British Journal of Pharmacology (2005) 146, 543–552
www.nature.com/bjp

Interactions of metoclopramide and ergotamine with human 5-HT_{3A} receptors and human 5-HT reuptake carriers

[1]Jan Walkembach, [2]Michael Brüss, [1]Bernd W. Urban & *,[1,2]Martin Barann

[1]Klinik und Poliklinik für Anästhesiologie und operative Intensivmedizin, Universitätskliniken Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany and [2]Institut für Pharmakologie und Toxikologie, Universität Bonn, Reuterstr. 2 b, D-53113 Bonn, Germany

1 The actions of metoclopramide and ergotamine, drugs which are used as a combined migraine medication, on human (h)5-HT_{3A} receptors and 5-HT reuptake carriers, stably expressed in HEK-293 cells, were studied with patch-clamp- and ([^{3}H]5-HT)-uptake techniques.

2 At clinical concentrations, metoclopramide inhibited peak and integrated currents through h5-HT_{3A} receptors concentration-dependently ($IC_{50} = 0.064$ and $0.076\,\mu$M, respectively) when it was applied in equilibrium (60 s before and during 5-HT ($30\,\mu$M) exposure). The onset and offset time constants of metoclopramide action were 1.3 and 2.1 s, respectively. The potency of metoclopramide when exclusively applied during the agonist pulse decreased more than 200-fold ($IC_{50} = 19.0\,\mu$M, peak current suppression).

3 Metoclopramide ($0.10\,\mu$M) did not alter the EC_{50} of 5-HT-induced peak currents. In contrast to the lack of competitive interaction between metoclopramide and 5-HT in this functional assay, metoclopramide inhibited specific [^{3}H]GR65630 binding to human h5-HT_{3A} receptors in a surmountable manner. This seeming discrepancy between functional studies and radioligand binding experiments may be accounted for by (1) the slow kinetics of inhibition of peak currents by metoclopramide compared with the fast onset and offset kinetics of 5-HT-induced currents and (2) the low efficacy of metoclopramide in inhibiting radioligand binding (e.g. only 20% binding inhibition compared to 79% peak current suppression by 200 nM metoclopramide).

4 At low concentrations (1–10 nM), ergotamine had no effect on 5-HT ($30\,\mu$M)-induced peak currents. Above clinical concentrations, ergotamine ($>3\,\mu$M) inhibited them.

5 When both drugs were applied together ($0.10\,\mu$M metoclopramide + 0.001 to $0.01\,\mu$M ergotamine), an inhibition of both, peak and integrated current responses was observed.

6 Neither metoclopramide ($\leqslant 30\,\mu$M) nor ergotamine ($\leqslant 30\,\mu$M) had an effect on the 5-HT reuptake carrier as they did not alter the citalopram-sensitive [^{3}H]5-HT uptake.

British Journal of Pharmacology (2005) **146,** 543–552. doi:10.1038/sj.bjp.0706351; published online 25 July 2005

Keywords: 5-HT_3 receptor; 5-HT reuptake carrier; metoclopramide; migraine; ergotamine; emesis

Abbreviations: Ergotamine, 12′-hydroxy-2′-methyl-5′α-(phenylmethyl)ergotaman-3′,6′,18-trione); GR65630, (3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; 5-HT, 5-hydroxy-tryptamine; metoclopramide, 4-amino-5-chloro-*N*-[2-diethyl-amino)ethyl]-2-methoxybenzamide

Introduction

Although largely superseded by triptans, the prokinetic and antiemetic drug metoclopramide is still administered together with ergotamine for the treatment of migraine, especially when accompanied by emesis (for a review, see Silberstein & McCrory, 2003). In addition to interactions with dopamine D_2 receptors (Woodward *et al*., 1994; Larson *et al*., 1995), both drugs also interact in more than one way with the serotonergic 5-hydroxy-tryptamine (5-HT) system (Hoyer *et al*., 1989; Gill *et al*., 1995; Haddjeri *et al*., 1998; for a review, see Silberstein & McCrory, 2003).

The 5-HT_3 receptor is the only 5-HT receptor-subtype that belongs to the superfamily of ligand-gated ion channels (for a review, see Costall & Naylor, 1999). It is permeable to Na^+, K^+ as well as Ca^{2+}. The pentameric 5-HT_3 receptor is a target for drugs, including general anesthetics and cannabinoids (Barann *et al*., 2000a, b; 2002). 5-HT_3 receptors are located in the periphery on vagal afferents and within the central nervous system in the area postrema, components that play a major role in the modulation of nausea and emesis (Carpenter *et al*., 1988; Minami *et al*., 2001). Unsurprisingly, the 5-HT_3-receptor is relevant in the physiology of emesis and nausea: 5-HT_3 receptors mediate substance-induced emesis, for example, in postoperative nausea and vomiting and they are targets for antiemetic drugs. The function of the 5-HT_3 receptor may be affected by drugs that modulate emesis by different mechanisms: currents through the 5-HT_3 channel can be inhibited by antagonists like ondansetron, which are potent antiemetic drugs (Cunningham *et al*., 1987) or potentiated, for example, by volatile

*Author for correspondence at: Klinik und Poliklinik für Anästhesiologie und operative Intensivmedizin, Universitätskliniken Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany.
E-mail: martin.barann@ukb.uni-bonn.de

anesthetics or alcohols, which may evoke emesis (Lovinger, 1991; Machu & Harris, 1994).

Ergotamine was the only specific anti migraine-medication until 1993, when the first triptan (sumatriptan) was introduced (Moskowitz & Cutrer, 1993). Today, the drug is used against acute attacks only. Ergot alcaloids such as dihydroergotamine affect the pathophysiology of migraine *via* 5-HT$_{1B/D}$-receptors on meningeal-vessels (Buzzi & Moskowitz, 1991). Ergotamine has been in use for migraine treatment since the late 19th century and besides 5-HT$_{1B/D}$ receptors it modulates the function of diverse receptors and proteins as well (Silberstein & McCrory, 2003). As a result different side effects of ergotamine are known, including nausea and emesis (Tfelt-Hansen *et al*., 2000). This led to the development of newer substances or the use of adjuvant medications, for example metoclopramide, as a prokinetic and antiemetic drug.

Metoclopramide has been in use as an antiemetic and prokinetic agent for many years. The drug is known for its D_2 (dopamine$_2$)-receptor antagonism but it also has some 5-HT$_3$ antagonistic property (Fozard & Mobarok Ali, 1978). Binding assays of metoclopramide on human 5-HT$_3$-receptors indicate IC_{50} values lower than 1 μM (Hirokawa *et al*., 2003); in addition, functional studies confirm that the 5-HT$_3$ receptor is a target for metoclopramide (Belelli *et al*., 1995; Brown *et al*., 1998; Lankiewicz *et al*., 1998).

We examined the actions of metoclopramide and of ergotamine on human 5-HT$_{3A}$ receptors using the patch-clamp technique on excised outside-out patches combined with a fast solution exchange system. The mechanism of action of metoclopramide was further evaluated in binding experiments using the specific 5-HT$_3$ receptor ligand [^{3}H]GR65630. In addition, the effects of metoclopramide and ergotamine on 5-HT reuptake carriers were studied as they control the concentration of serotonin in the 5-HT synaptic clefts and in plasma.

A preliminary account of this work has been published in abstract form (Walkembach *et al*., 2003).

