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The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4

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ABSTRACT

The influence of the selective 5-HT$_4$ receptor agonist prucalopride on acetylcholine release from cholinergic nerve endings innervating pig gastric circular muscle and the possible regulation of this effect by phosphodiesterases (PDEs) was investigated, as PDEs have been shown to control the response to 5-HT$_4$ receptor activation in pig left atrium. Circular muscle strips were prepared from pig proximal stomach and either submaximal cholinergic contractions or tritium outflow after incubation with [^{3}H]-choline, induced by electrical field stimulation, were studied. Prucalopride concentration-dependently increased the amplitude of submaximal cholinergic contractions and of acetylcholine release induced by electrical field stimulation. The effect of the highest concentration tested (0.3 μM) on cholinergic contractions was antagonized by the selective 5-HT$_4$ receptor antagonist GR113808 but not by granisetron or methysergide; the antagonism of prucalopride by GR113808 was confirmed in the release assay. The non-selective PDE-inhibitor 3-isobutyl-methyl-xanthine (IBMX) concentration-dependently reduced the amplitude of the cholinergic contractions; 3 μM IBMX reduced the cholinergic contractions maximally by 16% but it enhanced the facilitating effect of prucalopride from 51 to 83%. IBMX (10 μM) induced and enhanced the facilitating effect of prucalopride on electrically induced acetylcholine release. The selective inhibitors vinpocetine (PDE1), EHNA (PDE2) and cilostamide (PDE3) did not influence the effect of prucalopride on acetylcholine release but the PDE4-inhibitor rolipram (1 μM) enhanced the facilitating effect of prucalopride to the same extent as IBMX. These results demonstrate that 5-HT$_4$ receptors are present on the cholinergic nerves towards the pig gastric circular muscle, facilitating acetylcholine release; the intracellular transduction pathway of this facilitation is regulated by PDE4. Combination of a 5-HT$_4$ receptor agonist with selective inhibition of the PDE involved in this regulation of transmitter release might enhance the prokinetic effect of the 5-HT$_4$ receptor agonist.

1. Introduction

The highly selective 5-HT$_4$ receptor agonist prucalopride accelerates colonic transit and increases stool frequency in healthy volunteers (Emmanuel et al., 1998; Bouras et al., 1999) and is efficient in patients with severe chronic constipation resistant to laxative treatment (Camilleri et al., 2008; Tack et al., 2009). The main mechanism for these effects of prucalopride is thought to be interaction with facilitatory 5-HT$_4$ receptors on cholinergic nerve endings towards colonic smooth muscle, which have been shown in different species also at other levels of the gastrointestinal tract (Gershon and Tack, 2007). In human colon, these receptors were shown to be present on cholinergic nerve endings towards the longitudinal (Prins et al., 2000) and circular muscle (Leclere et al., 2005; Cellek et al., 2006). Untill it was withdrawn because of the potential of cardiac dysrhythmias, the non-selective 5-HT$_4$ receptor agonist cisapride was worldwide used for treatment of gastro-esophageal reflux and for increasing gastric emptying in gastroparesis, and less so for constipation. Facilitatory 5-HT$_4$ receptors are indeed also present on cholinergic nerve endings in human stomach circular muscle (Leclere and Lefebvre, 2002) similar to those described in the stomach of other species such as guinea pig (Takada et al., 1999) and dog (Prins et al., 2001a); prucalopride indeed accelerates gastric emptying in man (Bouras et al., 2001). In pig, which is a good model for the study of gastrointestinal issues in view of similar morphology and physiology of the gastrointestinal tract (Miller and Ullrey, 1987), we have shown before the presence of 5-HT$_4$ receptors on cholinergic neurons controlling longitudinal muscle but did not investigate circular muscle. The first aim of this

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study was therefore to investigate whether 5-HT_4 receptors also facilitate acetylcholine release from cholinergic neurons innervating the circularly oriented muscle in porcine stomach.

When comparing the effect of prucalopride on electrically induced cholinergic contractions in porcine proximal stomach longitudinal muscle with that in electrically paced porcine left atrial pectinate muscles, a clear and sustained enhancement of the cholinergic contractions in proximal stomach was observed but the positive inotropic effect of prucalopride was transient and very weak and only became prominent and sustained upon phosphodiesterase (PDE) inhibition with 3-isobutyl-1-methyl-xanthine (IBMX), illustrating a regulatory role of PDEs on 5-HT_4 receptor function in atrium (De Maeyer et al., 2006a,b), which has since been shown to be related to PDE3 and PDE4 activity (Galindo-Tovar et al., 2009). The robust and sustained effect of prucalopride in porcine stomach makes it unclear whether regulatory PDEs are involved. We did not test IBMX versus prucalopride in porcine stomach (De Maeyer et al., 2006a) but a recent detailed immunohistochemical study of PDE2A distribution in different species including dog, monkey and man, showed that the strongest immunoreactivity for PDE2A outside the central nervous system was present in enteric ganglia from the stomach to the colon (Stephenson et al., 2009). The second aim of this study was therefore to investigate whether PDEs control the effect of 5-HT_4 receptor stimulation on cholinergic neurotransmission in pig gastric circular muscle.

2. Methods

2.1. Animals

In the first part of the study, stomachs were obtained from approximately 6 months old healthy castrated male pigs, slaughtered at a local abattoir; the stomachs were transported to the laboratory in ice-chilled physiological salt solution. In the second part of the study (experiments with PDE-inhibitors), approximately 2 months old male piglets (Line 36, weighing approximately 20 kg) were obtained from Rattlerow Seghers (Lokeren, Belgium). On the morning of the experiment, the piglets were anesthetized with an intramuscular injection of 5 ml Zoletil 100 (containing 250 mg tiletamine and 250 mg zolazepam). After exsanguination, the entire stomach was dissected. The use of the piglets for this project was approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

For preparation of the smooth muscle strips, the stomach was cut open along the lesser curvature and placed in physiological salt solution (PSS) at room temperature (composition in mM: 112 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11.5 glucose and 25 $NaHCO_3$) as described by Mandrek and Milenov (1991; PSS I); or 118 NaCl, 4.69 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 2.51 $CaCl_2$, 11.1 glucose, 25 $NaHCO_3$ (Krebs-Henseleit; PSS II). After removal of the mucosa, 4 to maximum 12 muscle strips of approximately 1.5 cm in length and 0.3 cm in width were prepared from the proximal stomach in the direction of the circular muscle layer; up to 6 strips were obtained from the ventral side cutting from the great curvature towards the small one; the additional strips were prepared at the same level cutting in the direction of the circular muscle layer over the great curvature so that these strips were partially from the ventral and partially from the dorsal side. Strips used for release experiments were always obtained from the ventral side. All strips were used on the day of preparation. For functional experiments with measurement of contractility, the strips were mounted under a load of 2 g between 2 platinum plate electrodes in classic organ baths containing 10 ml of PSS I (first part of the study), or 5 (experiments with PDE-inhibitors other than IBMX) or 7 ml (experiments with IBMX) of PSS II (second part of the study) at 37 °C and gassed with carbogen (95% O_2/5% CO_2). Mechanical activity was recorded auxotonically via a Grass force-displacement transducer FT03 coupled in series with a 1 g cm^{-1} spring on a Graphtec linearcorder F WR3701 in the first part of the study; in the second part of the study, mechanical activity was recorded isometrically via a Grass force-displacement transducer FT03 (experiments with IBMX) or a MLT050/D force transducer from ADInstruments (experiments with other PDE-inhibitors) on a PowerLab/8 sp data recording system (ADInstruments) with Chart software. For release experiments, strips were mounted between 2 platinum wire electrodes under a load of 2 g in 2 ml organ baths containing PSS I, to which also 0.0015 mM choline and 0.057 mM ascorbic acid was added. Electrical field stimulation was performed by means of a Grass S88 stimulator with a constant voltage unit or a 4 channel custom-made stimulator.

