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Tissue dependent differences in G-protein coupled receptor kinases associated with 5-HT_4 receptor desensitization in the rat gastro-intestinal tract☆

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ABSTRACT

Desensitization of 5-HT_4 receptors is regulated by G-protein coupled receptor kinases (GRKs). However, the specific GRK(s) that regulates the desensitization of 5-HT_4 receptors in the in vivo setting is unknown. We investigated the in situ expression of 5-HT_4 receptors and the GRKs in the rat gastrointestinal tract using immunohistochemistry and their interaction using coimmunoprecipitation. 5-HT_4 receptors were expressed in the tunica muscularis mucosae of the oesophagus, longitudinal muscle, myenteric plexus, circular muscle, submucosal plexus and muscularis mucosae of both the proximal and distal colon. GRK2 was expressed in longitudinal muscle and occasionally in myenteric plexus whilst GRK5 showed limited expression in the nerve endings of the myenteric plexus and submucosal plexus of the colon. GRK3 was expressed in the tunica muscularis mucosae of the oesophagus, circular muscle, submucosal plexus and muscularis mucosae of the colon. GRK6 was expressed in the tunica muscularis mucosae of the oesophagus, longitudinal muscle, circular muscle, and muscularis mucosae of the colon. Stimulation of tunica muscularis mucosae of the oesophagus and distal colon using the 5-HT_4 receptor agonist, tegaserod, followed by analysis of the 5-HT_4 receptor antibody immunoprecipitate, revealed the coimmunoprecipitation of GRK6 with 5-HT_4 receptors in the tunica muscularis mucosae of oesophagus while GRK2 and GRK6 were coimmunoprecipitated with 5-HT_4 receptors in the distal colon. This study indicates that GRK6 may be involved in the regulation of the desensitization of 5-HT_4 receptors in the rat oesophagus whilst GRK2 and GRK6 may be involved in regulation of the desensitization of 5-HT_4 receptors in the distal colon.

1. Introduction

5-Hydroxytryptamine (5-HT) produces its physiological effects through the activation of several different receptor subtypes. The receptors are classified into seven distinct classes. These are 5-HT_1 (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1e}, 5-HT_{1F},), 5-HT_2 (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}), 5-HT_3, 5-HT_4, 5-ht_5, 5-HT_6 and 5-HT_7. With the exception of the 5-HT_3 receptor, which is a ligand gated ion channel receptor, all 5-HT receptors are G-protein coupled receptors (GPCRs). Several 5-HT_4 receptor splice variants have been cloned and characterized from different species including human. There are four C-terminal splice variants in rat (a, b, e, c1) [1–3], four in mouse (a, b, e, f) [4,5] and ten C-terminal (a, b, c, long c, d, e, f, g, i, n) and one internal (h) splice variants in human [6]. 5-HT_4 receptors mainly signal through the G-protein dependent Gs/cAMP/PKA pathway and also via the G-protein independent Src/ERK pathway [7]. 5-HT_4 receptors mediate many cellular functions, both in the central nervous system and at the periphery. In the gastrointestinal tract they are expressed in enteric neurons, interstitial cells of Cajal and smooth muscle cells [3,8,9]. Activation of 5-HT_4 receptors results in the release of neurotransmitters such as acetylcholine, from enteric neurons [10,11], which initiate the peristaltic reflex, facilitate propulsive activity in the colon and induce chloride secretion, increasing the amount of fluid in the lumen [12,13]. Functions such as modulation of intestinal secretion, stimulation of gastrointestinal motility, and modulation of muscle state (direct smooth muscle relaxation and/or inhibition of contractility, and

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T Nedi, HR Irving, IM Coupar and PJ White; GRK6 regulates the desensitization of 5-HT_4 receptors in the rat oesophagus. 16th World Congress of Basic and Clinical Pharmacology, Worldpharma 2010 July 17–23, 2010, Copenhagen, Denmark.

Abbreviations: ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCRs, G-protein coupled receptors; GRKs, G-protein coupled receptor kinases; 5-HT, 5-hydroxytryptamine; IPANs, intrinsic primary afferent neurones; PAGE, polyacrylamide gel; PBS, phosphate buffered saline; PBST, phosphate buffered saline and tween 20; RT-PCR, reverse transcription polymerase chain reaction.

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indirect neuron-mediated contraction) can all be attributed to stimulation of 5-HT_4 receptors [14,15]. Irritable bowel syndrome (IBS) is a common disorder where the behaviour of the large intestine (colon) changes with the result that patients typically experience symptoms of abdominal pain, discomfort, sensations of bloating and altered bowel habit [16]. The 5-HT_4 receptor agonist tegaserod was used for treatment of constipation predominant IBS until withdrawn in 2007 as it was associated with rare adverse cardiovascular effects [15,17]. Tegaserod has a relatively low therapeutic gain of 5–12% above placebo and is relatively poorly absorbed with about two-thirds of oral doses being eliminated in the faeces [18]. Tegaserod has been shown to desensitize the peristaltic reflex mediated by 5-HT release following mucosal stimulation in rats and it was suggested that high concentrations of tegaserod in the colonic lumen could blunt the peristaltic reflex [19]. We have previously demonstrated that chronic infusion of rats with 5-HT (5–10 days) results in residual desensitization of oesphageal 5-HT_4 receptors [20].

GPCRs can undergo two types of desensitization that have been shown to depend on different types of protein kinases. The nonspecific, or heterologous, desensitization is initiated by second messenger dependent protein kinases, such as protein kinase A or C [21]. On the other hand, the specific, or homologous mode of desensitization is triggered by GRKs. These serine/threonine kinases have the unique property of recognizing only agonist-occupied and activated receptors. Desensitization generally begins with agonist-induced phosphorylation of the GPCR by GRKs, then arrestins bind to the phosphorylated GPCRs. The arrestin-GPCR complex is internalized by endocytosis [22]. There are seven GRKs in humans, named GRK1-7, which are coded by seven different genes and classified into three sub-groups (visual GRKs: EC2.7.11.14; GRK2/3 family: EC2.7.11.15; and GRK4-6 family: EC2.7.11.16). Expression of GRK1 and GRK7 is confined to retinal rods and cones, respectively. By contrast, GRK2, GRK3, GRK5 and GRK6 are widely expressed in mammalian tissues, whereas GRK4 has very limited expression in the cerebellum, testis and kidney. Thus, most receptors in the body are thought to be regulated by one or more of the four widely expressed GRKs [21,23].

5-HT induces desensitization of its receptors at different rates depending on the receptor. Homologous desensitization has been reported for 5-HT_4 receptors in mouse colliculi neurons and rat oesophagus and colon [19,24,25] but the actual GRKs involved were not identified. Cell culture studies using co-expression of GRKs with different 5-HT_4 receptor splice variants showed a reduction in cAMP formation via GRK2, implicating it in the regulation of G-protein dependent Gs/cAMP/PKA signalling pathway [26]. More recently GRK5 has been shown to regulate the desensitization of the G-protein independent Src/ERK signalling pathway of 5-HT_4 receptors in heterologous cell systems [27]. However, there is no supporting evidence for the involvement of GRK2 and GRK5 in the regulation of the desensitization of 5-HT_4 receptors in the tissue. For 5-HT_4 receptors to be regulated by GRK2 and GRK5, it requires co-expression of the receptor with the regulatory protein in the same cells in the tissue. Therefore the aim of this study was to determine the GRK(s) that is co-expressed and interacts with the 5-HT_4 receptors in the rat oesophagus and colon.

