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Binding Sites for Bilobalide, Diltiazem, Ginkgolide, and Picrotoxinin at the 5-HT$_3$ Receptor

A. J. Thompson, R. K. Duke, and S. C. R. Lummis

Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom (A.J.T., S.C.R.L.); and Department of Pharmacology, University of Sydney, Sydney, New South Wales, Australia (R.K.D.)

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ABSTRACT

Bilobalide (BB), ginkgolide B (GB), diltiazem (DTZ), and picrotoxinin (PXN) are 5-hydroxytryptamine type 3 (5-HT$_3$) receptor antagonists in which the principal sites of action are in the channel. To probe their exact binding locations, 5-HT$_3$ receptors with substitutions in their pore lining residues were constructed (N−4′Q, E−1′D, S2′A, T6′S, L7′T, L9′V, S12′A, I16′V, D20′E), expressed in *Xenopus laevis* oocytes, and the effects of the compounds on 5-HT-induced currents were examined. EC$_{50}$ values at mutant receptors were less than 6-fold different from those of wild type, indicating that the mutations were well tolerated. BB, GB, DTZ, and PXN had pIC$_{50}$ values of 3.33, 3.14, 4.67, and 4.97, respectively. Inhibition by BB and GB was abolished in mutant receptors containing T6′S and S12′A substitutions, but their potencies were enhanced (42- and 125-fold, respectively) in S2′A mutant receptors. S2′A substitution also caused GB ligand trap. PXN potency was modestly enhanced (5-fold) in S2′A, abolished in T6′S, and reduced in L9′V (40-fold) and S12′A (7-fold) receptors. DTZ potency was reduced in L7′T and S12′A receptors (5-fold), and DTZ also displaced [^{3}H]granisetron binding, indicating mixed competitive/noncompetitive inhibition. We conclude that regions close to the hydrophobic gate of M2 are important for the inhibitory effects of BB, GB, DTZ, and PXN at the 5-HT$_3$ receptor; for BB, GB, and PXN, the data show that the 6′ channel lining residue is their major site of action, with minor roles for 2′, 9′, and 12′ residues, whereas for DTZ, the 7′ and 12′ sites are important.

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Introduction

Bilobalide (BB), ginkgolide B (GB), and picrotoxin (PTX) are noncompetitive inhibitors of GABA, glycine, and 5-HT$_3$ receptors (Sivilotti and Nistri, 1991; Pribilla et al., 1992; Huang et al., 2004; Hadley and Gaarder, 2005; Hawthorne et al., 2006; Thompson et al., 2011). Diltiazem (DTZ) is primarily a voltage-gated calcium-channel blocker but also inhibits 5-HT$_3$ and nicotinic acetylcholine receptors (Hargreaves et al., 1996; Houlihan et al., 2000; Chesnoy-Marchais and Cathala, 2001; Das et al., 2004). All of these compounds block the receptor channel, and mutations of the channel lining region have indicated specific interactions in GABA and glycine receptors (Hawthorne et al., 2006; Sedelnikova et al., 2006; Heads et al., 2008; Thompson et al., 2011).

The channels in Cys-loop receptors are lined by five (M2) α-helices (one from each subunit), and to simplify comparisons between receptors of this family, the amino acid residues that line this channel are referred to by an index number, with 0′ representing the conserved charged residue at the cytoplasmic side of the membrane, (e.g., Imoto et al., 1988). In the glycine receptor, GB inhibition is subunit-dependent, and a preference for the β-subunit can be attributed to residues at the 2′ position of the channel pore (Hawthorne and Lynch, 2005; Kondratskaya et al., 2005; Hawthorne et al., 2006). Experiments with BB and PTX on the same receptor show that the 6′ residue is particularly important, and the effects of 6′ substitutions on PTX in GABA and 5-HT$_3$ receptors show that this site of action is conserved across the family (Das and Dillon, 2005; Hawthorne and Lynch, 2005; Sedelnikova et al., 2006). However, there are differences between family members; 2′ mutations in 5-HT$_3$ receptors have limited effect on PTX inhibition but have a large effect at GABA and glycine receptors (Buhr et al., 2001; Yang et al., 2007). The actions of PTX at GABA and glycine receptors are further complicated by evidence of multiple actions; residues between 15′ and 19′ also influence the behavior of PTX in these receptors and either form a second binding site or have a role in the transduction of the PTX inhibitory effect (Dibas et al., 2002 and refs therein).

