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Pharmacological characterization of a rat 5-hydroxytryptamine type$_3$ receptor subunit (r5-HT$_{3A(b)}$) expressed in *Xenopus laevis* oocytes

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1 The present study has utilized the two electrode voltage-clamp technique to examine the pharmacological profile of a splice variant of the rat orthologue of the 5-hydroxytryptamine type 3A subunit (5-HT$_{3A(b)}$) heterologously expressed in *Xenopus laevis* oocytes.

2 At negative holding potentials, bath applied 5-HT (300 nM – 10 μM) evoked a transient, concentration-dependent ($EC_{50} = 1.1 \pm 0.1$ μM), inward current. The response reversed in sign at a holding potential of -2.1 ± 1.6 mV.

3 The response to 5-HT was mimicked by the 5-HT$_3$ receptor selective agonists 2-methyl-5-HT ($EC_{50} = 4.1 \pm 0.2$ μM), 1-phenylbiguanide ($EC_{50} = 3.0 \pm 0.1$ μM), 3-chlorophenylbiguanide ($EC_{50} = 140 \pm 10$ nM), 3,5-dichlorophenylbiguanide ($EC_{50} = 14.5 \pm 0.4$ nM) and 2,5-dichlorophenylbiguanide ($EC_{50} = 10.2 \pm 0.6$ nM). With the exception of 2-methyl-5-HT, all of the agonists tested elicited maximal current responses comparable to those produced by a saturating concentration (10 μM) of 5-HT.

4 Responses evoked by 5-HT at EC_{50} were blocked by the 5-HT$_3$ receptor selective antagonist ondansetron ($IC_{50} = 231 \pm 22$ pM) and by the less selective agents (+)-tubocurarine ($IC_{50} = 31.9 \pm 0.01$ nM) and cocaine ($IC_{50} = 2.1 \pm 0.2$ μM).

5 The data are discussed in the context of results previously obtained with the human and mouse orthologues of the 5-HT$_{3A}$ subunit. Overall, the study reinforces the conclusion that species differences detected for native 5-HT$_3$ receptors extend to, and appear largely explained by, differences in the properties of homo-oligomeric receptors formed from 5-HT$_{3A}$ subunit orthologues.

Keywords: 5-Hydroxytryptamine (5-HT); 5-HT$_3$ receptor; 5-HT$_{3A}$ receptor subunit; arylbiguanides; 2-methyl-5-HT; ondansetron; (+)-tubocurarine, cocaine

Introduction

The neurotransmitter 5-HT modulates neuronal activity within the central and peripheral nervous systems *via* multiple receptor subtypes, biochemical effectors and membrane conductances. Of the 14 genes currently known to encode 5-HT receptor subtypes, only one specifies a transmitter-gated ion channel subunit of the Cys-loop family (Barnard, 1996), that being the 5-HT$_3$ receptor (Maricq *et al*., 1991; Hoyer & Martin, 1997). At discrete locations within the nervous system, the activation of 5-HT$_3$ receptor populations elicits a transient increase in membrane conductance to cations, evoking neuronal depolarization and neurotransmitter release (Ropert & Guy, 1991; Kawa, 1994; McMahon & Kauer, 1997). In brain slices derived from the rat and ferret, 5-HT$_3$ receptor activation is documented to contribute to fast excitatory synaptic transmission in the lateral amygdala (Sugita *et al*., 1992) and developing visual cortex (Roerig *et al*., 1997), respectively. In addition, it has recently been shown that a selective 5-HT$_3$ receptor antagonist, ondansetron, modifies action potential frequency recorded extracellularly from hippocampal neurones of freely moving rats, attesting to the physiological relevance of 5-HT$_3$ receptor activation by endogenous 5-HT (Reznic & Staubli, 1997).

Recombinant 5-HT$_3$ receptors, assembled as a homo-pentameric complex of 5-HT$_{3A}$ (formerly termed 5-HT$_3$R-A; Maricq *et al*., 1991) subunits, function efficiently in heterologous expression systems and mimic many of the properties of the receptor endogenous to neurones (Maricq *et al*., 1991; Hope *et al*., 1993; Boess *et al*., 1995; Green *et al*., 1995). Indeed, the detection of only gene orthologues specifying the 5-HT$_{3A}$ subunit in mouse (Maricq *et al*., 1991; Hope *et al*., 1993), rat (Isenberg *et al*., 1993), guinea-pig (Lankiewicz *et al*., 1998) and human (Belelli *et al*., 1995; Miyake *et al*., 1995) might indicate that the native 5-HT$_3$ receptor is a homo-oligomer, a feature that would be consistent with relatively early evolutionary origin of this receptor class (Ortells & Lunt, 1995). Limited structural diversity occurs through alternative splicing of the pre-RNA encoding 5-HT$_{3A}$ subunits in mouse (Hope *et al*., 1993; Werner *et al*., 1994) and rat (Miquel *et al*., 1995), but not human (Belelli *et al*., 1995; Miyake *et al*., 1995) generating two subunit variants, renamed 5-HT$_{3A(a)}$ (formerly 5-HT$_3$R-A$_L$) and 5-HT$_{3A(b)}$ (alias 5-HT$_3$R-A$_S$) in line with NC-IUPHAR nomenclature recommendations (Vanhoutte *et al*., 1996). However, the biophysical and pharmacological properties of these isoforms are essentially identical (Downie *et al*., 1994; Werner *et al*., 1994).

