Influence of pH on the Binding of Diphenylmethylenepiperidines by 5-HT_{2B} Receptors in Rat Stomach Fundus

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Cyproheptadine is one of the compounds exhibiting the highest activity at 5-HT_{2B} receptors. In a previous work we analysed the relevance of the amino group in diphenylmethylenepiperidines (DPMP), which are open cyproheptadine analogues. Only compounds containing N–H or *N*-methyl motifs, showed significant 5-HT_{2B} activity. Surprisingly, the corresponding quaternary ammonium salt demonstrated a total lack of activity. Therefore, the question arises whether protonation favours the interaction of these compounds with 5-HT_{2B} receptors. Consequently, we studied the protonation influence (by varying the pH of the medium) on the antagonism of serotonin by some cyproheptadine analogues in rat stomach fundus. The main results were: 1) N-protonation increases the activity of DPMPs. 2) Alkaline pH facilitates the occurrence of a non-surmountable antagonism. 3) The contrast between the activity of protonated DPMPs and the lack of activity of the corresponding quaternary ammonium cation, suggests either that the latter is prevented from acting by steric hindrance, or that the mechanism by which protonation may increase the activity depends not only on the charge of the proton, but also on its ability to form hydrogen bonds.

Key words cyproheptadine; diphenylmethylenepiperidines; 5-HT_{2B} receptor; pH influence; protonation; binding mechanism

The 5-hydroxytryptamine$_2$ (5-HT_2) receptor class comprises three subtypes 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, which are similar in terms of their sequence, molecular structure, pharmacology and signal transduction pathways.[1] All of them belong to the G-protein-coupled-receptors superfamily. They are preferentially coupled to Gq/11 to increase the hydrolysis of inositol phosphates and elevate cytosolic calcium.[2]

The 5-HT_{2B} receptor, first found in rat stomach fundus,[3] was one of the latest to be characterised and cloned in mouse, rat and human.[4—7] As the rest of the 5-HT_2 receptors, 5-HT_{2B} mediates the release of phosphoinositides, although controversy persists on the possible involvement of other second messengers.[6—13] Interest in the 5-HT_{2B} receptor has mushroomed as the result of its presence in humans, in both the central nervous system and peripheral tissues.[6,7,14,15]

Cyproheptadine (Fig. 1) was chosen as the lead compound for this research project because of its high affinity at 5-HT_{2B} receptors in rat stomach fundus. In a previous work,[16] we demonstrated the relevance of the amino group in open cyproheptadine analogues. Only compounds having N–H or *N*-methyl motifs (compounds **1** and **2**: 4-diphenylmethylenepiperidine and 4-diphenylmethylene-1-methylpiperidine, respectively), showed significant 5-HT_{2B} antagonistic activity. This observation supports the previously postulated key role of a cationic amino group in the interaction of ligands at the active site of the aminergic G-coupled protein receptors, by interacting with a conserved anionic aspartate located in the third transmembrane α-helix (TMH) of the receptor.[17] However, a surprising result of our previous study was the total lack of activity of the corresponding quaternary ammonium ion (compound **3**: 4-diphenylmethylene-1,1-dimethylpiperidinium iodide).[16]

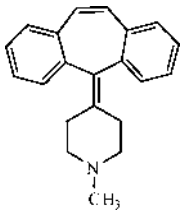

Fig. 1. Chemical Structure of Cyproheptadine

The pH of the biological medium is responsible for the changes in the potency of antagonists at some receptors.[18] A question therefore arises whether ligand protonation favours their interaction with 5-HT_{2B} receptors. To provide new insights on this issue, in this work we studied the influence of increasing the protonation of compounds **2** and **3** (by reducing the pH of the medium) on the antagonism of serotonin in rat stomach fundus preparations.

Results and Discussion

During the experiments described below, we verified that the pH of the solutions (6, 7.4, 8) remained unchanged over the duration of organ bath experiments (Fig. 2).