2.2. Contractility study

In the first part of the study, the PSS I continuously contained 4 μM guanethidine and 300 μM N^G-nitro-L-arginine methyl ester (L-NAME) to avoid noradrenergic and nitrergic responses respectively; additionally it contained 10 μM indomethacine to avoid spontaneous progressive contraction due to release of prostaglandins. After at least 1 h of equilibration with rinsing every 15 min, the tissues were contracted with 3 μM carbachol to test the contractile reactivity of the strip; this was followed by rinsing every 10 min during 30 min. Electrical field stimulation (EFS) was then applied twice at an interval of 5 min (10 s train at supramaximal voltage, 0.5 ms and 4 Hz). This yielded reproducible contractions after which 10 s trains of EFS were applied at 5 min interval with decreasing voltage until the voltage yielding a contraction amplitude of approximately 50% of that obtained at supramaximal voltage (V50%C) was reached. EFS was then stopped for 30 min with rinsing every 10 min. EFS was then started again and 10 s trains at V50%C, 0.5 ms and 4 Hz were repeated at 5 min interval until stabilization. After a further 5 trains, 0.03, 0.1 or 0.3 μM prucalopride was added to 3 parallel tissues and 10 further trains were registered; a fourth tissue received the solvent of prucalopride (control). To test antagonists versus the effect of prucalopride, the antagonist was added after 5 trains at V50%C; 6 further trains were then obtained before adding 0.3 μM prucalopride and registering 10 further trains; a parallel control strip received the solvent of the antagonist. To evaluate the neurogenic and cholinergic nature of the EFS-induced contractions, the influence of 3 μM tetrodotoxin and 1 μM atropine was tested. To test the possible influence of prucalopride on contractions induced by exogenous acetylcholine, a cumulative concentration-response curve to acetylcholine was constructed with half log unit ascending concentration increments from 1 nM onwards; after rinsing for 1 h at 10 min intervals, 0.03, 0.1 or 0.3 μM prucalopride was incubated for 15 min and the concentration-response curve to acetylcholine was repeated.

In the second part of the study, the PSS II continuously contained 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) and 1 μM indomethacine. The initial part of the protocol with carbachol and EFS to determine the V50%C was as described above except that trains of EFS were administered every 3 min. Once EFS was started again at V50%C (0.5 ms, 4 Hz, 10 s) and 5 stable responses were obtained, 2 types of experiments were performed. 1) The influence of the PDE-inhibitors IBMX, vinpocetine, EHNA, cilostamide and rolipram on the half maximal electrically induced contractions was investigated by adding them in half log unit ascending concentrations, starting after the 5th train and registering the response to 6 trains after addition of each concentration. The influence of cilostamide plus rolipram was tested by adding 1 μM cilostamide, registering 10 trains, then adding 1 μM rolipram and registering another 20 trains; in half of the tissues the order of administration was reversed. 2) The influence of IBMX and rolipram versus prucalopride was studied as follows. A total of 33–35 trains (10 s, V50%C, 0.5 ms, 4 Hz) was delivered at 3 min intervals. After 5 trains, 1, 3 or 10 μM IBMX was administered and after 15 trains 0.01 μM prucalopride; control tissues only received prucalopride or solvent. Similarly, 1 μM rolipram was added after 5 trains and 0.01, 0.03 or 0.1 μM prucalopride was added after 15 trains; in a small number of tissues, rolipram was added after 20 trains in the presence of prucalopride had been obtained.

2.3. Release study

The same method was used as described before (Leclere and Lefebvre, 2001). Strips were equilibrated for 1 h with superfusion of PSS I at 2 ml min^{-1} (Gilson Minipuls, France) and continuous EFS (40 V, 1 ms, 0.5 Hz) was applied for the last 20 min. Superfusion was stopped and the strips were incubated for 30 min with [^{3}H]-choline (5 μCi ml^{-1}) under continuous EFS (40 V, 1 ms, 2 Hz). EFS was stopped and the tissues were then superfused (2 ml min^{-1}) for 90 min to remove loosely bound radioactivity with PSS I, from now on also containing 10 μM hemicholinium-3 to prevent re-uptake of choline, 10 μM physostigmine to prevent hydrolysis of acetylcholine and 1 μM atropine to prevent auto-inhibition of acetylcholine release. After washout, the organ bath was filled with 1 ml of PSS. This was collected and replaced at 3 min intervals for a total of 37 samples. The strips were stimulated twice (S1 and S2) at 15 V, 1 ms and 4 Hz for 2 min starting at the 13th (sample 5) and 73rd (sample 25) min after the end of the washout period. Prucalopride (0.03, 0.1 or 0.3 μM) was added 15 min (sample 20) before S2. The 5-HT_4 receptor antagonist GR113808 (1, 10 or 100 nM) was tested versus 0.3 μM prucalopride by adding it 21 min (sample 13) before prucalopride. In the second part of the study, the influence of 10 μM IBMX, added from sample 13 onwards, was tested versus 0.01 or 0.03 μM prucalopride, added from sample 20 onwards. In the same protocol, the influence of 10 μM vinpocetine, 10 μM EHNA, 1 μM cilostamide and 1 μM rolipram was tested versus 0.01 μM prucalopride. At the end of the experiment, the tissues were blotted and weighed. For each sample, 0.5 ml was mixed with 2 ml of the scintillator containing solution Ultima Gold (Perkin Elmer, USA). Radioactivity of all samples was measured by liquid scintillation counting (Packard Tri-Carb 2100 TR, Packard Instrument Company, USA); external standardization was used to correct for counting efficiency. We have previously shown by separation with HPLC of the radioactive compounds in EFS-induced tritium outflow from pig stomach circular muscle strips after incubation with ^{3}H-choline, that changes in ^{3}H-acetylcholine parallel those in total tritium so that total tritium outflow can be considered as a marker for acetylcholine release (Leclere and Lefebvre, 2001).

2.4. Data analysis

In the contractility study, the average contraction to 5 trains of EFS just before treatment was taken as 100% and contractions induced by EFS in the presence of

treatment were related to this reference value. In experiments where prucalopride was added after 10 trains of EFS had been applied in the presence of IBMX, EFS-induced contractions in the presence of prucalopride were also expressed as % of the mean of the last 5 EFS-induced contractions in the presence of IBMX just before adding prucalopride (see Fig. 4B). In the release study, EFS-evoked an increase in tritium overflow not only in samples 5 (S_1) and 25 (S_2) but also in up to maximally the 6 subsequent samples. Basal tritium overflow during the period with stimulation-induced increase of tritium overflow was calculated by fitting a regression line through the 4 samples just before stimulation and the 4 values starting from where overflow had returned to basal values after stimulation. The stimulation-induced increase in tritium overflow was then determined by subtracting basal tritium overflow from the values in the samples with increased overflow. The S_2/S_1 ratio was then calculated.

Results are expressed as means ± SEM, *n* referring to tissues from different animals except for one series (see Fig. 7). Data obtained in parallel tissue groups were compared by an unpaired *t*-test (2 groups) or for more than 2 groups by ANOVA, followed by a post-hoc *t*-test corrected for multiple comparisons (Bonferroni). The influence of the increasing concentrations of the PDE-inhibitors on the electrically induced submaximal contractions was assessed by repeated measures ANOVA. *P* values of less than 0.05 were considered significant.

2.5. Drugs used

Acetylcholine chloride, L-ascorbic acid, atropine sulphate, choline chloride, guanethidine sulphate, indomethacine (Sigma), carbamoylcholin-chlorid (Fluka), hemicholinium-3-bromide (RBI or Sigma-Aldrich), 3-isobutyl-methyl-xanthine (Sigma-Aldrich), cilostamide, erythro-9-(2-hydroxyl-3-nonyl)adenine hydrochloride (EHNA), rolipram, vinpocetine (Tocris), [methyl-^{3}H]-choline chloride (NEN or Perkin Elmer), [1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1-H-indole-3-carboxylate maleate (GR113808; GlaxoWellcome), methysergide maleate (Sandoz), physostigmine salicylate (Federa or Sigma-Aldrich), N^G-nitro-L-arginine methyl ester (Sigma or Sigma-Aldrich), tetrodotoxin (Alomone Labs), prucalopride hydrochloride (Janssen Research Foundation), prucalopride succinate (Movetis). Drugs were dissolved in deionized water except for 3-isobutyl-methyl-xanthine (10 mM stock solution in 50% ethanol), cilostamide, rolipram, vinpocetine (dissolved in DMSO) and indomethacine (dissolved in 10% $NaHCO_3$ or DMSO before adding it to the physiological salt solution). For granisetron commercially available ampoules (Kytril, Roche) were used and diluted with 0.9% NaCl solution till the required concentration.