The orthologues of the 5-HT$_{3A}$ receptor demonstrate a high degree of amino acid sequence identity (81–95%, reviewed in Peters *et al*., 1997; Lankiewicz *et al*., 1998), yet species-dependent differences in the pharmacological profile of both recombinant and native receptors have been documented (e.g. Butler *et al*., 1990; Kilpatrick *et al*., 1991; Newberry *et al*., 1991; Peters *et al*., 1991). This situation is reminiscent of the pronounced species-dependent pharmacology encountered

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with certain G-protein-coupled 5-HT receptors, such as human and rat orthologues of the 5-HT$_{1B}$ receptor (Hamblin *et al*., 1992; Oksenberg *et al*., 1992). Whilst such differences potentially pose an impediment to receptor classification and drug development (Hall *et al*., 1993; Hoyer & Martin, 1997), they can also facilitate the detection of amino acid residues contributing to specific ligand binding domains upon the receptor complex (e.g. Oksenberg *et al*., 1992).

In the present study, we used the *Xenopus laevis* oocyte expression system to characterize the short form of the 5-HT$_3$ receptor (i.e. r5-HT$_{3A(b)}$) cloned from rat superior cervical ganglion neurones (Johnson & Heinemann, 1992; Isenberg *et al*., 1993) and rat brain (Miyake *et al*., 1995; Akuzawa *et al*., 1996). The short form of the rat and mouse 5-HT$_3$ receptors lack five and six consecutive amino acids respectively in the putative large intracellular loop. We focus particularly upon agonist and antagonist compounds that have previously revealed species differences in the pharmacological profile of 5-HT$_3$ receptors. These include representatives of the arylbiguanide class of agonist (i.e. phenylbiguanide (PBG) and several mono- and di-chloro substituted derivatives; Morain *et al*., 1994) and the antagonist (+)-tubocurarine (Yakel & Jackson, 1988; Peters *et al*., 1990). In addition, we have examined the actions of two dichloro-substituted derivatives of PBG upon human (h) and mouse (m) recombinant 5-HT$_3$ receptors for comparative purposes. A brief account of a part of this work has been published in abstract form (Mair *et al*., 1996).

Methods

The cDNA clone used routinely in the present study is a spliced version of the partial sequence (i.e. lacking bases corresponding to the signal peptide) cloned by Isenberg *et al*. (1993) from the rat superior cervical ganglion (SCG). Functional expression of r5-HT$_{3A(b)}$ was achieved by engineering the sequence encoding the signal peptide of m5-HT$_{3A(a)}$ into the SCG partial clone. The full length r5-HT$_{3A(b)}$ clone (a kind gift of Prof S. Heinemann) was also expressed in control experiments and yielded results identical to those obtained with the spliced clone. cRNA transcripts of the latter were synthesized following linearization of the cDNA using the restriction enzyme *Sac I*. A Riboprobe System II transcription kit (Promega Limited, U.K.) was used to produce capped *in vitro* transcripts using T3 RNA polymerase. DNA was removed by incubation with DNAase and protein contamination was minimized by use of phenol and chloroform. The quality and integrity of cRNA transcripts was assessed by spectrophotometric and electrophoretic techniques respectively. cRNA transcripts encoding the m5-HT$_{3A(b)}$ and h5-HT$_{3A}$ receptors were prepared according to the methods of Hope *et al*. (1993) and Belelli *et al*. (1995), respectively.

Xenopus oocytes were isolated as previously described (Hope *et al*., 1993) and incubated in Barth's solution comprising (in mM) NaCl, 88; KCl, 1; $NaHCO_3$ 2.4; $MgCl_2$, 1; $CaCl_2$, 1 and HEPES, 15 (pH 7.5). Stage VI oocytes were injected with 50 ng of cRNA transcripts in 50 nl of nuclease-free water using a Drummond Digital Microdispenser 510 (Drummond Scientific Co., U.S.A.). The injected oocytes were stored in individual wells of a 96 well microtiter plate in 200 μl of standard Barth's supplemented with gentamycin (100 μg ml^{-1}). Oocytes were incubated at 19°C prior to use, 2–7 days after injection.

Agonist-evoked currents were recorded at a holding potential of −60 mV using conventional two electrode voltage clamp (Geneclamp 500, Axon Instruments). The voltage sensing and current passing microelectrodes were filled with 3 M KCl and 3 M CsCl respectively and had resistances of 0.5–0.8 MΩ when measured in the Barth's solution detailed above or a modified solution in which $BaCl_2$ (1 mM) replaced $CaCl_2$. Oocytes were held in a bath of 300 μl volume and continually superfused with extracellular solution at a rate of 15 ml min^{-1}. Current and voltage signals from the voltage-clamped oocyte were analysed using the Strathclyde Electrophysiology Software WinWCP program (Dempster, 1997) and a National Instruments Lab-PC and laboratory interface board (National Instruments, Newberry, U.K). Timed pulses of drugs dissolved in Barth's solution were applied to oocytes *via* a BPS-4 bath perfusion system (Adams and List Associates, New York) with a four way manifold. Drug application was automatically controlled by the computer program during recording sweeps *via* the laboratory interface TTL digital outputs and solenoid-controlled pinch valves attached to the perfusion lines. Current-voltage curves for agonist induced currents were determined by the digital subtraction of sweeps consisting of 5 s voltage ramps from −140 to +30 mV applied both in the presence and absence of agonist. Antagonists were pre-applied for 1 min prior to co-application with agonist. All measurements were performed at ambient temperature (18–22°C).