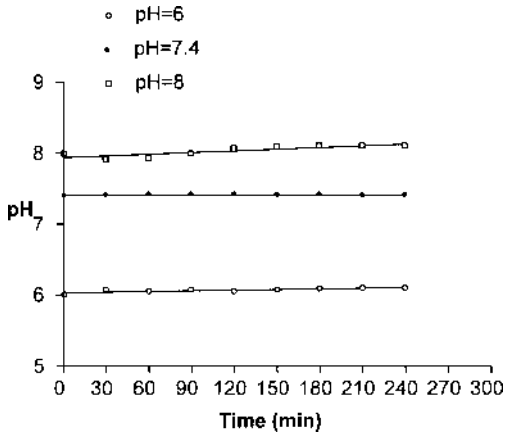

Fig. 2. pH Stability of Krebs Solutions over the Duration of Organ Bath Experiments

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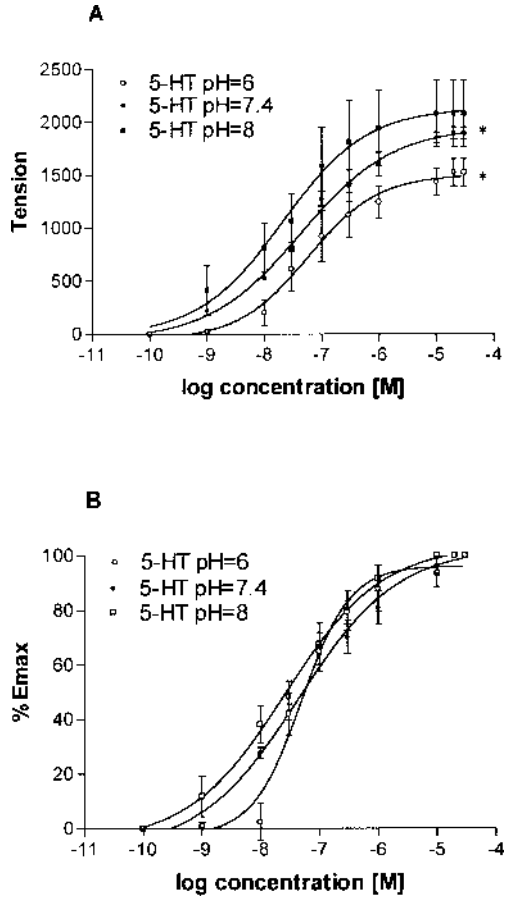
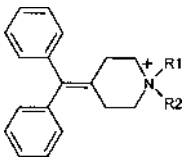


Fig. 3. Concentration–Response Curves to Serotonin at Three Different pH Values (6, 7.4, 8)

The response is expressed as a mg of tissue tension (A) or as percentage of the maximum response (B). $*p<0.05$.

Table 1. Antagonist Activity of Studied Compounds over Cumulative Concentration–Response Curves of Serotonin in Rat Stomach Fundus



Compound	R1	R2	pH	$pA_2 \pm$S.E.M.	Schild slope
1	H	H	7.4	6.18 ± 0.03[a)]	1.18 ± 0.05
2	Me	H	6	7.36 ± 0.03	0.91 ± 0.02
2	Me	H	7.4	6.12 ± 0.20	0.79 ± 0.17
2	Me	H	8	6.53 ± 0.18	0.54 ± 0.15
3	Me	Me	7.4	$pD'_2=5.54 \pm 0.10$ [b)]	

a) Data from Loza *et al*., 1992. *b*) No detectable activity.

