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European Journal of Pharmacology 402 (2000) 205–213

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Glycinergic potentiation by some 5-HT_3 receptor antagonists: insight into selectivity

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Received 13 April 2000; received in revised form 17 July 2000; accepted 21 July 2000

Abstract

The ability of various 5-HT_3 receptor antagonists to potentiate spinal glycine responses was investigated. Whereas (3-α-tropanyl)-1*H*-indole-3-carboxylate (ICS 205930), (3-α-tropanyl)-3,5-dichlorobenzoate (MDL 72222) and 1-methyl-*N*-(3-α-tropanyl)-1*H*-indazole-3-carboxamide (LY 278584) exhibited this property, even in identified motoneurones, several other chemically similar 5-HT_3 receptor antagonists did not. Introducing a methyl group on the nitrogen of the azabicyclo moiety of ICS 205930 greatly reduced the ability to potentiate glycine responses. Neither endo-1-methyl-*N*-(9-methyl-9-azabicyclo[3.3.1]non-3-yl)-indazole-3-carboxamide (granisetron), differing from LY 278584 by an additional carbon in this cycle, nor 2β-carbomethoxy-3β-benzoyloxytropane (cocaine), 1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazol-1-yl)-methyl]-4*H*-carbazol-4-one (ondansetron) and (*S*)-4-amino-*N*-(1-azabicyclo[2.2.2]oct-3-yl)-5-chloro-2-methoxy-benzamide ((*S*)-zacopride) could potentiate glycine responses. A pharmacophore model of the glycinergic potentiators was generated by molecular modelling using MDL 72222 as a template. According to this model, an aromatic ring, a carbonyl group and a tropane nitrogen atom are required for glycinergic potentiation, as previously described for 5-HT_3 receptor antagonism. However, the steric allowance at the glycine receptor site and the tridimensional arrangement of the pharmacophoric elements appear to be more restricted.

Keywords: Glycine receptor; 5-HT_3 receptor antagonist; Potentiation; Interaction; Pharmacophore model; Selectivity

1. Introduction

In a previous study (Chesnoy-Marchais, 1996), it was shown that three compounds known as antagonists of 5-HT_3 receptors can also potentiate the responses to low concentrations of glycine recorded in primary cultures of ventral spinal cord neurones. These compounds are (3-α-tropanyl)-3,5-dichlorobenzoate (MDL 72222, also named bemesetron and **1** in Fig. 4), (3-α-tropanyl)-1*H*-indole-3-carboxylate (ICS 205930, also named tropisetron and **2** in Fig. 4) and 1-methyl-*N*-(3-α-tropanyl)-1*H*-indazole-3-carboxamide (LY 278584, **3** in Fig. 4). It was argued, based on the following results, that these compounds' ability to potentiate glycine responses is independent of their ability to antagonise 5-HT_3 receptors. First, potentiation of glycine responses was observed during continuous, rapid perfusion of the neurone in the absence of serotoninergic agonist and in the presence of tetrodotoxin; second, the potentiators did not affect the baseline current recorded in the absence of glycine; third, the rank order of the compounds by potency for potentiating effects on glycine responses was different from their rank order for antagonist action on 5-HT_3 receptors. Indeed, to potentiate glycine responses, between 10 and 100 nM ICS 205930 and MDL 72222 was sufficient, whereas micromolar concentrations of LY 278584 were necessary. In contrast, the reported IC_{50}s for 5-HT_3 receptors are between 0.2 and 60 nM for ICS 205930 (Zifa and Fillion, 1992; Hoyer et al., 1994; see Newberry et al., 1991; Lankiewicz et al., 1998 for differ-

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00531-8

ences between species), between 3 and 60 nM for MDL 72222 (Zifa and Fillion, 1992; Hoyer et al., 1994; Hussy et al., 1994) and below 3 nM for LY 278584 (Zifa and Fillion, 1992; Glitsch et al., 1996).

Selective potentiators of glycine responses would be potentially useful as antispamodics, anticonvulsants and during anaesthesia. The potentiating effects of some 5-HT_3 receptor antagonists on glycine responses were previously observed using cultures which contained mainly spinal interneurones. As reported below, using cultures of identified and isolated purified motoneurones, we checked that these effects also concern the glycinergic inhibitory responses of the neurones which directly control muscle contraction. In view of the relative paucity of glycine receptor pharmacology (Becker and Langosch, 1998), the high affinity of some 5-HT_3 receptor antagonists for these receptors could encourage the search for selective modulators of glycine receptors, derived from the chemical structure of the active 5-HT_3 receptor antagonists. In the present paper, we investigate the ability of several other 5-HT_3 receptor antagonists to modulate glycine responses, using cultures of ventral spinal neurones. We confirm that the active compounds act independently to potentiate glycine responses and antagonise 5-HT_3 receptors. Furthermore, we compare the interactions of these molecules with the two families of receptors by a structure-activity analysis of the data, using molecular modelling.

2. Materials and methods

2.1. Electrophysiological study

The experiments were performed at room temperature (20–23°C) using the whole-cell configuration of the patch–clamp technique and primary cultures of purified motoneurones or primary cultures of ventral spinal cord neurones from E15 rat embryos.

The preparation of the ventral spinal cord cultures has been described previously (Chesnoy-Marchais, 1996) as well as the purification, identification and culture of motoneurones (Lévi et al., 1999). Recordings from purified motoneurones and from ventral spinal neurones were performed, respectively, between day 7 and day 9 and between day 12 and day 15 in culture.

