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Agonist actions of dihydroergotamine at 5-HT_{2B} and 5-HT_{2C} receptors and their possible relevance to antimigraine efficacy

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1 The pharmaceutical compound, dihydroergotamine (DHE) is dispensed to prevent and reduce the occurrence of migraine attacks. Although still controversial, the prophylactic effect of this drug is believed to be caused through blockade and/or activation of numerous receptors including serotonin (5-HT) receptors of the 5-HT_2 subtype.

2 To elucidate if 5-HT_2 receptors (5-HT_2Rs) may be involved in DHE prophylactic effect, we performed investigations aimed to determine the respective pharmacological profile of DHE and of its major metabolite 8′-hydroxy-DHE (8′-OH-DHE) at the 5-HT_{2B} and 5-HT_{2C}Rs by binding, inositol triphosphate (IP_3) or cyclic GMP (cGMP) coupling studies in transfected fibroblasts.

3 DHE and 8′-OH-DHE are competitive compounds at 5-HT_{2B} and 5-HT_{2C}Rs. 8′-OH-DHE interaction at (5-HT_{2B}Rs) was best fitted by a biphasic competition curve and displayed the highest affinity with a K_i of 5 nM. These two compounds acted as agonists for both receptors in respect to cGMP production with pEC_{50} of 8.32 ± 0.09 for 8′-OH-DHE at 5-HT_{2B} and 7.83 ± 0.06 at 5-HT_{2C}Rs.

4 Knowing that the antimigraine prophylactic effect of DHE is only observed after long-term treatment, we chronically exposed the recombinant cells to DHE and 8′-OH-DHE. The number of 5-HT_{2B}R-binding sites was always more affected than 5-HT_{2C}Rs. At 5-HT_{2B}Rs, 8′-OH-DHE was more effective than DHE, with an uncoupling that persisted for more than 40 h for IP_3 or cGMP. By contrast, the 5-HT_{2C}R coupling was reversible after either treatment.

5 Chronic exposure to 8′-OH-DHE caused a persistent agonist-mediated desensitisation of 5-HT_{2B}, but not 5-HT_{2C}Rs. This may be of relevance to therapeutic actions of the compound.
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Keywords: 5-HT2; cGMP; desensitisation; DHE; IP3; serotonin; receptors

Abbreviations: cGMP, cyclic GMP; DHE, dihydroergotamine; DOI, (±)1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; IP3, inositol triphosphate; mCPP, meta-chlorophenylpiperazine; NO, nitric oxide; PBS, phosphate buffer saline; 5-HT, serotonin, 5-hydroxytryptamine

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) receptors are highly heterogeneous and they have been regrouped within different families (5-HT_1–5-HT_7). With the exception of the 5-HT_3 receptors, which are ligand-gated ion channels, all others are G-protein-coupled receptors with each family sharing structural, pharmacological and transductional characteristics (Hoyer *et al*., 2002). 5-HT receptors (5-HTRs) have been implicated in the regulation of several psychiatric and neurological disorders related to serotonergic neurotransmission, and specific receptor subtypes have recently been associated with either the pathogenesis or the treatment of migraine headache (Villalon *et al*., 1997).

A role for 5-HT in migraine has been supported by sudden changes in circulating levels of 5-HT and its metabolites observed during the early phases of a migraine attack (Goadsby, 2000), along with the ability of 5-HT-releasing agents to induce migraine-like symptoms (Kalkman, 1994; Panconesi & Sicuteri, 1997). Migraine patients have a systemic disturbance of 5-HT metabolism, associated with low 5-HT plasma levels between attacks and increased levels and release of platelet 5-HT during attacks (Ferrari, 1998). Contrary to earlier belief, the rise of plasma 5-HT is not the cause of migraine but probably represents a self-defence mechanism. Earlier observations showed that injection of 5-HT can abort migraine attacks, but at the cost of significant side effects. These findings led to the hypothesis that selective stimulation of 5-HTRs might safely abort attacks (Ferrari, 1998). The development of 5-HTR agonists with efficacy in the clinic for the alleviation of migraine pain further implicates 5-HT as a key molecule in migraine (Goadsby *et al*., 2002).

Several theories regarding the aetiology of migraine have been proposed (Ferrari, 1998; Johnson *et al*., 1998; Goadsby *et al*., 2002). The vasodilatory theory of migraine suggested that extracranial arterial dilation during an attack was related to migraine pain; a theory supported when vasoconstrictors such as sumatriptan alleviated migraine pain. The neurological theory of migraine proposed that migraine resulted from

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abnormal firing in brain neurons. Cortical spreading depression, one facet of the neurological theory, could explain the prodrome of migraine. The neurogenic dural inflammation theory of migraine supposed that the dural membrane surrounding the brain became inflamed and hypersensitive due to release of neuropeptides from primary sensory nerve terminals. A role for 5-HT is supported by the efficacy in treating acute migraine pain and associated symptoms of 5-HTR ligands such as triptans, which are agonists at 5-HT$_{1D}$ and 5-HT$_{1B}$R subtypes. In addition, the prophylactic effect of several pharmaceuticals frequently dispensed to prevent the occurrence of migraine attacks has been suggested to be caused through blockade of 5-HTRs of the 5-HT$_2$ type (Kalkman, 1994; Panconesi & Sicuteri, 1997). Migraine headache is thought to be transmitted by the trigeminal nerve from the meninges and their blood vessels. Various human meningeal tissues express 5-HT$_{1B/1D}$, 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_4$ and 5-HT$_7$R mRNAs (Schmuck *et al*., 1996). 5-HT$_{2C}$R mRNA was found in neurons involved in the central processing of nociceptive neurotransmission (Molineaux *et al*., 1989).

