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Metoclopramide stimulates catecholamine- and granin-derived peptide secretion from pheochromocytoma cells through activation of serotonin type 4 (5-HT_4) receptors

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Introduction

The worldwide used gastroprokinetic and antiemetic agent metoclopramide is known to exert stimulatory effects on prolactin and aldosterone secretions in human subjects (Norbiato *et al.* 1977, Harrington *et al.* 1983). These two endocrine actions are usually attributed to the antidopaminergic properties of the compound (Fraser 1987, Molitch 1992). Metoclopramide has also been shown to induce acute hypertensive crisis and adrenergic myocarditis in patients with pheochromocytoma and

metoclopramide administration has been used as a provocative test in the diagnosis of pheochromocytoma (Plouin *et al.* 1976, Agabiti-Rosei *et al.* 1977, Sever 1977, Abe *et al.* 1984, Hsu *et al.* 1993, Maxwell *et al.* 2001, Leow & Loh 2005). In some cases, metoclopramide-induced adrenergic myocarditis presented as a dilated cardiomyopathy with profound left ventricular akinesia (Maxwell *et al.* 2001, Leow & Loh 2005). The mechanism of metoclopramide-evoked hypertensive crisis in these patients likely involves an increase in norepinephrine release via presynaptic D_2 receptor blockade, an inhibition of the vasodilatory effect of dopamine leading to potentiation of the hypertensive action of norepinephrine and epinephrine, and a direct stimulatory effect of the drug on pheochromocytoma cells (Plouin *et al.* 1976, Abe *et al.* 1984, Adler-Graschinsky *et al.* 1984). Although the occurrence of dopamine D_2 receptors has been clearly established in pheochromocytoma tissues (Wu *et al.* 2001), the molecular events involved in the action of metoclopramide on pheochromocytoma cells are not fully elucidated.

Beside its antagonistic action at D_2 receptors, metoclopramide is able to act as a partial agonist at the serotonin4 (5-HT_4) receptor type (Dumuis *et al.* 1989). The 5-HT_4 receptor is a 7-transmembrane domain receptor positively coupled to adenylyl cyclase (Bockaert *et al.* 2004, Brattelid *et al.* 2004). Its primary transcript is alternatively spliced to produce nine receptor isoforms that differ in the length and structure of their C-terminal tail (a–g, i, and n) and one variant that exhibits a 14-amino acid insertion within the second extracellular loop (h; Bockaert *et al.* 2004, Brattelid *et al.* 2004). It is now recognized that the 5-HT_4 receptor mediates metoclopramide-evoked aldosterone secretion as well as the abnormal response of plasma cortisol to metoclopramide observed in some patients with ACTH-independent macronodular adrenal hyperplasia (Lefebvre *et al.* 1993, Rizzi *et al.* 1997, Cartier *et al.* 2003, Christopoulos *et al.* 2005).

In the present report, a pheochromocytoma removed from a patient who experienced hypertension crisis after metoclopramide administration was used as a model for the study of the mechanism of action of metoclopramide on pheochromocytoma cells. *In vitro* experiments were conducted in order to examine the effect of metoclopramide on the secretion of catecholamine- and granin-derived peptides, including the secretogranin II-derived peptide EM66 and the chromogranin A-derived peptide WE14, by cultured pheochromocytoma cells. In addition, RT-PCR analysis was used to investigate the expression of *5-HT_4* receptor mRNA in the patient's tumor and a retrospective series of 17 pheochromocytomas.

Methods

Patients and materials

A 46-year-old woman was referred to our Department of Endocrinology for a recently discovered left adrenal tumor measuring 7 cm of diameter. She had a history of labile hypertension and paroxysmal spells of headache, palpitations, and cold sweating, and was therefore highly suspected of pheochromocytoma. Immediately after admission, she complained of nausea and unfortunately received 10 mg metoclopramide orally. Thirty minutes later, her blood pressure and pulse rose to 180/100 mmHg and 150/min respectively, and signs of cardiac insufficiency appeared. She was then transferred to the cardiological intensive care unit. Ventriculography showed profound hypokinesia and a large thrombus of the left ventricle. Treatment with low-molecular-weight heparin, aspirin, and prazosin led to recovery of normal ventricular function, regression of the ventricular thrombus, and normalization of blood pressure allowing surgical removal of the adrenal mass. Pathological examination of the tumor confirmed the diagnosis of pheochromocytoma. All clinical signs of catecholamine excess completely resolved after surgery.