3. Results

3.1. Influence of prucalopride on EFS-induced submaximal cholinergic contractions

The circular muscle strips of the pig proximal stomach did not show spontaneous phasic activity and basal tone remained constant during the course of the experiment. EFS-induced contractions at V50%C attained an amplitude of 67 ± 10% ($n = 6$) of that induced by 3 μM carbachol at the beginning of the experiment; the contractions were neurogenic and cholinergic as they were abolished by 3 μM tetrodotoxin ($n = 4$) and 1 μM atropine ($n = 4$). In control tissues, the amplitude of the EFS-induced contractions at V50%C remained stable upon repetitive stimulation (amplitude of the contraction by a 15th stimulation train was 100 ± 5% of the mean response to trains 1 to 5; $n = 6$). Prucalopride did not influence the basal tone of the strips but it progressively enhanced the amplitude of the EFS-induced contractions (Fig. 1A) coming close to the maximal effect for a given concentration at the 5th stimulation train in its presence; the facilitating effect of prucalopride was concentration-dependent for the concentration range studied (0.03, 0.1 or 0.3 μM; Fig. 1B). Cumulative administration of acetylcholine induced sustained and concentration-dependent contractions from 0.1 μM onwards; the contraction amplitude at 100 μM attained 95 ± 3% ($n = 4$) of that induced by 3 μM carbachol at the beginning of the experiment. Concentration-response curves of acetylcholine obtained in the presence of 0.03, 0.1 or 0.3 μM prucalopride did not differ from that in its absence within the same strips ($n = 4$ for each concentration). Before addition of 0.3 μM prucalopride, the EC50 value for the concentration-response curve of acetylcholine was 1.7 ± 0.7 μM and the Emax (as % of the response to 3 μM carbachol) was 96 ± 3%;

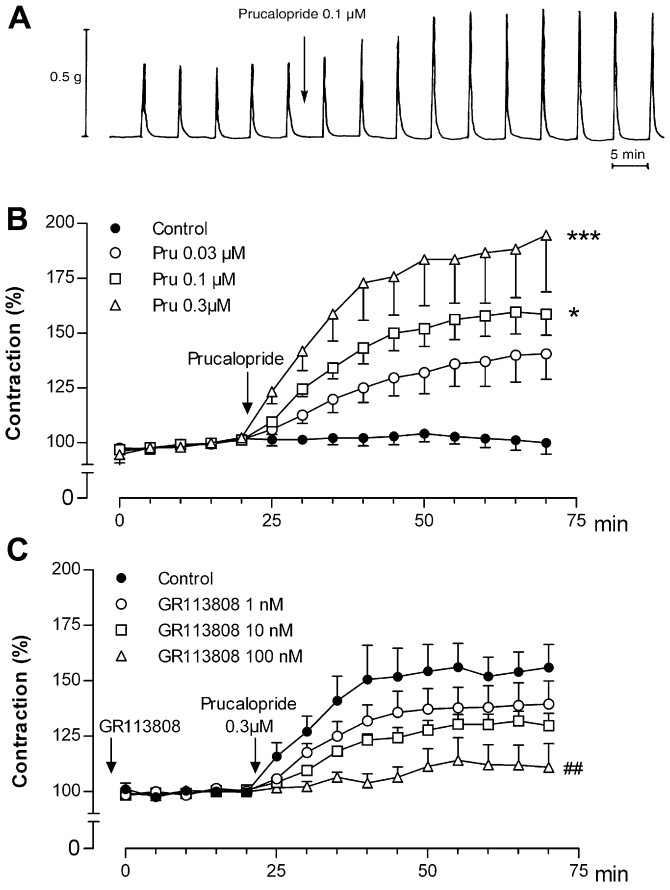

Fig. 1. A: Representative trace (auxotonic registration) showing the facilitating effect of 0.1 μM prucalopride on submaximal EFS-induced contractions in the presence of 300 μM L-NAME in a pig gastric circular muscle strip. B–C: Mean enhancing effect of increasing concentrations of prucalopride (Pru) on EFS-induced submaximal contractions (B) and concentration-dependent antagonism by GR113808 of the enhancing effect of 0.3 μM prucalopride (C). Responses are expressed as percentage of the mean of the 5 contractions before adding prucalopride. Means ± SEM of $n = 6$ tissues are shown. $^{***}P < 0.001$, $^{*}P < 0.05$: significant difference of the final response versus that in control tissues without prucalopride; $^{\#\#}P < 0.01$: significant difference of the final response versus that in control tissues only treated with prucalopride.

after adding prucalopride these values were 1.6 ± 0.8 μM and 96 ± 3% ($n = 4$).

GR113808 (1, 10 and 100 nM) per se did not influence the EFS-induced contractions but concentration-dependently inhibited the facilitating effect of 0.3 μM prucalopride (Fig. 2B). Granisetron (1 μM) per se did not influence the EFS-induced contractions nor did it influence the facilitating effect of 0.3 μM prucalopride, that enhanced the EFS-induced contractions to 125 ± 10% ($n = 6$) in the absence of granisetron and to 134 ± 7% ($n = 6$) in the presence of granisetron. In the series where methysergide was tested, 0.3 μM prucalopride alone increased the EFS-induced contractions to 159 ± 11% ($n = 6$). Methysergide (10 μM) per se enhanced the amplitude of the EFS-induced contractions to 162 ± 11% ($n = 6$) of that before it was added; when 0.3 μM prucalopride was added in the presence of methysergide, it further enhanced the amplitude of the EFS-induced contractions to 202 ± 19% ($n = 6$).

3.2. Influence of PDE-inhibitors per se on EFS-induced submaximal cholinergic contractions

The non-selective PDE-inhibitor IBMX induced a concentration-dependent reduction of the contractions from 3 μM onwards and in

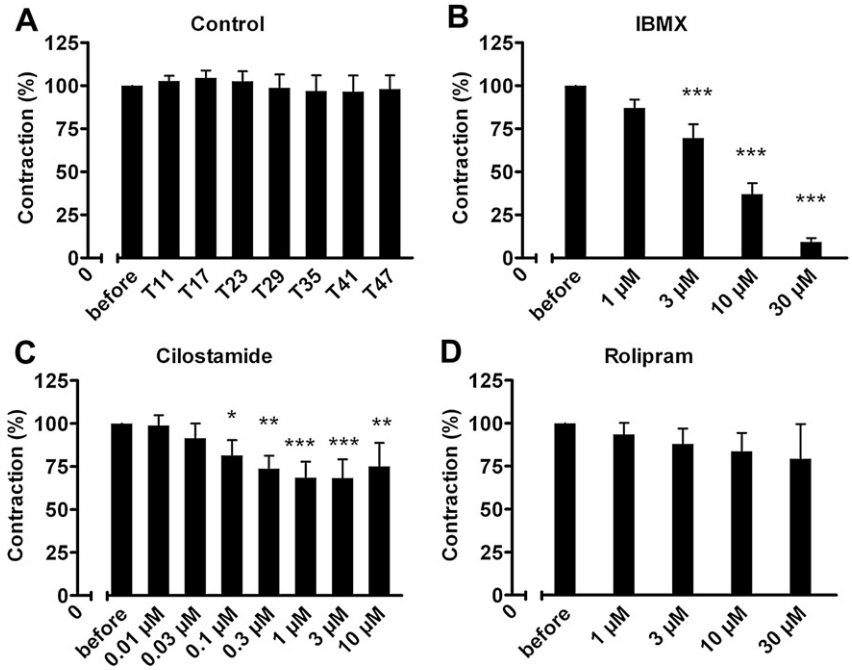

Fig. 2. Influence of increasing concentrations of the PDE-inhibitors IBMX (B), cilostamide (C) and rolipram (D) on EFS-induced submaximal contractions. Six trains of EFS were applied in the presence of each concentration of PDE-inhibitor and the response to the 6th train was expressed as percentage of the mean of the 5 contractions before adding the lowest concentration of the PDE-inhibitor. Control tissues (A) were stimulated 47 times and the response was measured at each 6th train from train 11 (T11) on. Means ± SEM of $n = 6–8$ tissues are shown. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$: significant difference versus the response before.