2. Materials and methods

2.1. Animals

All animal experiments and procedures conformed to Australian National Health and Medical Research Council's guidelines and were approved by the Victorian College of Pharmacy Animal Ethics Committee of Monash University.

2.2. cDNA synthesis and PCR amplification

Six adult male Sprague–Dawley rats weighing 485–575 g were killed by carbon dioxide asphyxiation. The oesophagus, jejunum, ileum and colon tissues were rapidly removed, and the tissues were immediately placed in tubes containing RNA later® solution (Ambion/Applied Biosystems, Melbourne, Australia) and stored at −20 °C. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Melbourne, Australia) according to the manufacturer's protocol. The concentration of RNA was determined using a Qubit™ fluorometer (Invitrogen, Melbourne, Australia). The reverse transcription (RT) reaction was carried out as follows: 1 μg of total RNA, 5 μM oligo-$(dT)_{15}$ primer, 0.5 mM each dNTP, 4 μl of (5×) first strand buffer, 5 mM dithiothreitol and 10 units Superscript™ III reverse transcriptase (Invitrogen) in a total volume of 20 μl in each reaction mixture. In order to determine if there was any DNA contamination, control reactions (-RT reaction) with the entire components except Superscript™ III reverse transcriptase (Invitrogen) were also carried out. The reaction components were mixed thoroughly by pipetting and were incubated at 50 °C for 59 min to synthesize the first strand cDNA in FTS-960 DNA Thermal sequencer (Corbett Research, Sydney, Australia). To assess the quality of cDNA produced, positive control experiments were undertaken to amplify cDNA for the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). No GAPDH reverse transcriptase polymerase chain reaction (RT-PCR) products were detected in the negative reverse transcriptase control reactions. Specific cDNA for the 5-HT_4 receptor splice variants was detected using a partial nested PCR amplification. Forward outer and inner primers common to all three splice variants were designed. Also PCR primers specific for the rat GRK2, GRK3, GRK5 and GRK6 were designed using the Primer3 program (http://frodo.wi.met.edu) and were synthesised by Micromon at the Department of Microbiology, Monash University. All primers are listed in Table 1. For all samples a standard PCR mixture contained 2.5 μl of (10×) PCR buffer, 1 μl cDNA (or negative RT reaction) template, 0.1 μM primers, 200 μM dNTPs, 4 μl of Q solution and 0.5 U Taq DNA polymerase (Qiagen) per 25 μl reaction mixture. Tubes were denatured at 94 °C for five min and then 35 cycles of amplification were performed (60 s denaturation at 94 °C, 50 s annealing at 60 °C for GAPDH, 60 s annealing at 55 °C for 5-HT_4 splice variants and GRKs and 60 s extension at 72 °C) with a final extension at 72 °C for eight min in FTS-960 DNA Thermal sequencer. Amplification products were separated by electrophoresis on 1.8% agarose gels and visualized by ethidium bromide staining. The identity of the PCR amplification products was confirmed by DNA sequencing. The PCR products were cleaned using UltraClean™ PCR clean up kit according to the manufacturer's protocol (MoBio Laboratories, Inc. Australia) and the samples together with forward primers were submitted for automated sequencing by Micromon (Monash University).

2.3. Primary antibodies

All GRK antibodies used in this study have been characterised by a number of other groups and used in different studies. Immunoblot analysis of oesophagus and colon tissue lysate using anti-GRK2 antibody revealed a strong immunoreactive protein of around 80 kDa as has been reported previously [28–30]. We also observed immunoreactive protein of the expected sizes for GRK3, GRK5 and GRK6 (Fig. 1). Similarly a previous characterisation study of anti-GRK3, anti-GRK5 and anti-GRK6 antibodies using recombinant purified rat GRK proteins revealed that the anti-GRK5 antibody was selective essentially labelling one band on the blot [31]. However, the anti-GRK3 antibody labels both GRK2 and GRK3 with much higher sensitivity to GRK3 than GRK2 [31–33]. The anti-

Table 1
List of primers used for PCR.

Gene	GenBank accession no.	Primers	Product size (bp)
GAPDH	AF106860	Forward 5′-CTCACTGGCATGGCCTTCCG-3′ Reverse 5′-ACCACCCTGTTGCTGTAGCC-3′	292
5-HT_4	U20906	Common Outer forward: 5′-TCTTTCAGACGTGCCTTCCT-3′ Inner forward 5′-ACTGTCCCCTGTTCAACCAC-3′	
5-$HT_{4(a)}$	U20906	Reverse 5′-AGGGACTCTGGGTCATTGTG-3′	122
5-$HT_{4(b)}$	U20907	Reverse 5′-GTGACACCGACTCTCCCATT-3′	93
5-$HT_{4(e)}$	AJ011370	Reverse 5′-AGGGACTCTGGGTCATTGTG-3′	185
GRK2	M87854	Forward 5′-ATGCATGGCTACATGTCCAA-3′ Reverse 5′-TGTGTCTCTTCCACCGACTG-3′	158
GRK3	M87855	Forward 5′-TGACCTATGCCTTCCACACA-3′ Reverse 5′-GCCAAGATCCGATATCCTCA-3′	242
GRK5	NM_030829	Forward 5′-TGGCACTCAATGAAAAGCAG-3′ Reverse 5′-ACAAGGCTCGTTCTTCCTCA-3′	180
GRK6	NM_031657	Forward 5′-AGCCAGTGACCAAAAACACC-3′ Reverse 5′-TCCAGGATCTGCTTCTCGTT-3′	181

GRK6 antibody labels both GRK5 and GRK6 proteins [31,33,34]. Due to the cross-reactivity of GRK3 and GRK6 antibodies we further confirmed the expression of the protein using western blot and mRNA by RT-PCR. The difference in the in situ expression and coimmunoprecipitation that has been observed in our study mainly depends on the selectivity of anti-GRK2 and anti-GRK5 antibodies. The expression of GRK3 is reported for histological layers that do not have GRK2 immunoreactivity and the expression of GRK6 is also reported for histological layers that do not have GRK5 immunoreactivity (see Fig. 2). In western blot analysis, we observed a major immunoreactive band of around 42 kDa and a second faint band of around 85 kDa which is possibly a receptor dimer as previously observed [9] for the 5-HT_4 receptor antibody.

2.4. Western blots

Six male Sprague–Dawley rats weighing 240–260 g were killed by carbon dioxide asphyxiation. The animals were quickly dissected and the lower third of the oesophagus proximal to the diaphragm, and the proximal and distal colon were excised. For the oesophagus tissue the outer muscularis externa was separated from the inner tunica muscularis mucosae. The tissues were washed three times with ice-cold phosphate buffered saline (PBS). Tissues were homogenized in 1:10 (w/v) of homogenization buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, complete protease inhibitor cocktail tablet) (Roche, Sydney, Australia) for 1 min. Debris was removed from the