The ergot alkaloids and their semisynthetic derivatives including dihydroergotamine (DHE) are families of chemical entities that have many different pharmacological effects. This diversity results from their interaction with multiple receptors, their variable receptor affinity and intrinsic activity, and their variable organ-specific receptor access. DHE exhibits alpha-adrenergic antagonist activity with only low arterial vasoconstriction potential. The vasoconstrictor activities of these compounds have long been believed to be the basis of their clinical effects, but recent evidence suggests that its antimigraine action may result in part from their inhibitory effects on neurogenic inflammation and neuronal transmission and in part from vasodilatation inhibition and/or correction (Hoskin *et al*., 1996; Panconesi & Sicuteri, 1997; Goadsby, 2000). The long duration of action of DHE appears to result from its major active metabolite 8′-OH-DHE which is present, in case of oral repeated administration, at a concentration five to seven times greater than DHE (C_{max} for DHE 5–35 nM) and its long half-life (10–13 h) has been proposed to account for the low rate of headache recurrence observed with DHE (Silberstein, 1997). The antimigraine drug DHE produces selective vasoconstriction in the external carotid artery (Villalon *et al*., 1999). Both sumatriptan and DHE are effective in aborting migraine headaches. However, headache recurrence is two and a half time as likely with sumatriptan as with DHE (Winner *et al*., 1996).

To elucidate if 5-HT$_2$Rs could be involved in the long-term antimigraine prophylactic action of DHE *via* its metabolite 8′-OH-DHE, we determined their pharmacological profiles toward the human 5-HT$_{2B}$ and 5-HT$_{2C}$Rs. We, then, investigated the effects of chronic exposure to these compounds of cells expressing these receptors. We observed different responses to these compounds, the 5-HT$_{2B}$Rs being the most responsive and chronically affected by 8′-OH-DHE.

Methods

Materials

DHE and 8′-OH-DHE were obtained from CEA (Saclay, France). A 1 mM stock solution of each of the two compounds was prepared by first dissolving in 200 μl ethanol and then making up the volume with distilled water. The subsequent dilutions were performed in the binding buffer. All other chemicals of the purest grade available were from classical commercial sources. [^{125}I]DOI (81.4 TBq mmol^{-1}) was from DuPont-New England Nuclear (Boston, U.S.A.).

Cell cultures

The previously described transfection method to obtain clones of human 5-HT$_{2B}$R-expressing LMTK$^-$ fibroblasts (Choi *et al*., 1994) was applied to obtain clones of human 5-HT$_{2C}$R-expressing fibroblasts. Stable clonal cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% 5-HT-depleted foetal calf serum (FCS). Single clones expressing similar amount of receptors were selected and used at similar passages.

Radioligand binding experiments

They were performed on crude membranes of transfected cells prepared using [^{125}I]DOI as radiolabelled compound as previously detailed (Loric *et al*., 1995; Kellermann *et al*., 1996). Briefly, to prepare crude membranes for binding assays, cells were washed twice with cold phosphate buffer saline (PBS), then harvested with a rubber policeman in PBS containing 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} antipain, 15 μg ml^{-1} benzamidine, and 0.1 mM phenylmethylsulphonyl fluoride. After centrifugation, the resulting pellet was frozen at -70°C before homogenisation. Frozen cell pellets were thawed at 37°C, resuspended with 10 ml of cold 4 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulphonyl fluoride, 10 mM imidazole buffer, pH 7.30, and centrifuged for 10 min at 5000 $\times$ g. The supernatant obtained from this centrifugation was collected, poured onto a 20% sucrose cushion, and then centrifuged for 90 min at 100,000 $\times$ g. The membrane-containing pellet was resuspended in 75 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole buffer, pH 7.30, for use in binding assays. Protein contents were determined using the bicinchoninic acid protein assay (Pierce, Chichester, U.K.). Binding experiments were carried out at room temperature under shaking in a total volume of 1 ml. The specific binding was defined as the binding that was inhibited by 1 μM levels of ritanserin. Assays were initiated by the addition of 50 μl of 50 mM Tris buffer, pH 7.40, containing 25 nM of radiolabelled ligand and appropriate competing ligands (12 different concentrations of compounds DHE, and 8′-OH-DHE) to 50 μl of membranes (20 μg of protein). A 30-min incubation period was followed by the addition of 5 ml of ice-cold 10 mM Tris buffer, pH 7.40. Samples were filtered on polyethyleneimine-treated filters and the radioactivity retained by the filters was determined as previously described (Bruns *et al*., 1983). This was repeated independently at least three times.