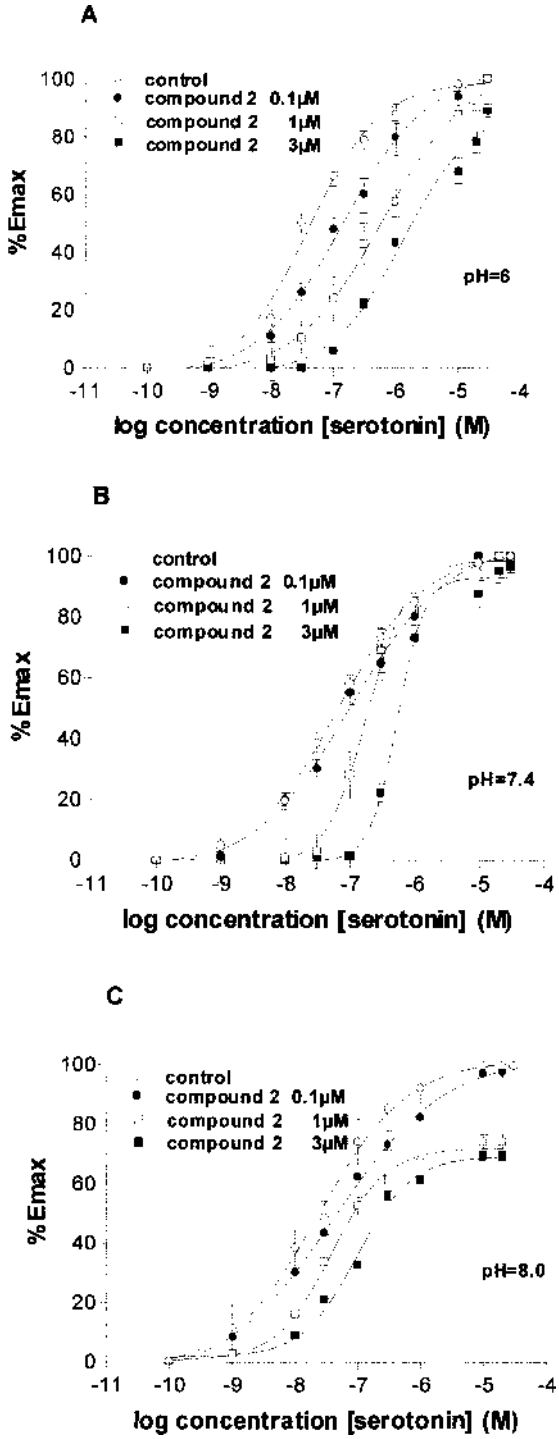


Fig. 4. Antagonistic Activity of Compound **2** over Cumulative Concentration–Response Curves of Serotonin in Rat Stomach Fundus at pH=6 (A), 7.4 (B) and 8 (C)

Serotonin concentration-response curves (CRC) recorded in the absence of antagonists showed that the response of the tissues fell with pH (Fig. 3A); the differences between the tensions recorded at pH 6 and pH 8 were statistically significant ($p<0.05$). The same phenomenon was observed when the effect of pH on calcium uptake in vascular smooth muscle cells was studied.[19] We have accounted for this phenomenon when evaluating the influence of pH on antagonism by normalising the response as a percentage of the maximum response obtained in the absence of antagonist. This mathematical transformation convert the curves shown in Fig. 3A into those depicted in Fig. 3B.

Table 1 summarizes the pharmacological behaviour of the studied compounds. At concentrations of 0.1—3.0 μM, compound **2** caused a dose-dependent right shift of the serotonin concentration–response curves (Fig. 4). Maximum response was always attained at pH 6 and 7.4, and the corresponding

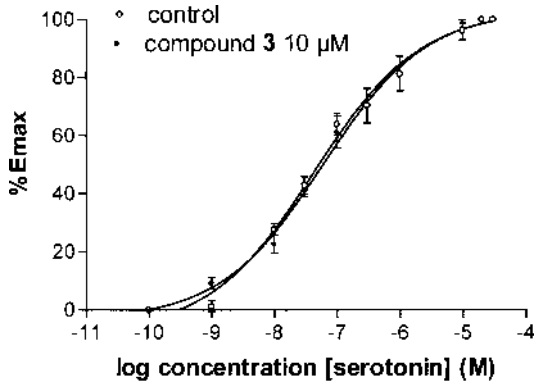


Fig. 5. Lack of Effect of Compound **3** on 5-HT Concentration–Effect Curves in Rat Stomach Fundus at pH=7.4

Schild plot slopes did not differ significantly from unity; pA_2 was 7.63 at pH 6 and 6.12 at pH 7.4. These observations suggested that protonation of the nitrogen atom of the antagonists increases the activity of compound **2** as it does the activity of many amino compounds at other serotonin receptors.[20] At pH 8 the compound **2** at the lowest concentration used (0.1 μM) elicited a shift to the right of the serotonin concentration-response curves which was slightly higher than the shift at pH=7.4. However, at pH 8, it was not possible to attain maximum response in the presence of antagonist concentrations of 1 μM or more, and the Schild plot indicated non-competitive antagonism with an apparent pA_2 of 6.53 and a pD_2' of 5.54. In previous studies of antagonism of serotonin in rat stomach fundus, the reduction of maximum response in the presence of high concentrations of antagonist has been attributed to antagonist-induced conformational alteration of the receptor.[21] Our results suggest that alkaline pH facilitates such alterations, which can be responsible for the small differences on the shifts of 5-HT CRC caused by compound **2** at pH 8 and 7.4.

