstable (18 out of 236 strips) or a technical defect occurred mainly a defect of electrodes (10 out of 236 strips) meaning that the results of 184 strips are given. This exclusion from data analysis also explains unequal group sizes within an experimental series.

Data are expressed as mean±SEM and n refers to the number of tissues obtained from different patients. As data were homogenous (Levene's test), they were compared by one-way ANOVA with unpaired *t* tests with Bonferroni correction for multiple comparisons; the Bonferroni corrected *t* tests were only conducted if the ANOVA showed significance. *P*-values less than .05 were considered statistically significant. Statistical analysis was performed with GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA).

2.5 | Drugs

L-ascorbic acid, atropine sulfate salt, choline chloride, guanethidine sulfate, hemicholinium-3 bromide, 3-isobutyl-1-methylxanthine (IBMX), eserine salicylate salt (physostigmine), roflumilast were obtained from Sigma-Aldrich (St. Louis, MO, USA); cilostamide, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) hydrochloride, rolipram, vinpocetine from Tocris Bioscience (Bristol, UK); [methyl-^{3}H]-choline chloride from Perkin Elmer (Boston, MA, USA) and prucalopride succinate from Selleck Chemicals (Houston, TX, USA).

Drugs were dissolved and diluted in distilled water, except for IBMX which was dissolved in ethanol, yielding a concentration of 0.05% in the organ bath, and vinpocetine, cilostamide, rolipram, and roflumilast which were dissolved in DMSO, yielding a concentration of, respectively 1%, 0.01%, 0.01%, and 0.003% in the organ bath.

3 | RESULTS

3.1 | Influence of IBMX on the effect of prucalopride (Figures 1 and 2)

We previously have shown that 0.3 μmol L^{-1} prucalopride induces a pronounced increase in EFS-induced acetylcholine release in human