Determination of intracellular cGMP levels

Cells were washed twice in fresh serum-free medium and incubated for various times at 37°C with 100 mM isobutylmethylxanthine and test agents. The reaction was stopped by aspiration of the medium followed by addition of 500 ml ice-cold 95% ethanol/5% formic acid (1 : 1, v/v). After 1 h at 4°C, the ethanolic phase was collected and lyophilised. cGMP was

quantified using an iodinated radioimmunoassay kit (cGMP RIA kit RPA 525, Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Desensitisation, receptor expression

Transfected cells maintained in DMEM containing 10% 5-HT-depleted FCS and 300 μg ml^{-1} of hygromycin were seeded in 12- or 24- well at a density of 4×10^4 cells cm^{-2}. After 24-h, cells were rinsed in HBSS and incubated 24-h in serum-free medium [DMEM/F-12 (1 : 1) with 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 30 nM selenium, 20 nM progesterone, and 100 μM putrescine]. Stably transfected mouse fibroblasts LMTK were exposed to saturating concentration of DHE (1 μM) (or 8′-OH-DHE) for various time (1, 2, 5, 10, 20 and 40 h) and the variation in receptor (B_{max}) was determined by binding of [^{123}I]DOI. Three to four independent experiments were performed, each measure determined in triplicate.

Desensitisation, receptor transduction

Transfected cells were rinsed, incubated 24-h in serum-free medium and exposed to a saturating concentration of DHE (1 μM) (or 8′-OH-DHE) for various time (1, 2, 5, 10, 20 and 40 h) and the cGMP levels were determined as described before (Manivet *et al*., 2000) using an iodinated radioenzymatic assay kit (cGMP RIA kit RPA 525, Amersham Pharmacia Biotech). Three to four independent experiments were performed with each measure determined in triplicate.

Similarly treated cells maintained in DMEM containing 10% 5-HT-depleted FCS and exposed to saturating concentration of DHE (1 μM) (or 8′-OH-DHE) for various time (1, 2, 5, 10, 20 and 40 h) were used to determine the IP_3 levels as described before (Loric *et al*., 1995) using an iodinated radio receptor assay kit (IP_3 kit TRK 1000, Amersham). Three to four independent experiments were performed, each measure determined in triplicate.

Data analysis

Binding data were analysed using the iterative nonlinear fitting software Graphpad-Prism 2.0. This allowed to determine IC_{50} values and to calculate inhibition constants (K_i) according to the Cheng–Prusoff equation. Data points were F-tested to fit a single- or two-site model with or without an additional parameter regarded as nonspecific binding.

Results

We have generated clones of stably transfected LMTK cells expressing human 5-HT$_{2B}$ or 5-HT$_{2C}$Rs to assess their respective affinity for DHE and 8′-OH-DHE by competition towards [^{125}I]DOI. Mouse fibroblasts, LMTK$^-$ cells, do not expressed endogenous 5-HTRs. After transfection, we selected clonal cell lines expressing similar physiological levels of receptors. Clones expressing 205 ± 2 fmol, (mean $\pm$ s.e.m., $n = 4$) of human 5-HT$_{2B}$R per mg of protein or 195 ± 3 fmol (mean $\pm$ s.e.m., $n = 4$) of human 5-HT$_{2C}$R per mg of protein were selected, when measured against [^{125}I]DOI on crude membrane extracts as previously described (Loric *et al*., 1995; Kellermann *et al*., 1996). Affinities for standard 5-HT$_2$R agonists (Table 1) were found similar to previously published values (Cussac *et al*., 2002), indicating that they are not affected by expression levels of these receptors in the cell lines at passages used in this study. DHE and 8′-OH-DHE interacted with these receptors at high affinity. However, iterative curve fitting suggested the presence of more than one site for 8′-OH-DHE (Hill number of 1.79, $P < 0.01$, Table 2) at 5-HT$_{2B}$Rs that displayed the highest affinity ($K_i = 8.85 \pm 0.13$) (Figure 1).

Table 1 Binding affinities (K_i, in nM)

K_i	*5-HT$_{2B}$R*	*5-HT$_{2C}$R*
DOI	24 ± 5	63 ± 6
mCPP	18 ± 2	126 ± 11
MK212	151 ± 18	891 ± 18
BW723C86	2.3 ± 0.4	115 ± 13

Affinities (K_i, values) were determined by competition binding experiments with [^{125}I]DOI. K_i values are expressed as means $\pm$ s.e.m. of four independent experiments performed in triplicates.

Table 2 Hill numbers determined by iterative curve fitting

H number	*5-HT$_{2B}$R*	*5-HT$_{2C}$R*
DHE	0.99	1.38
8′-OH-DHE	1.79*	0.89

Hill numbers determined by iterative curve fitting after competition binding experiments with [^{125}I]DOI. *$P < 0.01$.