The internal solution used to fill the recording electrode contained (in mM): 145 Cs methanesulfonate, 15 CsCl, 1 $MgCl_2$, 0.1 EGTA, 3 ATP–Mg, 0.3 GTP–Na and 10 HEPES–CsOH pH 7.2. Before recording, the culture medium was replaced by the external solution to be used during the recording. This solution contained (in mM): 150 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 20 glucose and 10 HEPES–NaOH pH 7.4 and was continuously perfused slowly in the dish. In addition, a fast perfusion system was used for rapid application of glycine and modulators. All solutions applied via this system contained 0.2 μM tetrodotoxin. As previously explained, the fast perfusion system was made of two glass barrels and lateral movements of the two barrels were controlled by a computer-driven motor in order to apply the solution of the desired barrel to the cell. In all cases, only one of the barrels contained glycine and the cell was continuously perfused with solution from one of the two barrels (either the control solution containing tetrodotoxin or a solution also containing a modulator and/or glycine, see Chesnoy-Marchais (1996) for more details concerning this perfusion system). All tubings were in Teflon, and traces of chemicals previously used were eliminated by extensive washing. At the low concentrations of glycine used in this study, successive responses recorded in the same solution were usually quite stable when tested every 40 s. Repetitive measurements of successive glycine responses were systematically performed in order to separate true modulatory effects from possible slow spontaneous changes in the response. In all the figures, "control" traces are means of traces recorded symmetrically before wash-in and after wash-out of the modulator. When a modulator of glycine response was applied "with preincubation", it was applied continuously between and during the successive glycine applications, that is in both barrels of the fast perfusion system; when applied "without preincubation", it was present only in the glycine-containing barrel. In some experiments, ICS 205930 was applied only during glycine applications, since its potentiating effect was previously shown to be identical whether it was applied without or with preincubation (see Fig. 5b in Chesnoy-Marchais, 1996). However, in most experiments, the compounds tested were continuously applied.

Patch–clamp micro pipettes were made from hard glass (Kimax 51); the shank of each pipette was covered with Sylgard and the tip was fire-polished. The resistance of these electrodes was between 5 and 10 MΩ. The cells were voltage-clamped by an EPC7 List amplifier, controlled by a TANDON 38620 computer, via a Cambridge Electronic Design (CED) 1401 interface, using CED software. The current monitor output of the amplifier was filtered at 0.3 kHz before being sampled on-line at 0.6 kHz. The bath was connected to the ground via an agar bridge. Membrane potentials were corrected for the junction potential of 10 mV amplitude that was measured between the recording pipette and the usual external solution. The series resistance (Rs) was between 10 and 20 MΩ. Experiments in which Rs changed suddenly were eliminated; the current modulations described occurred without any simultaneous change in Rs. The zero indicated on current traces is the absolute zero current level. All values are expressed as mean ± S.D. (number of observations).

All compounds tested were diluted just before use from a stock solution prepared the day of the experiment. Stock solutions of ICS 205930 (RBI) and LY 278584 (RBI) were prepared in distilled water at 1 and 10 mM, respectively.

MDL 72222 (RBI) was prepared at 3 mM in dimethyl sulfoxide (DMSO). When testing the effects of 0.1 μM MDL 72222, all the solutions applied by the fast perfusion system contained the same amount of DMSO (1/30,000). The quaternized derivative of ICS 205930, (3-α-tropanyl)-indole-3-carboxylate methiodide (Q-ICS 205930, RBI) and GR 38032F, 1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazol-1-yl)-methyl]-4*H*-carbazol-4-one hydrochloride dihydrate (ondansetron) were prepared at 1 mM in distilled water. BRL 43694, endo-1-methyl-*N*-(9-methyl-9-azabicyclo[3.3.1]non-3-yl)-indazole-3-carboxamide (granisetron), 2β-carbomethoxy-3β-benzoyloxytropane hydrochloride (cocaine, Sigma) and (*S*)-4-amino-*N*-(1-azabicyclo[2.2.2]oct-3-yl)-5-chloro-2-methoxy-benzamide ((*S*)-zacopride) were also prepared in distilled water at 10 or 2 mM (for (*S*)-zacopride). A given stock solution of glycine (Sigma), prepared at 10 mM in distilled water and kept at −20°C, was used for several weeks.

2.2. Molecular modelling

For all molecules, the three dimensional structure was generated using Insight II and Discover (Molecular Simulations, San Diego, CA) for minimisation. The cvff forcefield (MSI) was used for all calculations, assuming a dielectric constant of 80. The conformational flexibility of each molecule was investigated through a simulated annealing protocol (Bessis et al., 1999; Jullian et al., 1999). After initial minimisation, the temperature of the system was raised up to 900 K for 1 ps, and cooled to 600 and 300 K during 5 ps. The resulting conformation was minimised again, by using a combination of steepest descent (until derivative less than 5 kcal/mol) and conjugated gradient (derivative less than 0.05 kcal/mol) methods. This procedure was repeated 100 times for each molecule, and each final minimised conformation was archived (Bessis et al., 1999; Jullian et al., 1999). The overall superposition (Fig. 5) was obtained by matching homologous atoms from selected conformers. These atoms were the nitrogen of the tropane moiety (or homologous amino cycle), the methyl group of its equatorial *N*-methyl substituent (except for **8** and **9**), the two heteroatoms (O or N) from ester or amide functions, and the centroid of phenyl, pyrrole (indole) or pyrazole (indazole) rings.