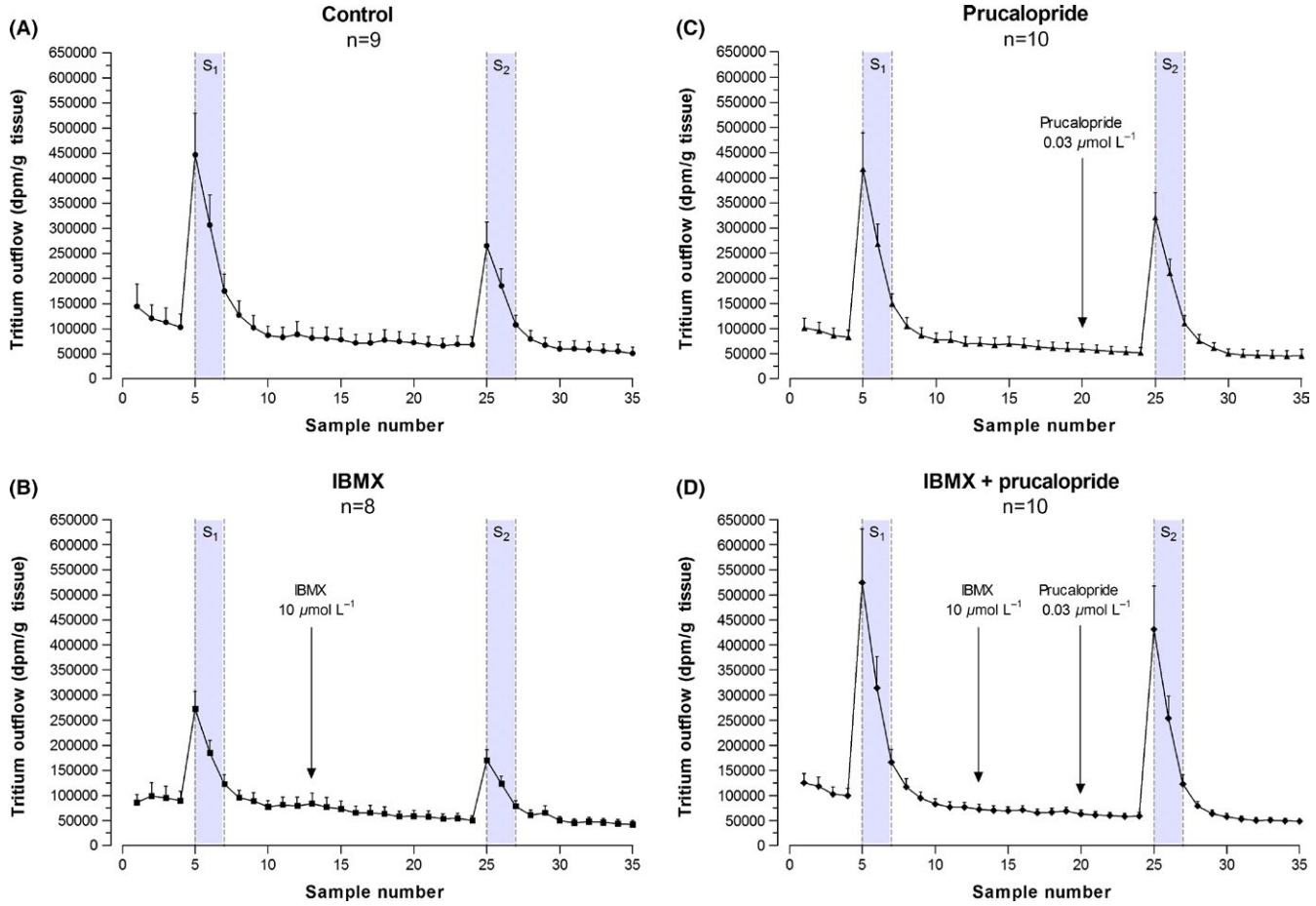

FIGURE 1 Influence of IBMX and prucalopride on basal and electrically induced tritium outflow. Influence of 10 μmol L^{-1} IBMX (B), 0.03 μmol L^{-1} prucalopride (C), and prucalopride in the presence of IBMX (D) on basal and electrically induced total tritium outflow; parallel control tissues are shown in (A). Samples were collected every 3 minutes for measurement of total tritium outflow. Tissues were stimulated twice (1 millisecond, 15 V, 4 Hz, 2 minutes) at the 5th (S_1) and 25th (S_2) sample inducing a stimulation-induced peak of the tritium outflow as indicated by grey shading; IBMX and prucalopride were added before S_2 as indicated by the arrows. Means±SEM are shown

colon.[12,26] A 10-fold lower concentration of prucalopride was therefore used in the first series of the actual study to test the influence of 10 μmol L^{-1} IBMX. EFS induced a clear-cut increase in tritium outflow above the basal level in up to three samples after EFS and at the 6th sample after EFS, tritium outflow returned to baseline values (Figure 1). The S_2-induced tritium outflow was lower than that induced by S_1 (Figure 1A), yielding a S_2/S_1 ratio of 0.60±0.03 in the control group (n=9; Figure 2A). Prucalopride and IBMX did not influence basal tritium outflow (Figure 1B and 1C); IBMX had no significant effect on the S_2-induced tritium outflow (n=8; Figures 1B and 2A). Prucalopride (0.03 μmol L^{-1}) however significantly enhanced EFS-induced tritium outflow (Figure 1C) yielding a S_2/S_1 ratio of 0.88±0.04 (n=10) vs 0.60±0.03 in control tissues (Figure 2A). No apparent regional differences in the effect of prucalopride were observed (see Table S1). Prucalopride (0.03 μmol L^{-1}) in the presence of IBMX (10 μmol L^{-1}) led to a S_2/S_1 ratio of 0.94±0.05 (n=10), which is somewhat higher than with prucalopride alone but this increase did not reach significance (Figure 2A).

IBMX (10 μmol L^{-1}) was therefore tested vs a 3-fold lower concentration of prucalopride (0.01 μmol L^{-1}). In this series, the S_2/S_1 ratio was 0.74±0.04 (n=7) in the control tissues, 0.76±0.05 (n=11) in the strips where IBMX alone was added and 0.86±0.03 (n=9) in the strips where prucalopride alone was added; this tendency to increase the S_2/S_1 ratio by 0.01 μmol L^{-1} prucalopride did not reach significance (Figure 2B). However, prucalopride (0.01 μmol L^{-1}) in the presence of IBMX (10 μmol L^{-1}) induced a pronounced increase in the S_2-induced tritium outflow with a S_2/S_1 ratio of 1.06±0.07 (n=10), being significantly different from controls and from strips treated with prucalopride alone (Figure 2B).