Methods

Transfection

HEK-293 cells at 20% confluence were stably transfected by the modified calcium phosphate method (Chen & Okayama, 1987) with the h5-HT$_{3A}$ receptor cDNA or human 5-HT transporter cDNA subcloned into the mammalian expression vector pCDNA3 (Invitrogen) under control of human cytomegalovirus promoter. At 2 days after transfection, stably transfected cells were selected by the addition of geneticin (800 μg ml^{-1}) to the culture medium. The medium was changed every 2 days and after occurrence of single-cell colonies these were separated by usage of cloning cylinders (Sigma). Single-cell colonies were further subcultured in 24-well plates (Falcon) until confluence. About 20–40 colonies from each transfection experiment were tested for stable transfection of the particular cDNA by [^{14}C]guanidiunium influx and binding of the selective 5-HT$_3$ receptor antagonist [^{3}H]GR 65630 [3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone] or specific [^{3}H]5-HT uptake. Colonies with best expression were used for further experiments.

Cell culture

HEK-293 cells were grown as monolayer on culture plates (Nunc) in DMEM Nutrient Mix F12 (1 : 1; v v^{-1}) medium containing 10% heat inactivated fetal calf serum, penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}), geneticin (0.75 mg ml^{-1}) and glutamine (292 μg ml^{-1}). Cells were cultured at 37° in humidified atmosphere (5% CO_2). For patch-clamp experiments cells were subcultured in monodishes (Nunc, 35 mm diameter) 7–11 days before an experiment; for reuptake assays experiment, cells were subcultured in 24-wells 2 days before the experiments.

Electrophysiology

Before starting patch-clamp recordings, the culture medium was replaced by 'extracellular' solution of the following composition (mM): NaCl 150; KCl 5.6; $CaCl_2$ 1.8; $MgCl_2$ 1; HEPES 10; D-Glucose 20; pH 7.4. In the extracellular solution used for superfusion of the excised patches D-Glucose was omitted. Patch pipettes with resistances of 2–4 MΩ were filled with 'intracellular' solution containing (mM): KCl 140; EGTA 10; $MgCl_2$ 5; HEPES 10; pH 7.4. Experiments were performed at room temperature (18–22°C).

Patch pipettes were manufactured from borosilicate glass capillaries (Kwik-Fil™, World Precision Instruments, U.S.A.) using a pipette puller (List L/M-3P-A). Outside-out patches were excised from cells possessing a smooth surface, angularly shape and having clear contact to neighbouring cells. The seal resistances were 1–6 GΩ. For current measurements we used a patch-clamp amplifier (EPC-7, List Electronic, Darmstadt, Germany) in combination with an external low pass filter set at 1 kHz (either Frequency Devices, MA, U.S.A. or LPBF-48DG, NPI Electronic, Tamm, Germany). Data were digitally recorded at a sampling rate of 2 kHz with a Digidata 1200 (Axon Instuments, Foster City, CA, U.S.A.) interface and Clampex-8 software (Axon) was used for recording-protocols. At 500 ms before 5-HT exposure, the membrane potential was stepped from a holding value of 0 to −100 mV. These conditions were chosen to optimize the stability of the excised patches and the reproducibility of results.

Fast solution exchange system

Two application systems were employed: (a) A two-tube system was used for concentration–response curves of tested adjuvants. Patches were moved into position ~0.5 mm from the outflow (0.3 mm diameter) of a two-arm teflon superfusion system (for details see Liu & Dilger, 1991). The speed of the solution exchange was tested with 'open patch-pipette' experiments: the resulting responses had on- and offset exchange times of less than 1 ms. (b) A multitube perfusion system having a slightly slower exchange rate (solution exchange below 2 ms (RSC 200, Biologic, France)) was used for experiments in which patches were pre-exposed to the respective drug for a variable amount of time before agonist application (Barann *et al*., 2000b). It was possible to apply the drug-containing wash solution for ⩾25 ms prior to the agonist (5-HT) application without oscillations. We used the RSC 200 for this experimental protocol because it has one application tube for each solution, thus allowing clean exchange between more than two solutions. The kinetics of 5-HT-induced

currents and the effects of metoclopramide and ergotamine were unchanged when the different superfusion systems were used. The drug application systems were equipped with inert materials such as teflon tubing and glass to avoid loss of hydrophobic drugs (Barann *et al*., 2000c).

Drug application modes

A concentration of 30 μM 5-HT was chosen for the experiments with ergotamine and metoclopramide (if not stated otherwise) because it elicited a near maximal response to 5-HT, which was reproducible and stable under control conditions. Using the two-tube fast solution exchange system three different protocols of drug application were used (Barann *et al*., 2000a):

(1) equilibrium application (++): continuous exposure to the drug before and during the application of 5-HT;
(2) open channel application (−+): no drug application prior to the 5-HT pulse, only simultaneous application of drug and 5-HT;
(3) closed channel application (+−): pre-exposure to the drug before but not during the 5-HT application.

The standard experimental procedure using outside-out patches has been described by us in detail before, using the identical equipment (Barann *et al*., 2000a; 2002).

Reuptake experiments

Experiments were carried out on 24-well cell culture cluster plates (Falcon), using HEK-293 cells stably transfected with the h5-HT-transporter at room temperature (18–22°C). After removal of the growth medium, cells were washed and preincubated (for 15 min) with incubation buffer of the following composition: NaCl 125.0, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, HEPES 25.0, Glucose 5.55, Ascorbic acid 1.02, $CaCl_2$ 1.3 and Pargyline 1.0 (in mM). Subsequently, the cells were incubated for 5 min with buffer (main incubation period), containing 10 nM [^{3}H]5-HT (specific activity 1 mCi ml^{-1}, main incubation period). When drugs were tested for inhibition of [^{3}H]5-HT uptake, they were present during a preincubation (15 min) period and the main-incubation (5 min) period. The incubation was terminated by removal of the incubation buffer and rapidly washing the cells with ice-cold drug-free incubation buffer. Thereafter the cells were solubilized in 0.5 ml Triton X-100 (0.1%) and the [^{3}H]5HT content of solubilized cells was determined by liquid scintillation counting. An aliquot of the cell lysate was used for determination of the protein content according to the method of Lowry *et al*. (1951). Specific uptake was defined as uptake (in pmol mg^{-1} protein) inhibited by 1 μM citalopram, a specific 5-HT reuptake inhibitor. The [^{3}H]5-HT uptake in the absence of any drug was taken as control. Eight to 10 experiments were carried out for each concentration in triplicates.

[^{3}H]GR65630 binding

Radioligand binding to membranes from HEK-293 cells stably transfected with the human 5-HT$_{3A}$ receptor cDNA were performed as described by us before (Barann *et al*., 2002).

Data analysis

Data analysis was performed with pClamp 8 software (Axon Instuments, Foster City, CA, U.S.A.). Graph Pad Prism 3.03 software (Graph Pad, California, U.S.A.) was used to create graphics. The concentration–response curves for 5-HT were fitted by the Hill equation,

$$i = 1 - [EC_{50}^n/(c^n + IC_{50}^n)]$$

where i is the initial peak current as fraction of the maximal (control) current, c is the 5-HT concentration, n is the Hill coefficient and EC_{50} is the 5-HT concentration inducing the half-maximal effect. Potencies (e.g. in Table 1) are expressed as pEC_{50} or pIC_{50} values plus standard error as calculated by Prism 3.03. This equation was analogously applied to determine the metoclopramide inhibition of 5-HT response. The 5-HT (30 μM)-induced currents were analyzed as follows (clampfit 8.2 software, Axon): peak currents were determined with a cursor; the time constants of activation (τ_{ON}) and inactivation (τ_{OFF}) were fitted to the current response by separate mono-exponential fits. The integrated current responses were calculated as 'areas over the current curves'. Differences between single data points were tested for significance with the F-test (Excel) or the *t*-test (prism 4.03). Differences were considered significant when *P*-values for the respective test were <0.05. Results are means ± standard deviation.