Here we use two-electrode voltage-clamp to study the effects of M2 amino acid substitutions on BB, GB, DTZ, and PXN inhibition of 5-HT$_3$ receptors expressed in *Xenopus lae-*

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ABBREVIATIONS: BB, bilobalide; GB, ginkgolide B; PTX, picrotoxin; DTZ, diltiazem; 5-HT, 5-hydroxytryptamine; PXN, picrotoxinin.

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vis oocytes. To identify potential binding sites for these compounds, we made substitutions to nine residues that line the proposed water-accessible face of the M2 α-helix. We report the effects that these substitutions have on the potency of the compounds, and we present a model of their binding locations.

Materials and Methods

Materials. All cell culture reagents were obtained from Invitrogen Ltd. (Paisley, UK), except fetal calf serum, which was from Labtech International (Ringmer, UK). PXN and picrotin were separated and purified by recrystallization after short column vacuum chromatography from PTX purchased from Sigma-Aldrich Pty. Ltd. (Sydney, NSW, Australia). BB and GB were isolated from the 50:1 *Ginkgo biloba* leaf extract purchased from Winshing (Australia) Pty. Ltd. (Sydney, NSW, Australia) and purified by short column chromatography and recrystallization. The ^{1}H and ^{13}C NMR spectra of the purified PXN, picrotin, BB, and GB were consistent with the published data (van Beek 2005; Perry et al., 2001) and also indicated purity >98% in all cases. 5-HT3A and 5-HT3B receptor subunit cDNA was kindly donated by J. Peters (University of Dundee, Dundee, Scotland, UK).

Cell Culture and Oocyte Maintenance. *X. laevis* oocyte-positive female frogs were purchased from NASCO (Fort Atkinson, WI) and maintained according to standard methods (Goldin, 1992). Harvested stage V–VI *X. laevis* oocytes were washed in four changes of ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5), defolliculated in 1.5 mg/ml collagenase type 1A for approximately 2 h, washed again in four changes of ND96, and stored in ND96 containing 2.5 mM sodium pyruvate, 50 mM gentamicin, and 0.7 mM theophylline.

Human embryonic kidney 293 cells were maintained on 90-mm tissue culture plates at 37°C and 7% CO_2 in a humidified atmosphere. They were cultured in Dulbecco's modified Eagle's medium/Nutrient Mix F12 (1:1) with GlutaMAX (Invitrogen) containing 10% fetal calf serum. For radioligand binding studies, cells in 90-mm dishes were transfected using polyethylenimine. Thirty microliters of polyethylenimine (1 mg/ml), 5 μl of cDNA, and 1 ml of DMEM were incubated for 10 min at room temperature, added dropwise to a 80 to 90% confluent plate, and incubated for 3 to 4 days before harvesting (Reed et al., 2006).

Receptor Expression. Human 5-HT3A (UniProt accession number P46098) and 5-HT3B (O95804) subunit cDNA were cloned into pGEMHE for oocyte expression (Liman et al., 1992) and pcDNA3.1 (Invitrogen) for expression in human embryonic kidney 293 cells. Mouse 5-HT3A (UniProt Q6J1J7) and 5-HT3A (UniProt Q9JHJ5) were cloned into pcDNA3.1. cRNA was in vitro transcribed from linearized pGEMHE cDNA template using the mMessage mMachine T7 Transcription kit (Ambion, Austin, TX). Stage V and VI oocytes were injected with 50 nl of ~500 ng/μl cRNA (~25 ng), and currents were recorded 1 to 4 days after injection. A ratio of 1:3 (A/B) was used for the expression of heteromeric 5-HT_3 receptors, which has been shown to yield good heteromeric expression (Thompson et al., 2007).