Intracellular levels of IP_3 have been reported to be increased in cells expressing 5-HT$_{2B}$ or 5-HT$_{2C}$Rs in response to agonist stimulation (Loric *et al*., 1995; Lucaites *et al*., 1996). As previously shown for 5-HT (Manivet *et al*., 2000), cGMP levels were elevated upon stimulation by DHE or 8′-OH-DHE of LMTK$^-$ cells expressing 5-HT$_{2B}$ or 5-HT$_{2C}$Rs, thus, both compounds behaved as agonists. The EC_{50} values observed corresponded to the high-affinity sites previously identified, the pEC_{50} of 8′-OH-DHE being 8.32 ± 0.09 at 5-HT$_{2B}$ and 7.83 ± 0.06 at 5-HT$_{2C}$Rs for cGMP. In addition, 8′-OH-DHE acts as a partial agonist towards 5-HT$_{2C}$Rs (Figure 2).

To assess the effect of long-term exposure to these compounds, stably transfected LMTK$^-$ cells expressing 5-HT$_{2B}$ or 5-HT$_{2C}$Rs were exposed to saturating concentration (1 μM) of DHE or 8′-OH-DHE for various time (0, 1, 2, 5, 10, 20 and 40 h) and the number of sites (B_{max}) was measured by competition against [^{125}I]DOI binding. This pharmacological evaluation of receptor expression indicated that 5-HT$_{2B}$ and 5-HT$_{2C}$Rs responded differently to these two compounds, the 5-HT$_{2B}$Rs being more sensitive to chronic stimulation by 8′-OH-DHE than 5-HT$_{2C}$Rs. After 2 h exposure to 8′-OH-DHE, 60% of the initial stimulated level of 5-HT$_{2C}$R was still detected, whereas only 43% of the 5-HT$_{2B}$R initial level remained. Moreover, after 40 h of exposure to 8′-OH-DHE, 5-HT$_{2C}$R expression level returned to the initial value but this level was only 86% for 5-HT$_{2B}$Rs (Figure 3). The number of sites of 5-HT$_{2B}$R and 5-HT$_{2C}$R was much less affected by chronic exposure to DHE and returned to initial values within 10–15 h. At 20 h, the number of sites of 5-HT$_{2B}$R and 5-HT$_{2C}$R was variably affected by exposure to other 5-HT$_2$R agonists (Table 3).

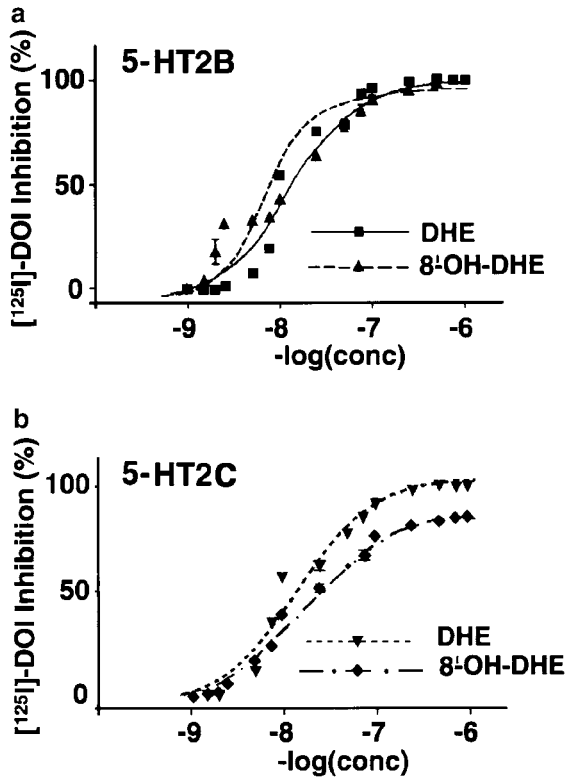

Figure 1 Competition curves against [^{125}I]DOI at 5-HT$_{2B}$ and 5-HT$_{2C}$Rs. Data points represent the percentage of specific [^{125}I]DOI binding inhibition at each concentration of DHE or 8′-OH-DHE at 5-HT$_{2B}$ (a) and 5-HT$_{2C}$ (b) receptors and are the average of at least three experiments performed in triplicate. Squares-plain line: 5-HT$_{2B}$R DHE; upper triangle-dashed line: 5-HT$_{2B}$R 8′-OH-DHE; lower triangle-dotted line: 5-HT$_{2C}$R DHE; diamond-dotted/dashed line: 5-HT$_{2C}$R 8′-OH-DHE.