Quantitative results are expressed as the arithmetic mean ± s.e.mean. Concentration-response data were fitted iteratively with the four parameter logistic equation:

$$\frac{I}{I_{max}} = \frac{A^{n_H}}{A^{n_H} + EC_{50}^{n_H}}$$

where I_{max} is the maximum inward current evoked by a saturating concentration of agonist, I is the inward current in the presence of agonist at concentration A, EC_{50} is the concentration of agonist evoking a half maximal response and n_H is the Hill coefficient. A similar equation, with antagonist concentration replacing A, inward current amplitude in the absence of agonist replacing I_{max}, and the concentration of antagonist (IC_{50}) producing a 50% block of the response replacing EC_{50} was used to analyse concentration-inhibition data obtained with antagonist compounds.

The drugs used were: 5-HT creatinine sulphate, (+)-tubocurarine chloride and cocaine hydrochloride (Sigma), 2-methyl-5-HT maleate (2-Me-5-HT), 1-phenylbiguanide (PBG) and 3-chlorophenylbiguanide dihydrochloride (3-CPBG; Research Biochemicals), ondansetron hydrochloride (Glaxo Research) and 2,5-dichlorophenylbiguanide (2,5-diCPBG) and 3,5-dichlorophenylbiguanide (3,5-diCPBG; Maybridge). All drugs were freshly dissolved as concentrated stock solutions in either double-distilled de-ionised water or Barth's solution and diluted into Barth's solution.

Results

Agonist pharmacology

The bath application of 5-HT (10 μM) to voltage-clamped oocytes previously injected with cRNA encoding the r5-HT$_{3A(b)}$ receptor elicited transient inward currents with peak amplitudes within the range 100 nA to 5 μA at a holding potential of −60 mV. The response elicited by very low concentrations (600 nM) of 5-HT demonstrated no measurable desensitization during the period of agonist application permitting a determination of the reversal potential ($E_{5\text{-HT}}$) of the response (−2.1 ± 1.6 mV; n = 4) to be made by the voltage-

ramp protocol described in Methods (not illustrated). This value is consistent with the activation of non-selective cation channels, as documented for 5-HT$_3$ receptors in numerous other preparations (Peters *et al*., 1992). 5-HT$_3$ receptors native to rat superior cervical ganglion neurones are permeable to Ca^{2+} (Yang *et al*., 1992). To assess the possibility that the Ca^{2+}-activated chloride conductance endogenous to oocytes contributes to the 5-HT evoked current, $E_{5\text{-HT}}$ was additionally determined in a modified Barth's solution wherein Ba^{2+} totally replaced Ca^{2+}. Little effect upon $E_{5\text{-HT}}$ (-5.8 ± 2.0 mV; $n=3$) was noted suggesting that Ca^{2+} influx, if it indeed occurs (see Gilon & Yakel, 1995), is insufficient to corrupt the response to 5-HT.

In normal Barth's solution, the minimal effective concentration of 5-HT was 300 nM and the response saturated at concentrations $\geqslant 10$ μM (Figure 1a). Analysis of the 5-HT concentration-response relationship yielded an EC_{50} value of 1.1 ± 0.1 μM ($n=4$) and a Hill coefficient (n_H) of 2.7 ± 0.3 ($n=4$; Figure 1b). Very similar values ($EC_{50}=0.70\pm0.02$ μM; $n_H=2.8\pm0.1$; $n=3$) where obtained with Barth's solution wherein Ba^{2+} replaced Ca^{2+}, providing further evidence that the secondary activation of a Ca^{2+}-dependent chloride conductance is not a confounding phenomenon under the conditions of the present study. The agonist potency of 5-HT is similar to that reported for the human and mouse orthologues of the 5-HT$_{3A}$ receptor (Downie *et al*., 1994; Belelli *et al*., 1995; Miyake *et al*., 1995; see Table 1). Similarly, the agonist potency of the 2-methyl derivative of 5-HT (2-Me-5-HT; $EC_{50}=4.1\pm0.2$ μM, $n_H=2.1$, $n=4$, Figure 1a and b) at the r5-HT$_{3A(b)}$ receptor was essentially identical to that previously found for the human orthologue but marginally higher than that determined at the m5-HT$_{3A(b)}$ splice variant (Downie *et al*., 1994; Belelli *et al*., 1995; Miyake *et al*., 1995; Table 1)

Phenylbiguanide (PBG), and several mono- and dichloro-substituted derivatives of the compound (Kilpatrick *et al*., 1990; Morain *et al*., 1994; Dukat *et al*., 1996), evoked inward current responses that were qualitatively similar to those elicited by 5-HT. For all such compounds studied, the rate of current activation and desensitization clearly increased with agonist concentration (Figure 1a). However, no attempt was made to quantify these parameters, because the geometry of the oocyte precludes solution exchanges of a rapidity sufficient for meaningful kinetic analysis. Phenylbiguanide was approximately 2.5 fold less potent than 5-HT ($EC_{50}=3.0\pm0.1$ μM, $n_H=2.1\pm0.2$; $n=4$) and elicited a maximal current response amounting to approximately 116% of that observed with a saturating concentration (10 μM) of 5-HT in the same population of oocytes (Figure 1b). The similarity in the EC_{50} values for 5-HT and PBG at