Drugs and solutions

Citalopram, 5-hydroxytryptamine creatinine sulfate, metoclopramide (monochloride) and pargyline were obtained from Sigma (München, Germany). Ergotamine tartrate was obtained from Tocris (Ellisville, U.S.A.). [^{3}H]5-HT was obtained from NEN (Boston, U.S.A.). [^{3}H]GR65630 (specific activity 64.8 Ci mmol^{-1}) was obtained from NEN DuPont (Dreieich, Germany).

Table 1 Parameters of the Hill equations for metoclopramide inhibition

Application mode	*Hill coefficient*	*pIC_{50} ± s.e.*[a]
Peak currents		
Equilibrium	1.2	7.20 ± 0.06
Closed channel	1.2	7.22 ± 0.03
Open channel	1.0	4.72 ± 0.19
Integrated current responses		
Equilibrium	0.6	7.12 ± 0.35
Closed channel	0.6	7.14 ± 0.24
Open channel	0.8	4.81 ± 10.17

[a]Calculated by prism 3.03.
Comparison of the parameters derived by Hill equations for the concentration–response curves of metoclopramide-induced inhibition of 5-HT (30 μM, −100 mV)-evoked peak currents and integrated current responses mediated by h5-HT$_{3A}$ receptors stably transfected in HEK-293 cells. The following drug application modes were used: equilibrium application, ++: metoclopramide was applied 60 s before plus during the 2 s 5-HT pulse; closed channel application, +−: application exclusively 60 s before 5-HT; open channel application, −+: application exclusively together with 5-HT.

5-HT and metoclopramide solutions were prepared daily from aqueous stock solutions (containing 50 and 10 mM, respectively). Ergotamine solutions were prepared daily from 10 mM stock in DMSO; the final DMSO concentration was $<0.01\%$, which was tested to have no effect on currents through 5-HT$_3$-receptors nor on the function of the 5-HT-transporter. Due to its low solubility and the vehicle used, ergotamine concentrations higher than 30 μM could not be prepared. All stocks were stored at -20°C.

Results

5-HT-induced currents

Application of 5-HT to excised patches for 2 s induced concentration-dependent inward currents ($EC_{50} = 10.3\ \mu$M; Hill coefficient = 1.5; Figure 1). The onset time constant of 5-HT (30 μM)-induced currents was 12 ± 6 and the offset time constant was 240 ± 179 (ms $\pm$ s.d., $n = 120$ different patches). We observed a slight effect of the duration of the 30 μM 5-HT pulse on the offset time constant. 1000 and 50 ms 5-HT (30 μM) pulses induced currents with time constants of current decay of 311 ms $\pm$ 141 (s.d.) and 269 ms $\pm$ 113 (s.d.), respectively, when current traces from seven patches (50 determinations) were compared. This effect was significant when a paired *t*-test (comparing traces from respective patches and sampling time) was performed ($P < 0.0001$).

Metoclopramide effects on 5-HT currents

Application of 0.01 to 10 μM metoclopramide in the absence of 5-HT did not induce any current in patches that subsequently responded to 30 μM 5-HT. Metoclopramide inhibited 5-HT (30 μM)-induced peak currents in a concentration-dependent manner. Figure 2 illustrates concentration–response curves of metoclopramide-induced inhibition. These measurements were carried out using three drug application modes: equilibrium, open-channel, and closed-channel application (see Methods and legend to Table 1). Following wash-periods of at least 60 s, the 5-HT-induced currents were recovered to the respective control values at the beginning of the experiment.

The concentration–response curves of peak current suppression by metoclopramide using the three application modes (described above) could be fitted to Hill equations (Table 1). When metoclopramide was applied using closed-channel application ($+-$), the inhibitory potency was almost identical to that obtained under equilibrium application (IC_{50} values: 0.060 and 0.064 μM, respectively). In contrast, when metoclopramide was applied using the open channel-application ($-+$), the resulting inhibitory potency was lower (IC_{50} approximately 19 μM).

When the measured current responses were integrated (area over the curves), the resulting potencies and their dependency on the drug application-mode were similar to those obtained for peak-current responses (Table 1). However, the steepness (Hill coefficient) of the concentration–response curves using either closed-channel or equilibrium application was higher when the currents were analyzed as peak-currents.

Figure 3a shows the effects of metoclopramide (0.05 μM) on currents through one single patch using the different application modes described above. As an additional effect of metoclopramide, the onset time constant (monoexponential fit) of 5-HT-evoked currents ($\tau_{on} = 12$ ms in control currents) was significantly prolonged when the drug was either applied continuously before and during the agonist pulse or when it was applied exclusively before the 5-HT pulse (Figure 3b). The effect of metoclopramide on the kinetics of current activation was concentration-dependent and it was not observed when the drug was applied exclusively during the 5-HT pulse ($-+$, open channel application; Figure 3a).

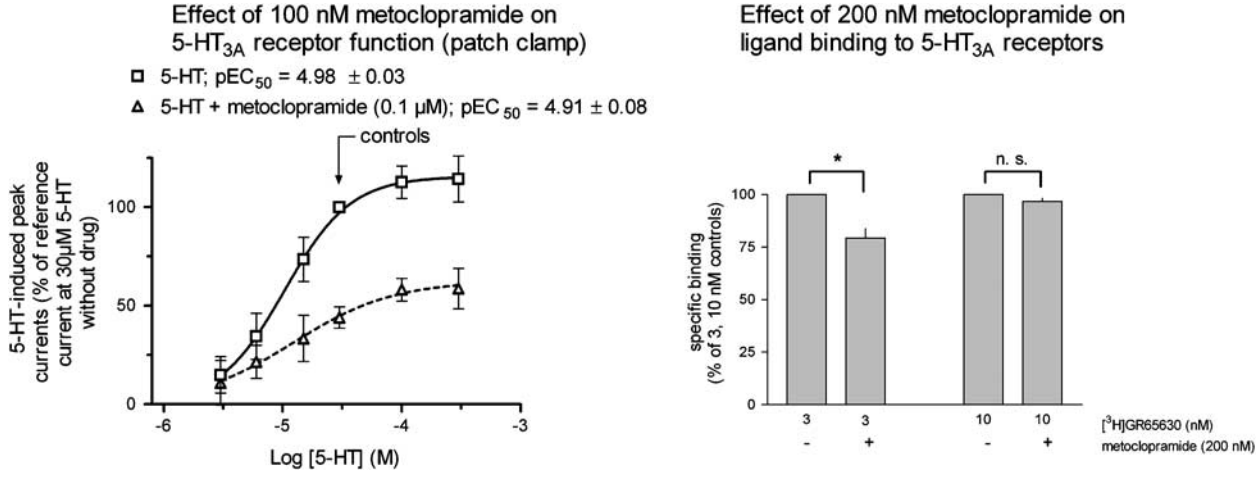

Figure 1 Left: concentration–response curves (-100 mV) of 5-HT at h5-HT$_{3A}$ receptors stably transfected in HEK-293 cells in the absence (squares) and presence (triangles) of metoclopramide (100 nM; applied in equilibrium mode, $++$). The data are normalized to the currents evoked by 30 μM 5-HT in the absence of metoclopramide (= controls). Apparently, the inhibition was unsurmountable by 5-HT. Shown are means $\pm$ s.d. of 3–10 different patches. Right: effect of metoclopramide (200 nM) on [^{3}H]GR65630 (3, 10 nM) binding. Apparently, the moderate but significant ($P < 0.01$, paired *t*-test) inhibition of 3 nM [^{3}H]GR65630 binding was surmountable by higher concentrations of [^{3}H]GR65630. Unspecific binding was determined by addition of 100 μM MDL72222 and substracted under each experimental condition. Means $\pm$ s.d. of three different experiments, performed in duplicates are shown.