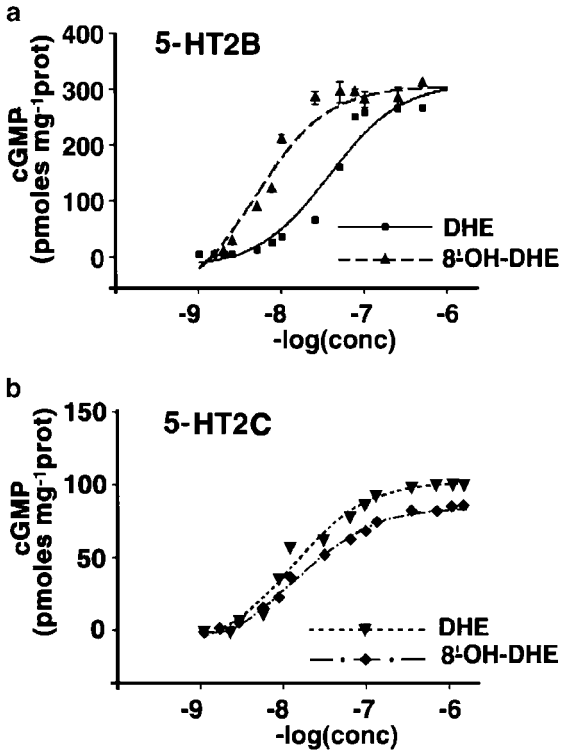

Figure 2 Concentration–response curves for cGMP production at 5-HT$_{2B}$ and 5-HT$_{2C}$Rs. Data points represent the intracellular cGMP level for each concentration of DHE or 8′-OH-DHE for 5-HT$_{2B}$ (a) and 5-HT$_{2C}$ (b) receptor-expressing cells. Values are expressed as pmol mg^{-1} of protein and are the average of at least three experiments performed in triplicate.

Stably transfected LMTK$^-$ cells expressing 5-HT$_{2B}$ or 5-HT$_{2C}$Rs were exposed to saturating concentration (1 μM) of DHE or 8′-OH-DHE for various time (0, 1, 2, 5, 10, 20 and 40 h) and the cGMP levels were assessed by radioimmunoassay. This experiment indicated that coupling of 5-HT$_{2B}$ and 5-HT$_{2C}$Rs to cGMP also behaved differently in response to these two compounds. The 5-HT$_{2B}$R stimulation of cGMP was more reduced after chronic stimulation by 8′-OH-DHE than 5-HT$_{2C}$Rs as previously observed for B_{max} (Figures 3 and 4). The lowest cGMP level was recorded after 2 h exposure of 5-HT$_{2B}$Rs-expressing cells to 8′-OH-DHE, in which only 14% of the initial stimulated level of cGMP remained, while 50% of the initial level of stimulated cGMP was detected in 5-HT$_{2C}$Rs-expressing cells. After 40 h of exposure to 8′-OH-DHE, the cGMP level returned to the initial value for 5-HT$_{2C}$Rs but this level was only 75% for 5-HT$_{2B}$Rs (Figure 4). The cGMP coupling of 5-HT$_{2B}$ and 5-HT$_{2C}$Rs was much less affected by chronic exposure to DHE than to 8′-OH-DHE, as initial desensitisation was subsequently completely reversed.

Since IP_3 is the classical transduction pathways to both receptors, we also evaluated the coupling to IP_3 production by stably transfected LMTK$^-$ cells expressing 5-HT$_{2B}$ or 5-HT$_{2C}$Rs after exposure to saturating concentration (1 μM) of DHE or 8′-OH-DHE for various time (0, 1, 2, 5, 10, 20 and 40 h). The IP_3 coupling of 5-HT$_{2B}$ and 5-HT$_{2C}$Rs was indeed found but with different responses to chronic exposure to these two compounds. The 5-HT$_{2B}$Rs stimulation of IP_3 levels was also more affected after chronic stimulation by 8′-OH-DHE than 5-HT$_{2C}$ as previously observed for B_{max} and cGMP. The lowest IP_3 stimulation level was recorded after 5 h exposure of 5-HT$_{2B}$R-expressing cells to 8′-OH-DHE, in which only 30% of the initial levels of IP_3 remained. In 5-HT$_{2C}$R-expressing cells, the lowest IP_3 level was 35% of the initial level and was detected after 2 h exposure to 8′-OH-DHE. Strikingly, after 40 h, 5-HT$_{2C}$R IP_3-coupling reached 86% of the initial value but this level remained stable at 50% of the initial values for 5-HT$_{2B}$Rs (Figure 5). As for cGMP, the IP_3 coupling of 5-HT$_{2B}$ and 5-HT$_{2C}$Rs was much less affected by chronic exposure to DHE than to 8′-OH-DHE, as initial desensitisation was subsequently completely reversed. These results indicate that chronic exposure to DHE leads to permanent impairment of the coupling efficiency of 5-HT$_{2B}$Rs through its metabolite 8′-OH-DHE.