3. Results

3.1. Some 5-HT_3 receptor antagonists also potentiate glycine responses from purified motoneurones

Isolated purified motoneurones have been shown to express functional glycine receptors with a voltage-sensitivity, a glycine-sensitivity and a strychnine-sensitivity similar to those of the receptors expressed in neurones cultured from the whole spinal cord (Lévi et al., 1999). We checked that the glycine responses of these identified

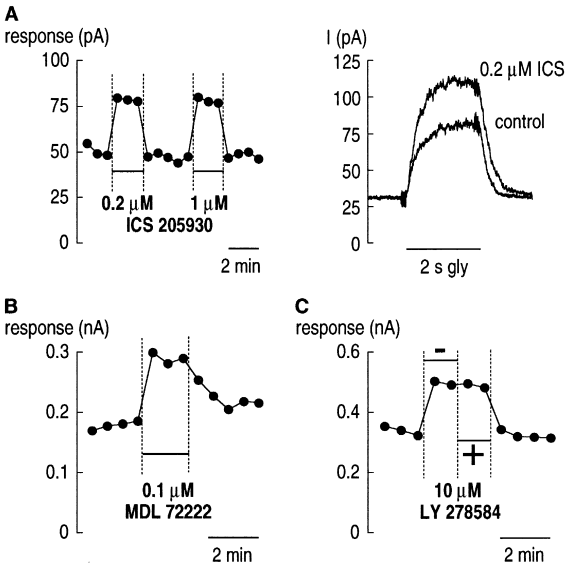

Fig. 1. Potentiation of the glycine responses of purified motoneurones by three 5-HT_3 receptor antagonists. (A) Potentiation of the response to 10 μM glycine by ICS 205930 successively applied (without preincubation) at 0.2 and 1 μM during periods indicated by the bars. Left plot, successive values of the response measured at the end of each application of glycine. Right traces, responses recorded in the absence or presence of 0.2 μM ICS 205930. (B), (C) Successive peak responses to 15 μM glycine in two different motoneurones and potentiation by 0.1 μM MDL 72222 (applied with preincubation, (B)) or by 10 μM LY 278584 (applied either without preincubation,"−", or with preincubation,"+", (C)). These experiments were performed at −30 mV in (A) and at −20 mV in (B) and (C).

motoneurones can also be potentiated by some 5-HT_3 receptor antagonists. At 0.2 μM, ICS 205930 (applied either without or with preincubation) reproducibly potentiated the responses of purified motoneurones to 10 μM glycine (by 60 ± 11% ($n = 4$) at −30 mV; Fig. 1A) and on the same cells, the potentiation induced by 1 μM ICS 205930 was similar (of 68 ± 5%; Fig. 1A). The responses to 15 μM glycine (measured at −20 mV) were also potentiated by 0.2 μM ICS 205930 (by 45% and 65%, respectively, in 2 motoneurones), by 0.1 μM MDL 72222 (by 48 ± 6% (3), application with preincubation; Fig. 1B) and by 10 μM LY 278584 (by 54 ± 1% (3), applications either without or with preincubation; Fig. 1C). Thus, the three 5-HT_3 receptor antagonists previously shown to potentiate glycine responses in ventral spinal cord cultures also potentiate the responses of identified motoneurones, in the same concentration ranges. These results encouraged us to perform the more complete pharmacological characterisation described below using ventral spinal cord cultures, which are much easier to prepare than purified motoneurones.

3.2. Comparison of several 5-HT_3 receptor antagonists as possible potentiators of glycine responses

Several molecules known as 5-HT_3 receptor antagonists were tested as possible modulators of responses to 10–15

μM glycine. Most experiments were performed at −30 mV. The molecules tested were applied with preincubation (see Section 2) in order to allow the detection of possible slow modulations.

The quaternized derivative of ICS 205930, Q-ICS 205930 (noted Q-ICS in Fig. 2), only differs from ICS 205930 by addition of a methyl group on the nitrogen atom of the tropane moiety and is slightly more potent than ICS 205930 on 5-HT_3 receptors (Watling et al., 1988; McKernan et al., 1990). As illustrated in Fig. 2, Q-ICS 205930 was a much less potent potentiator of glycine responses than ICS 205930; at 0.2 μM, Q-ICS 205930 did not potentiate the response whereas ICS 205930 induced a clear potentiation in the same neurone (Fig. 2A). This result was confirmed in eight experiments, four using 10 μM glycine (conditions under which, as already reported (Chesnoy-Marchais, 1999), 0.2 μM ICS 205930 induced a potentiation of 91 ± 34% (27)), four using 15 μM glycine, during which 0.2 μM ICS 205930 was also tested and induced a potentiation of 78 ± 12% (4). Q-ICS 205930 was also ineffective at 1 μM (4 cells) but, at 2 or 10 μM, it slightly potentiated the response to 15 μM glycine, by 16 ± 8% (6) and 31 ± 12% (9), respectively (Fig. 2B,C). The difference between the active concentrations of the two compounds is also illustrated by Fig. 2C. During the wash-out, the potentiation induced by 10 μM Q-ICS 205930 was immediately reversible; in contrast during the wash-out of high concentrations of ICS 205930, a larger potentiating effect was observed as a rebound (see also Chesnoy-Marchais, 1996). This difference in reversibility (observed in three cells on which both compounds were successively tested at 10 μM) confirms the specific potentiating effect of low concentrations of ICS 205930. We also observed the effect of Q-ICS 205930 to be slightly voltage-dependent, like that of ICS 205930 (Chesnoy-Marchais, 1996; Chesnoy-Marchais, 1999), which is also positively charged (by protonation of the nitrogen of the tropane). In four experiments, the effect of 10 μM Q-ICS 205930 (applied with preincubation) on the response to 15 μM glycine was successively tested at −30 and −90 mV; the percentage of potentiation was significantly larger at −90 than at −30 mV, by a factor 1.60 ± 0.14 (4) (paired *t*-test, $P = 0.005$; potentiation of 23.8 ± 5.4% and 37.9 ± 8.4% at −30 and −90 mV, respectively). This voltage-sensitivity was confirmed in four other experiments, in which the effect of 10 μM Q-ICS 205930 (here without preincubation and in the presence of 5 μM bicuculline) was successively tested at +10 and −90 mV. The percentage of potentiation of glycine responses was 1.92 ± 0.13 (4) times larger at −90 mV than at +10 mV ($P = 0.001$).