Fragments of the tumor were obtained at surgery and either immediately transported to the laboratory for culture experiments or frozen on dry ice and stored at −80 °C until RT-PCR experiments. RT-PCR studies were also performed in a retrospective series of 17 pheochromocytomas (nine benign and eight malignant) provided by a French endocrinological network for collection of adrenal tumors (Réseau COMETE) and stored at −80 °C until utilization. The tumors (mean diameter 64 ± 25 mm, range 30–120 mm) had been obtained from eight women and nine men (mean age 47.6 ± 16.5 year, range 13–68 year). Five tumors were of extra-adrenal localization. The diagnosis of benign tumor was assessed on the absence of any histological criteria of malignancy, mutation in the succinate dehydrogenase B (*SDHB*) gene, and tumor recurrence or metastatic diffusion during a follow-up of at least 2 years. Malignancy was established by the presence of at least one metastasis. Normal adrenomedullary tissue explants (control tissues) were obtained from two brain-dead organ donors. The adrenal medulla was carefully dissected from the cortex with a scalpel by scraping off the brown interdigited islets of chromaffin cells, as described previously (Cavadas *et al.* 2001). This method allows obtaining a degree of purity of 95% for chromaffin cells when the tissues are used for cell culture experiments. The protocols of collection of the tissues and the experimental procedures were

approved by the regional ethics committee and informed consent was obtained from all patients with pheochromocytoma.

Primary culture of pheochromocytoma cells

Pheochromocytoma fragments were immersed in culture medium (DMEM; Sigma–Aldrich), transported to the laboratory, and processed as described previously (Guillemot *et al*. 2006). Briefly, the tissues were minced with scissors, and tumor cells were enzymatically dispersed in DMEM containing 0.1% collagenase (Serlabo, Bonneuil-sur-Marne, France) and 30 U/ml DNase I (Sigma–Aldrich). Isolated cells were cultured in DMEM supplemented with 5% calf serum (Biowhittaker Europe, Verviers, Belgium), 10% horse serum (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (Life Technologies). Tumoral chromaffin cells were purified by overnight differential plating to remove adherent non-chromaffin cells and then plated at a density of 10^6 cells/ml per well. After 1 day, pheochromocytes were treated with metoclopramide (Sigma–Aldrich), dopamine (Sigma–Aldrich), cisapride (10^{-7} M; Laboratoire Janssen, Boulogne-Billancourt, France), domperidone (Sigma–Aldrich), and/or the 5-HT_4 receptor antagonist GR 113808 (10^{-7} M; GlaxoSmithKline, Greenford, England, UK). The cells were incubated with each secretagogue (three wells for each concentration tested) for 24 h at 37 °C. The culture medium was then collected and immediately frozen at −80 °C until HPLC or at −20 °C until RIA.

Catecholamine- and granin-derived peptide assays

Characterization and quantitation of norepinephrine and epinephrine in cell culture medium were carried out by HPLC combined with electrochemical detection after extraction on alumina, using a method adapted from that previously used for the measurement of plasma catecholamine concentrations (Joannides *et al*. 1998).

The concentrations of EM66 and WE14 were measured by RIA. Purified recombinant EM66 and synthetic [Tyr0] WE14 were iodinated by the chloramine-T method and separated from free iodine on Sep-Pak C18 cartridges using a gradient of acetonitrile (0–100%) in 0.1% trifluoroacetic acid (TFA), as described previously (Montero-Hadjadje *et al*. 2002, Guillemot *et al*. 2006). The RIA was performed in veronal buffer (pH 7.4) supplemented with 0.4% BSA (BSA; Roche Diagnostics) and 0.1% Triton X-100. The EM66 and WE14 antisera, used at a final dilution of 1:60 000 and 1:100 800 respectively, were incubated with 7000 c.p.m. tracer/tube in the presence of graded concentrations of standard (purified EM66 or synthetic WE14) or culture medium. After 2-day incubation at 4 °C, the antibody-bound fraction was immunoprecipitated by the addition of 100 μl of 20% bovine β-globulins and 1 ml of 5% polyethylene glycol 8000. After 20-min incubation at room temperature, the mixture was centrifuged (5000 ***g***, 30 min, 4 °C) and the pellet containing the bound fraction was counted on a γ-counter (LKB, Wallack, Rockville, MD). The standard curve was set up with concentrations of EM66 or WE14 ranging from 5 to 10 000 pg/tube.