the presence of 30 μM IBMX, the contractions were nearly abolished (Fig. 2B). None of the selective PDE-inhibitors was able to mimick the effect of IBMX. The PDE1-inhibitor vinpocetine (0.01–10 μM) and the PDE2-inhibitor EHNA (1–30 μM) did not significantly influence the submaximal cholinergic contractions ($n = 6$ for each agent; data not shown), nor did the PDE4-inhibitor rolipram (1–30 μM; Fig. 2D). The PDE3-inhibitor cilostamide (0.01–10 μM) reduced the contractions from 0.1 μM onwards but the maximal depression obtained was much smaller than with IBMX (reduction to 68 ± 11% with 3 μM cilostamide; Fig. 2C). When 1 μM cilostamide was added after 1 μM rolipram, it nearly abolished the electrically induced contractions (Fig. 3A); the response to the 10th stimulation train in the combined presence of rolipram and cilostamide only attained 13 ± 1% ($n = 4$) of the response before adding the PDE-inhibitors. Also when the order of administration was reversed, electrically induced contractions were as good as abolished. After first adding 1 μM cilostamide, the contraction decreased to 59 ± 13% at the 10th stimulation train in its presence; when further adding 1 μM rolipram, the contraction further decreased to 10 ± 5% ($n = 4$) at the 10th stimulation train in their combined presence.

3.3. Influence of PDE-inhibitors on the effect of prucalopride on EFS-induced submaximal cholinergic contractions

In the gastric circular muscle strips of piglets, prucalopride (0.01 μM) enhanced the EFS-induced contractions but this effect developed clearly slower than in the first part of the study (Fig. 4). IBMX, 1 and 3 μM, per se concentration-dependently decreased the EFS-induced contractions (maximally to 84 ± 2%, $n = 6$, in the presence of 3 μM IBMX; Fig. 4A). Using the decreased EFS-induced response in the presence of IBMX just before adding prucalopride as reference, a significant enhancement of the facilitating effect of prucalopride by 3 μM IBMX was evident (Fig. 4B). In an additional series, the influence of 10 μM IBMX was studied. This concentration of IBMX reduced the EFS-induced contractions by approximately 50%; in the tissues that received only IBMX, this reduction was maintained till the end of the experiment. Prucalopride (0.01 μM) enhanced EFS-induced contractions to 159 ± 13% ($n = 7$, $P < 0.05$ versus control: 97 ± 5%, $n = 6$). In the presence of 10 μM IBMX, prucalopride enhanced EFS-induced contractions to 185 ± 25% ($n = 8$); this was not significantly different from the response to prucalopride alone.

Rolipram (1 μM) was tested versus 0.01, 0.03 and 0.1 μM prucalopride (Fig. 5). In this series, the mean contractile response to the 10th stimulation train in the presence of rolipram tended to increase in comparison to the response before its administration: to 114 ± 8% ($n = 8$) before 0.01 μM prucalopride (Fig. 5A), 115 ± 8% ($n = 8$) before 0.03 μM prucalopride (Fig. 5B) and 122 ± 9% ($n = 8$) before 0.1 μM prucalopride (Fig. 5C). This was due to an increase in the response to stimulation in the presence of rolipram in some tissues. Eg in the tissues where 0.03 μM prucalopride was going to be added, the individual contractile response to the 10th stimulation in the presence of rolipram was 96, 111, 137, 155, 93, 102, 101 and 128%. Prucalopride alone significantly increased the electrically induced contractions to 162 ± 11% ($n = 7$; 0.01 μM; Fig. 5A), 171 ± 15% ($n = 8$; 0.03 μM; Fig. 5B) and 206 ± 10% ($n = 7$; 0.1 μM; Fig. 5C). When rolipram had been added before prucalopride, prucalopride increased the EFS-induced contractions to 181 ± 7% ($n = 8$; 0.01 μM), 206 ± 24% ($n = 8$; 0.03 μM) and 243 ± 23% ($n = 8$; 0.1 μM); these values were not significantly different from those in the presence of prucalopride alone. Still, when 1 μM rolipram was added after 20 stimulations in the presence of prucalopride had been obtained and the facilitating effect of prucalopride was stabilized, rolipram induced a clearcut further increase of the electrically induced contractions ($n = 2$ for the 3 concentrations of prucalopride; illustrated in Fig. 3B for 0.01 and in Fig. 3C for 0.1 μM prucalopride).

3.4. Influence of prucalopride on EFS-induced acetylcholine release

EFS caused a clearcut increase in tritium outflow above basal not only in the sample with stimulation but also in up to 6 further

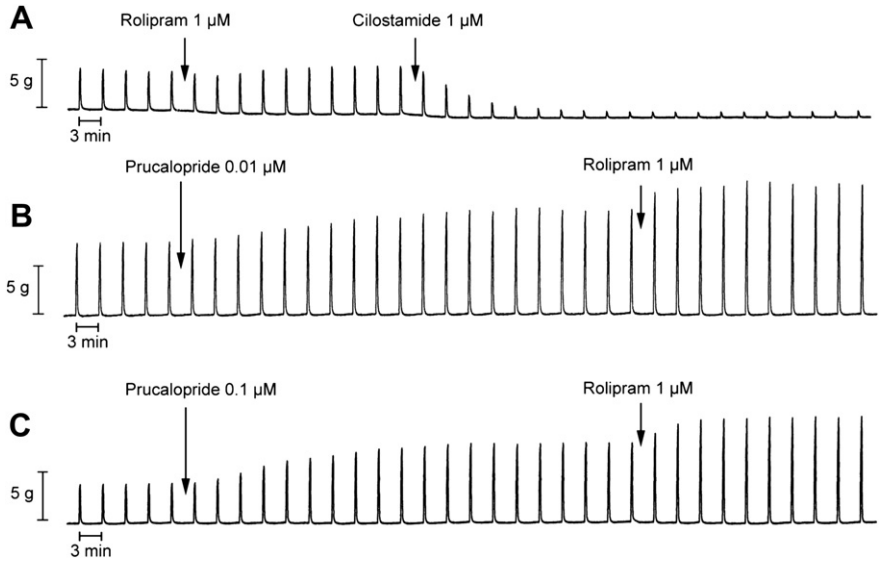

Fig. 3. Representative traces (isometric registration) showing the influence on submaximal EFS-induced contractions of consecutive administration of 1 μM rolipram and 1 μM cilostamide (A), and of 0.01 μM (B) or 0.1 μM (C) prucalopride and 1 μM rolipram.

samples. The response induced by the second stimulation train was less pronounced yielding a S2/S1 ratio of 0.7 (Table 1). Prucalopride (0.03, 0.1 and 0.3 μM) did not influence basal outflow but it enhanced the tritium outflow induced by the second stimulation train leading to a concentration-dependent increase of the S2/S1 ratio with an S2/S1 ratio of 1.05 for 0.3 μM prucalopride (Table 1). In an additional series, the influence of 1 μM prucalopride was tested but this did not induce a more pronounced effect than 0.3 μM prucalopride (S2/S1 ratio: 0.74 ± 0.05 for controls, $n = 5$; 1.04 ± 0.05 for 1 μM prucalopride, $n = 6$; $P < 0.01$). GR113808 (1, 10, 100 nM) did not influence basal outflow but concentration-dependently antagonized the facilitating effect of 0.3 μM prucalopride (Table 1).