3.2 | Influence of rolipram and roflumilast on the effect of prucalopride (Figures 3 and 4)

Rolipram (1 μmol L^{-1}) did not influence basal or EFS-induced tritium outflow (S_2/S_1 ratio of 0.72±0.02 [n=7], vs 0.72±0.03 [n=9] in controls; Figure 3). Prucalopride (0.01 μmol L^{-1}) alone did not significantly facilitate EFS-induced tritium outflow (S_2/S_1 ratio of 0.78±0.04, n=8). In the presence of rolipram, prucalopride (0.01 μmol L^{-1}) induced a pronounced increase in EFS-induced tritium outflow with a S_2/S_1 ratio of 0.95±0.03 (n=8), being significantly different

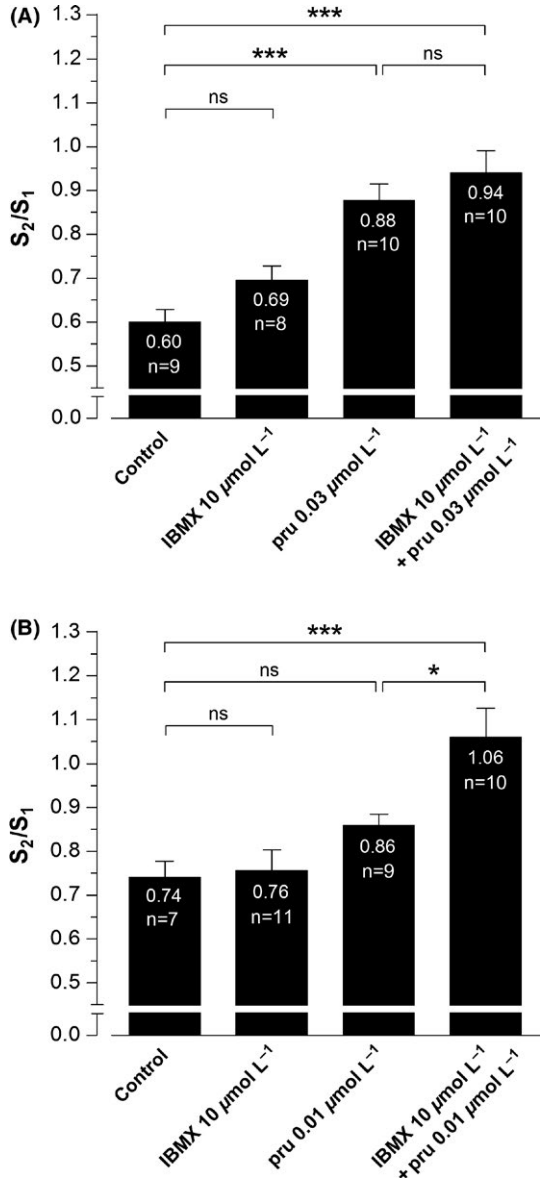

FIGURE 2 S_2/S_1 ratios illustrating the influence of IBMX on the effect of prucalopride. Influence of 10 µmol L^{-1} IBMX, 0.03 (A) and 0.01 (B) µmol L^{-1} prucalopride (pru), and 0.03 (A) and 0.01 (B) prucalopride in the presence of IBMX on the S_2/S_1 ratio of electrically induced total tritium outflow. Tissues were stimulated twice (S_1 and S_2; 1 millisecond, 15 V, 4 Hz, 2 minutes); IBMX was added 36 minutes and prucalopride 15 minutes before S_2. Mean S_2/S_1±SEM; one-way ANOVA followed by Bonferroni corrected *t* test with ns not significant, **P*<.05 and ****P*<.001