RNA extraction and real-time RT-PCR

Total RNA from the 18 pheochromocytomas including the patient's tumor and two normal adrenal medulla was extracted by the acid guanidium thiocyanate–phenol–chloroform procedure by using TRI reagent (Sigma). The concentration of total RNA was determined by measuring the optical density at 260 nm. Real-time RT-PCR analysis was carried out as described by Fink *et al*. (1998) in order to quantify *5-HT_4* receptor mRNA in both pheochromocytomas and normal adrenal medulla. The primers and fluorogenic TaqMan probe used for these experiments hybridized to all 5-HT_4 receptor splice variants as previously published (Cartier *et al*. 2005). Briefly, 1 μg total RNA from each tissue was converted to single-stranded cDNA using SuperScript II from Life Technologies with oligo (dT)12–18 primer (0.5 μg/ml), and the cDNA was diluted and aliquoted into microtitre plates. For each 25-μl TaqMan reaction, 5 μl cDNA was mixed with 1 μl water, 12.5 μl TaqMan Universal PCR Master Mix 2× (Applied Biosystems, Courtaboeuf, France), 2 μl sense primer (2 μM), 2 μl antisense primer (2 μM), and 2.5 μl TaqMan probe (2 μM). PCR parameters were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Parallel assays using the same cDNA pools were carried out using primers and probe to the housekeeping gene porphobilinogen deaminase. Quantitative RT-PCR was performed using an ABI Prism 7700 sequence detector system (Applied Biosystems) and analyzed using relative expression to porphobilinogen deaminase (PBGD), as previously described (Cartier *et al*. 2005). Briefly, the level of expression in each sample was normalized by dividing copies/ng total RNA of *5-HT_4* receptor gene by copies/ng total RNA of porphobilinogen deaminase gene, and expressed as a percentage. This mode of calculation allows correcting for both RNA quality and quantity.

Characterization of 5-HT_4 receptor isoforms by RT-PCR

Total RNA was extracted and reverse transcribed as described above. Amplification of the cDNAs encoding the different 5-HT_4 receptor C-terminal splice variants was performed by PCR using primer S1, which hybridizes to all 5-HT_4 receptor messengers, and splice variant-specific reverse primers as previously published (Cartier *et al*. 2005). The 5-$HT_{4(h)}$ variant was amplified using the forward primer Fwh, which is specific for cDNAs containing the 5-$HT_{4(h)}$ exon, and the reverse primer Revh, which hybridizes to all 5-HT_4 receptor messengers. All PCR-based procedures were performed in a final volume of 50 µl containing 10% reverse transcription mixture, 3 U DNA *Taq* Polymerase (Life Technologies), DNA Polymerase buffer (Life Technologies), 1.5 mM $MgCl_2$, 0.4 mM dNTP, and 20 pmol of each primer. The PCRs were performed for 40 cycles (94 °C, 40 s; 50 °C, 60 s; 72 °C, 90 s). The PCR products were analyzed in 1.5% agarose gels, blotted on a nylon membrane, and hybridized with the [32P]ATP-labeled oligonucleotide S2. In addition, PCR products were subcloned into pGEM-T (Promega) and sequenced, using the Thermosequenase kit (Amersham) on a Li-Cor 4200L DNA sequencer (ScienceTec, Les Ulis, France) using fluorescent T7 and T3 primers (MWG-Biotech, Courtaboeuf, France).

Statistical analysis

All results of cell incubation studies were expressed as mean ± S.E.M., and statistical analysis was performed using Dunnett's test after one-way ANOVA. Potencies and efficacies of the test substances are expressed as concentrations that produced half-maximum responses (EC_{50}) and maximum stimulations (E_{max}) respectively. Expression levels of *5-HT₄* mRNAs in the benign and malignant pheochromocytomas were compared using the Mann–Whitney *U*-test. Probability values less than 0.05 were considered significant. Data were analyzed with the Prism program (GraphPad Software, San Diego, CA, USA).