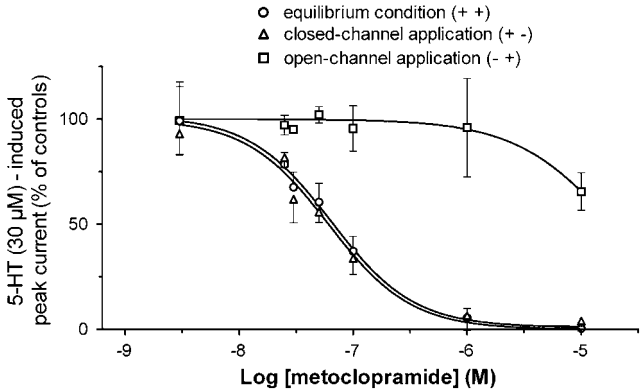

Figure 2 Inhibitory effects of metoclopramide on 5-HT (30 μM; −100 mV)-induced peak currents in excised patches of HEK-293 cells stably transfected with h5-HT$_{3A}$ receptors. The following drug application modes were used (see also legend to Table 1): equilibrium mode (+ +), closed channel mode (+ −), open channel mode (− +). The preapplication of metoclopramide to the patch (either in + + or + − mode) was a prerequisite of the full inhibitory potency (see also Table 1). Means ± s.d. of $n \geqslant 4$ experiments are shown.

In an additional series of experiments, the effect of metoclopramide (0.1 μM) on the concentration–response curve of 5-HT was determined to investigate whether the mechanism of block was of competitive nature (Figure 1). For this purpose, excised patches were exposed to different serotonin concentrations in the absence and in the presence of 0.1 μM metoclopramide (applied under equilibrium conditions). The 5-HT (30 μM)-evoked peak currents recorded in the absence of metoclopramide were defined as control values (see Figure 1). Metoclopramide changed only slightly the EC_{50}-value of 5-HT at human 5-HT$_{3A}$ receptors (10.4 μM *versus* 12.3 μM 5-HT), and the extent of inhibition by metoclopramide remained unchanged over a broad agonist concentration range (6–300 μM 5-HT), resulting in a reduced maximum response to 5-HT. The Hill factor of the concentration–response curves in the absence and presence of metoclopramide varied from 1.6 to 1.1, respectively.

To examine the onset (wash in) and offset (wash out) of the metoclopramide effects we pre-exposed patches to metoclopramide (0.1 μM) using different durations (0.1, 0.3, 1, 3, 5 and

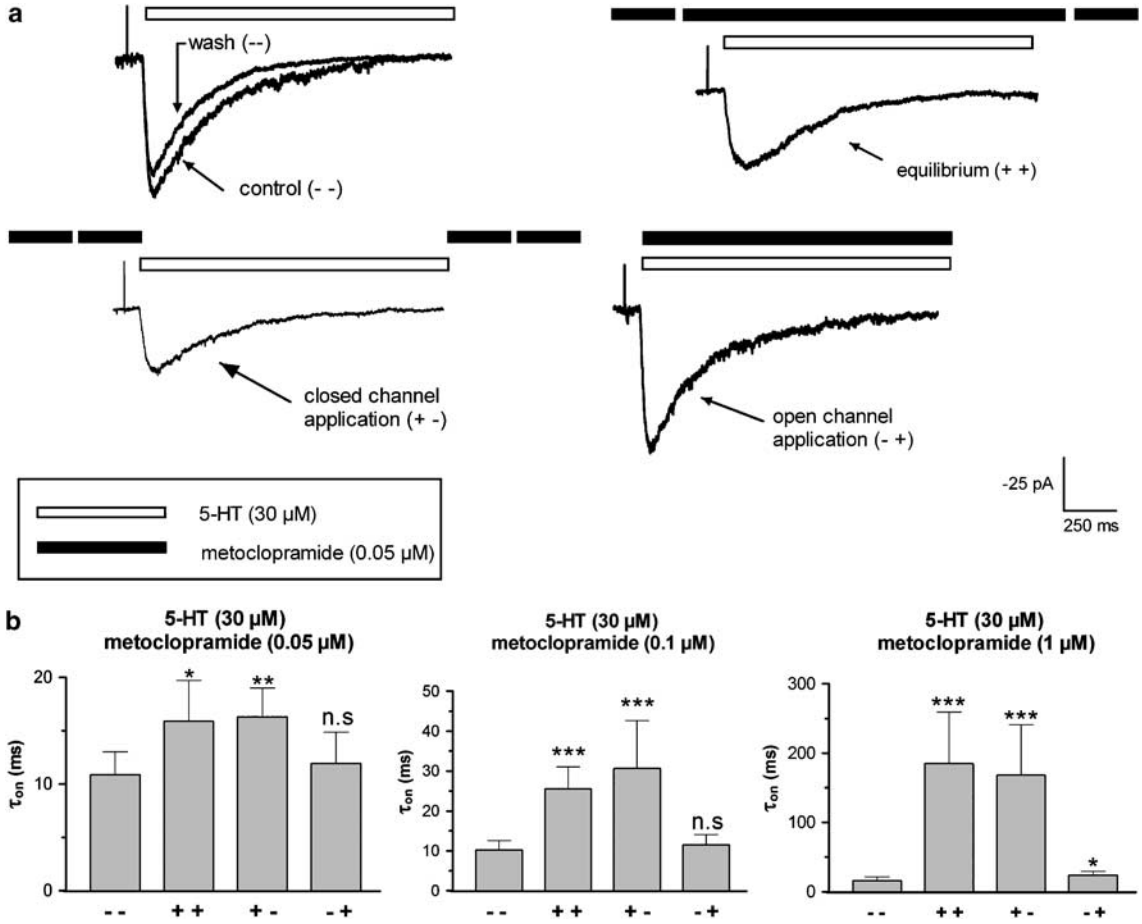

Figure 3 (a) Effect of metoclopramide (50 nM) on 5-HT (30 μM; −100 mV)-induced currents in one excised patch of a HEK-293 cell, stably transfected with h5-HT$_{3A}$ receptors. Results were obtained using three different modes of drug application (equilibrium = + +, closed channel = + −, open channel = − +; see also legend to Table 1). 'Wash' refers to the response to 5-HT 60 s after metoclopramide application. Data were digitally filtered at 500 Hz, shown are traces from one single patch. (b) Effect of metoclopramide on the onset kinetics (monoexponential fit, τ_{ON}) of 5-HT (30 μM; −100 mV)-induced currents in excised outside-out patches of HEK-293 cells, stably transfected with h5-HT$_{3A}$ receptors. Note the different scaling of the *Y*-axis. Obviously, a concentration-dependent metoclopramide-induced retardation of the current-onset (activation) is present when the drug is applied prior to the agonist (either in + + or + − mode). This effect is significant when compared to the control experiments in the absence of metoclopramide (***, $P<0.001$; **, $P<0.01$; *, $P<0.05$). Means ± s.d. of $n \geqslant 4$ experiments are shown. The effect is only modest at highest metoclopramide concentrations when the drug is applied exclusively during the 5-HT pulse (− + mode).