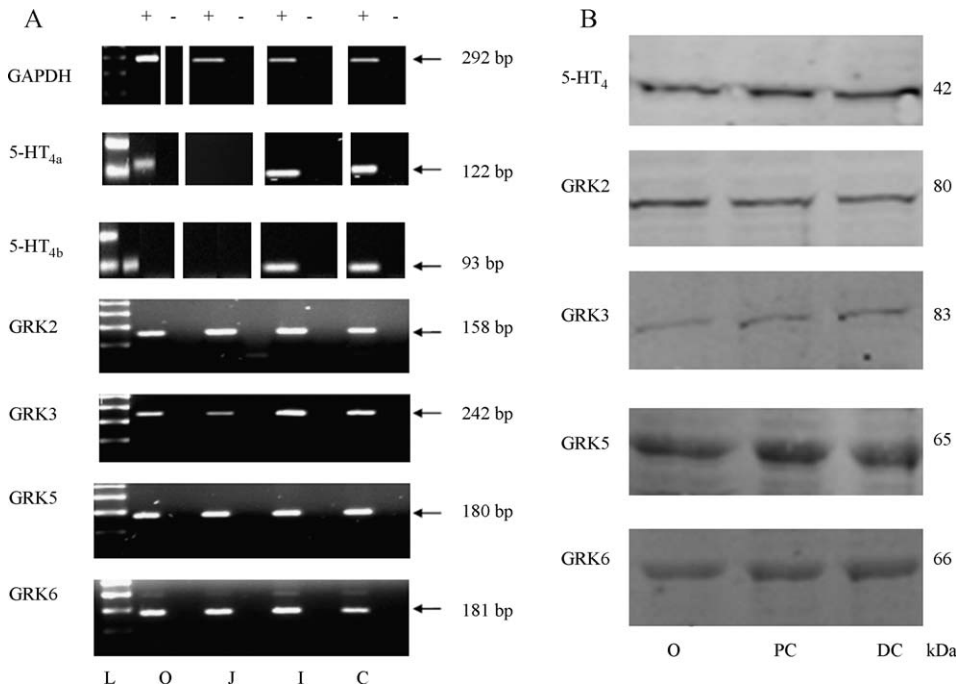

Fig. 1. Expression of 5-HT_4 receptors and GRKs in rat gastrointestinal tissue. (A) Examples of the RT-PCR analysis of GAPDH, 5-HT_4 receptor splice variants, and GRKs expressed in the rat digestive tract. The labelling is as follows: L: 100 bp ladder; O: oesophagus; J: jejunum; I: ileum and C: colon. Control amplification of the GAPDH gene (292 bp) from RNA samples with (plus) or without (minus) reverse transcriptase to monitor DNA contamination. Partial nested RT-PCR amplification of the 5-HT_4 receptor splice variants performed to amplify cDNA fragments of 122 bp, 93 bp and 185 bp representing isoforms a, b and e, respectively. RT-PCR amplification performed to amplify cDNA fragments of 158 bp, 242 bp, 180 bp and 181 bp representing GRK2, GRK3, GRK5 and GRK6, respectively. (B) Western blot analysis of the expression of 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 in the rat tunica muscularis mucosae of the oesophagus, proximal and distal colon. The labelling is as follows: O: oesophagus; PC: proximal colon and DC: distal colon.

homogenized suspensions by centrifugation at 12 000 × g for 10 min. Protein concentrations were determined using a Quant-It protein assay (Invitrogen) with the Qubit™ fluorometer and homogenates were stored at −80 °C until used.

Total protein (50 μg) was separated on 12% polyacrylamide gel (PAGE) (at 200 V for 1 h) and electrophoretically transferred (at 100 V for 1 h) to nitrocellulose membranes (Amersham Bioscience, Sydney, Australia). Nonspecific binding to the membrane was prevented by blocking with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, Nebraska USA) for 1 h. Membranes were probed with primary antibodies: anti-5-HT_4 receptor, goat polyclonal; anti-GRK2, mouse monoclonal; anti-GRK3, rabbit polyclonal; anti-GRK5, rabbit polyclonal; and anti-GRK6, rabbit polyclonal (Table 2) overnight at 4 °C and washed four times with PBS and 0.1% Tween 20 PBS (PBST). Membranes were incubated with fluorophore-conjugated secondary antibodies: donkey-anti-mouse IRDye 800CW (1:10 000 for GRK2), goat anti-rabbit IRDye 800CW (1:10 000 for GRK3, GRK5 and GRK6) and donkey-anti-goat IRDye 680 (1:10 000 for 5-HT_4 receptor) (LI-COR Biosciences) for 1 h at room temperature and were washed four times for five min using PBST. An Odyssey Infrared Imaging System (LI-COR Biosciences) was used to examine the immunoblots.

2.5. Immunohistochemistry

Three male Sprague–Dawley rats weighing 240–260 g were killed by carbon dioxide asphyxiation. The animals were quickly dissected and the lower third of oesophagus proximal to the diaphragm, and the proximal and distal colon were excised. The tissues were rinsed thoroughly with PBS pH 7.4 and immersion-fixed in 4% formaldehyde in PBS for 6 h at 4 °C. Fixed tissues were cleared of fixative with three 10 min washes in dimethylsulphoxide followed by three 10 min washes in PBS and placed in PBS-sucrose-azide (PBS containing 0.1% sodium azide and 30% sucrose as a cryoprotectant) for 36 h at 4 °C. Then the tissues were cut into small pieces and embedded in Tissue Tek and frozen in liquid nitrogen using isopentane and stored in −80 °C until used. Cryostat sections (10 μm) were cut, collected on 0.1% gelatinized slides and left for 1 h to dry at room temperature. Preparations were incubated in an antibody diluent solution containing 10% normal horse serum, 1% bovine serum albumin and 1% Triton X-100 in PBS for 30 min at room temperature and followed by overnight incubation at 4 °C with primary antibodies: anti-5-HT_4 receptor, rabbit polyclonal; anti-GRK2, mouse monoclonal; anti-GRK3, rabbit polyclonal; anti-GRK5, rabbit polyclonal; and anti-GRK6, rabbit polyclonal (Table 2). Combinations of rabbit anti-5-HT_4 receptor and mouse anti-GRK2 antibodies were used to study co-expression. Our immunohistochemistry protocol was optimised for rabbit anti-GRK3, anti-GRK5 and anti-GRK6 antibodies and the goat polyclonal anti-5-HT_4 receptor antibody did not work under these conditions. As a result we were not able to study the co-localisation of 5-HT_4 receptors with GRK3, GRK5 and GRK6. Tissue sections were washed three times for five min using PBS to remove unbound primary antibody and incubated with fluorophore-conjugated secondary antibodies: goat anti-rabbit Alexa 488 (Invitrogen, 1:500 for 5-HT_4 receptor); horse anti-mouse IgG Texas Red (Vector Laboratories, Burlingame, California USA, 1:400 for GRK2); and goat anti-rabbit IgG Fluorescein Isothiocyanate (FITC) (Vector Laboratories, 1:400 for GRK3, GRK5 and GRK6) for 1 h at room temperature. Preparations were washed three times for five min using PBS and then mounted using Vectashield mounting medium (Vector Laboratories) and incubated overnight at 4 °C. Images were taken using a conventional fluorescence microscope Olympus BX-61 (Olympus Australia, Melbourne Australia).

2.6. Concentration-effects of 5-HT and tegaserod on the rat oesophagus

Sprague–Dawley rats weighing 240–260 g were killed by carbon dioxide asphyxiation. The animals were quickly dissected and the lower third of the oesophagus proximal to the diaphragm was excised. The outer muscularis externa which is composed of longitudinal and circular muscle was separated from the inner tunica muscularis mucosae. The remaining tunica muscular mucosae (2 cm in length) was split into two by cutting longitudinally. Both mucosal preparations were mounted separately in 10 ml organ baths containing Krebs–Henseleit physiological solution which was composed of (in mM): 118.4 NaCl; 4.69 KCl; 2.5 $CaCl_2$; 1.2 $MgSO_4$; 25.0 $NaHCO_3$; 1.2 KH_2PO_4 and 11.0 D (±)-glucose, and aerated continuously with 95% O_2 and 5% CO_2 mixture and maintained at 37 °C. The tissue was maintained under a tension of 1 g and allowed to equilibrate for 1 h. Tension was recorded using an isometric transducer connected to a Power Lab/4sp (AD Instruments Pty Ltd, Castle Hill, Australia). Throughout the experiments, Krebs solution containing 3 μM indomethacin to inhibit prostaglandin synthesis and 1 μM ketanserin to block 5-HT_2 receptors, was added with every 15 min wash during the equilibration period. 30 μM cocaine was found to have no effect on the concentration effect curve of 5-HT and as a result it was not included in the desensitization study.