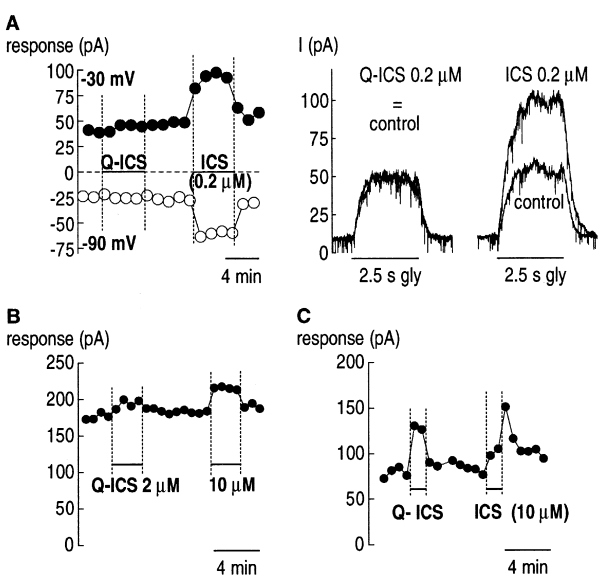

Fig. 2. Difference in potency between ICS 205930 and Q-ICS 205930. (A) The response of a ventral spinal neurone to 15 μM glycine is not modulated by 0.2 μM Q-ICS 205930 whereas it is clearly potentiated by 0.2 μM ICS 205930. The membrane was held at −30 mV and voltage-jumps to −90 mV of 4 s duration were applied every 80 s (and began 0.4 s before the glycine application). Left plot, successive values of the peak response to glycine at each membrane potential. Right traces, mean of two glycine responses recorded at −30 mV in the absence or presence of the compound tested. (B) Small and concentration-dependent potentiating effect of Q-ICS 205930 on the glycine response of another neurone maintained at −30 mV. (C) Comparison of the effects of 10 μM Q-ICS 205930 or ICS 205930 on the peak glycine response of a third neurone held at −20 mV. Note the difference in reversibility corresponding to the difference in potency between the two compounds.

The 5-HT_3 receptor antagonist granisetron (Zifa and Fillion, 1992; Hoyer et al., 1994) only differs from LY 278584 by an additional carbon in the azabicyclo moiety. At 1 μM (4 cells), 3 μM (6 cells), 6 μM (2 cells) or 10 μM (8 cells), granisetron did not affect the responses to 15 μM glycine. This is illustrated in Fig. 3 by two experiments during which the potentiating effect of either 0.2 μM ICS 205930 (Fig. 3A) or 10 μM LY 278584 (Fig. 3B) was subsequently observed on the same cells. Results similar to those of Fig. 3B were obtained in four experiments in which 10 μM LY 278584 induced a potentiation of 81 ± 16% (4). At 30 or 100 μM (Fig. 3C,D), granisetron reduced glycine responses by 21 ± 4% (5) and 47 ± 5% (4), respectively. This inhibitory effect did not require preincubation (Fig. 3C). Potentiation was never observed.

Two other potent 5-HT_3 receptor antagonists, ondansetron (see reference in Mair et al., 1998), applied at 0.1 μM (5 cells) or 1 μM (4 cells), and (*S*)-zacopride (Zifa and Fillion, 1992), applied at 0.1–0.2 μM (7 cells) or 1 μM (6 cells), did not affect glycine responses. At 10 μM, (*S*)-zacopride slightly reduced the response to 15 μM glycine, by 13 ± 6% (6) (data not shown).

Cocaine is a 5-HT_3 receptor antagonist in the micromolar range (see references in Mair et al., 1998) which was used to develop ICS 205930 and MDL 72222. Even though it is chemically related to these glycinergic potentiators, cocaine did not induce any significant modulation of glycine responses, whether applied at 0.1–0.2 μM (6 cells), 3 μM (3 cells) or 10 μM (3 cells) (data not shown).