Discussion

To our knowledge, this is the first functional characterisation of DHE and its major metabolite 8′-OH-DHE at the 5-HT$_{2B}$ and 5-HT$_{2C}$Rs described to date. Unfortunately, it is not possible to study receptors in native tissues due to the lack of complete segregation of receptors in discrete areas. The use of recombinant expression systems is therefore a useful approach, and also allows the study of human receptors. The two human receptor subtypes, 5-HT$_{2B}$ and 5-HT$_{2C}$Rs were transfected into

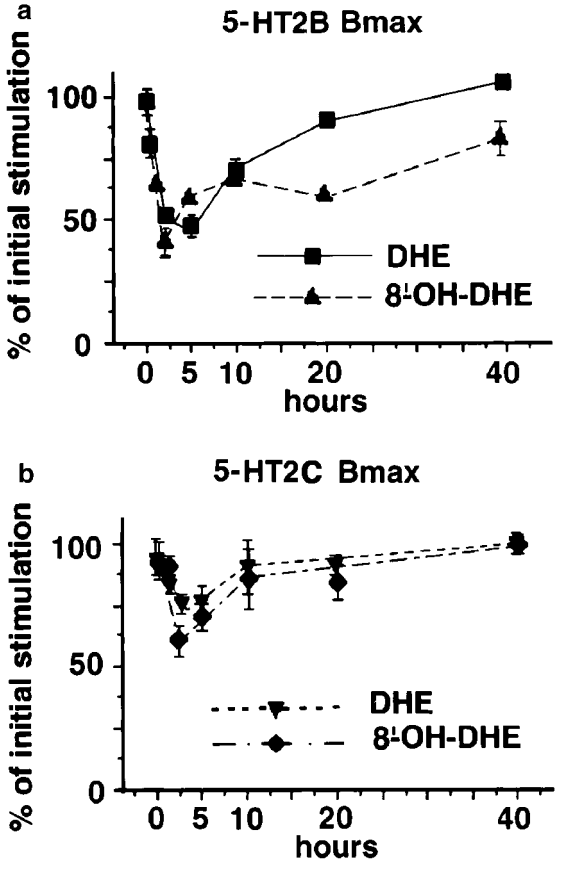

Figure 3 Time course of the B_{max} obtained at 5-HT$_{2B}$ and 5-HT$_{2C}$Rs after chronic exposure. Data points expressed as per cent of the initial stimulation (maximum) represent the average of the number of specific binding sites remaining for 5-HT$_{2B}$ (a) and 5-HT$_{2C}$ (b) receptor-expressing cells at each time point after exposure to saturating concentrations of DHE or 8′-OH-DHE and are the average of at least three experiments performed in triplicate.

Table 3 Number of sites (B_{max} after 20 h in per cent of 0 h)

B_{max}	*5-HT$_{2B}$R*	*5-HT$_{2C}$R*
5-HT	26±7	54±4
DOI	38±4	56±5
mCPP	93±6	51±5
MK212	36±9	46±5
BW723C86	54±4	25±6

Number of sites (B_{max}) were determined by competition binding experiments with [^{125}I]DOI after 20 h of stimulation by saturating concentrations of the different agonists and are expressed in percent of the initial B_{max} at 0 h. Values are expressed as means±s.e.m. of four independent experiments performed in triplicates.

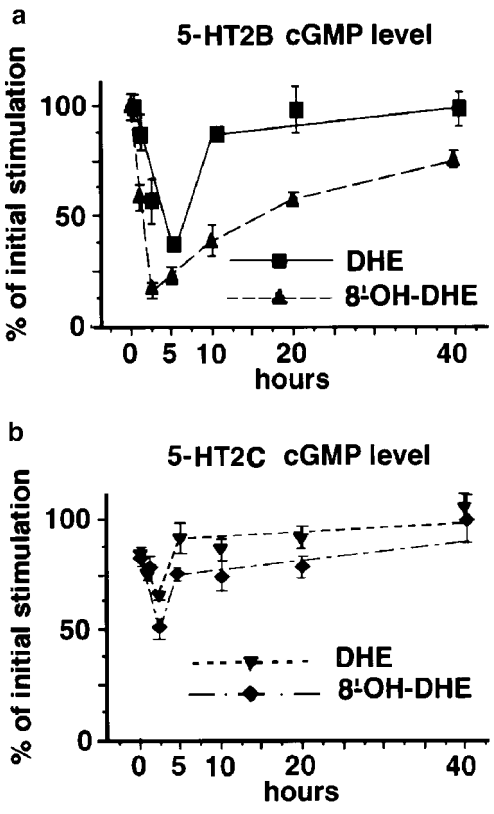

Figure 4 Time course of the cGMP levels obtained on 5-HT$_{2B}$ and 5-HT$_{2C}$Rs-expressing cells after chronic exposure. Data points expressed as per cent of maximal stimulation represent the average of the cGMP level measured at 5-HT$_{2B}$ (a) and 5-HT$_{2C}$ (b) receptor-expressing cells at each time point after exposure to saturating concentrations of DHE or 8′-OH-DHE and are the average of at least three experiments performed in triplicate.