3.5. Influence of PDE-inhibitors on the effect of prucalopride on EFS-induced acetylcholine release

The influence of IBMX (10 μM) was first tested versus 0.01 μM prucalopride, a concentration expected to be minimally effective on acetylcholine release. Indeed, 0.01 μM prucalopride did not significantly increase EFS-induced tritium outflow versus control tissues (Fig. 6); the S2/S1 ratio was 0.68 ± 0.04 in tissues where 0.01 μM prucalopride was administered before S2 ($n = 6$) versus 0.59 ± 0.01 in control tissues ($n = 6$; Fig. 7A). IBMX (10 μM) per se did not influence basal outflow (Fig. 6) nor did it influence EFS-induced tritium outflow (Fig. 7A). However, when IBMX was administered before prucalopride, a clearcut significant increase in EFS-induced tritium outflow was obtained (Fig. 7A). In a second series, 0.03 μM prucalopride per se significantly enhanced EFS-induced tritium outflow. IBMX (10 μM) per se again did not influence EFS-induced tritium outflow significantly but it further increased the facilitating effect of prucalopride (Fig. 7B).

EHNA (10 μM) did not influence basal nor EFS-induced tritium outflow; it did also not induce a facilitating effect of 0.01 μM prucalopride (S2/S1 ratio in control tissues: 0.53 ± 0.02; with 10 μM EHNA: 0.51 ± 0.05; with 0.01 μM prucalopride: 0.63 ± 0.04; with EHNA and prucalopride: 0.58 ± 0.03; $n = 4–6$). A small series was then started where 0.01 μM prucalopride was added before S2, either alone or preceded by vinpocetine (10 μM), cilostamide (1 μM) or rolipram (1 μM). None of these PDE-inhibitors influenced basal tritium outflow but in the presence of rolipram and prucalopride, the S2/S1 ratio (0.98 ± 0.02) was significantly enhanced ($P < 0.01$) versus that in the presence of prucalopride alone (0.70 ± 0.03); the S2/S1 ratio for vinpocetine plus prucalopride was 0.64 ± 0.05; for cilostamide plus prucalopride 0.69 ± 0.06 ($n = 4$ for each series). A series was then set-up including control tissues and the addition of rolipram alone before S2. Rolipram (1 μM) per se tended to increase the S2/S1 ratio but this was not significant. The S2/S1 ratio in the presence of rolipram plus 0.01 μM prucalopride (0.98 ± 0.07; $n = 6$) was significantly enhanced versus that in the presence of prucalopride alone (0.65 ± 0.03; $n = 6$; Fig. 8) similar to the level obtained in the presence of IBMX plus 0.01 μM prucalopride (Fig. 7A).

4. Discussion

4.1. Facilitation of cholinergic neurotransmission by 5-HT$_4$ receptor activation in pig gastric circular muscle

The data presented illustrate that cholinergic nerves in pig gastric circular muscle are endowed with facilitating 5-HT$_4$ receptors promoting acetylcholine release and cholinergic contractions. Neuronally induced submaximal cholinergic contractions, as evidenced by their blockade with the neuronal action potential propagation inhibitor tetrodotoxin and the muscarinic receptor antagonist atropine, were concentration-dependently enhanced by prucalopride, while cholinergic contractions induced by direct stimulation of muscarinic receptors with acetylcholine were not influenced. This suggests a presynaptic site of action of prucalopride with facilitation of acetylcholine release, which was confirmed by measuring acetylcholine release directly. The concentration range used lies within the EC$_{50}$–EC$_{100}$ region of the concentration-response curve for the enhancing effect of prucalopride through presynaptic 5-HT$_4$ receptors on electrically induced submaximal cholinergic contractions in canine and pig gastric longitudinal muscle preparations and the enhancing effect of the highest concentration tested (0.3 μM; 95%; see Fig. 2A) corresponds to an enhancing effect of 80–90% with the same concentration in these 2 preparations (Prins et al., 2001b; De Maeyer et al., 2006a). The enhancing effect of 0.3 μM prucalopride both on contractions and

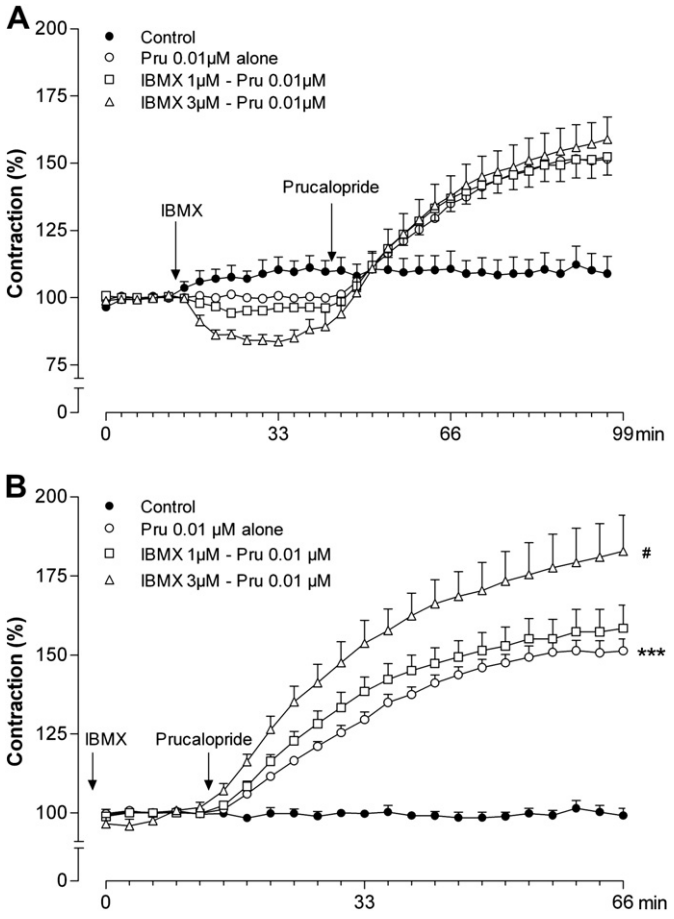

Fig. 4. Influence of IBMX (1 or 3 μM) on the enhancing effect of 0.01 μM prucalopride (Pru) on EFS-induced submaximal contractions. In (A) responses are expressed as % of the mean of the first 5 responses i.e., the contractions induced before adding IBMX. In (B) responses are expressed as % of the mean of the 5 contractions before adding prucalopride. Means ± SEM of $n = 6$ tissues are shown. In panel A, the SEM is not shown on all mean data points for clarity. $^{***}P < 0.001$: significant difference of the final response versus that in control tissues without prucalopride; $^{\#}P < 0.05$: significant difference of the final response versus that in tissues only treated with prucalopride.