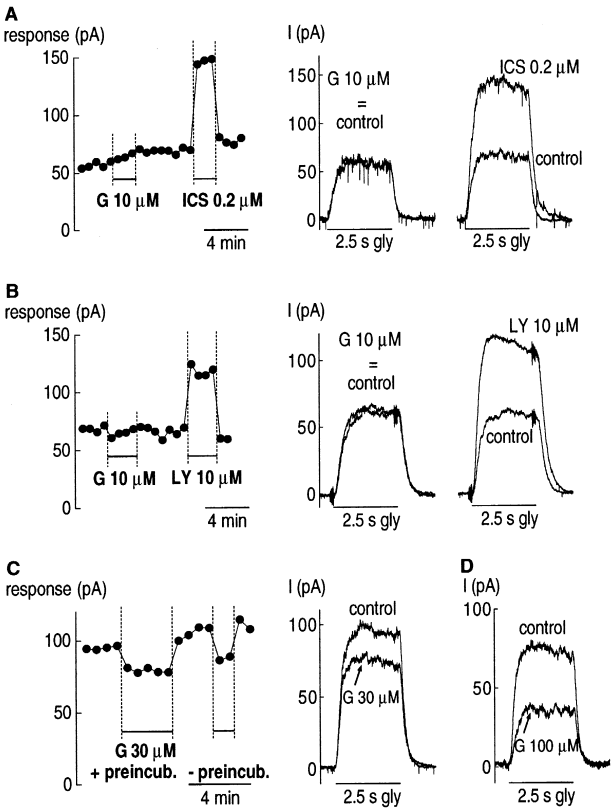

Fig. 3. Granisetron did not potentiate glycine responses. (A), (B) At 10 μM, granisetron (G) did not affect the glycine responses of two different neurones, whereas these responses were potentiated by either 0.2 μM ICS 205930 (A) or 10 μM LY 278584 (B). (C), (D) At 30 (C) or 100 (D) μM, granisetron reduced the glycine responses of two other neurones. In (C), granisetron was successively applied with and without preincubation. The left plots in (A), (B) and (C) give the successive values of the peak response to 15 μM glycine recorded at −30 mV, whereas the right traces in (A), (B), (C) and the traces illustrated in (D) are mean of glycine responses obtained in the absence (control) or presence of the modulator (two traces averaged in (A), four in the other cases).

3.3. Interpretation of the structure–activity relationship by molecular modelling

The structural similarities between the molecules displayed in Fig. 4 allow us to assume that the active ones bind at the same site. The pharmacophoric elements are analogous to those of 5-HT_3 receptor antagonists: an aromatic ring, a carbonyl group or its isostere and a basic nitrogen site in a tropane moiety. The spatial disposition of these elements has been defined (Hibert et al., 1990; Evans et al., 1991; Rival et al., 1998) and reviewed (Gozlan and Langlois, 1992; Chilmonczyk, 1995) for 5-HT_3 receptors. However there is no obvious reason for the disposition to be similar for glycinergic potentiators.

The compounds we used for our structure–activity analysis are displayed in Fig. 4. We included the potent 5-HT_3 receptor antagonist zatosetron (LY 277359, endo-5-chloro-2,3-dihydro-2,2-dimethyl-*N*-(3-α-tropanyl)-7-benzofuran-carboxamide; Robertson et al., 1992), even though it was not tested electrophysiologically, because recent binding data (Maksay, 1998) indicate that it may be also a glycinergic potentiator. All molecules were submitted to a high temperature dynamic procedure and for each of them, a hundred minimised conformations were generated, corresponding to the most stable possible states (Bessis et al., 1999; Jullian et al., 1999). These conformations were sorted according to several criteria, the dihedral angle between the aromatic ring and the carbonyl group (χ_1 = C_2, C_3, C_4, O_5, Fig. 4), the position of the tropane nitrogen above or below the aromatic ring and the axial or equatorial position of the methyl substituent of the tropane nitrogen. The possible values of each of these parameters were determined by the minimisation process. Examination of the first parameter (χ_1) for the nine molecules revealed three groups. In the first one comprising **1**, **4**, **7**, **8**, **9**, the aromatic ring and the carbonyl group are always coplanar ($\chi_1 = 0°$ or $\pm 180°$). In the second group, comprising **3** and **6**, the aromatic ring and the carbonyl group are never coplanar, the absolute value of χ_1 ranging from 30° to 60°. In the third group (**2** and **5**) both situations are encountered. The χ_1 parameter also determines the relative position of the phenyl ring and the carbonyl oxygen

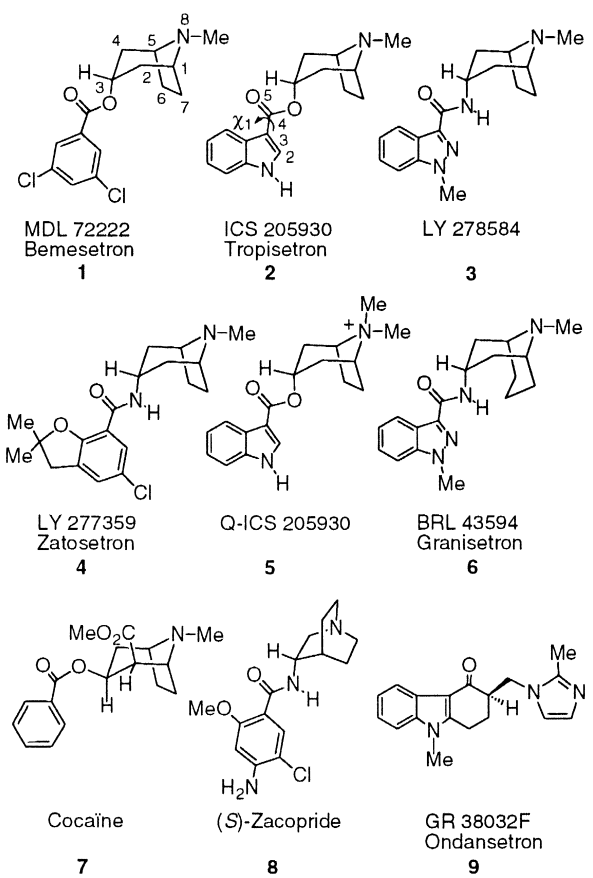

Fig. 4. Structure of the 5-HT_3 receptor antagonists assayed as glycinergic potentiators and used for construction of the model.