At 3 μM, carbachol was determined to induce submaximal contraction of the tunica muscularis mucosae. When the contraction stabilized, a cumulative concentration of 5-HT (1 nM–10 μM in half log increments) was added every 2 min and the relaxation recorded. The response to the cumulative addition of 5-HT was expressed as the percentage maximal 5-HT induced relaxation and the control concentration effect curve constructed. Upon completion of the recording, the tissue was washed with fresh Kreb's four times every five min followed by another four washes every ten min for a total duration of 1 h before the second concentration effect curve for 5-HT and tegaserod was constructed (test period). The relaxant effect was expressed as percentage of maximal 5-HT induced relaxation.

2.7. Desensitization of 5-HT_4 receptors

The desensitization study was performed as described previously by Rondé et al., [25]. A control cumulative concentration effect curve to 5-HT was constructed using the precontracted tunica

Table 2
Primary antibodies used for immunohistochemistry (Immuno) and western blot (WB).

Antigen	Host species	Dilution		Sources	References
		Immuno	WB		
5-HT_4 receptor	Goat		1:500	Santa Cruz; sc-32564	
5-HT_4 receptor	Rabbit	1:500		Novus Biological; NLS656	
GRK2	Mouse	1:100	1:500	Santa Cruz; sc-13143	[28–30]
GRK3	Rabbit	1:100	1:300	Santa Cruz; sc-563	[31–33]
GRK5	Rabbit	1:100	1:500	Santa Cruz; sc-565	[31–33]
GRK6	Rabbit	1:100	1:300	Santa Cruz; sc-566	[31,33,34]

muscularis mucosae of the rat oesophagus and washed as described above (control period). Before constructing the second concentration effect curve the tissue was incubated with the 5-HT_4 receptor agonist tegaserod (10 μM) for different desensitization periods (5, 10 and 20 min). The tissue was washed four times every min and a test cumulative concentration effect curve to 5-HT was constructed.

2.8. Coimmunoprecipitation and western blot analysis

Sprague–Dawley rats weighing 240–260 g were killed by carbon dioxide asphyxiation. The animals were quickly dissected and the lower third of oesophagus proximal to the diaphragm and the distal colon were excised. For the oesophagus tissue the outer muscularis externa was separated from the inner tunica muscularis mucosae. The distal colon was cut along the mesenteric border and then parallel to the long axis of the circular muscle to obtain strip preparations. The tunica muscularis mucosae and the distal colon preparations were mounted in 10 ml organ baths containing Krebs–Henseleit physiological solution which was aerated continuously with 95% O_2 and 5% CO_2 mixture and maintained at 37 °C. The tissues were allowed to equilibrate for 1 h with every 15 min wash with fresh Kreb's solution and incubated with and without 10 μM of tegaserod for 5 min. The incubation was terminated with four washes every min with Kreb's solution and followed by another three washes every min with ice-cold PBS. In order to prepare total tissue lysate, 70–100 mg of the tunica muscularis mucosae and distal colon tissues were homogenized and cleared as described above. Protein concentrations were determined and homogenates were stored at −80 °C until used.

Tunica muscularis mucosae and distal colon tissue homogenates (3.3% of the immunoprecipitation fraction) were analysed by western blot analysis for the presence of 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 proteins as described above. As the immunoprecipitation required more antibody than was feasible with anti-5-HT_4 receptor rabbit polyclonal antibody, we used anti-5-HT_4 receptor goat polyclonal antibody. Total protein (3 mg per sample) was precleared with 50 μl protein A agarose suspension (Roche) overnight and incubated with 5 μg of affinity purified anti-5-HT_4 receptor, goat polyclonal antibody by rocking gently for 8 h at 4 °C. Protein A agarose suspension (50 μl) was added and incubated overnight at 4 °C by rocking gently. The agarose beads were collected by centrifugation (20 s, 12 000 × *g*) and washed twice with 1 ml of PBS. After discarding the final wash, 25 μl of SDS-PAGE sample buffer was added and heated for 3 min at 100 °C. The supernatant was collected by centrifugation at 12 000 × *g* for 20 s. Equal volumes (12 μl) of anti-5-HT_4 receptor immune complex were analysed by western blot analysis for the presence of 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 proteins as described above.

2.9. Data analysis

Data for each treatment group was expressed as mean ± SEM. Concentration-effect curves were fitted with sigmoidal dose response curve using GraphPad Prism 5 (GraphPad Software, La Jolla, California USA). Differences in the E_{max} due to exposure of the tissue for different desensitization period were determined using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison post hoc test. A P value < 0.05 was considered to be statistically significant.

2.10. Drugs

5-Hydroxytryptamine creatinine sulfate monohydrate, carbachol, ketanserin tartarate, cocaine hydrochloride were all from Sigma (Sydney, Australia) and dissolved in distilled water. Tegaserod maleate (Sequoia Research Products Ltd, Berkshire, UK) was dissolved in dimethylsulfoxide (DMSO) while indomethacin (Sigma) was dissolved in 0.5% (w/v) sodium bicarbonate. All drugs were prepared at 10 mM stock concentration and then serially diluted in water. Tegaserod was prepared at 10 mM stock concentration in DMSO and then serially diluted in water. The final DMSO concentration in the test did not exceed 0.1% (v/v).

3. Results

3.1. Expression of 5-HT_4 receptors and GRKs

3.1.1. RT-PCR and western blot

Partial nested RT-PCR experiments revealed variations in the distribution of the 5-HT_4 receptor splice variants in the rat gastrointestinal tract (Fig. 1A). Two PCR products of the expected size of 122 bp and 93 bp representing 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ receptor splice variants, respectively were detected in oesophagus, ileum and colon tissues. The 5-$HT_{4(e)}$ splice variant was not detected in any tissue. No expression of any of the 5-HT_4 splice variants was observed in the jejunum ($n = 6$). Sequence analysis of PCR products confirmed the products as 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ receptor splice variants. To confirm the validity of our partial nested PCR reactions, cDNA from the hippocampus tissue was amplified as it has been reported to contain the 5-$HT_{4(e)}$ receptor splice variant [35]. A band of the expected size for the 5-$HT_{4(e)}$ receptor splice variant (185 bp) was detected (data not shown). RT-PCR performed with the RNA extracted from the different regions of the rat gastrointestinal tract revealed a wide distribution of GRK2, GRK3, GRK5 and GRK6. All the GRKs were detected and confirmed by sequence analysis in oesophagus, jejunum, ileum and colon tissues. The immunoblot analysis of the total tissue lysate of oesophagus, proximal and distal colon confirmed the expression of proteins of sizes corresponding to 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 (Fig. 1B).