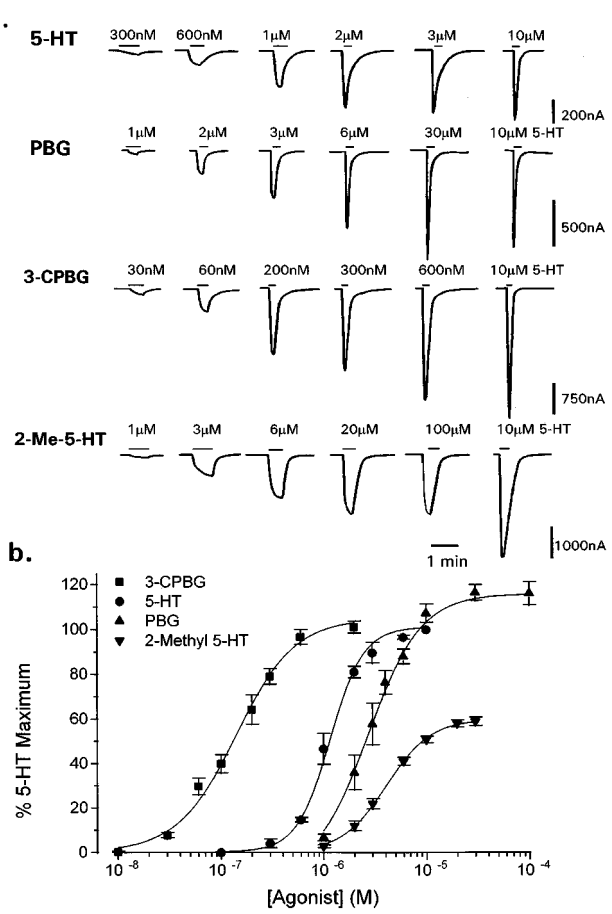

Figure 1 The agonist pharmacology of the rat 5-HT$_{3A(b)}$ subunit expressed in *Xenopus laevis* oocytes. (a) Representative traces illustrating concentration-dependent inward currents evoked by bath applied 5-HT, 1-phenylbiguanide (PBG), 3-chlorophenylbiguanide (3-CPBG) and 2-methyl-5-HT (2-Me-5-HT). Note that for all agonists, the kinetics of current activation and desensitization increase with agonist concentration. The trace to the extreme right of each row indicates the maximal inward current response evoked by a saturating concentration (10 μM) of 5-HT. Periods of drug application in this and all subsequent figures are indicated by horizontal bars above each trace. All currents were recorded at a holding potential of -60 mV. (b) Graph depicting the relative potencies and efficacies of 5-HT, 1-phenylbiguanide (PBG), 3-chlorophenylbiguanide (3-CPBG) and 2-methyl-5-HT (2-Me-5-HT). Peak current amplitude (ordinate, linear scale) is expressed as a percentage of the maximal current response to 5-HT (10 μM) and plotted against the concentration of agonist in the medium (abscissa; logarithmic scale). The maximal response to 5-HT was determined for each oocyte examined. Data points are the mean of at least four observations made on different oocytes. Vertical bars indicate the s.e.m. Curves were fitted as described in Methods.

Table 1 Comparative pharmacology of rat, human and mouse 5-HT$_{3A}$ receptor orthologues

Compound	*r5-HT$_{3A(b)}$*			*h5-HT$_{3A}$*			*m5-HT$_{3A(b)}$*			*EC$_{50}$ or IC$_{50}$ ratio*	
	pEC$_{50}$ or pIC$_{50}$	*n$_H$*	*α*	*pEC$_{50}$ or pIC$_{50}$*	*n$_H$*	*α*	*pEC$_{50}$ or pIC$_{50}$*	*n$_H$*	*α*	*h/r*	*m/r*
5-HT	5.79	2.7	1.00	5.51*	1.9*	1.00*	5.63†	2.2†	1.0†	1.9	1.4
PBG	5.57	2.1	1.16	4.07*	2.1*	0.68*	4.47†	2.0†	0.73†	31.6	12.6
3-CPBG	6.91	1.9	1.00	5.44*	1.8*	0.81*	6.08†	1.8†	0.88†	29.5	6.8
3,5-diCPBG	7.84	1.6	0.99	6.90	2.6	0.96	6.85	1.8	0.93	8.7	9.8
2,5-diCPBG	7.99	2.4	1.00	6.93	2.9	0.91	7.04	2.4	0.87	11.5	8.9
2-Me5-HT	5.39	2.1	0.66	5.27*	2.1*	0.87*	4.82†	2.2†	0.09†	1.3	3.7
Ondansetron	9.63	−1.0	–	9.51*	−1.0*	–	9.52†	−1.0†	–	1.3	1.3
(+)-Tubocurarine	7.52	−1.7	–	5.59*	−0.9*	–	8.85†	−0.9†	–	85.1	0.047
Cocaine	5.46	−2.1	–	6.12*	−1.1*	–	5.64†	−1.1†	–	0.22	0.66

*Data from Belelli *et al*. (1995) or †Downie *et al*. (1994). α: Ratio of maximal peak inward responses induced by saturating concentrations of test agonist and 5-HT. pEC$_{50}$ and pIC$_{50}$ values reported in this study are the mean calculated from experiments performed on 3–4 separate oocytes.

the r5-HT$_{3A(b)}$ receptor is concordant with data obtained with 5-HT$_3$ receptors endogenous to rat peripheral neurones (Ireland & Tyers, 1987; Newberry & Gilbert, 1989), but contrasts markedly with the much lower relative potency of PBG at the human or mouse orthologues of the 5-HT$_{3A}$ receptor (Downie *et al*., 1994; Belelli *et al*., 1995; Lankiewicz *et al*., 1998; Table 1).