for the compounds containing an indole ring (**2**, **3**, **5**, **6**): with $-180° < \chi_1 < -90°$ or $90° < \chi_1 < 180°$, the phenyl ring and the carbonyl oxygen are located on the same side, whereas with $-90° < \chi_1 < 90°$, they are opposite. We first examined the two most active molecules (**1** and **2**) and noted that **1** could only adopt two symmetrical conformations with the tropane nitrogen above (lowest energy conformer) or below the aromatic/carbonyl plane. The conformation with tropane equatorial *N*-methyl substituent was slightly more stable than the one with axial substituent ($\Delta E = 0.17$ kcal). The most stable conformation of **1** was thus chosen as a template. In comparison, **2** was much more flexible, and a stable conformation (with planar disposition, the tropane nitrogen above the plane and the *N*-methyl substituent equatorial) which fits perfectly the pharmacophoric points of **1** could be found (Fig. 5). A similar result was obtained from analysis of **5** and an analogous conformation could be selected and superimposed to **1** (Fig. 5). Zatosetron **4** also adopts similar conformations; interestingly 99% of its conformations locate their furane ring on the same side as the carbonyl group, whereas the opposite situation which would favour an intramolecular hydrogen bond is found to be much less stable ($\Delta E = 6.0$ kcal). This is also the case when the dynamic process is run with an epsilon value of 4 that would mimic a hydrophobic environment and favour intramolecular coulombic interactions. The lowest energy conformer could be perfectly superimposed to the template (Fig. 5A). As mentioned above, LY 278584 **3** is never found with indazole ring and carbonyl group coplanar. All conformations display an angle of 35–60°, with the tropane nitrogen above or below the indazole ring. The phenyl ring of the indazole is always found opposite to the carbonyl oxygen. A conformer with $\chi_1 = -35°$ was selected and superimposed to **1** (Fig. 5). However, the quality of the superposition was not as high as with the above com-