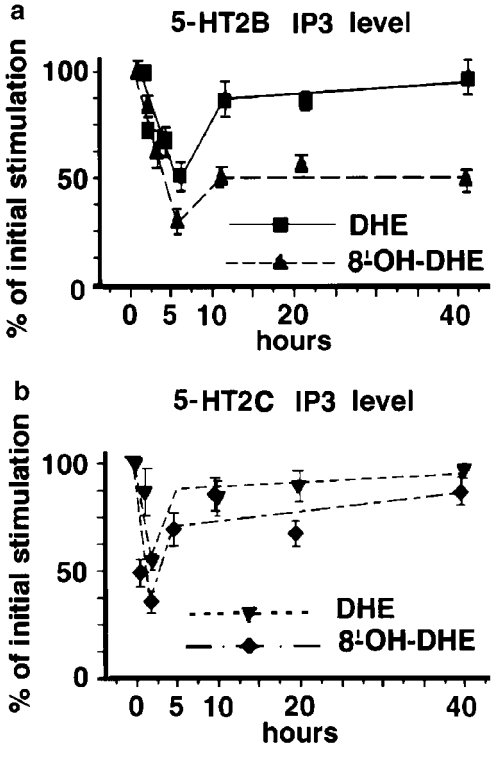

Figure 5 Time course of the IP$_3$ levels obtained on 5-HT$_{2B}$ and 5-HT$_{2C}$R-expressing cells after chronic exposure. Data points expressed as per cent of maximal stimulation represent the average of the IP$_3$ level measured at 5-HT$_{2B}$ (a) and 5-HT$_{2C}$ (b) receptors at each time point after exposure to saturating concentrations of DHE or 8′-OH-DHE and are the average of at least three experiments performed in triplicate.

LMTK$^-$ cells thus ensuring they were studied in an identical background. Parental nontransfected cells when examined under an identical experimental paradigm did not exhibit a concentration-dependent rise in second messengers in response to agonists. The above cell lines were therefore chosen as they express a single receptor of interest at relatively low and close to physiological levels and they were used at nearly similar passages to ensure stable expression levels. DHE had previously been reported to exhibit alpha-adrenergic antagonist and 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, and 5-HT$_{1F}$R agonist activity (EC_{50} around 1.5, 10, 0.5, and 10 nM, respectively) (Buzzi & Moskowitz, 1991; Boess & Martin, 1994; Silberstein, 1997). No data about 8′-OH-DHE have been published. Observed values for 5-HT$_{2B}$R are within the same range suggesting that its stimulation is physiologically likely.

External carotid vasoconstrictor responses to DHE are mediated by 5-HT$_{1B/1D}$Rs as well as alpha2-adrenoceptors (Villalon *et al*., 1999). The moderate vasodilator response of cranial arteriovenous anastomoses to the ergot derivatives seems to be mediated, at least in part, by 5-HT$_{1B/1D}$Rs (De Vries *et al*., 1998). DHE does not contract isolated rat aorta rings, but insurmountably blocks contractions in response to 5-HT. As this response has been reported to be potently (pK_B 8.1) and competitively antagonised by the selective 5-HT$_{2A}$R antagonist, ketanserin (Kalkman & Schneider, 1996), it is thought to be mediated by the HT$_{2A}$R. Thus, DHE appears to be an antagonist of the HT$_{2A}$R. The 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$Rs share a high degree of sequence homology and have very similar pharmacological profiles (Rothman *et al*., 2000). DHE inhibits [^{3}H]-mesulergine binding to HT$_{2C}$Rs in piglet choroid plexus with a pK_D of 7.1, rather less than that of 5-HT (pK_D 8.1) and is a full agonist (pEC_{50} 7.6) but with potencies similar to that of 5-HT (pEC_{50} 7.7) (Brown *et al*., 1991). Concerning the 5-HT$_{2B}$R, there was only indirect information originating from pharmacological studies of endothelium-mediated relaxation of pig pulmonary or cerebral arteries, which show a pharmacological profile very close to that observed on transfected cells expressing the 5-HT$_{2B}$R (Glusa & Roos, 1996; Schmuck *et al*., 1996; Glusa & Pertz, 2000). The 5-HT$_{2B}$ and 5-HT$_{2C}$R-expressing cell lines used in the present study display very similar affinities for previously characterised agonists (Table 1) (Cussac *et al*., 2002). However, the 8′-OH-DHE presents complex interactions toward the 5-HT$_{2B}$Rs with a Hill number of 1.79, suggesting multiple interaction sites (Table 2), the highest affinity site for 5-HT$_{2B}$Rs being the most efficient. Our results clearly show that DHE and for the first time 8′-OH-DHE act as agonists vs both 5-HT$_{2B}$ and 5-HT$_{2C}$Rs.

Although it is also generally believed that the cellular signal transduction mechanisms activated by the 5-HT$_2$Rs are indistinguishable, recent data suggest also significant differences in their signalling cascades. 5-HT-stimulated IP_3 production has been previously observed at the 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$Rs (Loric *et al*., 1995; Briddon *et al*., 1998). In response to 5-HT stimulation, 5-HT$_{2A}$R-mediated cGMP generation was reported together with calcium (Ca^{2+}) mobilisation in C6 glioma cells through NO-dependent pathway (Kagaya *et al*., 1995; Miyoshi *et al*., 2001). The 5-HT$_{2C}$Rs expressed in the choroid plexus trigger phosphoinositide turnover and also the formation of cGMP formation in a calcium-sensitive manner (Kaufman *et al*., 1995). Concerning the 5-HT$_{2B}$R, the pharmacological study of endothelial-mediated relaxation of pig pulmonary arteries (Glusa & Roos, 1996; Glusa & Pertz, 2000) showed that DHE, as well as 5-HT and alpha-methyl-5-HT, elicited a reversible endothelium-dependent relaxation of precontracted arterial ring segments, associated with an increase in intracellular cGMP production through direct activation of NO synthase (Manivet *et al*., 2000). The present results clearly identify DHE and 8′-OH-DHE as agonist for both IP_3 and cGMP second messengers at 5-HT$_{2B}$ and 5-HT$_{2C}$Rs.