Results

Effect of metoclopramide on catecholamine- and granin-derived peptides from cultured pheochromocytoma cells

Administration of graded concentrations of metoclopramide (10^{-9} to 10^{-6} M) to pheochromocytoma cells in primary culture induced a dose-dependent increase in norepinephrine and epinephrine secretion (Fig. 1A and B). The potencies (EC_{50}) and efficacies (E_{max}) of metoclopramide to stimulate catecholamine production were 1.44×10^{-8} M and +44 ± 12% respectively for norepinephrine, and 1.23×10^{-8} M and +43 ± 12% respectively for epinephrine. Interestingly, a concentration of

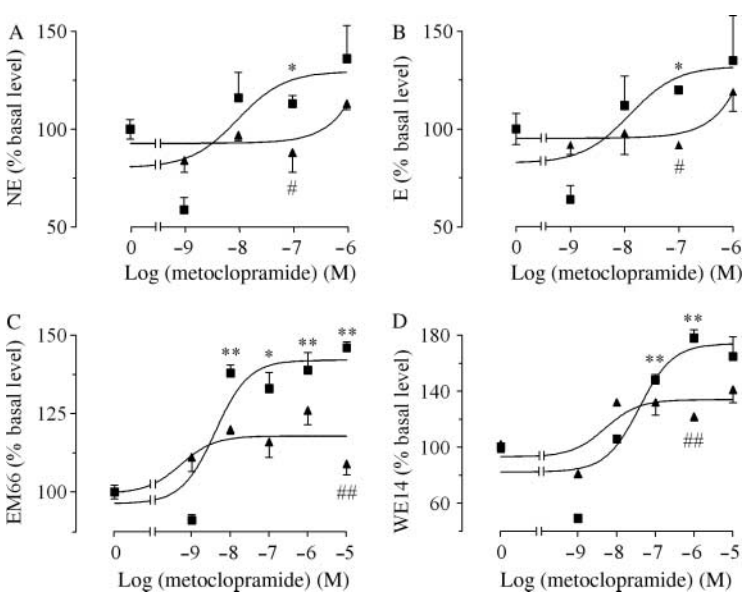

Figure 1 Effect of metoclopramide on (A) norepinephrine (NE), (B) epinephrine (E), (C) EM66, and (D) WE14 production from pheochromocytoma cells. Graded doses of metoclopramide (10^{-9} to 10^{-5} M) were incubated with cultured pheochromocytoma cells in the absence (■) or presence (▲) of the 5-HT_4 receptor antagonist GR 113808 (10^{-7} M). Each point represents the mean of at least three determinations, expressed as percentages of basal level (control). Bars denote S.E.M. *$P<0.05$ and **$P<0.01$ versus control; #$P<0.05$ and ##$P<0.01$ versus metoclopramide alone.

10^{-7} M metoclopramide, which corresponds to the level of metoclopramide commonly observed in plasma after oral intake of 10 mg of the drug (Lamparczyk *et al.* 2001), was sufficient to trigger a significant increase (i.e., $+20\pm4\%$ for norepinephrine and $+23\pm4\%$ for epinephrine) in catecholamine secretion. Metoclopramide also induced a concentration-dependent increase in EM66 and WE14 productions (Fig. 1C and D) with EC_{50} reaching respectively 4.03×10^{-9} and 3.84×10^{-8} M, and E_{max} of $+46\pm4\%$ and $78\pm12\%$ respectively. The norepinephrine, epinephrine, EM66, and WE14 responses to metoclopramide were all inhibited by GR 113808 (10^{-7} M), a 5-HT_4 receptor antagonist devoid of any dopaminergic properties (Grossman *et al.* 1993; Fig. 1).

Effect of cisapride and domperidone on catecholamine- and granin-derived peptides from cultured pheochromocytoma cells

Incubation of cultured pheochromocytoma cells with the 5-HT_4 receptor agonist cisapride at the concentration observed in plasma (C_{max}) after oral administration of the drug, i.e., 10^{-7} M (Gross *et al.* 1999), induced a significant stimulation of norepinephrine ($+23\pm4\%$), epinephrine ($+24\pm1\%$), EM66 ($+43\pm3\%$), and WE14 ($+63\pm15\%$) production (Fig. 2). Cisapride-induced catecholamine- and granin-derived peptide secretions were inhibited by GR 113808 (10^{-7} M). In contrast to cisapride, domperidone (10^{-6} M), a dopaminergic D_2 receptor antagonist devoid of any agonistic activity at the 5-HT_4 receptor, had no significant effect on catecholamine- and granin-derived peptide release (Fig. 2).