60 s) of application time before the agonist pulse (Figure 4). The time-constant of drug onset was $\tau_{on} = 1.3$ s (mono-exponential fit). After 60 s exposure to metoclopramide, we applied drug-free buffer to patches for various durations. The wash out time constant was $\tau_{off} = 2.1$ s (Figure 4; mono-exponential fit). Calculating the rate constants k_{OFF} (0.48 s^{-1}) and k_{ON} ($7.7 \times 10^6\ s^{-1}\ M^{-1}$) from these time constants, a K_D for metoclopramide of 62 nM was determined, which is nearly identical to the IC_{50}. The calculation of the kinetic K_D assumes that the action of metoclopramide is independent of the state of the channel.

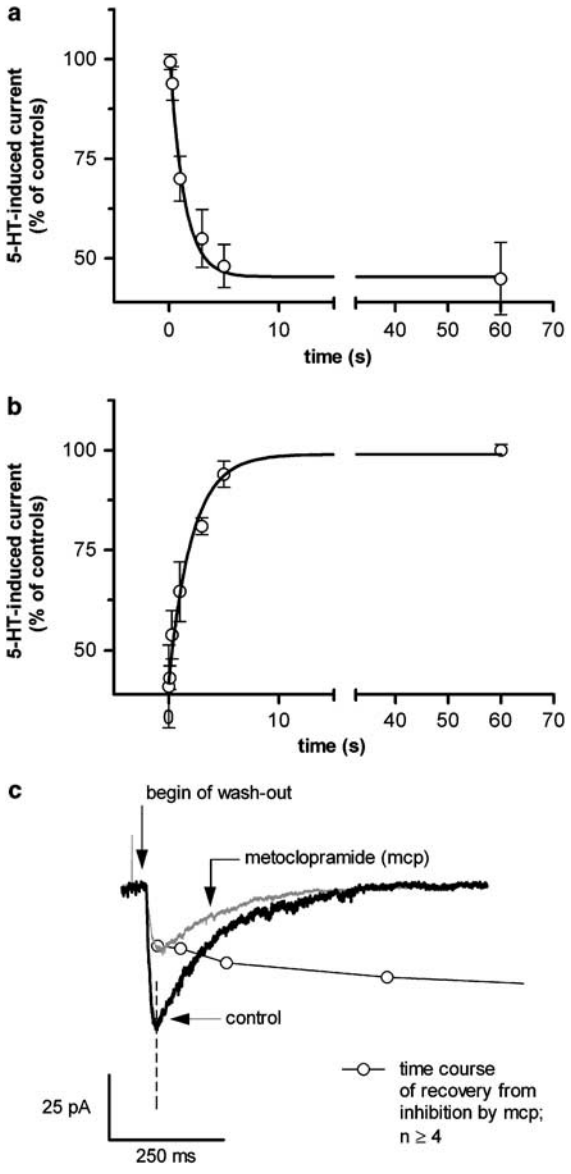

Figure 4 (a) The amplitude of 5-HT (30 μM; −100 mV)-induced peak currents through h5-HT$_{3A}$ receptor channels as a function of the duration of metoclopramide (100 nM) application prior to the 5-HT pulse (wash-in). (b) The amplitude of 5-HT-induced peak currents through h5-HT$_{3A}$ receptor channels as a function of the duration of removal of metoclopramide (wash-out). Monoexponential fits with time constants of 1.3 and 2.1 s, respectively are shown; means ± s.d. of $n \geq 4$ experiments. (c) Comparison of recovery (open circles) from inhibition by metoclopramide (100 nM applied for 60 s, upper trace) and kinetics of the respective control current induced by 5-HT (30 μM, lower trace).

Ergotamine effects on 5-HT-induced currents

Application of 0.001–30 μM ergotamine in the absence of 5-HT did not induce any current in patches that subsequently responded to 30 μM 5-HT. Figure 5 illustrates concentration–response curves for ergotamine effects on 5-HT (30 μM)-induced peak currents when the drug was applied in equilibrium (++, see Table 1). Low concentrations of 1–10 nM ergotamine had no effect. Above 3 μM, ergotamine caused an inhibition (Figure 5), but a complete concentration–response curve could not be established (concentrations >30 μM could not be prepared, see Methods). The inhibition of peak currents was reversible after washout from the drug for at least 60 s. All three application modes (Table 1) of ergotamine to the patches were used, similar to the metoclopramide experiments. However, in contrast to metoclopramide, no dependence of ergotamine effects on the application mode was found (not shown). Thus, the effects of ergotamine on currents evoked by 30 μM 5-HT differ from that of metoclopramide in two aspects: (1) an inhibition was observed only at high concentrations (>3 μM), and (2) no dependence on the drug application mode was detected.

Ergotamine and metoclopramide administered together

Since metoclopramide and ergotamine are used in combination during migraine therapy, both drugs were administered simultaneously to patches in an additional series of experiments with the standard concentration of 5-HT (30 μM). A clinical metoclopramide concentration of 100 nM (close to its IC_{50}) was chosen, while for ergotamine the clinically relevant low range (0.001 and 0.01 μM) was selected. The inhibition of currents were compared to the effects of 100 nM metoclopramide administered in the absence of ergotamine. The inhibition by metoclopramide was comparable to the effects of 100 nM metoclopramide administered alone (see Figure 6).

Effects of metoclopramide on [^{3}H]GR65630 binding

In competition binding experiments, 5-HT inhibited concentration-dependently the specific binding of the selective 5-HT$_3$ receptor ligand [^{3}H]GR65630 to membranes of HEK 293 cells

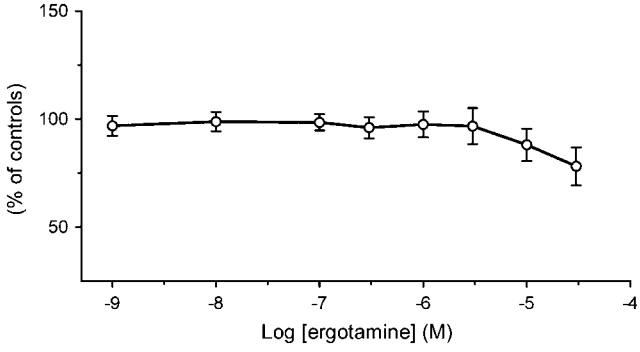

Figure 5 Effects of ergotamine, applied in equilibrium (++, see Table 1) on 5-HT (30 μM; −100 mV)-induced currents through h5-HT$_{3A}$ receptor channels. Peak–current responses are shown. Means ± s.d. of $n \geq 4$ experiments are shown.

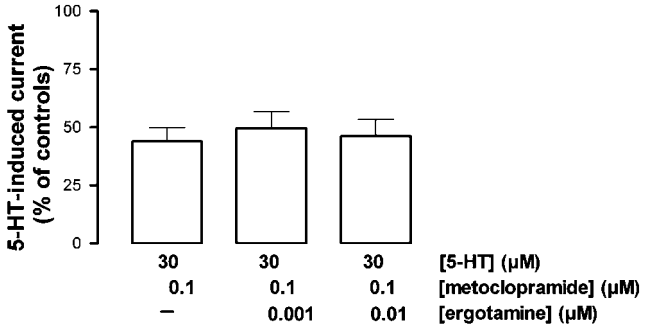

Figure 6 Inhibition by metoclopramide (0.1 μM) of 5-HT (30 μM; −100 mV)-induced peak currents, mediated by h5-HT$_{3A}$ receptors in the absence and in the presence of ergotamine (0.001, 0.01 μM). Both drugs were applied 60 s prior plus during the 5-HT pulse (=equilibrium condition, ++). Means±s.d. of $n=7$ experiments are shown.