3.1.2. Immunohistochemistry

The tunica muscularis mucosae of the oesophagus is a longitudinal smooth muscle separated from the stratified squamous epithelium by the lamina porpria. Obtained from the rat, it is a standard tissue for studying 5-HT_4 receptor mediated relaxation by 5-HT_4 agonists [36,37]. Immunohistochemical analysis showed 5-HT_4 receptor immunoreactivity on the tunica muscularis mucosae of the oesophagus. Immunoreactive cells were also found for GRK3 and GRK6, while there were no immunoreactive cells for GRK2 and GRK5 (Fig. 2).

The colon was divided into proximal and distal regions to determine whether a similar pattern of expression at the different histological layers occurs. In the proximal colon (Fig. 3), 5-HT_4 receptor immunoreactivity was observed in longitudinal muscle, myenteric plexus, circular muscle, submucosal plexus and muscularis mucosae while GRK2 immunoreactivity was mainly restricted to longitudinal muscle and occasionally on the myenteric plexus. The apparent immunoreactive labelling of GRK2 seen on the circular muscle is due to a high background of the colon tissue under the red channel. 5-HT_4 receptors were detected in the same tissue regions as GRK2 on the longitudinal muscle and occasionally in the myenteric plexus. GRK3 was expressed in circular muscle, submucosal plexus and muscularis mucosae. However as discussed in Section 2.3, the immunoreactivity observed on the longitudinal muscle and myenteric plexus may be non-specific binding to GRK2. The immunoreactivity of GRK5 was restricted to the nerve endings of both myenteric plexus and submucosal plexus. The immunoreactivity was predominantly observed in the cell bodies of nerves in ganglia. GRK6 was expressed in the longitudinal muscle, circular muscle, and

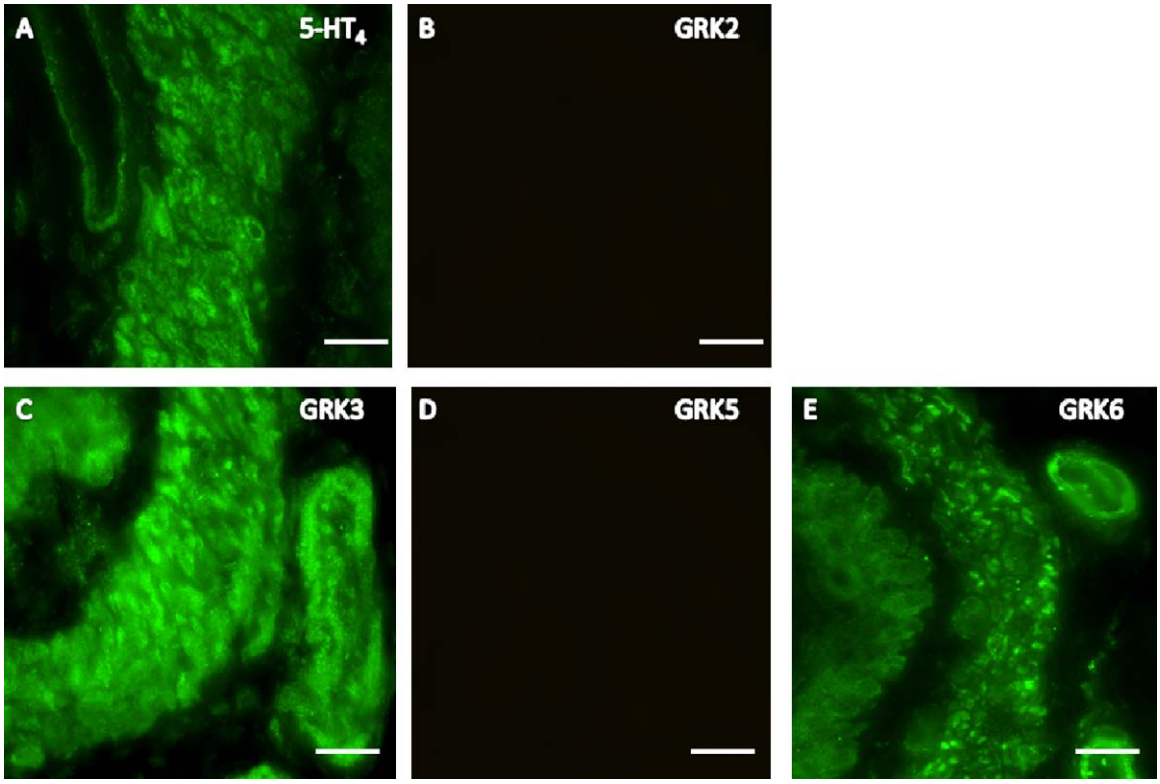

Fig. 2. Localization of 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 immunoreactivity in the tunica muscularis mucosae of the rat oesophagus. (A, C and E) Representative immunoreactivity of 5-HT_4 receptors, GRK3 and GRK6, respectively. (B and D) No immunoreactive cells were detected for GRK2 and GRK5. Scale bars are 20 μm.

muscularis mucosae. However the immunoreactivity observed at myenteric and submucosal plexus may be non-specific binding to GRK5. In the distal colon (Fig. 3), immunoreactivity to 5-HT_4 receptors was similar to that of the proximal colon. GRK2 immunoreactivity was observed on the longitudinal muscle and occasionally on the cell bodies of myenteric plexus where immunoreactivity to 5-HT_4 receptors was also present. The immunoreactivity of GRK3, GRK5 and GRK6 was also similar to that observed in the proximal colon.

3.2. Homologous desensitization of 5-HT_4 receptors

Carbachol (3 μM) produced a reproducible contraction of the tissue strips during the control and test period. 5-HT and tegaserod (1 nM–10 μM) induced concentration-dependent relaxation of carbachol-precontracted tunica muscularis mucosae of the rat oesophagus upon cumulative addition (Fig. 4). The mean EC_{50} of 5-HT was 88 nM (95% confidence limits: 66–119 nM) ($n = 6$) while that of tegaserod was 608 nM (95% confidence limits: 294–1256 nM) ($n = 6$). The intrinsic activity of tegaserod was 86.5 ± 6.2% ($n = 6$) of that of the full agonist 5-HT-induced relaxation.

The E_{max} for 5-HT induced-relaxation of carbachol precontracted tunica muscularis mucosae during the test period was 100.80 ± 2.12% ($n = 6$) compared with the 5-HT-induced maximal relaxation during the control period (see Fig. 5A for protocol). As shown in Fig. 5B, the concentration-response curve of 5-HT at the control period was similar to that in test period. Pre-incubation of the tunica muscularis mucosae of the rat oesophagus with 10 μM tegaserod produced a time dependent decrease in the E_{max} of the 5-HT response during the test period (Fig. 5C). The decrease in the E_{max} was statistically significant at 20 min pre-incubation with 10 μM tegaserod (76.62 ± 5.73 ($n = 6$); $P < 0.05$ one way ANOVA followed by Dunnett's multiple comparison test). The E_{max} value is the average ($n = 6$) which is slightly different from the value estimated for the best-fit upper asymptote (Fig. 5C). There were no significant differences in the mean EC_{50} of tegaserod values (241 nM (95% confidence limits: 158–367 nM) at 0 min; 151 nM (74–307 nM) at 20 min).