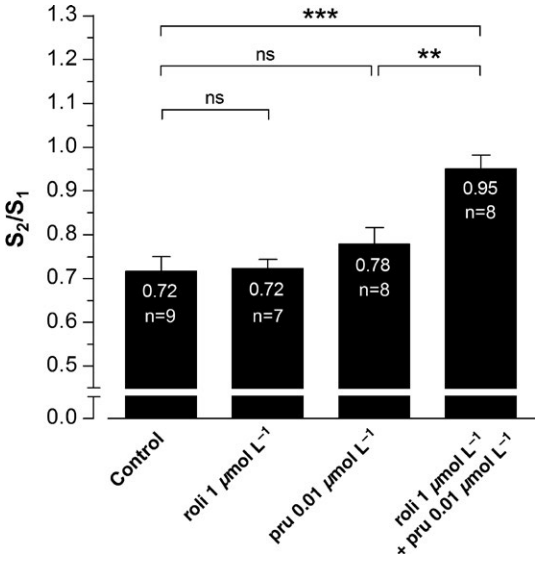

FIGURE 3 S_2/S_1 ratios illustrating the influence of rolipram on the effect of prucalopride. Influence of 1 µmol L^{-1} rolipram (roli), 0.01 µmol L^{-1} prucalopride (pru) and prucalopride in the presence of rolipram on the S_2/S_1 ratio of electrically induced total tritium outflow. Tissues were stimulated twice (S_1 and S_2; 1 millisecond, 15 V, 4 Hz, 2 minutes); rolipram was added 36 minutes and prucalopride 15 minutes before S_2. Mean S_2/S_1±SEM; one-way ANOVA followed by Bonferroni corrected *t* test with ns not significant, ***P*<.01 and ****P*<.001

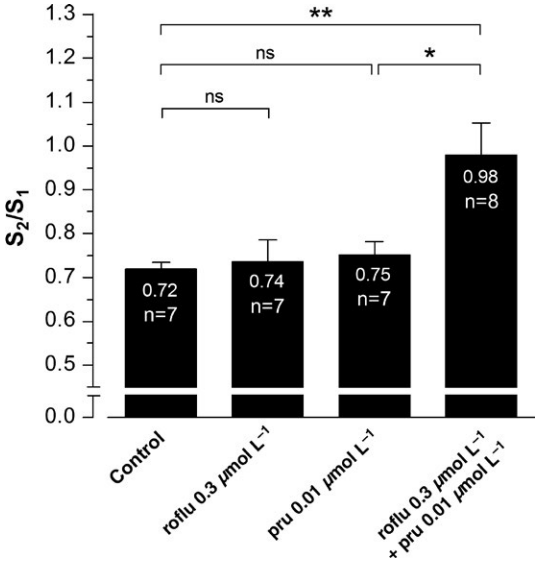

FIGURE 4 S_2/S_1 ratios illustrating the influence of roflumilast on the effect of prucalopride. Influence of 0.3 µmol L^{-1} roflumilast (roflu), 0.01 µmol L^{-1} prucalopride (pru) and prucalopride in the presence of roflumilast on the S_2/S_1 ratio of electrically induced total tritium outflow. Tissues were stimulated twice (S_1 and S_2; 1 millisecond, 15 V, 4 Hz, 2 minutes); roflumilast was added 36 minutes and prucalopride 15 minutes before S_2. Mean S_2/S_1±SEM; one-way ANOVA followed by Bonferroni corrected *t* test with ns not significant, **P*<.05 and ***P*<.01

from that in control strips and in strips where prucalopride alone was administered.

This was confirmed with another selective PDE4 inhibitor roflumilast that also did not influence basal tritium outflow. Roflumilast (0.3 µmol L^{-1}) and prucalopride (0.01 µmol L^{-1}) alone had no significant influence on the EFS-induced tritium outflow with S_2/S_1 ratios of respectively 0.74±0.05 (n=7) and 0.75±0.03 (n=7) vs 0.72±0.02 (n=7) in control tissues (Figure 4). Prucalopride in the presence of roflumilast significantly enhanced the S_2/S_1 ratio up to 0.98±0.07 (n=8), when compared with control tissues and tissues receiving prucalopride alone. This enhancement in the effect of prucalopride by roflumilast is of similar magnitude as that found with rolipram.