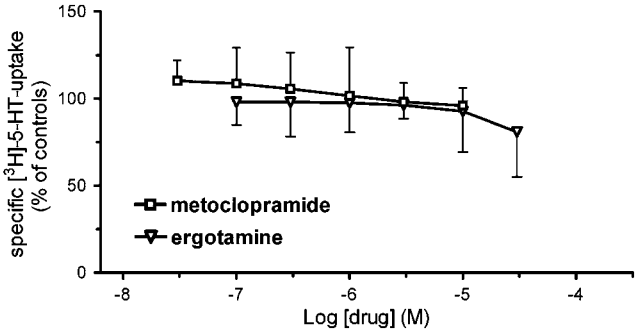

Figure 7 Metoclopramide and ergotamine effects on the h5-HT carrier-mediated [^{3}H]5-HT uptake (10 nM; 5 min) in stably transfected HEK-293 cells. The specific signal was calculated as difference between the uptake in the absence and presence of citalopram (1 μM). Shown are means±s.d. of 8–10 different experiments.

stably transfected with the h5-HT$_{3A}$ receptor cDNA (see Figure 5 in Barann *et al*., 2002). Metoclopramide (0.2 μM) significantly ($P<0.01$, paired *t*-test) reduced this specific [^{3}H]GR65630 (3 nM) binding by 21% ($n=3$ in triplicates, see Figure 1, right panel). The same metoclopramide concentration remained without significant effect when the [^{3}H]GR65630 concentration was raised to 10 nM, indicating that the specific inhibition was surmountable.

Effects on 5-HT reuptake carrier

To study whether the function of the 5-HT-transporter was modified by metoclopramide or ergotamine at concentrations that affected h5-HT$_{3A}$ receptors, we performed [^{3}H]5-HT reuptake assays. Figure 7 illustrates that the [^{3}H]5-HT uptake was not significantly inhibited over a broad concentration range. Only at very high concentrations, ergotamine (30 μM) reduced the citalopram-sensitive [^{3}H]5-HT uptake into HEK-293 cells.

Discussion

A major aim of the present study was to characterize pharmacologically the interactions of metoclopramide and ergotamine with human 5-HT$_{3A}$ receptors. As these receptors mediate emesis and pain (Aapro, 1991; Voog *et al*., 2000), their response to this common antimigraine drug combination may be relevant in addition to its well-characterized interaction with dopamine D$_2$ receptors. Effects of metoclopramide and ergotamine on the 5-HT reuptake carrier were also investigated; they may become relevant as the carrier regulates the extracellular and plasma serotonin concentration which is subject to sudden changes at the onset of a migraine attack (for a review, see Hamel, 1999).

The receptors studied here were pharmacologically and biophysically characterized in our previous work using identical experimental setups (Barann *et al*., 2000a; 2002).

Effects of metoclopramide on human 5-HT$_{3A}$ receptors

Concentrations of metoclopramide that inhibited 5-HT-induced currents in a concentration-dependent manner (Figures 1 and 2) are similar to those obtained during therapy (30–60 μg l^{-1} in blood, equivalent to 0.1–0.2 μM, McEvoy, 1992). Metoclopramide binds only moderately to plasmaproteins (13–30%). High levels of metoclopramide are reached within the area postrema, a region which contains 5-HT$_3$ receptors at high density (McEvoy, 1992). Although the drug is not much metabolized and 20–25% are eliminated unchanged, the existence of an active metabolite is considered a possibility (Fichtl *et al*., 1998). Since both peak current and integrated current responses were suppressed by metoclopramide with similar potency (Table 1), peak current suppression is sufficient to calculate the overall potency. The result that a preapplication of metoclopramide is necessary to obtain the full inhibitory potency implies that the effect of metoclopramide is slower than the typical opening and closing kinetics of 5-HT$_3$ receptor currents. This is confirmed by the determination of the exact time courses of onset and offset of inhibition by metoclopramide.

5-HT concentration–response curves recorded in the absence and in the presence of 0.1 μM metoclopramide had similar EC$_{50}$ values (Figure 1), while the maximum of the control curve was halved. This result is in contrast to a competitive interaction of metoclopramide at the 5-HT binding site(s) suggested by radioligand binding studies (Hamik & Peroutka, 1989; Hirokawa *et al*., 2003). In order to rule out species differences we performed, in addition to the functional studies, radioligand binding experiments; we confirmed binding competition between metoclopramide and the selective 5-HT$_3$ receptor antagonist [^{3}H]GR65630 in our receptor preparation. This seeming discrepancy between functional studies and radioligand binding experiments may be accounted for by (1) the slow kinetics of metoclopramide compared with the fast onset and offset kinetics of 5-HT-induced currents (for example, the wash out of pre-equilibrated metoclopramide is 150 times slower than the onset of 5-HT-induced currents, see Figure 4, lower panel) and (2) the low efficacy of metoclopramide in inhibiting radioligand binding (e.g. only 20% binding inhibition compared to 79% peak current suppression by 200 nM metoclopramide).

Although the exclusive analysis of peak current suppression has difficulty in detecting competitive action of metoclopramide at the 5-HT binding sites, a closer inspection of the

patch-clamp data shows a concentration–dependent prolongation of current onset when metoclopramide was applied to the receptor prior to 5-HT exposure (Figure 3b). This then is consistent with some competitive displacement of the drug by 5-HT also in the functional study.

Therefore, persuasive evidence that metoclopramide inhibits radioligand binding of specific 5-HT$_3$ receptor antagonists and contrasting reports of 5-HT$_3$ antagonists acting *via* noncompetitive mechanisms (for a review, see Costall & Naylor, 1999) need not be a contradiction. As the discussion above shows it may simply reflect different functional endpoints being examined by different experimental methods.

Previously it had already been reported that metoclopramide did not only show affinity for dopamine receptors (Imafuku, 1987) but also – to a lesser extent – for 5-HT$_3$ receptors of different species (Barann *et al*., 1993). The IC$_{50}$ values reported in the present study are similar to results from functional studies on murine 5-HT$_3$ receptors (Gill *et al*., 1995), and human 5-HT$_3$ receptors on whole cells (Belelli *et al*., 1995; Brown *et al*., 1998; Lankiewicz *et al*., 1998). Comparisons of affinities of metoclopramide to dopamine D$_2$- and 5-HT$_3$ receptors are inconclusive, some studies report equal or even higher potencies at 5-HT$_3$ receptors (Hamik & Peroutka, 1989; Hirokawa *et al*., 2003).

The low IC$_{50}$ value reported here supports the conclusion that metoclopramide is at least as potent on human 5-HT$_3$ receptors as on dopamine D$_2$ receptors. Effects of metoclopramide on 5-HT$_3$ receptors have not only been suggested to contribute to its antiemetic potency but also to result in some intrinsic migraine-releaving potency (Schwarzberg, 1994). However, the studies on a potential role of 5-HT$_3$ receptor antagonists for the treatment of migraine remained controversial in the early 1990s (Couturier *et al*., 1991; Chapell *et al*., 1994; for a review, see Ferrari, 1991); today, this group of drugs is no longer considered for use as a single migraine medication.

Effects of ergotamine on human 5-HT$_{3A}$ receptors

The results point to fundamental differences between the actions of ergotamine and metoclopramide: in contrast to the potency of metoclopramide (IC$_{50}$ = 60 nM) a significant inhibition by ergotamine was observed only at concentrations $\geqslant 30\,\mu$M. A faster onset of ergotamine inhibition when compared to metoclopramide was suggested by the finding that ergotamine inhibition was independent on whether or not the drug was present before the agonist application. Thus, depending on its concentration, ergotamine either weakly inhibits or does not affect 5-HT-induced peak currents. Ergotamine is known to be a drug with many effects on different transmitter systems (for a review, see Tfelt-Hansen *et al*., 2000; Silberstein & McCrory, 2003), including dopamine receptors, several 5-HT receptors and adrenoceptors (Leysen & Gommeren, 1984).