3.3. Coimmunoprecipitation and western blot analysis

Coimmunoprecipitation studies were undertaken to test which GRKs were likely to be involved in the desensitization process. Since GRK association with the receptors is an early event in the desensitization process [21,38], we opted to use tissues that had been treated for 5 min. Firstly, western blot analysis was performed with the total tissue lysate of both unstimulated and stimulated (tegaserod 10 μM, 5 min) tunica muscularis mucosae of the rat oesophagus to confirm the presence of proteins of sizes corresponding to the 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 in the lysate (Fig. 6A left). The tissue lysate, confirmed to have all the above proteins, was immunoprecipitated using the anti-5-HT_4 receptor antibody. Western analysis using anti-5-HT_4 receptor antibody revealed immunoprecipitation of a band of around 42 kDa as expected for 5-HT_4 receptors. The immunoprecipitate was also analysed for coimmunoprecipitation of the GRKs and revealed the presence of GRK6 in the stimulated tissue for each of 4 animals (representative blot shown in Fig. 6A right). No bands were detected for GRK2, GRK3 and GRK5.

As the histological expression of 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 was similar for both proximal and distal colon, we used only the distal colon tissue in the coimmunoprecipitation study. The presence of proteins of sizes corresponding to 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 proteins in the lysate of both unstimulated and tegaserod (10 μM) stimulated distal colon lysate was confirmed by immunoblot analysis (Fig. 6B left). Western blot analysis of the 5-HT_4 receptor immunoprecipitate using antibodies against the different GRKs revealed the coimmunoprecipitation of both GRK2 and GRK6 in both unstimulated and

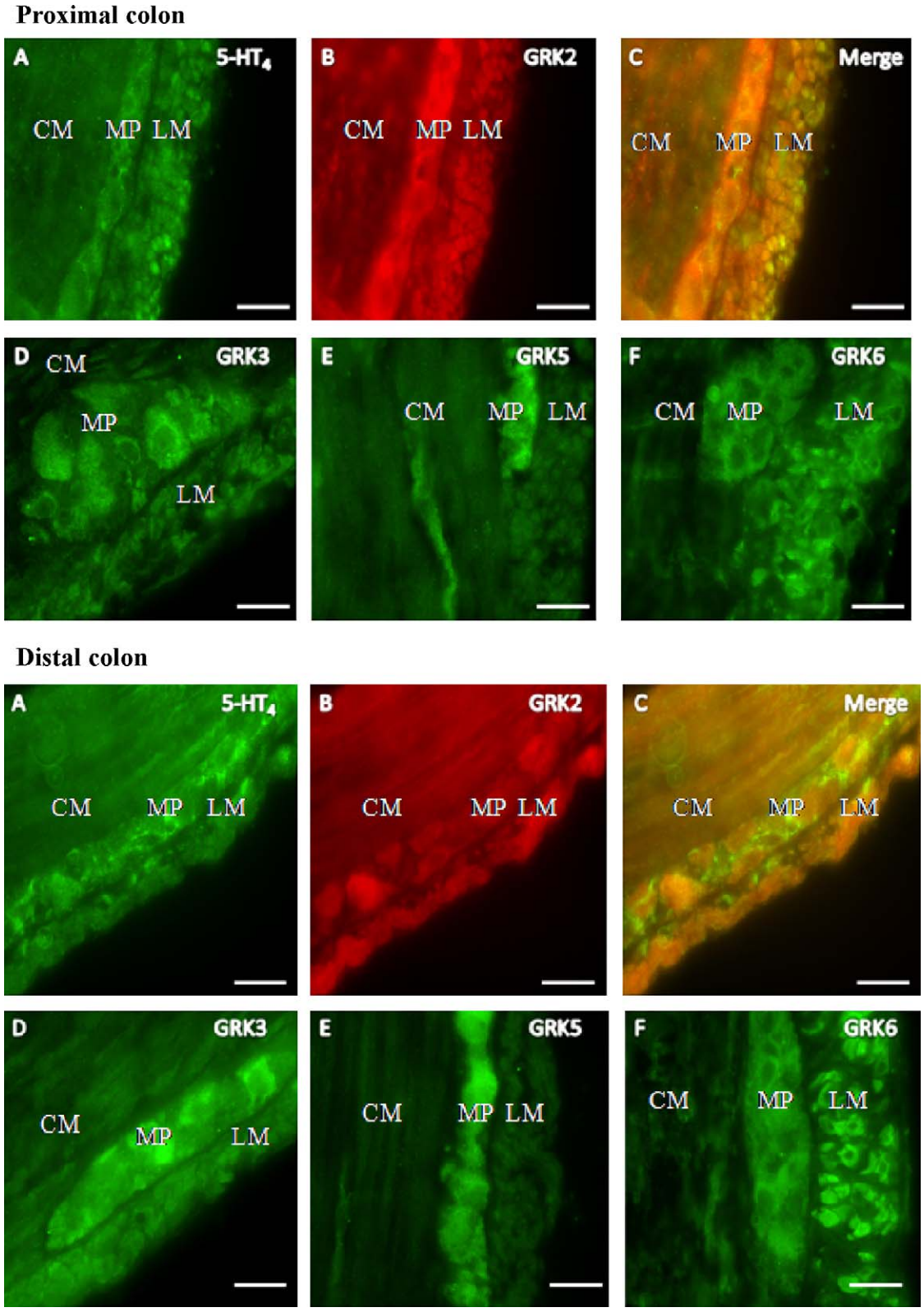

Fig. 3. Localization of 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 in the different histological layers of the rat proximal and distal colon. (A) Representative immunoreactivity of 5-HT_4 receptors in the longitudinal muscle (LM), myenteric plexus (MP) and circular muscle (CM). (B) Immunoreactivity of GRK2 in the longitudinal muscle and myenteric plexus. (C) Merged image of the co-expression of 5-HT_4 receptors and GRK2 in the longitudinal muscle and myenteric plexus. (D) Representative immunoreactivity of GRK3 in the longitudinal muscle, myenteric plexus and circular muscle. (E) Immunoreactivity of GRK5 in the myenteric plexus and submucosal plexus. (F) Representative immunoreactivity of GRK6 in the longitudinal muscle, myenteric plexus and circular muscle. Scale bars are 20 μm.

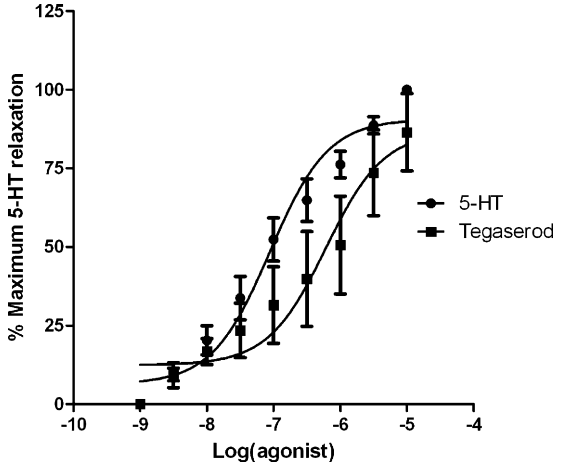

Fig. 4. Concentration-effect curves of the relaxation of tunica muscularis mucosae of the rat oesophagus by 5-HT (●) and tegaserod (■). Results are expressed as mean ± SEM ($n = 6$) percentage of maximal 5-HT-induced relaxation.