Knowing that DHE is generally prescribed for long-term use, we investigated the effect of chronic treatments of cells expressing 5-HT$_{2B}$ or 5-HT$_{2C}$Rs exposed to saturating concentrations (1 μM) of these compounds to ensure their complete desensitisation. Desensitisation is an adaptive mechanism in biological systems thought to facilitate responsiveness of the cell to successive multiple extracellular stimuli over time. The receptor, first uncoupled from the G protein is then sequestered in an intracellular compartment. This step is now viewed as a mechanism that allows dephosphorylation by specific phosphatases and resensitisation of the receptor. Degradation of the protein and reduction of the steady-state mRNA (mostly due to decreased stability of the mRNA) are the concomitant mechanisms leading to the decrease of receptor number (downregulation) (Chuang *et al*., 1996). *In vivo*, when the stimulation is chronically persistent, the receptor transcription is downregulated, but due to the use of a strong promoter containing expression vectors this step cannot be observed in transfected cells. To date, little is known about 5-HT$_{2B}$Rs desensitisation. The human 5-HT$_{2A}$ and 5-HT$_{2C}$Rs have been both reported to exhibit receptor uncoupling at the level of IP_3, with the 5-HT$_{2C}$R being more sensitive than the 5-HT$_{2A}$R (Briddon *et al*., 1998). More recently, the desensitisation characteristics of recombinant human 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$Rs stably expressed in CHO-K1 cells, investigated by calcium fluorimetry showed that 5-HT$_{2B}$Rs exhibited the most dramatic degree of desensitisation to 5-HT, with a rapid time course. The partial agonists meta-chlorophenylpiperazine (mCPP) and DOI also elicited desensitisation, generally in line with their relative efficacies at each receptor (Berg *et al*., 2001; Porter *et al*., 2001). For the first time, we have studied long-term effects of DHE exposure and found that 5-HT$_{2B}$Rs exhibit the most dramatic degree of desensitisation in respect to both IP_3 and cGMP coupling by its metabolite 8′-OH-DHE, which is more effective and permanent. Further experiments are necessary to elucidate the molecular mechanism involved in this phenomenon.

Both neuronal and vascular mechanisms have been proposed on the basis of actions of 5-HT in migraine. Blockade of neural transmission and the neurogenic inflammatory response provides a mechanism by which sumatriptan and ergot alkaloids alleviate vascular headaches (Moskowitz, 1992). The vasodilatory theory of migraine suggests that extracranial arterial dilation during an attack is related to migraine pain, whereas in the neurogenic dural inflammation theory of migraine, inflammation of the dural membrane surrounding the brain is due to release of neuropeptides from primary sensory nerve terminals. Substance P, calcitonin gene-related peptide and NO all play a role in the dural inflammatory cascade. Pretreatment with the antimigraine drugs DHE and sumatriptan markedly attenuates plasma protein extravasation

induced by electrical trigeminal ganglion stimulation (Buzzi & Moskowitz, 1991; Hoskin *et al*., 1996). Pharmacological agents such as mCPP and NO donors have also been used to induce dural extravasation in animals.

NO is suspected to play a key role in migraine since NO donors cause a dose-dependent headache with several migrainous characteristics. A cause of migraine could be increased amounts and/or affinity of an enzyme in the NO-triggered cascade of reactions (Olesen *et al*., 1994). NO, which may be released from blood vessels, perivascular nerve endings or from brain tissue, is an important molecular trigger mechanism in spontaneous headache pain (Thomsen & Olesen, 2001). It has been shown that 5-HT$_{2B}$Rs stimulate the NO production in cell lines (Manivet *et al*., 2000) and relaxation of the pig cerebral artery (Schmuck *et al*., 1996). Thus, 5-HT$_{2B}$Rs located on endothelial cells of meningeal blood vessels have been proposed to trigger migraine headache through the formation of NO. Recent data are consistent with this hypothesis, since activation by mCPP of peripheral 5-HT$_{2B}$Rs initiates dural plasma protein extravasation *via* NO (Johnson *et al*., 2003).

In conclusion, the DHE long half-life may account for the low rate of headache recurrence at least partially through permanent inhibition of vascular 5-HT$_{2B}$-dependent second messengers (NO) *via* its major active metabolite 8′-OH-DHE. Whether the antimigraine action of DHE involves a combination of acute agonist action at 5-HT$_{1B/1D}$Rs, a long-lasting uncoupling of 5-HT$_{2B}$Rs *via* 8′-OH-DHE and an insurmountable antagonist action at 5-HT$_{2A}$Rs remains to be established on cerebral vasculature.

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