It has previously been shown that the introduction of a 3-chloro substituent into PBG (i.e. 3-CPBG or *meta*-CPBG; Kilpatrick *et al*., 1990) greatly increases the agonist potency of the compound in functional studies of 5-HT$_3$ receptors expressed in the rat (Kilpatrick *et al*., 1990) and, to a lesser extent, the mouse (Sepúlveda *et al*., 1991; Boess *et al*., 1992; Morain *et al*., 1994). The r5-HT$_{3A(b)}$ receptor is similar in this respect, since 3-CPBG ($EC_{50}=140\pm10$ nM, $n_H=1.9\pm0.1$; $n=4$) demonstrated approximately 9 and 22 fold greater potency over 5-HT and the parent compound respectively (Figure 1a and b). When compared with results obtained with the h5-HT$_{3A}$ receptor (Belelli *et al*., 1995), 3-CPBG and PBG display an identical ($\sim$30 fold) selectivity for the rat orthologue (Table 1). A less pronounced and non-identical selectivity is apparent between r5-HT$_{3A(b)}$ and m5-HT$_{3A(b)}$ subunits (Table 1).

The agonist potencies of the di-chloro-substituted compounds 2,5-dichlorophenylbiguanide, (2,5-diCPBG; $EC_{50}=10.2\pm0.6$ nM, $n_H=2.4\pm0.2$, $n=4$) and 3,5-dichlorophenylbiguanide (3,5-diCPBG; $EC_{50}=14.5\pm0.4$ nM, $n_H=1.6$, $n=4$) at the r5-HT$_{3A(b)}$ receptor were similar and exceeded that of 3-CPBG by approximately 8–12 fold (Figure 2a and b). In the present study, a direct comparison of the effect of these two agonists upon the h5-HT$_{3A}$ receptor revealed their agonist potencies to be essentially identical (2,5-diCPBG; $EC_{50}=118\pm7$ nM, $n_H=2.9\pm0.2$, $n=4$ *versus* 3,5-diCPBG; $EC_{50}=125\pm10$ nM, $n_H=2.6\pm0.2$, $n=4$) and approximately 9–11 fold lower than determined for the r5-HT$_{3A(b)}$ receptor (Table 1; Figure 2a and b). The potencies of 2,5-diCPBG ($EC_{50}=90\pm9$ nM; $n_H=2.4\pm0.2$; $n=3$) and 3,5-diCPBG ($EC_{50}=140\pm25$ nM; $n_H=1.8\pm0.2$; $n=3$) at the m5-HT$_{3A(b)}$ receptor (data not illustrated) indicate a similar degree of selectivity for the rat receptor and little preference between human and mouse orthologues (Table 1). Thus, although the arylbiguanide derivatives examined here demonstrate an identical rank order of potency at human and rat 5-HT$_{3A}$ receptor orthologues, they convincingly discriminate between them in terms of absolute potency. Finally, in contrast to several reports concerning 5-HT$_3$ receptors endogenous to rat neurones (Ireland & Tyers, 1987; Kilpatrick *et al*., 1990; Todorovic & Anderson, 1990), none of the arylbiguanides examined displayed the properties of a partial agonist (Table 1; Figure 2a and b).

Antagonist pharmacology

In order to substantiate the differences in the pharmacological profiles of human and rat recombinant 5-HT$_3$ receptors suggested by the results obtained with the arylbiguanide class of agonist, a limited number of antagonists were additionally examined, focusing upon compounds such as (+)-tubocurarine (Yakel & Jackson, 1988; Peters *et al*., 1990) and cocaine (Malone *et al*., 1991; Peters *et al*., 1991) that have previously revealed inter-species differences in the properties of native and recombinant 5-HT$_3$ receptors. (+)-Tubocurarine produced a concentration-dependent and readily reversible blockade of the response evoked by 5-HT bath applied at EC_{50} (i.e. 1 μM). From the data presented in Figure 3a and b, an IC_{50} of 31.9 ± 0.01 nM and an n_H of -1.7 ± 0.1 ($n=4$) were calculated. In comparison to data published for the m5-HT$_{3A(b)}$ receptor (Downie *et al*., 1994), (+)-tubocurarine is approximately 20 fold less effective as an antagonist of the rat orthologue (Table 1). However, the antagonist demonstrates an 85 fold selectivity for r5-HT$_{3A(b)}$ over h5-HT$_{3A}$ (Belelli *et al*., 1995; Table 1). Conversely, the IC_{50} of 2.1 ± 0.2 μM ($n_H=-2.1\pm0.1$; $n=4$) obtained in this study for the reversible antagonist cocaine, indicates the compound to be 5 fold less potent in antagonizing the rat *versus* the human 5-HT$_{3A}$ receptor (Figure 3a and b; Table 1). Cocaine does not discriminate between r5-HT$_{3A(b)}$ and m5-HT$_{3A(b)}$ orthologues (Table 1). Finally, the selective 5-HT$_3$ receptor antagonist ondansetron, which produced only a slowly reversible blockade of the response mediated by the r5-HT$_{3A(b)}$ receptor ($IC_{50}=231\pm22$ pM; $n_H=-1.0$; $n=4$) did not discriminate between the rat, human or mouse receptor orthologues (Figure 3a and b; Table 1).