Electrophysiology. Using two-electrode voltage clamp, *X. laevis* oocytes were routinely clamped at −60 mV using an OC-725 amplifier (Warner Instruments, Hamden, CT), Digidata 1322A (Molecular Devices, Sunnyvale, CA), and the Strathclyde Electrophysiology Software Package (http://spider.science.strath.ac.uk/sipbs/showPage.php?page=software_ses). Currents were filtered at a frequency of 1 kHz and sampled at 5 kHz. Microelectrodes were fabricated from borosilicate glass (Harvard Apparatus, Edenbridge, Kent, UK) using a two-stage horizontal pull (Sutter Instrument Company, Novato, CA) and filled with 3 M KCl. Pipette resistances ranged from 1.0 to 2.0 MΩ. Oocytes were perfused with saline at a constant rate of 15 ml/min. Drug application was via a simple gravity fed system calibrated to run at the same rate. Extracellular saline contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM mM HEPES, pH adjusted to 7.4 with NaOH.

Analysis and curve fitting was performed using Prism (ver. 3.02; GraphPad Software, San Diego, CA). Concentration-response data for each oocyte was normalized to the maximum current for that oocyte. A 2-min wash was typically used between drug applications. The mean and S.E.M. for a series of oocytes were plotted against agonist or antagonist concentration and iteratively fitted to the following equation:

$$I_A = I_{min} + \frac{I_{max} - I_{min}}{1 + 10^{n_H(\log A_{50} - \log A)}}$$

where A is the concentration of ligand present; I_A is the current in the presence of ligand concentration A; I_{min} is the current when $A = 0$; I_{max} is the maximal current, A_{50} is the concentration of A that evokes a current equal to $(I_{max} + I_{min})/2$; and n_H is the Hill coefficient. Comparisons were made by subtracting wild-type log A from mutant log A (i.e., the ratio of EC_{50} or IC_{50} values). K_B was estimated from IC_{50} values using the Cheng-Prusoff equation with the modification by Leff and Dougall (1993).

$$K_B = \frac{IC_{50}}{((2 + ([L]/[EC_{50}])^{n_H})^{n_H}) - 1}$$

where IC_{50} is the concentration of antagonist required to halve the maximal response and [L] is the agonist concentration.

Radioligand Binding. Transfected human embryonic kidney 293 cells were scraped into 1 ml of ice-cold HEPES buffer (10 mM, pH 7.4) and frozen at −20°C. After thawing, they were washed with HEPES buffer and homogenized, and 50 μg of crude cell membranes were incubated in 0.5 ml of HEPES buffer containing 1 nM [^{3}H]granisetron (~K_d) in the presence or absence of BB (0.25 nM–2.5 mM), GB (0.25 nM–2.5 mM), DTZ (0.1 nM–10 mM) or PXN (0.5 nM–5 mM). Nonspecific binding was determined using 1 mM quipazine. Reactions were incubated for at least 1 h at 4°C and terminated by vacuum filtration, using a Brandel cell harvester, onto GF/B filters presoaked in 0.3% polyethylenimine. Radioactivity was determined by scintillation counting using a liquid scintillation counter (LS6500; Beckman Coulter, Fullerton, CA). Competition binding (eight point) was performed on at least three separate plates of transfected cells from which the average IC_{50} was calculated. Each data set was analyzed by iterative curve fitting using Prism v3.02, according to the equation:

$$B_L = B_{min} + \frac{B_{max} - B_{min}}{1 + 10^{n_H(\log L_{50} - \log L)}}$$

where L is the concentration of ligand present; B_L is the binding in the presence of ligand concentration L; B_{min} is the binding when $L = 0$; B_{max} is the binding when $L = \infty$, L_{50} is the concentration of L that gives a binding equal to $(B_{max} + B_{min})/2$; and n_H is the Hill coefficient.