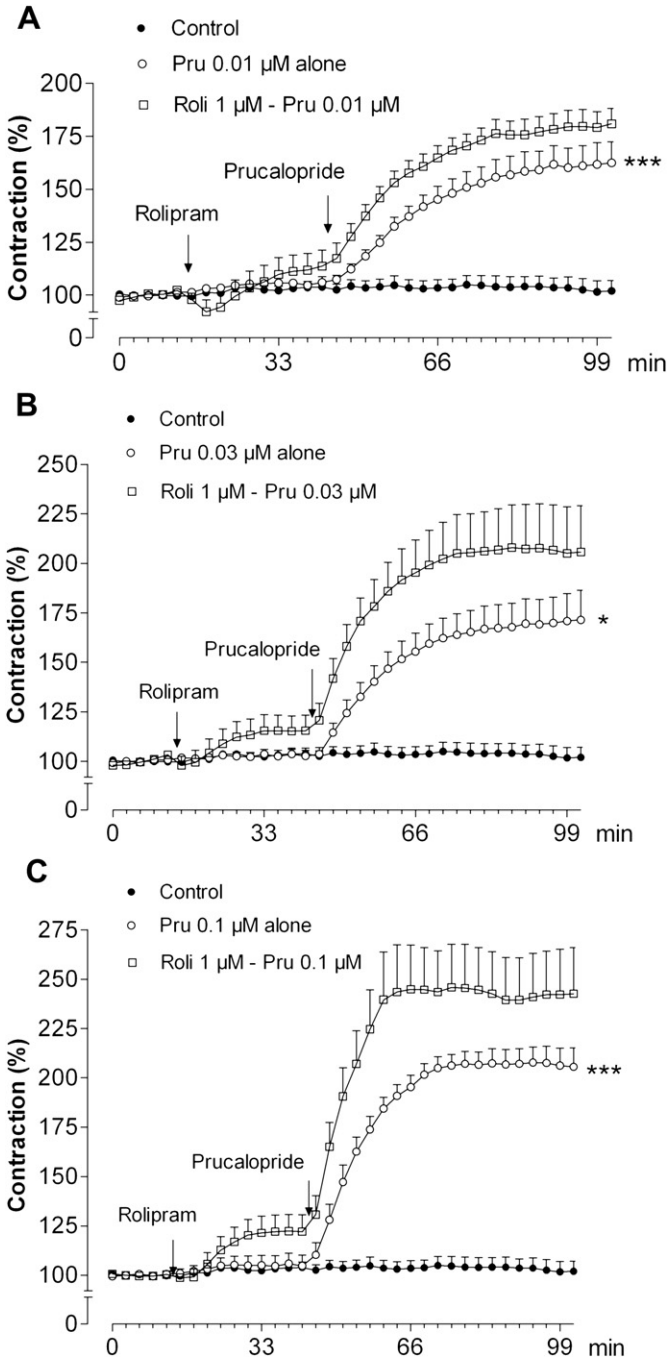

Fig. 5. Influence of 1 μM rolipram on the enhancing effect of 0.01 (A), 0.03 (B) and 0.1 (C) μM prucalopride on EFS-induced submaximal contractions. Responses are expressed as percentage of the mean of the 5 contractions before adding rolipram. Means ± SEM of $n = 7–8$ tissues are shown. $^{***}P < 0.001$, $^{*}P < 0.05$: significant difference of the final response versus that in control tissues without prucalopride.

acetylcholine release was concentration-dependently antagonized by the 5-HT_4 receptor antagonist GR113808 (Gale et al., 1994). GR113808 has a high affinity for 5-HT_4 receptors with reported pK_B estimates of 9.1 at 5-HT_4 receptors on cholinergic nerve endings in canine stomach (Prins et al., 2001b) and of 9.36 at 5-HT_4 receptors on human colon circular muscle cells (Irving et al., 2007). At 100 nM GR113808 can thus be expected to close to abolish the effect of a near maximal concentration of a 5-HT_4 receptor agonist which was the case with 0.3 μM prucalopride in both the functional and release assays corroborating its interaction with 5-HT_4 receptors. This was further underlined by the lack of effect of the 5-HT_3 receptor antagonist granisetron and the 5-HT_1, 5-HT_2, 5-ht$_5$, 5-HT_6 and 5-HT_7 receptor antagonist methysergide on the enhancement of cholinergic contractions by prucalopride. We have no clearcut explanation for the effect per se of methysergide on the electrically induced cholinergic contractions, that we also observed before in pig gastric longitudinal muscle (De Maeyer et al., 2006a). Further investigation of this effect of methysergide was, however, not within the aim of this study.

Similar to the human colon, the pig stomach thus has 5-HT_4 receptors on the cholinergic nerve endings towards both the circular and longitudinal muscle layer and pig gastric circular muscle can thus be considered as a model for the 5-HT_4 receptors on the cholinergic nerves innervating circular muscle in the human stomach (Leclere and Lefebvre, 2002). The longitudinal muscle layer of the stomach contracts in 1:1 correlation with the circular muscle layer (Sarna, 1993) but it can be expected that the thicker circular muscle layer contributes most to changes in intragastric pressure and gastric motility, so that it might be preferable to investigate the effect of new 5-HT_4 receptor agonists, developed for stimulating gastric motility and emptying, at 5-HT_4 receptors in the most important muscle layer.

Table 1
EFS-induced outflow of total radioactivity.

Influence of prucalopride				
S1	50,952 ± 3496	68,328 ± 11,006	91,698 ± 24,563	61,343 ± 11,445
Prucalopride (μM)	- (Control)	0.03	0.1	0.3
S2	35,494 ± 3025	62,398 ± 8272	91,877 ± 21,668	61,498 ± 9813
S2/S1	0.70 ± 0.04	0.97 ± 0.14	1.02 ± 0.03*	1.05 ± 0.09*
Influence of GR113808 on effect of prucalopride				
S1	50,543 ± 3791	42,314 ± 3744	45,180 ± 10,235	49,850 ± 8210
GR113808 (nM)	- (Control)	1	10	100
Prucalopride (μM)	0.3	0.3	0.3	0.3
S2	52,591 ± 2950	43,860 ± 4122	39,273 ± 9533	47,590 ± 8293
S2/S1	1.05 ± 0.03	1.05 ± 0.08	0.86 ± 0.04	0.74 ± 0.05##

Total radioactivity (tritium) is expressed in dpm g^{-1} tissue. For S1 and S2, the sum of radioactivity above baseline in sample 5 (S1) and sample 25 (S2), respectively, and the following samples with values above baseline is given. Means ± SEM of $n = 5$ to 6 tissues are given. *$P < 0.05$ versus control without prucalopride; ##$P < 0.01$ versus control without addition of GR113808 before prucalopride.

4.2. Regulatory control by PDEs of the 5-HT$_4$ receptor on cholinergic neurons in pig gastric circular muscle

5-HT$_4$ receptors are adenylyl cyclase coupled receptors generating cAMP and the facilitating effect of the 5-HT$_4$ receptor agonist renzapride on acetylcholine release from guinea pig small intestinal myenteric neurons was reported to be related to activation of the adenylyl cyclase-protein kinase A pathway (Ren et al., 2008). Cellular cyclic nucleotide levels are regulated by PDEs which catalyse their breakdown. The positive inotropic effect of 5-HT$_4$ receptor agonists in porcine left atrium becomes only prominent and sustained under conditions of PDE inhibition (De Maeyer et al., 2006b; Galindo-Tovar et al., 2009) illustrating an important role of PDEs in the control of 5-HT$_4$ receptor-induced cardiac cAMP levels. The enhancing effect of prucalopride on submaximal cholinergic contractions in pig gastric longitudinal (De Maeyer et al., 2006a) and circular (this study) muscle is sustained in the absence of PDE inhibition but this does not exclude a regulatory role of PDEs. A recent very thorough evaluation of the mRNA distribution for the 11 PDE isoenzymes in human peripheral tissues showed all PDE isoenzymes to be expressed in the stomach except for PDE6A (Lakics et al., 2010). Also in gastrointestinal smooth muscle, cyclic nucleotides are essential mediators of relaxation and their intracellular concentration is regulated by PDEs; a major part of the PDE mRNA expressed in human stomach may thus have been derived from the smooth muscle cells. In our experiments, the non-selective PDE-inhibitor IBMX concentration-dependently reduced the amplitude of the electrically induced cholinergic contractions (Fig. 2). This corresponds to the inhibitory effect of non-selective PDE-inhibitors versus contractions induced by exogenously administered agonists (Barnette et al., 1993; Barbier and Lefebvre, 1995; Tomkinson and Raeburn, 1996) and electrical field stimulation (Park et al., 2003) in gastrointestinal muscle preparations and illustrates that also in porcine gastric circular muscle, PDEs are controlling the cyclic nucleotide concentrations.