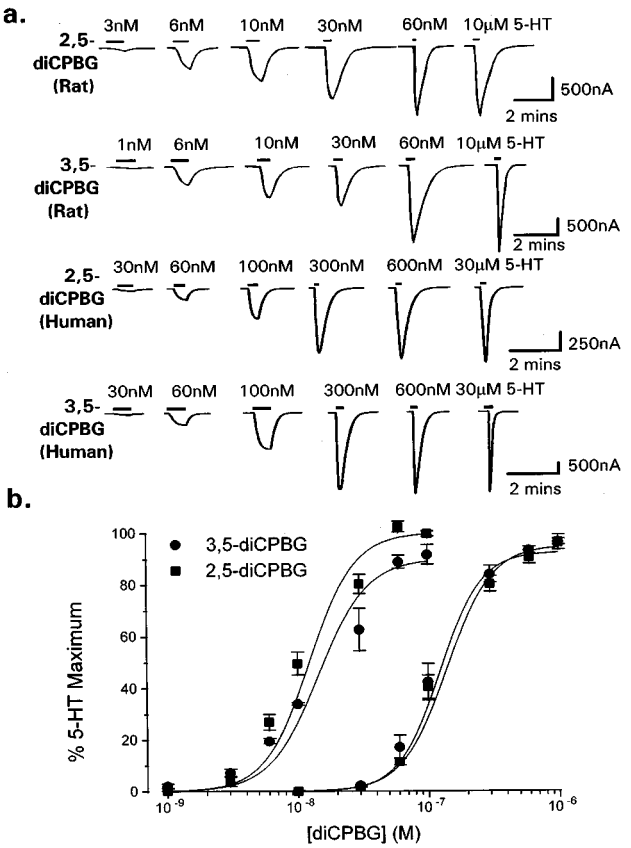

Figure 2 A comparison of the effects of dichloro-substituted derivatives of phenylbiguanide upon rat 5-HT$_{3A(b)}$ and human 5-HT$_{3A}$ receptor subunits expressed in *Xenopus laevis* oocytes. (a) Representative traces illustrating concentration-dependent inward currents evoked by 2,5-dichlorophenylbiguanide (2,5-diCPBG) and 3,5-dichlorophenylbiguanide (3,5-diCPBG) bath applied to oocytes expressing the rat (upper two rows) or human (lower two rows) subunit orthologues. The trace to the extreme right of each row indicates the maximal inward current response evoked by a saturating concentration (10–30 μM) of 5-HT. (b) Graph depicting the relative potencies and efficacies of 2,5-dichlorophenylbiguanide (2,5-diCPBG) and 3,5-dichlorophenylbiguanide (3,5-diCPBG). Peak current amplitude (ordinate, linear scale) is expressed as a percentage of the maximal current response to 5-HT (rat 10 μM; human 30 μM) and plotted against the concentration of agonist in the medium (abscissa; logarithmic scale). The maximal response to 5-HT was determined for each oocyte examined. Data points are the mean of at least four observations made on different oocytes. Vertical bars indicate the s.e.m. Curves were fitted as described in Methods.

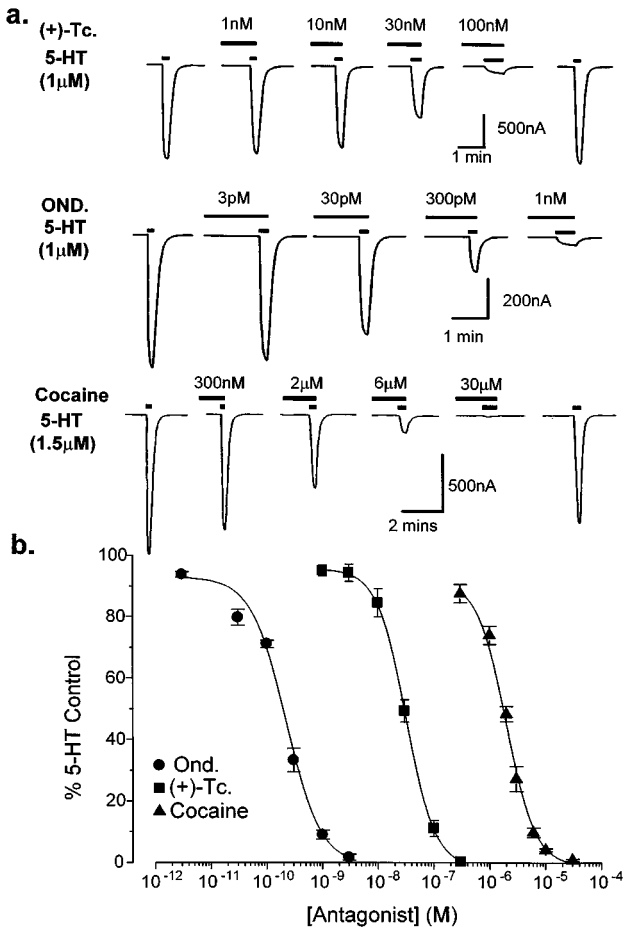

Figure 3 Antagonist pharmacology of the rat 5-HT$_{3A(b)}$ receptor subunit expressed in *Xenopus laevis* oocytes. (a) representative traces illustrating the concentration dependent suppression by (+)-tubocurarine ((+)-Tc), ondansetron (Ond) and cocaine of inward current responses evoked by 5-HT at EC_{50} (1–1.5 μM). Antagonist compounds were pre-applied for 1 min prior to co-application with 5-HT. Note that antagonism by (+)-tubocurarine and cocaine was readily reversible, whereas the effect of ondansetron was not. (b) Graph depicting the relative potencies of (+)-tubocurarine ((+)-Tc), ondansetron (Ond) and cocaine as antagonists of the inward current response to 5-HT. Peak current amplitude (ordinate, linear scale) is expressed as a percentage of the control current response to 5-HT at EC_{50} and plotted against the concentration of antagonist in the medium (abscissa; logarithmic scale). Data points are the mean of at least four observations made on different oocytes. Vertical bars indicate the s.e.m. Curves were fitted as described in Methods.