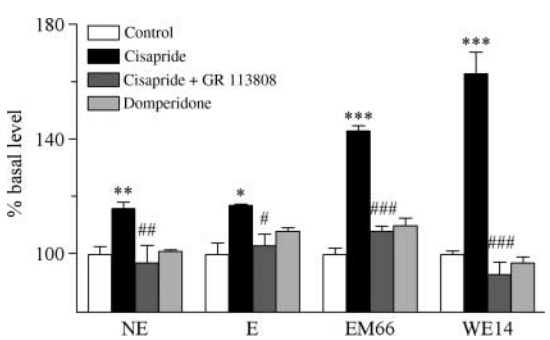

Figure 2 Effects of the 5-HT_4 receptor agonist cisapride and the dopamine D_2 receptor agonist domperidone on norepinephrine (NE), epinephrine (E), EM66, and WE14 production from pheochromocytoma cells. Cisapride (10^{-7} M) was incubated with cultured pheochromocytoma cells in the absence (black bars) or presence (dark grey bars) of GR 113808 (10^{-7} M). Domperidone was incubated with pheochromocytoma cells at the concentration of 10^{-6} M (light grey bars). Each point represents the mean of at least three determinations, expressed as percentages of basal level (control, open bars). Bars denote S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ versus control; $\#P<0.05$, $\#\#P<0.01$, and $\#\#\#P<0.001$ versus cisapride alone.

Effect of metoclopramide on the catecholamine- and granin-derived peptide responses to dopamine from cultured pheochromocytoma cells

Incubation of pheochromocytoma cells with graded concentrations of dopamine (10^{-9} to 10^{-6} M) induced a dose-dependent inhibition of catecholamine production reaching $-49\pm2\%$ for norepinephrine and $-51\pm3\%$ for epinephrine (Fig. 3A and B). Interestingly, the inhibitory effect of dopamine on norepinephrine and epinephrine secretions was not significantly influenced by metoclopramide (10^{-6} M; Fig. 3A and B). In contrast to its negative action on catecholamine release, dopamine dose dependently stimulated EM66 and WE14 production with maximum efficacies of $+28\pm10\%$ and $+30\pm6\%$ (Fig. 3C and D).

Quantitative expression of 5-HT_4 receptors in normal adrenal medulla and pheochromocytomas

We have investigated the quantitative expression of *5-HT_4* receptor mRNA by the tissues by real-time PCR using oligonucleotides hybridizing to all receptor variants. *5-HT_4* receptor mRNAs were detected in all the tissues (Fig. 4). When expressed as arbitrary units normalized to porphobilinogen deaminase, 5-HT_4 receptor expression levels were 3.61 and 1.23% in the two normal medulla explants, 0.13–207% with a median value of 2.01% in benign pheochromocytomas, and 0.28–25.4% with a median value of 2.22% in malignant pheochromocytomas. There was no significant difference in the expression rates of benign versus malignant pheochromocytomas. The patient's pheochromocytoma was one of the tissues that expressed the highest levels of *5-HT_4* receptor mRNA, i.e., 29.7%.

Characterization of 5-HT_4 receptor isoforms by RT-PCR in normal adrenal medulla and pheochromocytomas

RT-PCR amplification was applied to characterize 5-HT_4 receptor isoforms in both normal adrenal medulla and pheochromocytomas (Table 1). None of the known isoforms was detected in adrenomedullary explants. By contrast, pheochromocytoma tissues were found to frequently express isoforms (a), (b), (c), (i), and (n). Isoform (g) was present in two tumors, i.e., one benign (no. 3) and one malignant (no. 10). Two pheochromocytomas (nos. 6 and 17) exclusively expressed isoform (b). There was no significant difference between the profiles of expression of 5-HT_4 receptor isoforms in benign and malignant pheochromocytomas, respectively.