K_i values were estimated from IC_{50} values using the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + [L]/K_d}$$

where IC_{50} is the concentration of antagonist that blocks half of the specific binding, [L] is the free concentration of radioligand, and K_d is the equilibrium dissociation constant of the radioligand.

Results

To find the molecular determinants of BB, GB, DTZ, and PXN binding, a series of mutants was generated at each of the nine channel-lining M2 residues of the human 5-HT_3A receptor subunit (Fig. 1). Residues were mostly mutated to amino acids with similar chemical properties to prevent complications arising from changes in channel gating. Conserva-

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tive changes minimize the impact on receptor structure and function, but still reveal specific interactions between the receptor and antagonist (e.g., Thompson et al., 2005). Wild-type and mutant receptors (except for S2′T and S12′T, which were nonfunctional) responded to 5-HT in a concentration-dependent manner, with EC_{50} and n_H values of mutants differing <6-fold from wild type (Table 1; Fig. 2).

Effects of Compounds. None of the compounds elicited a response when applied alone. At wild-type receptors, BB, GB, DTZ, and PXN caused a concentration-dependent inhibition of the 5-HT EC_{50} response (Tables 2–5; Fig. 3). Inhibition was unaltered by pretreatment of the compounds.

Significant effects on BB inhibition were seen at residues Ser2′, Thr6′, Leu7′, Leu9′, and Ser12′ (Table 2; Fig. 3A). The most dramatic effects were at T6′S and S12′A, where inhibition by 1 mM BB was completely abolished. Of the remaining mutants that were inhibited by BB, S2′A exhibited the greatest change in potency, with a 42-fold enhancement. L7′T showed a 6-fold enhancement and L9′V showed a 3-fold reduction in potency.

Inhibition by 1 mM GB was abolished by 6′ and 12′ mutations, but at all other mutants, except N−4′, potency was increased (Table 3; Fig. 3B). Decreases in IC_{50} were generally small (<5-fold), with the exception of S2′A, which displayed a decrease of 125-fold.

PXN inhibition was eliminated by T6′S substitution (Table 4; Fig. 3C). Significant pIC_{50} changes were also seen at positions 4′, 1′, 2′, 9′, and 12′, but effects on potency were generally small (<5-fold), with the exceptions of L9′V and S12′A, which showed reductions of 38- and 6-fold, respectively.

The relative changes in DTZ inhibition were less than with the other three compounds (Table 5; Fig. 3D). Most of the substitutions had no effect, although mutation of Leu7′ and Ser12′ caused 7-fold decreases in potency.

S2′A Mutants Must Reopen to Recover from GB Inhibition. In the S2′A mutant receptor, a striking increase in potency of both BB (42-fold) and GB (125-fold) was observed. For GB, but not BB, this was accompanied by ligand trap: after a coapplication of GB and 5-HT, the next response to 5-HT alone had a reduced current amplitude that returned to the pretreatment amplitude only with a second 5-HT application. This effect is shown in Fig. 4, which is a representative example of eight similar experiments on different oocytes. Peak currents were stable with 5-HT alone (Fig. 4A), but, after GB inhibition, recovery was independent of the time interval between the first (5-HT + GB) and second (5-HT alone) applications (Fig. 4, B–E), showing that the effect was not the consequence of prolonged recovery from desensitization. After the initial increase in current amplitude, further applications of 5-HT showed no additional increases (Fig. 4E). Application of GB in the absence of 5-HT did not change the peak response of subsequent 5-HT applications but was altered if GB was added at any time during 5-HT application (Fig. 4, F–H).