Although the nitrogen atom of the diphenylmethylenepiperidine quaternary ammonium cation **3** bears permanently a positive charge, like that of compound **2** when protonated, **3** failed to antagonise serotonin at pH 7.4 (Fig. 5): EC_{50} for serotonin was 4.36×10^{-8} in the absence of **3** and 5.18×10^{-8} in its presence. This negative result is in keeping with the fact that quaternary ammonium salts have so far only exhibited activity at receptors linked to ion channels, such as the $5HT_3$ serotonin receptor.[22] The contrast between the activities of **2** and **3** suggests that the binding of protonated **2** to the rat stomach fundus serotonin receptor (and presumably the binding of other protonated ligands to other aminergic receptors) not only requires a positive charge on the amino group but also other features not present in quaternary ammonium compounds.

Conclusions

(1) The activity of the diphenylmethylenmethylpiperidine **2** at the rat stomach fundus serotonin receptor increases as pH falls from 7.4 to 6.0, a finding which suggests that protonation of the amino group increases the activity of diphenylmethylenepiperidines.

(2) A particular antagonism behaviour of compound **2** at pH 8 has been observed, since it exhibits a non surmountable antagonism at concentrations above 1 μM, which suggest that alkaline pH facilitates the alteration of receptor protein and/or its interaction with the studied ligands.

(3) The contrast between the activity of compound **2** and the lack of activity of the diphenylmethylenepiperidine quaternary ammonium cation **3** suggests either that the latter is prevented from acting by steric hindrance, or that the mechanism by which protonation may increase the activity of protonated DPMPs depends not only on the charge of the proton, but also on its ability to form hydrogen bonds.

Experimental

Drugs and Chemicals Compounds **1**—**3** were synthesized as described by Loza *et al.* (1993).[16] 5-HT hydrochloride and cyproheptadine hydrochloride were supplied by Sigma. All other reagents were of the highest purity available. Aqueous solutions of all drugs were prepared daily using distilled water. All drug concentrations mentioned below are expressed as final molar concentrations in the tissue bath.

Functional Experiments Male 250—300 g Sprague-Dawley rats were killed by cervical dislocation, and their stomachs were dissected out and immersed in modified Krebs solutions having the following compositions (mM): $NaCl_2$, 118; KCl, 4.7; $MgSO_4\cdot7H_2O$, 1.2; $CaCl_2\cdot2H_2O$, 2.5; KH_2PO_4, 1.18; glucose, 11; $NaHCO_3$, variable to obtain the desired pHs. Strips of stomach fundus were prepared by Vane's (1957)[23] method and mounted in organ baths containing 10 ml of the relevant Krebs solution, maintained at 37 °C with aeration by carbogen (95% O_2, 5% CO_2). Before addition of drugs, the tissue strips were equilibrated for 1 h under a 1 g load. Isometric contractions were recorded during cumulative addition of serotonin using a Grass transducer FTO3C and a Grass polygraph 7D.

Concentration–response curves for serotonin were constructed as per Van Rossum[24] at pH 6, 7.4 and 8. In initial control runs stable contractions were achieved throughout the serotonin concentration range 0.01 nM—10 μM. Two control runs giving identical curves were followed by test runs with increasing concentrations of antagonist. Between runs, the tissues were washed and allowed to rest for 60 min. If antagonist was to be used in the next run, it was added to the medium at this point and the tissues were left in this solution for 45 min.

Expression of Results and Statistical Analysis Affinity of the agonists was measured in terms of EC_{50} ($1/K_A$). Agonist CRC were fitted to the following equation: $E=E_{max}\,[A]^s/EC_{50}+[A]^s$. E_{max}, $[A]$ and s represent the maximum response, agonist concentration and curve slope, respectively. EC_{50} is the concentration of agonist that produces 50% of the maximal response.

Antagonist potency was measured, following Arunlaksana and Schild,[25] in terms of pA_2 ($-\log$ concentration of antagonist required to maintain a constant response when the agonist concentration is doubled), or in terms of pD_2' obtained using the Van Rossum's method.[24]

All the data presented are the average of 4—6 experiments. Pharmacological calculations were performed by the Pharmacological Calculation System Program.[26]

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