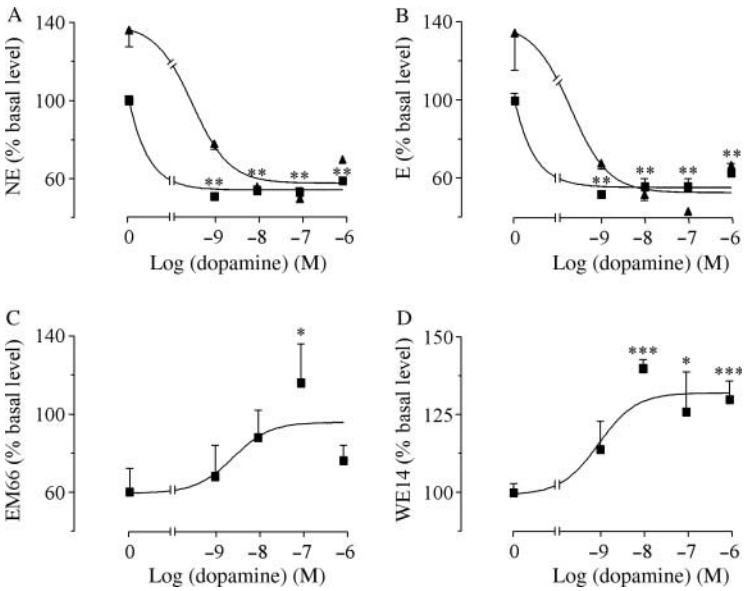

Figure 3 Effect of dopamine on (A) norepinephrine (NE), (B) epinephrine (E), (C) EM66, and (D) WE14 production from pheochromocytoma cells. Graded concentrations of dopamine (10^{-9} to 10^{-6} M) were incubated with cultured pheochromocytoma cells in the absence (■) or presence (▲) of metoclopramide (10^{-6} M). Each point represents the mean of at least three determinations, expressed as percentages of basal level (control). Bars denote S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ versus control.

Discussion

In the present study, we have used pheochromocytoma tissue removed from a patient who experienced hypertension crisis and acute adrenergic myocarditis after metoclopramide administration, as an *in vitro* model for the study of the mechanism of action of metoclopramide on pheochromocytoma cells. It was indeed probable that metoclopramide may have triggered catecholamine secretion from the tumor tissue, leading to severe hypertensive paroxysm. Consistent with this hypothesis, our results showed that metoclopramide dose dependently stimulated catecholamine production from pheochromocytoma cells. It was also physiopathologically relevant to note that the concentration of metoclopramide usually observed in the plasma after oral intake of the drug (10^{-7} M) was sufficient to stimulate catecholamine release by tumor cells. Moreover, culture experiments showed that metoclopramide potently stimulated the production of two granin-derived peptide release, i.e., EM66 and WE14, that have previously been detected in pheochromocytoma tissues (Montero-Hadjadje *et al.* 2002, Yon *et al.* 2003, Guillemot *et al.* 2006).

The observation that metoclopramide-induced catecholamine- and granin-derived peptide productions were reduced by the specific 5-HT_4 antagonist GR 113808 indicates that the stimulatory effect of metoclopramide on pheochromocytes is at least partly mediated by 5-HT_4 receptors. The presence of functional 5-HT_4 receptors in the patient's pheochromocytoma tissue was confirmed by the following results: cisapride, another 5-HT_4 receptor agonist devoid of any antidopaminergic properties, activated catecholamine, and granin-derived peptide secretion; the stimulatory effect of cisapride was completely inhibited by GR 113808. As D_2 receptors are expressed in pheochromocytoma tissues (Wu *et al.* 2001), it was conceivable that metoclopramide may have also activated tumor cells through its antagonistic D_2

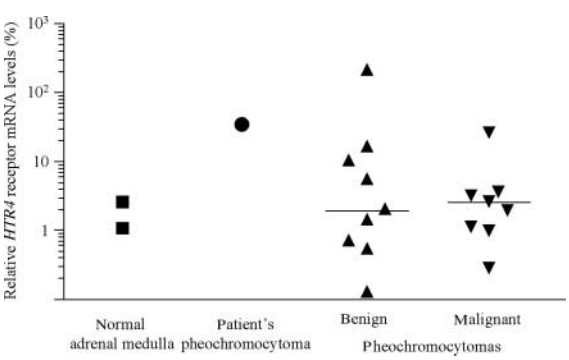

Figure 4 Expression of *5-HT₄* receptor mRNA in pheochromocytoma tissues. *5-HT₄* receptor mRNA level was quantified in normal adrenal medulla (■), the patient's pheochromocytoma (●), benign (▲), and malignant (▼) pheochromocytomas by the use of real-time RT-PCR. The horizontal bars represent the median values observed in the groups of benign and malignant pheochromocytomas. The level of expression in each sample was normalized by dividing copies/ng total RNA of *5-HT₄* receptor gene by copies/ng total RNA of porphobilinogen deaminase gene, and expressed as a percentage.