Competition Binding. In a previous study, we showed that BB, GB, and PXN do not compete with granisetron (a high-affinity competitive antagonist) at 5-HT$_3$A or 5-HT$_3$AB receptors (Thompson et al., 2011). Here, we extended these studies to include DTZ. The pK_d of [^{3}H]granisetron was 9.04 ± 0.05 ($n = 7$, $K_d = 0.91$ nM) for 5-HT$_3$A receptors and

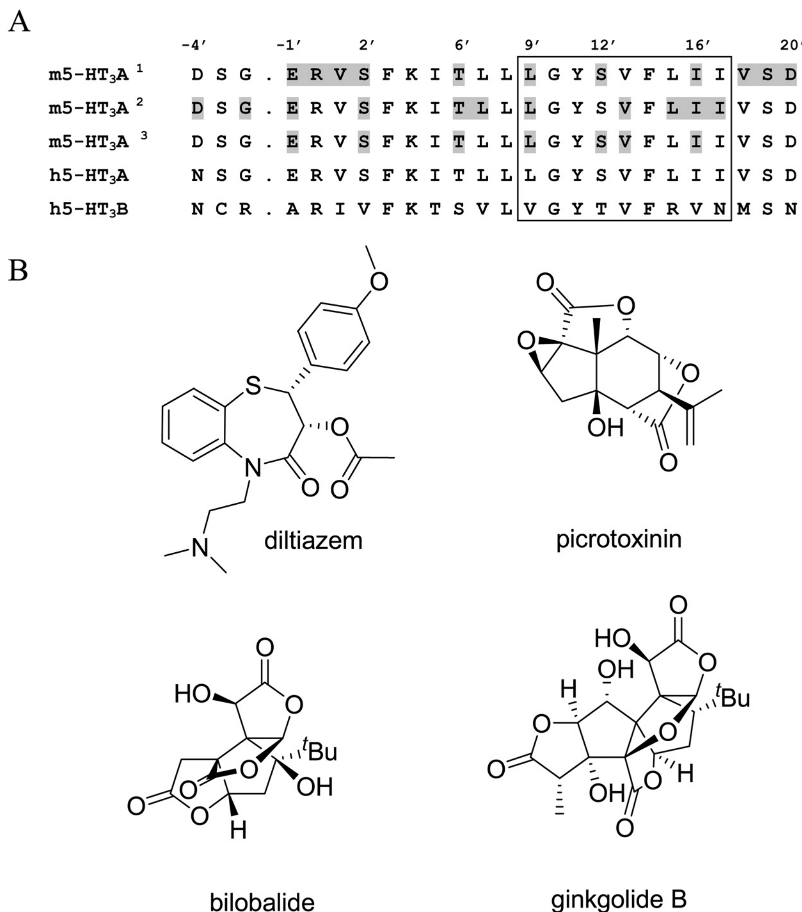

Fig. 1. A, M2 channel lining residues of mouse and human 5-HT$_3$ receptors. The probable "gate" region of the channel (~8 Å long) is boxed (Miyazawa et al., 2003). The M2 amino acid sequence for the murine A subunit (UniProt accession number Q6J1J7) is shown, with residues that are water accessible channel lining highlighted in gray. B, structure of the compounds used in this study. 1, Kaneez and White (2004); 2, Reeves et al. (2001); 3, Panicker et al. (2002); m, mouse; h, human.

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9.09 ± 0.05 (n = 4, K_d = 0.80 nM) for 5-HT_3AB receptors, consistent with previous reports (Brady et al., 2001). At concentrations close to K_d [^{3}H]granisetron was displaced by DTZ with pIC_{50} values of 3.52 ± 0.07 (IC_{50} = 340 μM, n = 12) for 5-HT_3A receptors and 3.44 ± 0.01 (IC_{50} = 366 μM, n = 3) for 5-HT_3AB receptors. These yielded K_i values of 171 and 183 μM, respectively (eq. 4). Hill slopes were 0.84 ± 0.05 and 1.14 ± 0.20 for 5-HT_3A and 5-HT_3AB receptors, respectively.