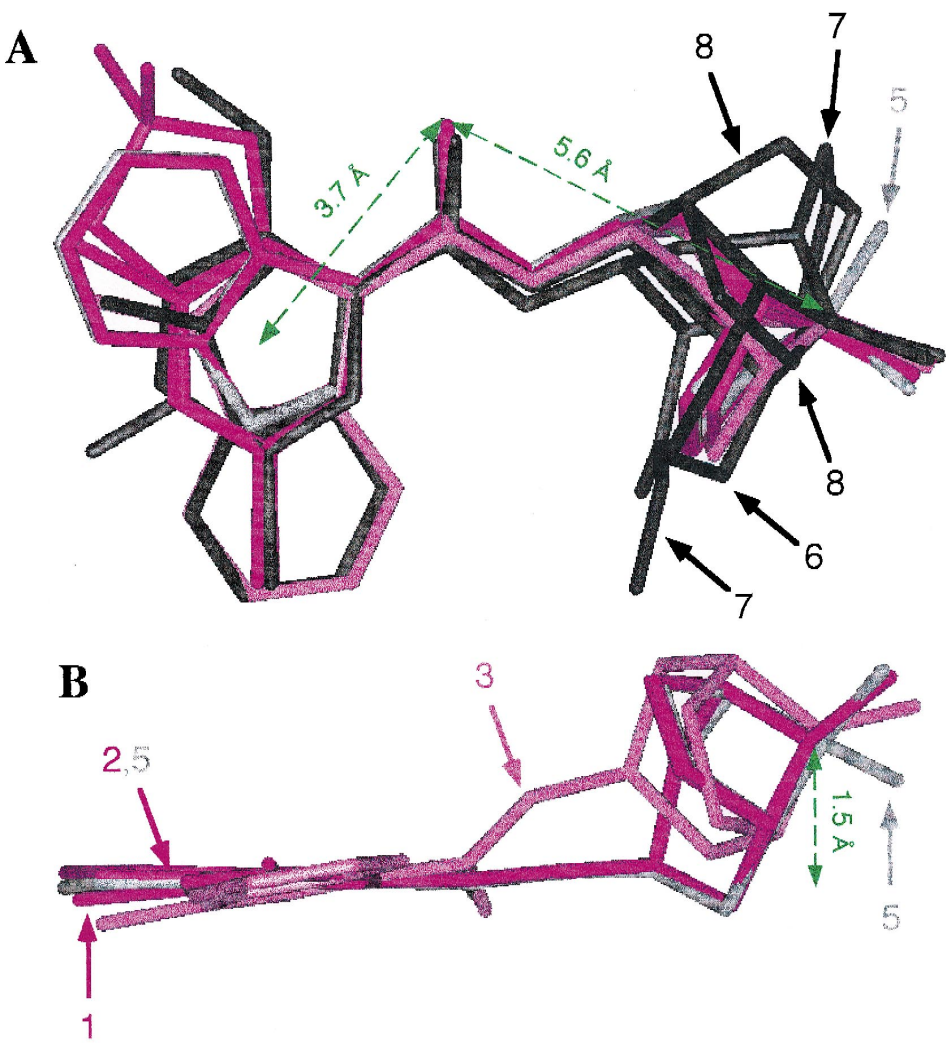

Fig. 5. Superposition of the compounds assayed as glycinergic potentiators in their conformation best fitting MDL 72222 **1** template. This molecule was chosen as a superposition pattern because it is one of the most potent ligand and adopts one most stable conformation. Ligands are coloured according to their activity: magenta (potent) for **1**, **2**, **4**, pink (efficient but of low potency) for **3**, grey (of low activity even at 10 μM) for **5** and black (inactive) for **6**–**8**. Ondansetron **9** is not displayed for clarity. Pharmacophoric distances are indicated. (A) Front view; (B) Side view (only **1**, **2**, **3** and **5** are shown).

pounds (the RMS value between **3** and **1**, mean distance calculated for four homologous superimposed atoms, is 0.45, whereas it is 0.07 between **2** and **1**; Fig. 5B). With granisetron **6**, conformations similar to those of **3** were calculated and a similar superposition was obtained (Fig. 5). Conversely (*S*)-zacopride **8**, cocaine **7** and ondansetron **9** (not shown in Fig. 5) behave as **1** and their selected conformer displayed a good superposition of the pharmacophoric elements. The racemic mixture of ondansetron **9** was assayed, thus both enantiomers were submitted to the high temperature dynamic procedure. Since the bemesetron **1** template holds a tropane nitrogen above the aromatic plane, conformers of the (*R*)-enantiomer fitted better the model. The (*S*)-zacopride methoxy substituent was chosen with the same orientation as the furane ring of **4**. Cocaine is the only tropane derivative of Fig. 4 which is *exo* substituted (aromatic ester on the same side as the tropane nitrogen); all the others are *endo* substituted (aromatic ester or carboxamide opposite to this nitrogen). The conformer of cocaine which best fits the model does not align its tropane ethano bridge (between carbons 6 and 7, see Fig. 4) with previous ones (Fig. 5A). The final superposition of compounds **1**–**8** is shown in Fig. 5. Ondansetron is not displayed for clarity. The molecules are coloured according to their activity as glycinergic potentiators: magenta for **1**, **2** and **4** (both potent and efficient), pink for **3** (efficient but less potent), grey for **5** (less active than **3**), and black for the inactive compounds (**6**–**8**). All selected conformations are either at global minimum or less than 1 kcal/mol above.

4. Discussion

The 5-HT_3 receptor antagonists which had been shown to potentiate glycine responses in cultures of ventral spinal cord neurones (Chesnoy-Marchais, 1996) were shown here to induce similar modulations of glycine responses from purified motoneurones cultured in the absence of spinal interneurones. This reinforces the idea that an understanding of the glycinergic modulatory activity of this class of molecules might prove useful toward development of more selective compounds, that could be used for example as antispasmodics. By expression of recombinant glycine receptors in Xenopus oocytes, it was shown recently that only the responses of heteromeric receptors can be potentiated by submicromolar concentrations of ICS 205930 (Chesnoy-Marchais and Supplisson, 1999; Supplisson and Chesnoy-Marchais, 2000). Thus, whereas spinal glycine receptors are often considered as being mainly $\alpha 2$ homomers in the embryo and $\alpha 1/\beta$ heteromers in the adult (Becker and Langosch, 1998), the results obtained here from purified motoneurones indicate that these cells already express heteromeric glycine receptors after 1 week in culture (conclusion already drawn from the low picrotoxinine-sensitivity of the glycine responses of these motoneurones (Lévi et al., 1999), since picrotoxinine is much more potent on homomeric receptors than on heteromeric receptors (Pribilla et al., 1992)). In the present paper, using cultured neurones showing reproducibly the potentiations studied, we attempted to find properties that could discriminate the interaction of the ligands studied with glycine receptors from their interaction with 5-HT_3 receptors.

Recently, the displacement of [^{3}H]strychnine binding from rat spinal cord membranes by various 5-HT_3 receptor antagonists and chemically related substances has been measured without or with 10 μM glycine (Maksay, 1998). Interestingly, with glycine (but not without), [^{3}H]strychnine binding could be displaced by submicromolar concentrations of the compounds which potentiate (in the same concentration range) the electrophysiological response to glycine (ICS 205930 and MDL 72222 (Chesnoy-Marchais, 1996)); on the contrary, the compounds which reduce these responses (5-hydroxytryptamine; *m*-chlorophenylbiguanide, D-tubocurarine (Chesnoy-Marchais, 1996)) could only displace [^{3}H]strychnine at very high concentrations (Maksay, 1998). In addition, the ability of glycine to displace [^{3}H]strychnine has been compared in the absence or presence of these substances: potentiating agents seemed to slightly increase glycine affinity, whereas inhibitory agents decreased it. These correlations between binding data and previous electrophysiological data have led Maksay (1998) to classify a series of chemicals as "gly-positive", "gly-negative" or "gly-neutral" agents. The electrophysiological data reported in the present paper agree qualitatively with this classification. Granisetron, classified as a "gly-negative" agent at 8 μM, is shown here to be unable to potentiate glycine responses and to be inhibitory above 10 μM (Fig. 3); ondansetron and cocaine, classified as "gly-neutral" agents at 10 and 200 μM, respectively, are also shown here to be unable to potentiate glycine responses, and were both reported (at high concentrations) to inhibit electrophysiological glycine responses of hippocampal neurones (Ren et al., 1999; Ye et al., 1999). The present study has reinforced the classification proposed by Maksay (1998) and has evaluated the glycinergic modulatory activity of Q-ICS 205930 and (*S*)-zacopride. It seems legitimate to combine electrophysiological and binding data to try to understand why only some 5-HT_3 receptor antagonists can be glycinergic potentiators. The results of the molecular modelling analysis illustrated by Fig. 5 provides some elements of answer.