Table 1 Expression of 5-HT_4 receptor isoforms in normal adrenal medulla and pheochromocytomas

	a	b	c	d	e	f	g	h	i	n
N1	−	−	−	−	−	−	−	−	−	−
N2	−	−	−	−	−	−	−	−	−	−
PP	−	+	+	−	−	−	−	−	+	+
1	+	+	+	−	−	−	−	−	+	+
2	+	+	+	−	−	−	−	−	+	+
3	+	+	+	−	−	−	+	−	+	+
4	+	+	+	−	−	−	−	−	+	+
5	−	+	−	−	−	−	−	−	+	−
6	−	+	−	−	−	−	−	−	−	−
7	+	+	+	−	−	−	−	−	+	+
8	+	+	+	−	−	−	−	−	+	+
9	+	+	+	−	−	−	−	−	+	+
10	+	+	+	−	−	−	+	−	+	+
11	+	+	+	−	−	−	−	−	+	+
12	+	+	+	−	−	−	−	−	+	+
13	−	+	+	−	−	−	−	−	+	+
14	+	+	+	−	−	−	−	−	+	+
15	+	+	+	−	−	−	−	−	+	+
16	+	+	+	−	−	−	−	−	+	+
17	−	+	−	−	−	−	−	−	−	−

N1-2, normal adrenal medulla; PP, patient's pheochromocytoma; 1–9, benign pheochromocytomas; 10–17, malignant pheochromocytomas; +, expression; −, no detectable expression.

receptor properties. In fact, it is well documented that metoclopramide stimulates prolactin secretion by block-ing the inhibitory action of dopamine on pituitary lactotrophs (Molitch 1992). In agreement with this hypothesis, we noted that dopamine potently decreased norepinephrine and epinephrine release, as previously reported in sympathetic nerve terminals and adrenal medulla (Mannelli *et al.* 1995). However, metoclopra-mide had no action on dopamine-evoked inhibition of catecholamine release indicating that metoclopramide does not stimulate catecholamine production by antag-onizing the effect of dopamine on pheochromocytes. These results also suggest that the inhibitory action of dopamine on norepinephrine and epinephrine secretion is not mediated by D_2 receptors. As the presence of D_4 receptor in pheochromocytomas has been clearly established (Wu *et al.* 2001), it is possible that the dopaminergic regulation of catecholamine production by pheochromocytoma cells may involve this dopamine receptor type for which metoclopramide exhibits a low affinity (Rizzi *et al.* 1997). The observation that metoclopramide and dopamine effects on catecholamine release were independent of the D_2 receptor was also consistent with the lack of significant action of domperidone, a well-known D_2 receptor antagonist devoid of any serotonin antagonistic properties, on norepinephrine and epinephrine secretions by the patient's tumor cells. In contrast to its inhibitory effect on norepinephrine and epinephrine release, dopamine acted as a potent stimulator of granin-derived peptides production, indicating that metoclopramide could not activate granin secretion through its dopamine antagonistic properties. These data also show that dopamine can differentially regulate catecholamine- and granin-derived peptide productions from pheochromocytoma cells. The processes involved in these complex regulations likely involve selective release of secretory granule contents through kiss-and-run and/or piecemeal degranulation (Crivellato *et al.* 2006).

The occurrence of functional 5-HT_4 receptors in the patient's pheochromocytoma led us to investigate quantitative and qualitative expressions of *5-HT_4* receptor mRNAs in two normal adrenomedullary tissues and benign and malignant pheochromocytomas. Using primers hybridizing to the common region of all 5-HT_4 receptor isoforms, we observed the presence of *5-HT_4* receptor mRNAs in all tissues studied, including the patient's tumor, which was one of the tissues that expressed the highest levels of the receptor. There were no statistically significant differences between the expression levels of benign versus malignant pheochromocytomas. The fact that normal adrenomedullary explants expressed *5-HT_4* receptor mRNA at levels similar to those observed in pheochromocytoma tissues was unexpected with regard to the observation that the potency of metoclopramide to stimulate catecholamine secretion *in vivo* and to elevate blood pressure is much higher in pheochromocytoma than in