However, in contrast to the metoclopramide-induced inhibition, the ergotamine effect discussed here occurs well above clinical plasma concentrations (0.1–10 nM, Maassen-VanDenBrink *et al*., 1997). The oral bioavailability of ergotamine is low (<5%) when compared to the intramuscular application (30–60%). Metabolism of ergotamine is dependent on cytochrome *P*450 complex and active metabolites are unknown (Tfelt-Hansen *et al*., 2000). As the human 5-HT$_{3A}$ receptor is most likely to be unaffected by ergotamine during migraine therapy, it remains fully functional within a neural network *mediating* emesis (Barnard, 2002).

Effects of metoclopramide in combination with ergotamine on human 5-HT$_{3A}$ receptors (patch clamp)

The inhibition of human 5-HT$_{3A}$ receptors by metoclopramide (0.1 μM) was unaffected by low (clinical) ergotamine concentrations (0.001–0.01 μM) and thus it will still be present during combined therapy. It is important to note that the metoclopramide concentration of 0.1 μM used here is similar to the one resulting from a typical *in vivo* dose when used in combination with ergot alkaloid treatment (10 mg (Edwards *et al*., 2001; Silberstein & McCrory, 2003) resulting in $\sim 60\,\text{ng ml}^{-1}$ which is equal to $\leqslant 0.2\,\mu$M when 20% plasma albumin binding is assumed).

Effects of metoclopramide and ergotamine on [^{3}H]5-HT uptake

We tested the effects of both drugs on the citalopram-sensitive [^{3}H]5-HT uptake over a broad concentration range (0.03–10 μM; Figure 7). The rationale for these experiments was the following: changes in the available 5-HT concentration might modulate effects on 5-HT$_3$ receptors observed when patients are treated with metoclopramide plus ergotamine. For example, if ergotamine inhibited the carrier at low concentrations, this would increase the plasma 5-HT levels (the carrier is expressed at high densitiy on platelets) and this could affect the function of 5-HT$_3$ receptors, which mediate emesis. Such a situation is described as 'serotonin syndrom' during an intoxication by selective serotonin reuptake inhibitors like citalopram. In addition, it is reported that during a migraine attack sudden changes of 5-HT plasma levels occur (Hamel, 1999), which the intact reuptake carrier is expected to buffer. In addition, any suppression of the transporter by metoclopramide would counteract its 5-HT$_3$ receptor antagonism. Our results suggest that the pharmacological mechanisms of metoclopramide and ergotamine do not directly involve the 5-HT reuptake carrier, ruling out the above-mentioned actions.

Summary and conclusions

The results indicate that metoclopramide at clinical plasma concentrations is a potent antagonist of human 5-HT$_3$ receptors, which is compatible with its use as an antiemetic. The inhibition by metoclopramide is unaffected by clinical concentrations of ergotamine, thus this effect will be present during the combined therapy with both drugs. In addition, metoclopramide does not suppress the function of the 5-HT reuptake carrier, which otherwise could have counteracted its antiemetic potency. In contrast to metoclopramide, ergotamine did not alter the function of 5-HT$_{3A}$ receptors at clinical concentrations.

This study was supported by the Deutsche Forschungsgesellschaft (BA 1454). We thank Dr H Bönisch for supplying us with HEK-293 cells, stably transfected with human 5-HT reuptake carriers. We thank Ms I Linden for some experimentation.

References

AAPRO, M.S. (1991). 5-HT$_3$ receptor antagonists. An overview of their present status and future potential in cancer therapy-induced emesis. *Drugs,* **42,** 551–568.

BARANN, M., DILGER, J.P., BÖNISCH, H., GÖTHERT, M., DYBEK, A. & URBAN, B.W. (2000b). Inhibition of 5-HT$_3$ receptors by propofol: equilibrium and kinetic measurements. *Neuropharmacology,* **39,** 1064–1074.

BARANN, M., FRIEDERICH, P., RETZMANN, K., DYBEK, A. & URBAN, B.W. (2000c). Loss of propofol during *in-vitro* experiments. *Anesthesiology,* **93,** 309–310.

BARANN, M., GÖTHERT, M., FINK, K. & BÖNISCH, H. (1993). Inhibition by anaesthetics of ^{14}C-guanidinium flux through the voltage-gated sodiumchannel and the cation channel of the 5-HT$_3$ receptor of N1E-115 neuroblastomacells. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **347,** 125–132.

BARANN, M., MEDER, W., DORNER, Z., BRÜSS, M., BÖNISCH, H., GÖTHERT, M. & URBAN, B.W. (2000a). Recombinant human 5-HT$_{3A}$ receptors in outside-out patches of HEK293 cells: basic properties and barbiturate effects. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **362,** 255–265.

BARANN, M., MOLDERINGS, G., BRÜSS, M., BÖNISCH, H., URBAN, B.W. & GÖTHERT, M. (2002). Direct inhibition by cannabinoids of human 5-HT$_{3A}$ receptors: probable involvement of an allosteric modulatory site. *Br. J. Pharmacol.,* **137,** 589–596.

BARNARD, E.A. (2002). The range of channel structures and their potential sites for anesthetic interaction. In: *Molecular and Basic Mechanisms of Anesthesia*, eds. Urban, B.W. & Barann, M., pp. 44–51. Lengerich: Pabst Science Publishers.

BELELLI, D., BALCAREK, J.M., HOPE, A.G., PETERS, J.A., LAMBERT, J.J. & BLACKBURN, T.P. (1995). Cloning and functional expression of human 5-Hydroxytryptamine type 3AS receptor subunit. *Mol. Pharmacol.,* **48,** 1054–1062.

BROWN, A.M., HOPE, A.G., LAMBERT, J.J. & PETERS, J.A. (1998). Ion permeation and conduction in a human recombinant 5-HT$_3$ receptor subunit (h5-HT$_{3A}$). *J. Physiol.,* **507,** 653–665.

BUZZI, M.G. & MOSKOWITZ, M.A. (1991). Evidence for 5-HT1B/1D receptors mediating the antimigraine effect of sumatriptan and dihydroergotamine. *Cephalalgia,* **11,** 165–168.

CARPENTER, O., BRIGGS, D.B., KNOX, A.P. & STROMINGER, N. (1988). Excitation of area postrema neurons by transmitters, peptides, and cyclic nucleotides. *J Neurophysiol,* **59,** 358–369.

CHAPELL, A.S., BAY, J.M., BOTZUM, G.D. & COHEN, M.L. (1994). Zatosetron, a 5-HT$_3$ receptor antagonist in a multicenter trial for acute migraine. *Neuropharmacology,* **33,** 509–513.

CHEN, C. & OKAYAMA, H. (1987). High-efficacy transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.,* **7,** 2745–2752.

COSTALL, B. & NAYLOR, R.J. (1999). Neuropharmacology of 5-HT$_3$ receptor ligands. In: *Serotoninergic Neurons and 5-HT Receptors in the CNS*, eds. Baumgarten, H.G. & Göthert, M., pp. 409–438. Berlin: Springer Science Publishers.

COUTURIER, E.G., HERING, R., FOSTER, C.A., STEINER, T.J. & CLIFFORD ROSE, F. (1991). First clinical study of the selective 5-HT3 antagonist, granisetron BRL 43694), in the acute treatment of migraine headache. *Headache,* **31,** 296–297.

CUNNINGHAM, D., HAWTHORN, J., POPLE, A., GAZET, J.C., FORD, H.T., CHALLONER, T. & COOMBES, R.C. (1987). Prevention of emesis in patients receiving cytotoxic drugs by GR38032F, a selective 5-HT3 receptor antagonist. *Lancet,* **27,** 1461–1463.