To probe whether DTZ has an allosteric effect on the binding site, we also examined its effects on L7′T and S12′A-containing mutant receptors. Neither the affinity for [^{3}H]granisetron (L7′T: pK_d = 9.00 ± 0.03, K_d = 1 nM, n = 5; S12′A: pK_d = 9.01 ± 0.07, K_d = 0.97 nM, n = 4) or the potency with which diltiazem displaces this radioligand (L7′T: pIC_{50} = 3.37 ± 0.04, IC_{50} = 426 μM, n = 3; S12′A: pIC_{50} = 3.36 ± 0.07, IC_{50} = 436 μM, n = 4) were significantly different (Student's t test, $p > 0.05$).

Effects on Heteromeric Receptors. 5-HT_3AB receptors had a pEC_{50} (Table 1) that was similar to values shown elsewhere (Hapfelmeier et al., 2003; Thompson et al., 2007). Inhibition of the EC_{50} response by BB, GB, and PXN has been presented by us previously (Thompson et al., 2011), and the values are included in Tables 2 to 4. Here we extended these studies to include DTZ (Table 5). IC_{50} values for all of the compounds were higher (between 5- and 14-fold) at 5-HT_3AB receptors than those at 5-HT_3A receptors.

TABLE 1
Changes in the parameters of 5-HT concentration-response curves caused by M2 substitutions
Values are presented as mean ± S.E.M.

Receptor	pEC_{50}	EC_{50}	Hill Slope	n
		μM		
5-HT_3A	5.76 ± 0.03	1.7	2.56 ± 0.31	6
D20′E	6.07 ± 0.02	0.8	3.47 ± 0.45	5
I16′V	5.55 ± 0.02	2.8	2.51 ± 0.33	5
S12′T	N.F.			12
S12′A	5.03 ± 0.04	9.3	1.54 ± 0.04	5
L9′V	5.31 ± 0.02	4.9	1.76 ± 0.15	4
L7′T	5.09 ± 0.02	8.0	2.11 ± 0.18	5
T6′S	5.88 ± 0.03	1.3	2.15 ± 0.30	4
S2′T	N.F.			15
S2′A	5.66 + 0.03	2.2	2.37 ± 0.03	5
E−1′D	5.61 ± 0.02	2.5	2.79 ± 0.30	5
N−4′Q	5.60 ± 0.02	2.5	3.31 ± 0.38	5
5-HT_3AB[a]	4.55 ± 0.05	28	1.00 ± 0.12	8

N.F., nonfunctional.
[a] Values taken from Thompson et al. (2011) were recorded at the same time as the current results and can be directly compared.

Discussion

Mutagenesis is widely used to probe the interactions of ligands with their receptors. Here we made substitutions of nine 5-HT_3 receptor channel-lining residues and studied their effects on BB, GB, DTZ, and PXN inhibition. Inhibition by BB, GB, and PXN was abolished by T6′S substitution. BB and GB were also highly sensitive to changes at Ser2′ and Ser12′, and PXN to changes at Leu9′. DTZ showed both competitive and noncompetitive antagonism, the noncompetitive component of which was eliminated by substitutions at Leu7′, and Ser12′.