3.3 | Influence of vinpocetine, EHNA and cilostamide on the effect of prucalopride (Figure 5)

In order to be able to study the three PDE inhibitors in one series, there were no control strips without addition of active compounds before S_2 included, but the strips where prucalopride alone was administered were used for comparison. In the presence of prucalopride (0.01 μmol L^{-1}) a S_2/S_1 value of 0.74±0.03 (n=5) was obtained. This was not influenced by previous addition of vinpocetine (100 μmol L^{-1}), EHNA (30 μmol L^{-1}), and cilostamide (1 μmol L^{-1}) where S_2/S_1 ratios of respectively 0.72±0.07 (n=4), 0.72±0.05 (n=6), and 0.72±0.03 (n=6) were obtained (Figure 5). A one-way ANOVA did not reach significance. Vinpocetine, EHNA, and cilostamide had no effect on the basal tritium outflow.

3.4 | Influence of ethanol and DMSO on the effect of prucalopride

The solvents were tested in two small series. Ethanol (0.05%) did not influence basal tritium outflow. In the presence of ethanol, the S_2/S_1 ratio of 0.64±0.06 (n=3) was the same as that in controls (0.64±0.03; n=3). Prucalopride (0.01 μmol L^{-1}) alone led to a S_2/S_1 ratio of 0.68±0.07 (n=3), which was not influenced by previous addition of ethanol (S_2/S_1 ratio of 0.65±0.08; n=3).

DMSO (1%) had no effect on basal and EFS-induced tritium outflow with a S_2/S_1 ratio of 0.72±0.05 (n=4) vs 0.70±0.02 (n=4) in controls. In the presence of prucalopride (0.01 μmol L^{-1}) the S_2/S_1 ratio was 0.81±0.07 (n=4), which is comparable to 0.83±0.05 (n=4) measured for prucalopride in the presence of DMSO.

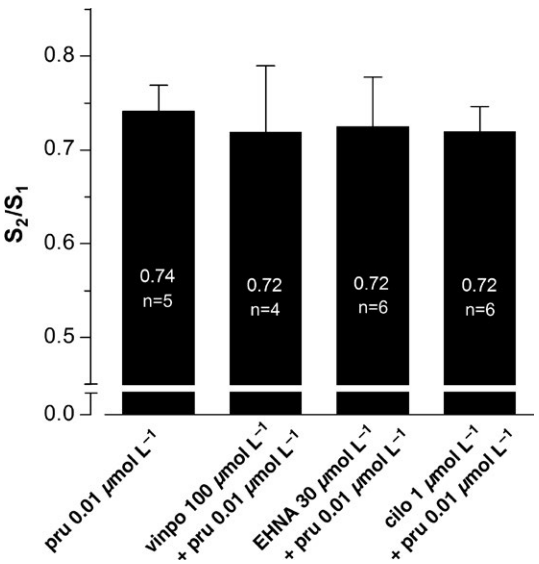

FIGURE 5 S_2/S_1 ratios illustrating the influence of vinpocetine, EHNA and cilostamide on the effect of prucalopride. Influence of 0.01 μmol L^{-1} prucalopride (pru) alone or in the presence of 100 μmol L^{-1} vinpocetine (vinpo), 30 μmol L^{-1} EHNA or 1 μmol L^{-1} cilostamide (cilo) on the S_2/S_1 ratio of electrically induced total tritium outflow. Tissues were stimulated twice (S_1 and S_2; 1 millisecond, 15 V, 4 Hz, 2 minutes); vinpocetine, EHNA and rolipram were added 36 minutes and prucalopride 15 minutes before S_2. Mean S_2/S_1±SEM; one-way ANOVA did not reach significance