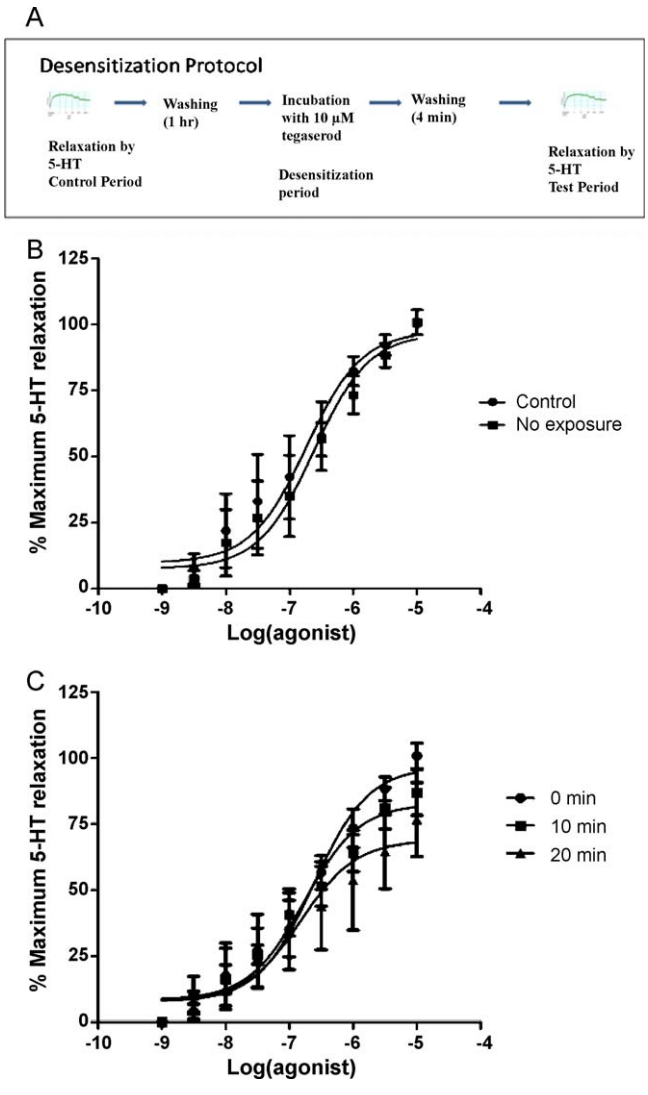

Fig. 5. (A) Desensitization protocol for 5-HT_4 receptors. (B) Reproducibility of the concentration-effect curves of 5-HT induced relaxation of tunica muscularis mucosae of the rat oesophagus at the control period (●) and at test period without exposure to tegaserod (■). C. Desensitization of 5-HT_4 receptors mediated relaxation upon exposure to 10 μM tegaserod for 10 (■) and 20 (▲) min. Results are expressed as mean ± SEM ($n = 6$) percentage of maximal 5-HT-induced relaxation.

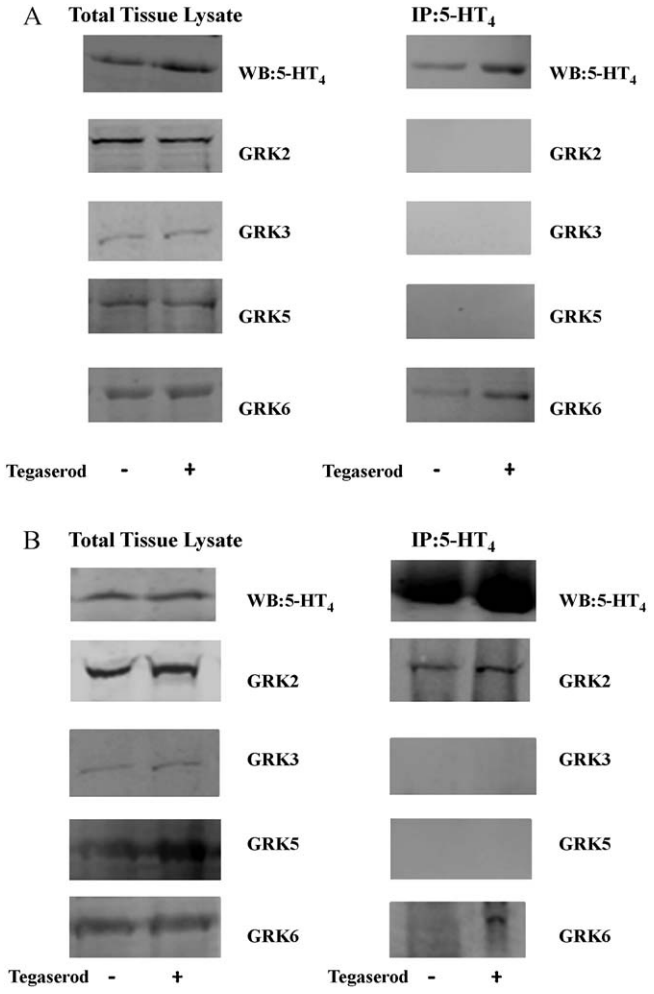

Fig. 6. Representative western analyses of coimmunoprecipitations in rat oesophagus tunica muscularis mucosae and distal colon. (A) Left, western analyses of 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 in the total tissue lysate from rat tunica muscularis mucosae without (−) and with (+) stimulation by 10 μM tegaserod (5 min). Right, Western analyses of anti-5-HT_4 receptor immunoprecipitate fraction of total tissue lysate from rat tunica muscularis mucosae for 5-HT_4 receptor, GRK2, GRK3, GRK5 and GRK6. (B) Left, western analyses of 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6 in the total tissue lysate from rat distal colon without and with stimulation by 10 μM tegaserod. Right, Western analyses of anti-5-HT_4 receptor immunoprecipitate fraction for 5-HT_4 receptor, GRK2, GRK3, GRK5 and GRK6 from the total tissue lysate of the rat distal colon.

tegaserod stimulated tissues ($n = 4$). However, GRK3 and GRK5 were not detected in the 5-HT_4 receptor immunoprecipitates (Fig. 6B right).

4. Discussion

This study was designed to investigate the expression of 5-HT_4 receptors and GRKs, and their possible interaction in the rat gastrointestinal tract. The tunica muscularis mucosa of the rat oesophagus is composed of smooth muscle cells known to express 5-HT_4 receptors. The precontracted tunica muscularis mucosae relaxes upon activation with 5-HT_4 receptor agonists in a concentration dependent manner [36,37]. Hence, the tunica muscularis mucosae of the rat oesophagus is used as a standard tissue for assay of 5-HT_4 receptor agonists and antagonists. In the colon 5-HT_4 receptors are expressed in enterochromaffin cells, enteric neurons (intrinsic primary afferent neurons (IPANs), interneurones, efferent neurons of the myenteric and submucosal plexus) interstitial cells of Cajal and smooth muscle cells [8,9,39–

43]. Activation of 5-HT_4 receptors results in the release of neurotransmitters such as acetylcholine, from enteric neurons [10,11], which initiate the peristaltic reflex, facilitate propulsive activity in the colon and induce chloride secretion, increasing the fluid in the lumen [12,13]. Therefore, we selected the tunica muscularis mucosae of the oesophagus, and also the proximal and distal colon as suitable tissues to study the co-expression of 5-HT_4 receptors and GRKs, and if they interacted.

The detection of the 5-HT_4 receptor splice variants required nested RT-PCR which indicates that they were expressed at low levels in the tissues of the oesophagus, ileum and colon. The 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ splice variants were widely expressed and were detected in all tissues except jejenum while 5-$HT_{4(e)}$ was not detected in any tissues. This distribution of the mRNA of 5-HT_4 receptors is similar to that shown in previous studies of rat intestine [1–3]. Messenger RNA encoding for GRK2, GRK3, GRK5 or GRK6 was detected in all the tissues studied without the need of nested RT-PCR. This shows a relatively high level of mRNA expression of GRKs when compared to the 5-HT_4 splice variants. Western blot analysis of the tissue homogenates of the tunica muscularis mucosae of the oesophagus, proximal and distal colon confirmed the expression of proteins of sizes corresponding to 5-HT_4 receptors, GRK2, GRK3, GRK5 and GRK6.