The superposition of the nine selected conformers (Fig. 5) reveals the spatial disposition of the common elements which are probably recognised by the glycine receptor. The present model is a working hypothesis since it is only based on a few active compounds and needs to be further confirmed with new active molecules. The aromatic region, the carbonyl group and the tertiary amine site are laid out as in the template molecule **1**: the carbonyl group and the aromatic cycle are coplanar, the tropane nitrogen is situated above the plane, characteristic distances are phenyl

ring centroid–carbonyl oxygen = 3.7 Å and carbonyl oxygen–tropane nitrogen = 5.6 Å, tropane nitrogen–phenyl plane = 1.5 Å. This model is very similar to the 5-HT_3 one (Hibert et al., 1990; Evans et al., 1991; Gozlan and Langlois, 1992; Chilmonczyk, 1995; Rival et al., 1998). Inspection of the colour distribution in Fig. 5, black or grey for molecules which are inactive or little active as glycinergic potentiators, magenta or pink for efficient glycinergic potentiators, shows that for glycinergic potentiation, a rather large aromatic region can be tolerated whereas the region allowed around the tropane nitrogen is much more restricted than for the interaction with 5-HT_3 receptors. Indeed the marked difference between the modulatory effects of ICS 205930 **2** and Q-ICS 205930 **5** on glycine receptors is related to the additional methyl substituent on the tropane nitrogen of **5** (Fig. 5A), since **2** and **5** adopt similar conformations. Moreover substituents from **6**, **7** and **8** located in this region would be responsible for their lack of activity on glycine receptors (Fig. 5A). The reduced potency of **5** compared to **2** as a glycinergic potentiator might result from steric hindrance or from the loss of a hydrogen bond between the protonated nitrogen and the glycine receptor. The weaker potency of **3** compared to **1**, **2** and **4** (Chesnoy-Marchais, 1996; Maksay, 1998) might be connected with the lack of indazolecarboxamide coplanar conformations. Repulsive interaction between indazole or indole group and amide hydrogen prevents flatness. In contrast, it is not the case with esters. The selected **3** conformer is thus not well suited to the model (Fig. 5B) and would not adapt optimally to the receptor binding site. The non-coplanar molecule **3** is more potent on 5-HT_3 receptors than the coplanar molecule **1** (Zifa and Fillion, 1992). These observations would suggest that the coplanar disposition of the aromatic and carbonyl group is more strictly required for glycinergic potentiators than for 5-HT_3 receptor antagonists. However the region localised around the tropane nitrogen seems to be the most critical one, explaining why only some 5-HT_3 receptor antagonists potentiate glycine responses. We have suggested that the inability of other 5-HT_3 receptor antagonists to potentiate glycine responses result from their incapacity to bind to the potentiating site. However, we cannot exclude yet that these inactive compounds also bind to this site without allowing the conformational change responsible for the potentiation. Ideally, binding studies using 5-HT_3 radioligands and a heterologous system expressing a high density of heteromeric glycine receptors (allowing to assess the affinity of these ligands for glycine receptors) would clarify this point. In any case, the structural features pointed out by the present modelling study would be responsible for their inactivity. In order to design compounds showing the reverse selectivity (potentiation of glycine responses without interaction with 5-HT_3 receptors), it would be tempting to explore new substitutions in the aromatic region, keeping a restricted bulk around the tropane.

A few amino acids have been identified as contributing to the binding of some 5-HT_3 receptor antagonists on 5-HT_3 receptors: a negatively charged residue, glutamate 106 of the mouse 5-HT_3 A_L receptor (Boess et al., 1997), and a few tryptophan residues, which are likely to interact with the positively charged amine in the tropane of these antagonists by a cation-Π interaction (Venkataraman et al., 1999; Yan et al., 1999; Spier and Lummis, 2000). Interestingly, in the glycine receptor subunits present in spinal neurones ($\alpha 1$, $\alpha 2$ and β), the position corresponding to the 5-HT_3 A_L E106 is also a glutamate, and the positions corresponding to the important tryptophans are either tryptophan or phenylalanine, which are both able to participate in cation-Π interactions (Dougherty, 1996). Future identification of the binding site of the potentiators on glycine receptors would allow to further evaluate the present model by docking it into the tridimensional structure of the binding pocket. This should help to design new active molecules.

Acknowledgements

This work was supported by the European Commission (BMH4-CT97-2374) and by l'Association Française contre les Myopathies (MNM97). The molecular modelling study was supported by grants from the CNRS (PCV97-115) and RETINA France. We wish to thank SmithKline Beecham Pharmaceuticals for granisetron, Synthélabo Recherche for (*S*)-zacopride, Glaxo Group Research Limited for ondansetron. We also thank D. Lévy for the ventral spinal cord cultures and R. Hoffmann (MSI), P. Ascher and J. Bradley for useful discussions.

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