4 | DISCUSSION

Prucalopride (Resolor™), marketed for constipation, stimulates whole gut transit and colonic transit in healthy humans[27,28] and increases stool frequency and decreases stool consistency in patients with constipation.[29,30] The underlying mechanism for these prokinetic properties of prucalopride and other 5-HT_4 receptor agonists is thought to be interaction with stimulating 5-HT_4 receptors localized on cholinergic neurons toward GI smooth muscle, facilitating acetylcholine release and cholinergic contraction in the GI tract. This mechanism was clearly shown in human stomach[11] and colon[12] as prucalopride increased electrically induced acetylcholine release, which was antagonized by selective 5-HT_4 receptor antagonists confirming interaction with 5-HT_4 receptors; the facilitating effect of 0.3 μmol L^{-1} prucalopride, which is 10- to 30-fold higher than used in this study, was antagonized by 1 nmol L^{-1} SB204070 in human stomach[11] and by 0.01 μmol L^{-1} GR 113808 in human colon.[12] Cardiovascular adverse events were reported with the 5-HT_4 receptor agonists cisapride and tegaserod, but these adverse events are not 5-HT_4 receptor-related (cisapride by interaction with human ether-a-go-go-related gene potassium channels and tegaserod possibly by interaction with 5-HT_1 and/or 5-HT_2 receptors[31]). These cardiovascular side effects should thus not occur with the new generation of highly selective 5-HT_4 receptor agonists such as prucalopride. Prucalopride was indeed found to be cardiovascularly safe in several randomized placebo-controlled double-blind trials in patients with chronic constipation;[32] nevertheless combination therapies are explored to lower the dose and risk of adverse events. Combination of a 5-HT_4 receptor agonist with the acetylcholinesterase inhibitor neostigmine had a synergistic effect on cholinergic activity in vitro in human colon[5] and on colonic transit time in vivo in rat;[33] the synergistic effect on cholinergic activity in vitro in human colon was confirmed with the acetylcholinesterase inhibitor donepezil.[34] Combination of a 5-HT_4 receptor agonist with a PDE4 inhibitor might also be considered. 5-HT_4 receptors are G_s-protein coupled receptors linked to adenylate cyclase and the generation of the second messenger cAMP, and PDEs are the sole family of isozymes degrading cAMP. A tight controlling role of the signal transduction of cardiac 5-HT_4 receptors was shown: by PDE3 plus PDE4 in the porcine heart[16,17] and by PDE3 in the human heart.[18] As 5-HT_4 receptors enhancing cholinergic neurotransmission in porcine stomach and colon are controlled by PDE4, we proposed that combining a 5-HT_4 receptor agonist with a PDE4 inhibitor might enhance its gastroprokinetic effect.[9,22,23] The actual study therefore investigated whether 5-HT_4 receptors enhancing cholinergic neurotransmission in human large intestine are also controlled by PDEs, by studying the effect of PDE inhibition on the facilitating effect of prucalopride on acetylcholine release.

In order to be able to observe a possible facilitating influence of PDE inhibition on the effect of prucalopride, the influence of 10 μmol

L^{-1} of the non-selective PDE inhibitor IBMX, which was shown to be effective in enhancing the effect of prucalopride on acetylcholine release in porcine gastric circular muscle,[9] was first tested vs 0.03 μmol L^{-1} prucalopride, a 10-fold lower concentration than the one shown before to clearly enhance electrically induced acetylcholine release in human colon.[12] However this concentration of prucalopride still induced a pronounced facilitation of the electrically induced acetylcholine release with a S_2/S_1 ratio of 0.88 (vs 0.60 in the control group) which is in the range of the effect previously reported with 0.3 μmol L^{-1} prucalopride in human colon circular muscle;[12,26] the effect of 0.03 μmol L^{-1} prucalopride was not significantly enhanced by IBMX. When further decreasing the prucalopride concentration to 0.01 μmol L^{-1}, a non-significant increase in S_2/S_1 ratio to 0.86 (vs 0.74 in the control group) was observed. But in the presence of IBMX, that had no influence on the electrically induced acetylcholine release, 0.01 μmol L^{-1} prucalopride significantly enhanced the S_2/S_1 ratio to 1.06. This degree of potentiation with IBMX is in agreement with the level obtained in porcine stomach circular muscle.[9] The effect of IBMX is not due to an effect of its solvent ethanol, given the non-effect of ethanol on electrically induced acetylcholine release and its potentiation by prucalopride. The intraneuronal pathway of 5-HT_4 receptors on cholinergic neurons, facilitating acetylcholine release, in human large intestinal circular muscle is thus also regulated by PDEs.