non-pheochromocytoma patients (Hsu *et al*. 1993). This apparent discrepancy may be explained by differences in the functional properties of 5-HT_4 receptors respectively expressed by the normal adrenal medulla and pheochromocytoma tissues. Indeed, although real-time RT-PCR experiments using primers specific to the common region of all 5-HT_4 receptor variants had shown that the normal adrenal medulla actually expresses the receptor, no specific cDNA band could be detected after RT-PCR amplification of mRNAs encoding the currently known isoforms of the 5-HT_4 receptor in medulla extracts. These results show that adrenomedullary cells express 5-HT_4 receptor isoforms different from the already-recognized splicing variants. It is conceivable that these unidentified isoforms may be less efficiently activated by metoclopramide than the variants that were detected in pheochromocytomas explants. In addition, recent studies have shown that 5-HT_4 receptors, like other G-protein-coupled receptors, can form homo- and/or heterodimers that modulate receptor function (Berthouze *et al*. 2005). It is thus possible that differences in the expression profiles of 5-HT_4 receptor splice variants among tissues could lead to a weak responsiveness to metoclopramide in normal adrenal medulla in comparison with pheochromocytomas. Conversely, we cannot exclude that metoclopramide stimulates catecholamine secretion from the normal adrenal medulla as efficiently as it does in pheochromocytoma tissues but the low mass of catecholamine-secreting tissue in non-pheochromocytoma patients prevents them from experiencing metoclopramide-evoked hypertension crisis.

The different patterns of expression of 5-HT_4 receptor isoforms observed respectively in the normal adrenal medulla and pheochromocytoma tissues also suggest that maturation of 5-HT_4 receptor primary transcripts is altered in adrenomedullary tumors in comparison with normal adrenal chromaffin tissue. Nevertheless, processing of the *5-HT_4* receptor mRNA does not seem to depend on the degree of differentiation of tumors since expression profiles of 5-HT_4 receptor isoforms were not significantly different in benign versus malignant tumors.

Pheochromocytoma crisis is a rare life-threatening emergency, with a high mortality rate, i.e., reaching 85% in some studies (Brouwers *et al*. 2003). Preventing paroxysmal crises and their potentially life-threatening consequences, such as acute heart failure and seizures, is a major goal of the preoperative management of patients with pheochromocytoma. On the other hand, it is well known that hypertensive episodes may be precipitated by various drugs that potentiate the action of catecholamines or directly stimulate pheochromocytoma tissues and are therefore contraindicated in patients with pheochromocytoma (Keiser 2001, Eisenhofer *et al*. 2007). However, drug-induced paroxysms are often unpredictable with new compounds whose action on adrenomedullary cells is generally unknown. In this respect, several new potent 5-HT_4 receptor agonists, like tegaserod, mosapride, and prucalopride, are currently developed or have already been commercialized in several countries as digestive tract prokinetic agents. Since decreased gastrointestinal motility is one of the non-specific features of pheochromocytoma (Keiser 2001), some patients with unrecognized pheochromocytoma may potentially be treated with these compounds. Although 5-HT_4 receptor agonist-evoked hypertension crisis has not yet been reported, the present study, which shows that adrenomedullary tumors express functional 5-HT_4 receptors, suggests that administration of these prokinetic agents to pheochromocytoma patients would lead to a high risk of paroxysmal crisis. This assumption is supported by the observation that the expression rates of the 5-HT_4 receptor in pheochromocytoma tissues were similar to or higher than those previously noted in the gastrointestinal tract and adrenal cortex, two organs that are well known to respond *in vivo* to the stimulatory action of 5-HT_4 receptor agonists (Cartier *et al*. 2005, van Lelyveld *et al*. 2007). It seems therefore reasonable to recommend to systematically eliminate the diagnosis of pheochromocytoma by the use of urinary or plasma metanephrine measurements before prescribing 5-HT_4 receptor agonists when decreased gastrointestinal motility is associated with hypertension and/or signs of catecholamine excess.

In summary, the present *in vitro* study shows that metoclopramide stimulates catecholamine- and granin-derived peptide secretions from pheochromocytoma tissue by activating serotonin 5-HT_4 receptors rather than antagonizing D_2 receptors. Our data also suggest that all 5-HT_4 receptor agonists, including the newly developed compounds tegaserod, mosapride, and prucalopride, might better be avoided in patients with proven or suspected pheochromocytoma.

Declaration of interest

All authors have nothing to disclose.

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