Sensitivity to BB and GB was abolished by substitutions at the 6′ residue, similar to observations at the glycine receptor (Hawthorne et al., 2006). Inhibition was also affected by 2′ substitutions in this receptor, and, in combination with evidence from mutant cycle analysis, suggests that ginkgolides bind close to both positions 2′ and 6′ (Hawthorne et al., 2006). Similar 2′ sensitivity was shown in the present study, with an enhancement of 42-fold for BB and 125-fold for GB. This enhancement of GB sensitivity was accompanied by ligand trap at the 2′ mutant, a phenomenon that has also been described for BB, GA, and PXN at glycine receptors (Hawthorne and Lynch, 2005; Hawthorne et al., 2006; Bali and Akabas, 2007; Wang et al., 2007). In our previous work, GB displayed use dependence at wild-type 5-HT_3 receptors, which is similar to the reports of BB at $GABA_C$ and ginkgolide C at glycine receptors (Ivic et al., 2003; Huang et al., 2006; Thompson et al., 2011). These data imply that GB acts as an open-channel blocker of the 5-HT_3 receptor, and, when the channel gate is closed, is trapped in the pore of the high-affinity S2′A mutant. The opposite A2′S mutation in insect GABA receptors is responsible for resistance to cyclodiene insecticides and PTX and, in light of this evidence, the binding site of these compounds seems to be broadly conserved across the Cys-loop family (Ffrench-Constant et al., 1993).

In $GABA_A$ receptors the 6′ position is the main (hydrogen bonding) interacting residue and the 2′ position acts as a secondary (hydrophobic) site for PTX binding (Erkkila et al., 2008). Our results support the role of Thr6′ as a major determinant of PXN binding, but the effect of our conserved mutation (inhibition was abolished) was more pronounced than those reported for the $GABA_A$ receptor, where changing all five of the 6′ Thr residues to Ser only caused a 48-fold shift in the PTX IC_{50} (Erkkila et al., 2008). The effects of the 2′ and 6′ substitutions indicate that BB, GB, and PXN can penetrate beyond the central "hydrophobic girdle," which forms the "gate" of the channel (Leu9′–Val13′; Miyazawa et al., 2003), and our observation that

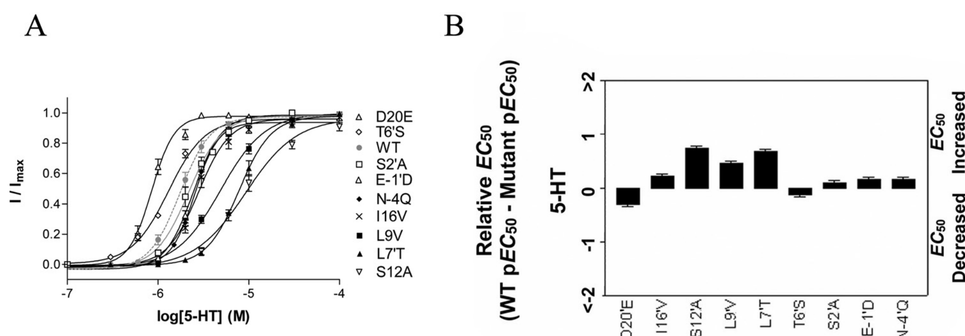

Fig. 2. Wild-type and mutant 5-HT_3 receptor concentration-response curves from which 5-HT pEC_{50} values were determined. (A, data are presented as mean ± S.E.M.; n can be found in Table 1). The difference between wild-type and mutant 5-HT_3 receptors is shown in B. Data are presented as mean ± S.E.D.

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GB is trapped in the high-affinity S2′A mutant is consistent with this hypothesis. 2′ Residues have been implicated in PTX inhibition in $GABA_A$, $GABA_C$, and glutamate-gated Cys-loop receptors, with some small effects at glycine and $5\text{-}HT_3A$ receptors (Gurley et al., 1995; Wang et al., 1995; Xu et al., 1995; Zhang et al., 1995; Etter et al., 1999; Shan et al., 2001; Das and Dillon, 2005). We propose that the decreased IC_{50} values we observed for BB, GB, and (to a lesser extent) PXN in the S2′A mutant may arise from the more hydrophobic side-chain of Ala enabling the ligands to adopt a more energetically favorable position within the channel, possibly via interactions with their lipophilic side chains. Ala substitutions at Ser12′ also had large effects on BB and GB (inhibition abolished), although only moderately effected PXN. If we assume that the 6′ residue is important for ligand interactions, Ser12′ may act as a secondary binding site or interact with antagonists as they descend into the pore.