EDWARDS, K.R., NORTON, J. & BEHNKE, M. (2001). Comparison of intravenous valproate *versus* intramuscular dihydroergotamine and metoclopramide for acute treatment of migraine headache. *Headache,* **41,** 976–980.

FERRARI, M.D. (1991). 5-HT$_3$ receptor antagonists and migraine therapy. *J. Neurol.,* **238** (Suppl 1), S53–S56.

FICHTL, B., FÜLGRAFF, G., NEUMANN, H.G., WOLLENBERG, P., FORTH, W., HENSCHLER, D. & RUMMEL, W. (1998). Allgemeine Pharmakologie und Toxikologie. In: *Allgemeine und spezielle Pharmakologie und Toxikologie*, ed. Forth, W., Henschler, D., Rummel, W. & Starke, K., 7th edn, pp. 3–102. Heidelberg: Spektrum akademischer Verlag.

FOZARD, J.R. & MOBAROK ALI, A.T. (1978). Blockade of neuronal tryptamine receptors by metoclopramide. *Eur. J. Pharmacol.,* **49,** 109–112.

GILL, C.H., PETERS, J.A. & LAMBERT, J.J. (1995). An electrophysiological investigation of the properties of a murine recombinant 5-HT$_3$ receptor stably expressed in HEK-293 cells. *Br. J. Pharmacol.,* **114,** 1211–1221.

HADDJERI, N., SELETTI, B., GILBERT, F., DE MONTIGNY, C. & BLIER, P. (1998). Effect of ergotamine on serotonin-mediated responses in the rodent and human brain. *Neuropsychopharmacology,* **19,** 365–380.

HAMEL, E. (1999). The biology of serotonin receptors: focus on migraine pathphysiology and treatment. *Can. J. Neurol. Sci.,* **26** (Suppl. 3), S2–S6.

HAMIK, A. & PEROUTKA, S.J. (1989). Differential interactions of traditional and novel antiemetics with dopamine D$_2$ and 5-hydroxytryptamine$_3$ receptors. *Cancer Chemother. Pharmacol.,* **24,** 307–310.

HIROKAWA, Y., FUJIWARA, I., SUZUKI, K., HARADA, H., YOSHIKAWA, T., YOSHIDA, N. & KATO, S. (2003). *N*-(1-ethyl-4-methylhexahydro-1,4-diazin-6-yl)pyridine-3-carboxamides with potent serotonin 5-HT$_3$ and dopamine D$_2$ receptor antagonistic activity. *J. Med. Chem.,* **46,** 702–715.

HOYER, D., GOZLAN, H., BOLANOS, F., SCHLECHTER, L.E. & HAMON, M. (1989). Interaction of psychotropic drugs with central 5-HT$_3$ recognition sites: fact or artifact? *Eur. J. Pharmacol.,* **171,** 137–139.

IMAFUKU, J. (1987). The characterization of [^{3}H]sulpiride binding sites in rat striatal membranes. *Brain Res.,* **402,** 331–338.

LANKIEWICZ, S., LOBITZ, N., WETZEL, H.R., RUPPRECHT, R., GISSELMAN, G. & HATT, H. (1998). Molecular cloning, functional expression, and pharmacological characterization of 5-hydroxytryptamine$_3$ receptor cDNA and its splice variants from guinea pig. *Mol. Pharmacol.,* **53,** 202–212.

LARSON, B.T., SAMFORD, M.D., CAMDEN, J.M., PIPER, E.L., KERLEY, M.S., PATERSON, J.A. & TURNER, J.T. (1995). Ergovaline binding and activation of D$_2$ dopamine receptors in GH4ZR7 cells. *J. Anim. Sci.,* **73,** 1396–1400.

LEYSEN, J.E. & GOMMEREN, W. (1984). The dissociation rate of unlabelled dopamine antagonists and agonists from the dopamine-D$_2$ receptor, application of an original filter method. *J. Recept. Res.,* **4,** 817–845.

LIU, Y. & DILGER, J.P. (1991). Opening rate of acetylcholine receptor channels. *Biophys. J.,* **60,** 424–432.

LOVINGER, D.M. (1991). Ethanol potentiation of 5-HT$_3$ receptor-mediated ion current in NCB-20 neuroblastoma cells. *Neurosci Lett.,* **122,** 57–60.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

MAASSENVANDENBRINK, A., REEKERS, M., BAX, W.A., FERRARI, M.D. & SAXENA, P.R. (1997). Coronary side-effect potential of current and prospective antimigraine drugs. *Circulation,* **98,** 25–30.

MACHU, T.K. & HARRIS, R.A. (1994). Alcohols and anesthetics enhance the function of 5-hydroxytryptamine3 receptors expressed in *Xenopus laevis* oocytes. *J. Pharmacol. Exp. Ther.,* **271,** 898–905.

MCEVOY, G.K. (1992). *Clin Pharm Drug Data Base: AHFS Drug Information*. Bethesda (U.S.A.): American Society of Hospital Pharmacists Inc., 2363pp.

MINAMI, M., ENDO, T., YOKOTA, H., OGAWA, T., NEMOTO, M., HAMAUE, N., HIRAFUJI, M., YOSHIOKA, M., NAGAHISA, A. & ANDREWS, P.L. (2001). Effects of CP-99, 994, a tachykinin NK(1) receptor antagonist, on abdominal afferent vagal activity in ferrets: evidence for involvement of NK(1) and 5-HT(3) receptors. *Eur. J. Pharmacol.,* **428,** 215–220.

MOSKOWITZ, M.A. & CUTRER, F.M. (1993). SUMATRIPTAN: a receptor-targeted treatment for migraine. *Annu. Rev. Med.,* **44,** 145–154.

SCHWARZBERG, M.N. (1994). Application of metoclopramide specificity in migraine attacks therapy. *Headache,* **34,** 439–441.

SILBERSTEIN, S.D. & MCCRORY, D.C. (2003). Ergotamine and dihydroergotamine: history, pharmacology, and efficacy. *Headache,* **43,** 144–166.

TFELT-HANSEN, P., SAXENA, P.R., DAHLOF, C., PASCUAL, J., LAINEZ, M., HENRY, P., DIENER, H., SCHOENEN, J., FERRARI, M.D. & GOADSBURY, P.J. (2000). Ergotamine in the acute treatment of migraine: a review and European consensus. *Brain,* **123,** 9–18.

WALKEMBACH, J., URBAN, B.W. & BARANN, M. (2003). Characterization of metoclopramide and ergotamine interaction with human 5-HT$_{3A}$ receptors. *Soc. Neurosci.,* **33,** 798.1.

WOODWARD, R., DANIELL, S.J., STRANGE, P.G. & NAYLOR, L.H. (1994). Structural studies on D_2 dopamine receptors: mutation of a histidine residue specifically affects the binding of a subgroup of substituted benzamide drugs. *J. Neurochem.,* **62,** 1664–1669.

WYNN, R.L., ESSIEN, E. & THUT, P.D. (1993). The effects of different antiemetic agents on morphine-induced emesis in ferrets. *Eur. J. Pharmacol.,* **241,** 47–54.

VOOG, O., ALSTERGEN, P., LEIBUR, E., KALLIKORM, R. & KOPP, S. (2000). Immediate effects of the serotonin antagonist granisetron on temporomandibular joint pain in patients with systemic inflammatory disorders. *Life Sci.,* **68,** 591–602.

(Received April 27, 2005
Revised June 16, 2005
Accepted June 23, 2005
Published online 25 July 2005)