Discussion

The present study represents an initial description of the pharmacological properties of the rat orthologue of the 5-HT$_{3A(b)}$ subunit heterologously expressed in *Xenopus laevis* oocytes. Although characterization of this species homologue of the 5-HT$_{3A(b)}$ receptor has previously been achieved utilizing the binding of the 5-HT$_3$ receptor antagonist [^{3}H]-ramosetron (YM060) to membrane homogenates prepared from cDNA transfected COS-1 cells (Miyake *et al*., 1995; Akuzawa *et al*., 1996), functional expression has been noted only in abstract form (Johnson & Heinemann, 1992). The data available from binding studies suggests homo-oligomeric receptors composed of rat 5-HT$_{3A(b)}$ subunits to possess a pharmacological profile that is essentially identical to that of the 5-HT$_3$ receptor native to rat cerebral cortex (Akuzawa *et al*., 1996). Similarly, the pharmacological properties of human and mouse recombinant 5-HT$_{3A}$ receptors largely mirror those of their endogenous counterparts (Maricq *et al*., 1991, Downie *et al*., 1994; Belelli *et al*., 1995; Bonhaus *et al*., 1995; Miyake *et al*., 1995), although discrepancies in the efficacies of certain partial agonists, such as 2-Me-5-HT, dopamine and RS-056812-198, have been reported (van Hooft & Vijverberg, 1997; van Hooft *et al*., 1997). Notwithstanding the latter, the species-dependent pharmacology established for 5-HT$_3$ receptors native to neuronal tissues extends, in the main, to the orthologues of the cloned 5-HT$_{3A}$ subunit (see also below).

Arylbiguanides, exemplified by PBG, have long been known to mimic the action of 5-HT at 5-HT$_3$ receptors (e.g. Wallis *et al*., 1982) and recent detailed studies have established structure activity relationships for biguanide- and arylguanidine-derivatives as selective agonists of 5-HT$_3$ receptors endogenous to clonal cell lines derived from the mouse (Morain *et al*., 1994; Dukat *et al*., 1996). The present study adds to this information by providing data for rat, human and mouse clonal 5-HT$_3$ receptors studied under voltage-clamp conditions. In comparison to previous results obtained with the human 5-HT$_{3A}$ subunit under identical conditions of recording and expression (Belelli *et al*., 1995), PBG and 3-CPBG are approximately 30 fold more potent as agonists of the rat 5-HT$_{3A(b)}$ receptor. A direct comparison of the potencies of the dichloro-substituted compounds, 3,5-diCPBG and 2,5-diCPBG, at the human and rat receptor orthologues in this study revealed a qualitatively similar, though less pronounced (i.e. approximately 10 fold), selectivity for the rodent receptor. More modest differences (i.e. approximately 3–4 fold) in the potency of PBG and 3-CPBG acting at mouse and human 5-HT$_{3A}$ receptor orthologues have been reported by Belelli *et al*. (1995) and subsequently confirmed by Lankiewicz *et al*. (1998). The present results indicate that di-chloro substitution, as in 3,5-diCPBG and 2,5-diCPBG, essentially abolishes this narrow margin of selectivity between m5-HT$_{3A(b)}$ and h5-HT$_{3A}$ receptors. It is notable that the EC_{50} values determined for the arylbiguanides acting at the m5-HT$_{3A}$ receptor differ little from those reported by Morain *et al*. (1994) in voltage-clamp recordings performed on mouse N1E-115 neuroblastoma cells, providing further evidence for strong pharmacological similarity between native and recombinant 5-HT$_3$ receptors within a species. Lankiewicz *et al*. (1998) have very recently demonstrated the inactivity of PBG at guinea-pig 5-HT$_{3A(a)}$ and 5-HT$_{3A(b)}$ subunits, complementing results obtained with tissues native to this species (Butler *et al*., 1990; Newberry *et al*., 1991). However, 3-CPBG was an agonist at guinea-pig 5-HT$_{3A}$ receptor subunits, albeit with a potency >100 fold less than that found in the present study (Lankiewicz *et al*., 1998).

The pronounced differences in the agonist potencies of arylbiguanide compounds across cloned 5-HT$_{3A}$ receptor orthologues do not extend to the indoles 5-HT or 2-Me-5-HT which, from the results of the present and comparable studies, appear to be approximately equipotent at rat, mouse (Downie *et al*., 1994), human (Belelli *et al*., 1995, Miyake *et al*., 1995) and guinea-pig receptor homologues (Lankiewicz *et al*., 1998). Thus, overall, the electrophysiological data suggest a preferential interaction between arylbiguanide compounds and the rat 5-HT$_3$ receptor. Accordingly, studies employing radioligand binding techniques have demonstrated PBG and 3-CPBG to possess a considerably higher affinity for 5-HT$_3$ receptors native to rat than those present in mouse, guinea-pig, rabbit or human tissues (e.g. Kilpatrick *et al*., 1991; Bufton *et al*., 1993; Wong *et al*., 1993). A similar trend is apparent from binding studies conducted upon rat, guinea-pig and human recombinant 5-HT$_3$ receptors (Miyake *et al*., 1995; Akuzawa *et al*., 1996; Hope *et al*., 1996; Lankiewicz *et al*., 1998).