5-HT_4 receptor immunoreactive cells were found in the tunica muscularis mucosae of the oesophagus, longitudinal muscle, myenteric plexuses, circular muscle, submucosal plexuses and muscularis mucosae of both proximal and distal colon, which is in agreement with previous studies [3,8,9]. An interesting finding was the lack of any immunoreactive cells to antibodies against GRK2 and GRK5 in the smooth muscle of the tunica muscularis mucosae of the oesophagus whilst there were immunoreactive cells to GRK3 and GRK6. The detection of GRK2 and GRK5 immunoreactivity in the immunoblot and mRNA in RT-PCR may be due to their expression on the nerve endings, which was difficult to identify in the muscularis mucosae using immunohistochemistry. This was surprising as cell culture studies have shown that GRK2 and GRK5 are involved in the regulation of the desensitization of G-protein dependent Gs/cAMP/PKA signalling pathway [26] and G-protein independent Src/ERK signalling pathway of 5-HT_4 receptors [27], respectively. Our findings perhaps highlight differences between cell types, or alternatively differences between cultured cells and those under native in vivo conditions. In the colon we observed a similar pattern of expression of GRKs in both proximal and distal regions. GRK2 immunoreactivity was found mainly in longitudinal muscle and occasionally on myenteric plexuses, whilst GRK5 immunoreactivity was restricted to nerve endings of myenteric and submucosal plexus. GRK3 was expressed in circular muscle, submucosal plexus and muscularis mucosae, whilst GRK6 was expressed in the longitudinal muscle, circular muscle, and muscularis mucosae. The difference in the pattern of expression observed for the different GRKs as compared to the expression of 5-HT_4 receptors makes it difficult to attribute the regulation of 5-HT_4 receptors to any particular GRK(s) studied.

To obtain clues about the GRK(s) involved in 5-HT_4 receptor desensitization in the rat oesophagus we established a desensitization protocol using the 5-HT_4 receptor agonist tegaserod. Tegaserod desensitized the 5-HT_4 receptor-mediated relaxation of the tunica muscularis mucosae of the rat oesophagus. A statistically significant reduction in E_{max} was observed at 20 min exposure, but it took longer than in a previously reported study [25]. This discrepancy is difficult to explain, but one possibility is that it is due to differences in the drugs used. In our study we used tegaserod which is a less potent 5-HT_4 receptor agonist, while Rondé et al. [25] used 5-HT which is a more potent agonist. Based on desensitization studies of β_2-adrenoceptors and M_3 muscarinic receptors, it is often generalized that partial agonists induce less desensitization of GPCRs than full agonists [44]. However, the capacity of agonists to induce 5-HT_4 receptor desensitization was shown to depend more on the activation potency of the drug than its efficacy [24]. Therefore the lower potency of tegaserod may contribute to the difference in the onset of desensitization.

To determine which GRKs were associated with 5-HT_4 receptors, immunoprecipitation experiments were conducted on tissue homogenates using the anti-5-HT_4 receptor antibody. A protein band of the expected size for the 5-HT_4 receptors was detected in the immunoprecipitate when probed with anti-5-HT_4 receptor antibody. GRK6 was the only GRK coimmunoprecipitated with the 5-HT_4 receptors upon stimulation of the tunica muscularis mucosae of the oesophagus with a 5-HT_4 receptor agonist tegaserod. This result suggests that tegaserod binding to the 5-HT_4 receptor has triggered the desensitization process preferentially involving GRK6. This is in agreement with our immunohistochemistry result that showed the expression of GRK6 along with 5-HT_4 receptors on the tunica muscularis mucosae.

Previously tegaserod has been shown to induce functional 5-HT_4 receptor desensitization in rat colonic tissue [19] within 10 min. Hence we used our desensitization protocol to examine which GRKs associated with the 5-HT_4 receptor in the distal colon. In the distal colon both GRK2 and GRK6 were coimmunoprecipitated with 5-HT_4 receptors in both unstimulated and tegaserod stimulated tissues. This finding was unexpected as GRKs specifically bind agonist-activated receptors [21]. However, it could be due to desensitization in response to endogenous 5-HT release. Circular and longitudinal muscle strips of the distal colon spontaneously generate contractions with low frequency and high amplitude (tonic contraction) and contractions with high frequency and low amplitude (rhythmic phasic contractions). 5-HT acts through 5-HT_4 receptors on the IPANs that in turn activate cholinergic motor neurons that contract the circular and longitudinal muscle [45,46]. We observed spontaneous contractions during the equilibration period of the distal colon. This spontaneous contraction may initiate the desensitization of 5-HT_4 receptors in unstimulated tissues and the coimmunoprecipitation of GRK2 and GRK6 with 5-HT_4 receptors.

From this study we propose that GRK2 may be involved in the regulation of the desensitization of 5-HT_4 receptors at sites where it is co-expressed with 5-HT_4 receptors as in the case of longitudinal muscle and myenteric plexuses. However, when GRK2 is absent, GRK6 may be involved in the regulation of 5-HT_4 receptors. The coimmunoprecipitation of GRK2 with 5-HT_4 receptors in the distal colon partly supports the major role of GRK2 in 5-HT_4 receptors desensitization revealed in cell culture studies [26]. However, the situation in tissue is obviously more complex. Activation of neuronal 5-HT_4 receptors, which are located exclusively presynaptically on the terminals of IPANs, at synapses within the myenteric plexus and at the neuromuscular junction, results in prokinetic activity throughout the GI tract. 5-HT_4 receptors enhance the secretion of acetylcholine and calcitonin gene-related peptide from stimulated submucosal IPANs which potentiate the excitatory neurotransmission. Potentiation of acetylcholine release also occurs at nerve-nerve synapses in the myenteric plexus and at motor neuron-smooth muscle synapses. These neurotransmitters connect via interneurones to ascending excitatory (releasing acetylcholine and/or tachykinins) and descending inhibitory (releasing nitric oxide and/or vasoactive intestinal peptide and/or ATP) motor neurones, resulting in ascending contraction and descending relaxation [12,39,47,48]. The presynaptic localization of neuronal 5-HT_4 receptors at synapses within the myenteric plexus and exclusive localisation of GRK5 on the nerve endings of both myenteric and submucosal plexus require additional research.

Previous studies of M_2 muscarinic receptors showed that in the CNS and lung, they appear to be regulated primarily by GRK5

despite cardiac M_2 receptors being regulated normally in GRK5 knockout mice [49,50]. The difference in patterns of co-expression and coimmunoprecipitation observed in our study supports the idea that desensitization of receptors may be regulated by different GRKs in different tissues [21]. In addition, our results underscore that regulation of particular GPCRs by GRKs differs based upon the specific cell composition, tissue and organ studied [51].

In conclusion, our study has shown a different pattern of expression of 5-HT_4 receptors and GRKs in the tunica muscularis mucosae of rat oesophagus and the different histological layers of the colon. In addition, 5-HT_4 receptors were coimmunoprecipitated with GRK6 in the rat oesophagus; while they were coimmunoprecipitated with GRK2 and GRK6 in distal colon. This indicates that GRK6 may be involved in the regulation of the desensitization of 5-HT_4 receptors in the rat oesophagus whilst GRK2 and GRK6 may be involved in regulation of the desensitization of 5-HT_4 receptors in the distal colon.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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