Still, a careful study of PDE2A distribution by immunohistochemistry revealed prominent expression in enteric ganglia from stomach to colon (Stephenson et al., 2009). We therefore investigated the influence of the non-selective PDE-inhibitor IBMX on the facilitating effect of prucalopride on cholinergic contractions in the functional assay. As the pig stomach provision from the slaughter house was no longer available by then, commercially available piglets were now used. In order to be able to observe a possible facilitating influence of IBMX on the effect of prucalopride, 0.01 μM prucalopride (3 times lower than the lowest concentration in part I) was used expecting a mild influence on submaximal cholinergic contractions. However, this concentration increased the cholinergic contractions to approximately 150% in the gastric circular muscle strips of the piglets; this higher sensitivity might be related to the younger age of the animals. The time interval before reaching a stable effect of prucalopride was also increased but this might be related to the smaller interval in between stimulation trains (3 min in stead of 5); cfr our results reported for pig gastric longitudinal muscle where trains of EFS at 3 min interval were shown to induce stable contractions (De Maeyer et al., 2006a), we had reduced the train interval to 3 min for the second part of the study. The interpretation of the results when studying IBMX versus prucalopride in the functional assay was hampered by the relaxing effect of IBMX per se. This functional antagonism by IBMX was maintained throughout the experiment as evident from the study of the effect of 10 μM IBMX alone. When prucalopride was added in the presence of IBMX, it was able to enhance the electrically induced contractions, the effect of prucalopride in the presence of IBMX being more pronounced than with prucalopride alone, reaching significance for 3 μM IBMX (Fig. 4B). This suggests that, notwithstanding the negative influence on the effect of prucalopride by functional antagonism of released acetylcholine at the muscular level, IBMX has a positive influence on the facilitating effect of prucalopride at the cholinergic nerves leading to a higher enhancement of acetylcholine release.

This was confirmed in the release assay. IBMX had no influence per se on basal outflow and on electrically induced acetylcholine release but it induced a significant facilitating effect on a per se subeffective concentration of prucalopride (0.01 μM) on acetylcholine release and it further increased the already significant effect of 0.03 μM prucalopride. In the release assay, 0.01 μM prucalopride indeed only tended to increase the acetylcholine release while it had a clearcut effect on submaximal cholinergic contractions in the functional assay. However, one should realize that the release assay only measures overflow from acetylcholine out of the tissue in the organ bath medium while the functional assay measures the direct smooth muscle response to acetylcholine released from the cholinergic nerves. Additionally, acetylcholine release was measured after 15 min of incubation with prucalopride while the maximal enhancing effect on cholinergic contractions (50% increase) in part II of the functional study was measured after a total incubation of prucalopride for 54 min (18 trains of EFS at 3 min interval). Our results indicate that the stimulatory effect of 5-HT$_4$ receptors on acetylcholine release from cholinergic nerves innervating circular muscle of the pig stomach is regulated by neuronal PDEs. This does not correlate with data of Kilbinger et al. (1995), who reported that in longitudinal muscle-myenteric plexus preparations of guinea pig ileum, IBMX did not increase the enhancing effect of 5-HT$_4$ receptor agonists on acetylcholine release. In contrast, Yau et al. (1987) reported that the adenylate cyclase activator forskolin increased acetylcholine release in the same set-up and this effect was enhanced in the presence of the non-selective PDE-inhibitors IBMX and theophylline suggesting

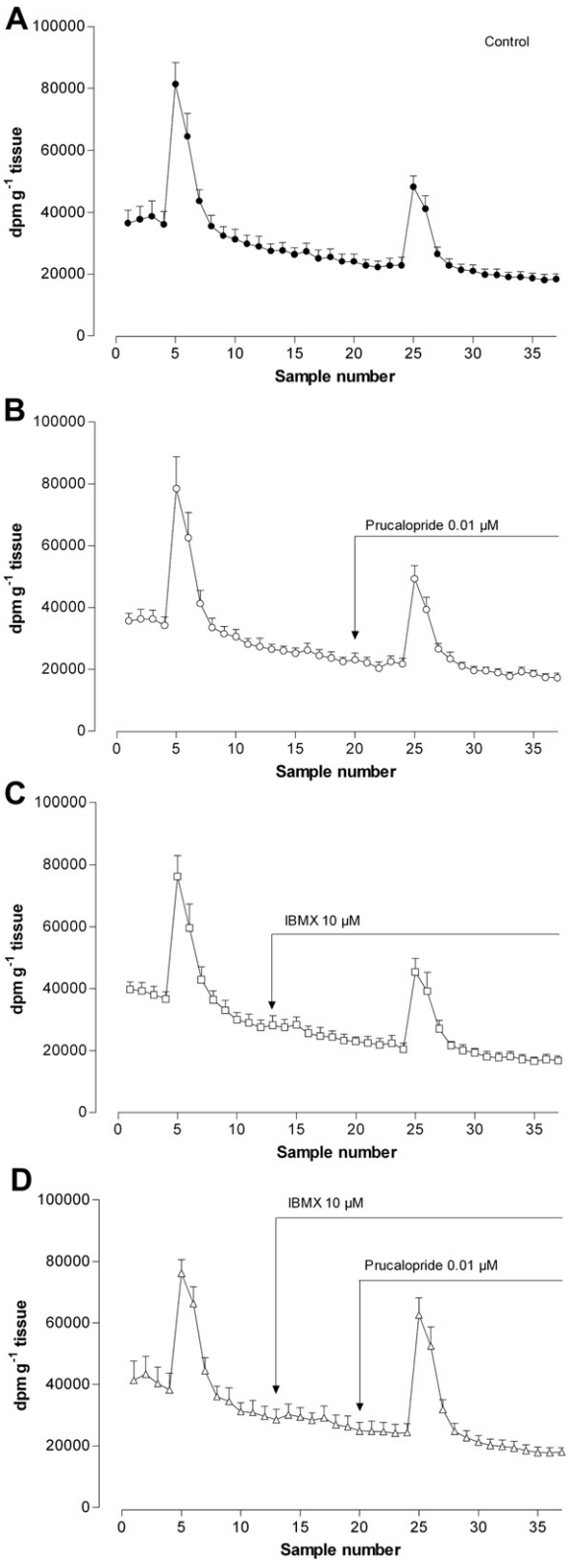

Fig. 6. Influence of prucalopride (0.01 μM, B), IBMX (10 μM, C) and prucalopride in the presence of IBMX (D) on EFS-induced release of total radioactivity; in A, parallel control tissues are shown. The content of the organ bath was collected in 3 min samples for measurement of total radioactivity (37 samples in total). Tissues were stimulated twice (15 V, 1 ms, 4 Hz, 2 min), at the 5th (S1) and 25th (S2) sample. Prucalopride and IBMX were added before S2 at the time points indicated. Means ± SEM of $n = 6$ tissues are shown.

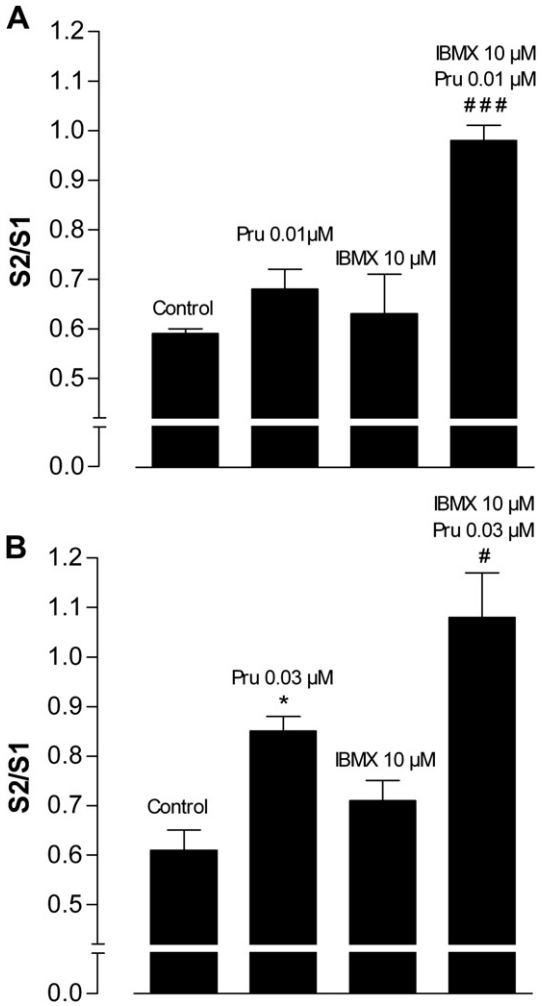

Fig. 7. Influence of prucalopride (Pru; 0.01 μM, A; 0.03 μM, B), IBMX and prucalopride in the presence of IBMX on the S2/S1 ratio of EFS-evoked total radioactivity release. Tissues were stimulated twice (S1 and S2; 15 V, 1 ms, 4 Hz, 2 min); IBMX was added 36 min and prucalopride 15 min before S2. The EFS-induced efflux of total radioactivity above baseline by S2 is expressed as a ratio of that by S1. Means ± SEM of $n = 5$ to 6 tissues are shown. $^{*}P < 0.05$: significantly different from control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$: significantly different from prucalopride alone.