A large difference (up to 100 fold) exists between the K_i values commonly found for agonists in competition with antagonist radioligands for binding to native (e.g. Wong *et al*., 1993; Akuzawa *et al*., 1996) or rat recombinant 5-HT$_3$ receptors (e.g. Akuzawa *et al*., 1996) and the agonist EC_{50} values reported here. Similar observations have been made in studies performed on mouse 5-HT$_3$ receptors endogenous to N1E-115 neuroblastoma cells, where agonist EC_{50} values determined under voltage-clamp suggest apparent affinities much lower than those determined by radioligand binding (Sepúlveda *et al*., 1991; Morain *et al*., 1994; Delagrange *et al*., 1996). One possible explanation of this discrepancy is that the relatively prolonged exposure to agonist necessary to achieve equilibrium under the conditions of the radioligand assays promotes a desensitized conformation of the receptor which recognizes agonists with high affinity (Sepúlveda *et al*., 1991). Consistent with this interpretation, the superfusion of agonist at concentrations insufficient to evoke a discernible current response in voltage-clamp studies appears to convert 5-HT$_3$ receptors into a desensitized state(s) refractory to activation by the same, or other, agonist subsequently applied at relatively high concentration (Neijt *et al*., 1988; Bartrup & Newberry, 1996; van Hooft & Vijverberg, 1996). The IC_{50} for such an effect corresponds more closely to agonist K_i values determined in ligand-binding studies (Bartrup & Newberry, 1996; van Hooft & Vijverberg, 1996). Interestingly, the K_i and EC_{50} values determined for 2-Me-5-HT and 3-CPBG acting at the guinea-pig recombinant 5-HT$_3$ receptor differ by only 2 fold (Lankiewicz *et al*., 1998). This may correlate with the unusually slow kinetics of receptor desensitization in this species (Lankiewicz *et al*., 1998).

In addition to the arylbiguanide agonists, (+)-tubocurarine clearly discriminates between the rat, human and mouse orthologues of the 5-HT$_{3A}$ receptor subunit. The antagonist potency of (+)-tubocurarine determined in the present study ($pIC_{50} = 7.52$), which is in reasonable correspondence with that found for the 5-HT$_3$ receptor of rat superior cervical ganglion neurones (Newberry *et al*., 1991; Yang *et al*., 1992), is intermediate to the high and low affinities obtained in electrophysiological assays conducted upon mouse ($pIC_{50} \sim 8.8$; Hope *et al*., 1993, Downie *et al*., 1994, Hussy *et al*., 1994; Gill *et al*., 1995) and human ($pIC_{50} \sim 5.6$; Belelli *et al*., 1995; Brown *et al*., 1998) recombinant 5-HT$_{3A}$ receptors respectively. This rank order of potency corresponds to that found in binding and functional assays conducted upon 5-HT$_3$ receptors native to tissues of the relevant species (e.g. Newberry *et al*., 1991; Bufton *et al*., 1993; Wong *et al*., 1993). Such studies also indicate that the receptors endogenous to the guinea-pig and human possess a comparable affinity for (+)-tubocurarine (Bufton *et al*., 1993; Wong *et al*., 1993). By contrast to the discriminative properties of (+)-tubocurarine, the present results indicate that ondansetron, a prototypical 5-HT$_3$ receptor antagonist, does not select between rat, mouse (Downie *et al*., 1994), or human (Belelli *et al*., 1995), 5-HT$_{3A}$ receptor subunit orthologues. A similar conclusion applies to cocaine which shows only modest ($\sim 3-5$ fold) selectivity for the human *versus* rat and mouse receptor orthologues. However, cocaine, unlike ondansetron, does exhibit some selectivity for the 5-HT$_3$ receptor endogenous to rabbit sensory neurones (Malone *et al*., 1991; Peters *et al*., 1991) More generally, only minor differences in K_i values have been noted for a range of 5-HT$_3$ antagonists (e.g. ramosetron, granisetron, tropisetron and metoclopramide) in binding assays performed on rat, mouse and human 5-HT$_{3A}$ subunits (reviewed by Peters *et al*., 1997) However, as found for 5-HT$_3$ receptors native to guinea-pig tissues (Butler *et al*., 1990, Malone *et al*., 1991; Newberry *et al*., 1991), it might be anticipated that both ondansetron and cocaine will display considerably reduced affinity for the homo-oligomeric receptors assembled from the guinea-pig 5-HT$_{3A}$ subunit orthologue. Indeed, such information already exists for tropisetron and metoclopramide (Lankiewicz *et al*., 1998).

In conclusion, the present study provides an initial description of the pharmacological properties of the rat 5-HT$_{3A(b)}$ subunit expressed in *Xenopus laevis* oocytes and demonstrates that arylbiguanides act as full agonists with an atypically high agonist potency in comparison to their effect upon human, mouse and particularly guinea-pig 5-HT$_{3A}$ receptor orthologues. The antagonist potency of (+)-tubocurarine permits further discrimination between such species homologues. The high degree of amino acid sequence identity between 5-HT$_{3A}$ subunit orthologues (81–95%) will undoubtedly aid the identification of individual residues that contribute to, or impinge upon, the ligand binding site(s) of the 5-HT$_3$ receptor.

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