To identify the responsible PDE subtype, selective PDE inhibitors were investigated. As cAMP is the cyclic nucleotide involved in the 5-HT_4 receptor pathway, only the cAMP degrading classic PDE subtypes were investigated: PDE1, 2 and 3 degrade both cAMP and cGMP and PDE4 is cAMP specific[35,36] and are selectively inhibited by respectively vinpocetine, EHNA, cilostamide, and rolipram.[37] 100 μmol L^{-1} vinpocetine, 30 μmol L^{-1} EHNA and 1 μmol L^{-1} cilostamide did not influence the effect of prucalopride on electrically induced acetylcholine release. These concentrations were definitely high enough to inhibit their respective PDE subtype given the reported K_i-values of 14 μmol L^{-1}, 1 μmol L^{-1}, and 0.02 μmol L^{-1} [36] and IC_{50} values of 8-50 μmol L^{-1} [38-40] 1-5 μmol L^{-1} [41-43] and 0.005-0.13 μmol L^{-1} [35,41,44-46] for, respectively, vinpocetine, EHNA, and cilostamide. Rolipram in a concentration of 1 μmol L^{-1}, which is able to inhibit all PDE4 isozymes,[47] did not influence the electrically induced acetylcholine release, but significantly enhanced the effect of prucalopride; this is not related to its solvent DMSO that was without effect. The increase in S_2/S_1 ratio from 0.78 with prucalopride alone to 0.95 with prucalopride in the presence of rolipram is of the same magnitude as obtained in the series with IBMX (from 0.86 to 1.06). Roflumilast is another selective PDE4 inhibitor which is already used to suppress exacerbations of chronic obstructive pulmonary disease.[24,48,49] Roflumilast, inhibiting the four PDE4 isozymes with an IC_{50} of about 1 nmol L^{-1},[50] reached a maximal effect in a concentration of 0.3 μmol L^{-1} when tested for its enhancement of prucalopride-induced facilitation of acetylcholine release in porcine stomach.[23] In human large intestinal circular smooth muscle, this concentration of roflumilast did not influence acetylcholine release, but potentiated the facilitating effect of prucalopride to the same extent as rolipram with an increase in S_2/S_1 ratio from 0.75 with prucalopride alone to 0.98 with prucalopride in the presence of roflumilast. The results with rolipram and roflumilast illustrate that the intraneuronal pathway of acetylcholine release facilitating 5-HT_4 receptors, localized on cholinergic neurons in human large intestinal circular muscle, is regulated exclusively by PDE4, just as in the porcine stomach and colon. From their functional study in human colon circular muscle, Cellek et al.[5] suggested possible poor coupling of the 5-HT_4 receptor to acetylcholine release; this might be related to the regulation of the cAMP response to receptor activation by PDE4. In the porcine stomach, rolipram or roflumilast alone enhanced electrically induced acetylcholine release[23] suggesting that PDE4 constitutively regulates the basal content of cAMP in porcine stomach cholinergic neurons. This is not the case in human large intestine, as rolipram or roflumilast alone did not influence acetylcholine release.

The 5-HT_4 receptor pathway in cholinergic neurons in human large intestinal circular muscle is thus also regulated by PDE4. If the synergy between 5-HT_4 receptor agonism and PDE4 inhibition as shown here on acetylcholine release can also be obtained on EFS-induced cholinergic contraction, in vivo combination therapy of prucalopride or another selective 5-HT_4 receptor agonist with a selective PDE4 inhibitor can be considered for constipation. This combination therapy might allow to use a low dose of the 5-HT_4 receptor agonist, while the prokinetic effect is maintained. The recommended dose of prucalopride is 2 mg once daily. In healthy volunteers, taking 2 mg prucalopride per os once daily for 7 days, pharmacokinetic steady state was attained within 3 days and the C_{max} plasma concentration on day 7 was 8.09 ng mL^{-1}, at a median time of 2 hours postdose; C_{min} and $C_{average}$ were 2.21 and 4.32 ng mL^{-1} respectively.[51] Similar values were obtained in elderly patients with constipation. Upon intake of 0.5, 1 or 2 mg prucalopride once daily for 4 weeks, steady state was reached between days 4 and 7 of treatment and peak plasma concentrations were attained 2-3 hours postdose;[52] the near C_{max} plasma concentrations determined at 3 hours postdose on day 7 were 2, 4 and 7 ng mL^{-1}, corresponding to 0.005, 0.011 and 0.019 μmol L^{-1}. The prucalopride concentration of 0.01 μmol L^{-1} that shows synergy with PDE4 inhibition in the actual study thus correlates to the C_{max} obtained upon repeated dosing with 1 mg. When combining a 5-HT_4 receptor agonist with a PDE4 inhibitor, the action of the 5-HT_4 receptor agonist at the level of the cardiac 5-HT_4 receptors will not be influenced, because in the human atrium the 5-HT_4 receptor pathway is under sole control of PDE3.[16] However, the role of PDEs in controlling the cyclic nucleotide concentrations at the level of the smooth muscle cells in human large intestinal circular muscle must be taken in account. PDE inhibition might increase the basal level of cyclic nucleotides inducing relaxation. If the PDE involved is PDE4, the PDE4 inhibitor in the combination therapy might induce relaxation, counteracting the prokinetic effect in the large intestine. In porcine colon circular muscle the predominant PDE is PDE3.[22] In human rectal circular muscle, PDE1, 2, 3, and 4 inhibitors all reduced histamine-induced tone suggesting a role for several PDEs.[53] In human colon circular muscle, both IBMX and rolipram concentration-dependently reduced basal tone, rolipram having a smaller maximal effect; other PDE inhibitors