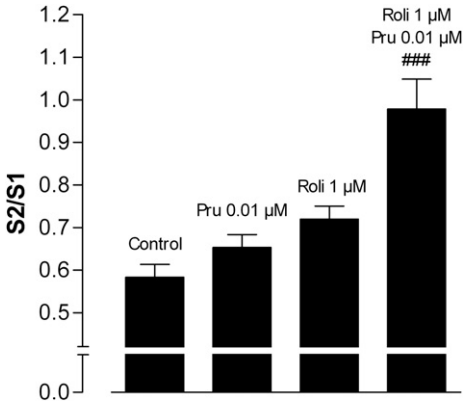

Fig. 8. Influence of prucalopride (Pru, 0.01 μM), rolipram (Roli, 1 μM) and prucalopride in the presence of rolipram on the S2/S1 ratio of EFS-evoked total radioactivity release. Tissues were stimulated twice (S1 and S2; 15 V, 1 ms, 4 Hz, 2 min); rolipram was added 36 min and prucalopride 15 min before S2. The EFS-induced efflux of total radioactivity above baseline by S2 is expressed as a ratio of that by S1. Means ± SEM of $n = 6$ tissues are shown. $^{\#\#\#}P < 0.001$: significantly different from prucalopride alone.

that cAMP-mediated influences on acetylcholine release are also in guinea pig ileum regulated by PDEs.

4.3. PDE subtype involved in the regulatory control of the 5-HT_4 receptor on cholinergic neurons in pig gastric circular muscle

Of the classic PDE subtypes 1–5 (Beavo and Reifsnyder, 1990; Maurice et al., 2003), PDE4 is cAMP specific, PDE5 is cGMP specific and PDE1, 2 and 3 have dual enzymatic activity, so that PDE1, 2, 3 or 4 might be involved in the control of the adenylate cyclase-cAMP-linked 5-HT_4 receptor on the cholinergic neurons in pig gastric circular muscle. We therefore studied the influence of selective PDE-inhibitors and first concentrated on PDE2 in view of its distribution in gastric enteric ganglia of different species (Stephenson et al., 2009). However EHNA, which has been shown to selectively inhibit PDE2 with an IC50 in the low micromolar range (Rivet-Bastide et al., 1997), did not influence the effect of prucalopride on electrically induced acetylcholine release in a concentration of 10 μM.

To evaluate the possible role of PDE1, 3 or 4, we investigated the influence of the inhibitors vinpocetine (PDE1), cilostamide (PDE3) and rolipram (PDE4) (Alexander et al., 2009). None of these PDE-inhibitors per se mimicked the near abolition of electrically induced contractions seen with IBMX, as vinpocetine and rolipram were without significant effect, while cilostamide inhibited the contractions maximally by 32%. As IBMX also inhibits the cGMP specific PDE5 and this enzyme can be present in gastrointestinal muscle, inhibition of PDE5 by IBMX might contribute to its pronounced inhibitory influence on electrically induced contractions; but the PDE5-selective inhibitor zaprinast (0.01–10 μM) did not influence the cholinergic contractions (results not shown). When cilostamide and rolipram were added together, near full inhibition of the electrically induced contractions was obtained, suggesting a redundant role of PDE3 and PDE4 in the control of the cyclic nucleotide levels in pig gastric circular muscle with PDE3 being predominant as cilostamide alone had some effect. The PDEs involved in the control of cyclic nucleotide levels and thus the contractile degree of gastrointestinal smooth muscle differs between species and regions of the gastrointestinal tract (see Barnette et al., 1990, 1993; Tomkinson and Raeburn, 1996). The role of both PDE3 and PDE4 in pig gastric circular muscle resembles the joint role of these 2 PDE subtypes in the control of canine and human respiratory smooth muscle (Torphy et al., 1991; Schmidt et al., 2000) and of the 5-HT_4 receptor-mediated inotropic response to 5-HT in porcine atrium (Galindo-Tovar et al., 2009).

When studying the influence of vinpocetine, cilostamide and rolipram versus the effect of prucalopride on acetylcholine release, only rolipram facilitated prucalopride. In a concentration of 1 μM, which is able to inhibit all PDE4 isozymes (Wang et al., 1997), it potentiated the effect of 0.01 μM prucalopride on acetylcholine release to the same extent as IBMX, suggesting that PDE4 is the sole PDE subtype controlling the response to 5-HT_4 receptor activation in the cholinergic neurons of pig gastric circular muscle. As rolipram per se did not reduce electrically induced cholinergic contractions in porcine gastric muscle, but enhanced the facilitating effect of prucalopride on acetylcholine release, it was expected to also enhance the facilitating effect of prucalopride on electrically induced cholinergic contractions in the functional assay. When rolipram was added before prucalopride, a tendency to enhanced facilitation of the cholinergic contractions by the 3 concentrations of prucalopride (0.01–01 μM) was observed; this enhancement was also visible when rolipram was added after prucalopride. Surprisingly, in this series, rolipram per se enhanced cholinergic contractions mildly to moderately in some tissues. This might be related to a borderline role of PDE4 in the control of acetylcholine release per se from cholinergic nerves in pig gastric circular muscle. In equine trachea, IBMX per se in high concentrations enhanced acetylcholine release from cholinergic nerves induced by electrical field stimulation (Zhang et al., 1996). Although we did not observe a significant influence of IBMX and rolipram on electrically induced acetylcholine release, a tendency to enhancement of the S2/S1 ratio was seen (Figs. 7B and 8).

In conclusion, our results show that 5-HT_4 receptors are present on the cholinergic nerves towards the pig gastric circular muscle and their stimulation facilitates acetylcholine release. Although stimulation leads to a maintained response, the intracellular pathway of facilitation is under the influence of PDE4 illustrating that also in peripheral neurons PDEs can be involved in the regulation of neurotransmitter release. Combination of 5-HT_4 receptor activation with acetylcholinesterase inhibition has been shown to have synergistic gastrointestinal prokinetic effects and this combination was proposed as a possible therapeutic approach for conditions with slow gastrointestinal transit (Cellek et al., 2008; Campbell-Dittmeyer et al., 2009). Another way to increase the gastroprokinetic effect of 5-HT_4 receptor activation might be to combine a 5-HT_4 receptor agonist with selective inhibition of the PDE regulating the transmission of the 5-HT_4 receptors in peripheral cholinergic neurons. The actual study illustrates that in pig gastric circular muscle, this effect can be obtained with a PDE4-selective inhibitor. Extrapolation to humans will of course require to investigate the PDE subtypes active in cholinergic neurons versus those in smooth muscle cells in gastric tissue as well as colonic tissue, another human target tissue of 5-HT_4 receptor agonists. If confirmed that PDE4 is regulating the intracellular pathway of 5-HT_4 receptors on cholinergic nerves in human gastric and colonic tissue, one could combine PDE4-inhibition with 5-HT_4 receptor agonists to enhance their gastrointestinal kinetic effect without influencing their cardiac effects, as in human heart, only PDE3 induces fade of 5-HT_4 receptor-mediated responses (Galindo-Tovar et al., 2009).

Conflict of interest

J.H. De Maeyer is employed by Shire-Movetis NV (prucalopride belongs to the portfolio of Shire-Movetis NV). R.A. Lefebvre is scientifically involved in a Research and Development Project of Shire-Movetis NV on extra-gastrointestinal effects of 5-HT_4 receptor agonists, financially supported by IWT (Agency for Innovation by Science and technology).

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