were not tested.[54] An elaborated in vitro functional study investigating the effect of PDE inhibitors on cholinergic contractions by electrically released acetylcholine and by an exogenous muscarinic agonist in human large intestinal circular muscle can clarify the muscular role of PDEs. However, even when the PDE inhibitor alone induces relaxation, it is possible to enhance the 5-HT_4 receptor-mediated facilitation of cholinergic contraction in a selected concentration range as shown before with IBMX in porcine stomach.[9] In human colon 5-HT_4 receptors have also been described on circular smooth muscle cells and rolipram, in a concentration not influencing basal tone, facilitated the relaxant effect of 5-HT via these receptors.[54] The possible contribution of these relaxant receptors to the clinical effect of 5-HT_4 receptor agonists such as prucalopride at colonic level, that in vivo is clear-cut prokinetic,[27,28] is not well understood. If relevant, the prokinetic effect of a 5-HT_4 receptor agonist might depend on a fine balance between its excitatory and inhibitory effects, and facilitation of both effects by rolipram might be beneficial. It should be stated that the presence of relaxant 5-HT_4 receptors in human colon circular muscle could not be systematically confirmed;[10] they were also not observed in porcine colon.[8]

5 | CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, the intraneuronal pathway of 5-HT_4 receptors facilitating cholinergic neurotransmission in human large intestinal circular muscle is, just as in the porcine GI tract, controlled by PDE4; PDE4 inhibition leads to an enhancement of the facilitating effect of prucalopride on acetylcholine release. If this synergy is confirmed in a functional assay with electrically induced cholinergic contractions, the in vivo prokinetic effect of 5-HT_4 receptor agonists in the large intestine might be promoted by combination with a selective PDE4 inhibitor; this can be further explored as possible combination therapy for gastrointestinal hypomotility disorders such as constipation as an alternative option to combination with acetylcholinesterase inhibitors. For both combination strategies safety will have to be evaluated. Muscarinic effects as diarrhea, nausea and vomiting and nicotinic ones as muscle cramps can occur with acetylcholinesterase inhibitors. The most frequently reported adverse events in clinical studies with the PDE4 inhibitor roflumilast for chronic obstructive pulmonary disease also included diarrhea and nausea along with weight loss and headache.[55]

ACKNOWLEDGMENTS

The authors thank Mrs. Inge Van Colen for technical support and Mrs. Saskia De Groote, Mrs. Inge Vandenbroucke, and Mrs. Eva Pape from the Department of Gastrointestinal surgery of Ghent University Hospital for help in obtaining the human specimens.

CONFLICT OF INTEREST

No competing interest declared.

AUTHOR CONTRIBUTION

RL designed the study. WC organized the collection of human tissue. VP performed experiments and data analysis. RL and VP interpreted the findings and prepared the manuscript and all authors contributed to its completion.

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SUPPORTING INFORMATION

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How to cite this article: Pauwelyn V, Ceelen W, Lefebvre RA. Synergy between 5-HT_4 receptor stimulation and phosphodiesterase 4 inhibition in facilitating acetylcholine release in human large intestinal circular muscle. *Neurogastroenterol Motil*. 2017;e13162. https://doi.org/10.1111/nmo.13162