BB, GB, DTZ, and PXN inhibition is subunit dependent at the $5\text{-}HT_3$ receptor, similar to the actions of these compounds at other Cys-loop receptors (Das and Dillon, 2003; Hawthorne and Lynch, 2006; Hawthorne et al., 2006; Erkkila et al., 2008; Gonzales et al., 2008; Thompson et al., 2011). $5\text{-}HT_3AB$ receptors were less sensitive to PXN than $5\text{-}HT_3A$ receptors, and mutation of the $5\text{-}HT_3A$ 6′ residue to its B subunit counterpart (T6′S) completely abolished inhibition by BB, GB, and PXN. Because the B subunit 6′Ser does not abolish inhibition in the heteromeric wild-type $5\text{-}HT_3$ receptor, the other two to four A subunit 6′ residues (Barrera et al., 2005; Lochner and Lummis, 2010, which combine with one to three B subunits to constitute this receptor) must be sufficient for PXN binding. In the $GABA_A$ receptor, PTX sensitivity also decreases as the number of mutated subunits is increased from one to five (Erkkila et al., 2008).

BB and PTX have mixed competitive and noncompetitive behaviors in a range of Cys-loop receptors, but at least one site of action is in the receptor channel (Smart and Constanti, 1986; Lynch et al., 1995; Wang et al., 1995; Huang et al., 2006). We have previously shown that BB, GB, and PXN do not compete with [^{3}H]granisetron, and both these data and our current results are consistent with these compounds binding solely in the $5\text{-}HT_3$ receptor pore

TABLE 2

Bilobalide inhibition parameters at wild-type and mutant $5\text{-}HT_3$ receptors

Values are presented as mean ± S.E.M.

Receptor	pIC_{50}	IC_{50}	Hill Slope	*n*
		μM		
$5\text{-}HT_3A$	3.33 ± 0.03	468	1.48 ± 0.16	6
D20′E	3.21 ± 0.04	600	1.25 ± 0.12	4
I16′V	3.12 ± 0.04	200	1.43 ± 0.15	5
S12′A	N.I.*	N.I.	N.I.	3
L9′V	2.79 ± 0.06*	1600	0.84 ± 0.09	8
L7′T	4.11 ± 0.09*	78	0.96 ± 0.09	5
T6′S	N.I.*	N.I.	N.I.	3
S2′A	4.94 ± 0.04*	11	0.86 ± 0.04	3
E−1′D	3.20 ± 0.12	630	0.67	4
N−4′Q	3.33 ± 0.10	470	1.09 ± 0.20	5
$5\text{-}HT_3AB$[a]	2.51 ± 0.15*	3100	0.68 ± 0.13	4

N.I., no inhibition at 1 mM.

[a] Values taken from Thompson et al. (2011) were recorded at the same time as the current results and can be directly compared.

*$P < 0.05$, significantly different from wild type (ANOVA with Dunnett's post test).

(Thompson et al., 2011). In contrast, the difference between K_B (34 μM) and K_i (171 μM) for DTZ indicates that competitive inhibition cannot completely account for its effects, suggesting DTZ inhibition includes both competitive and noncompetitive behaviors. Similar differences have also been reported for DTZ at mouse $5\text{-}HT_3$ receptors (Hargreaves et al., 1996; Gunthorpe and Lummis, 1999), and mefloquine has mixed inhibitory behavior at human $5\text{-}HT_3$ receptors (Thompson et al., 2007). In our experiments, DTZ potency was significantly changed by the 12′ mutation, although Lee et al. (2009) have previously shown that a 13′ mutation in mouse receptors reduces DTZ potency, whereas the 12′ residue is unaffected. The contrast in our results suggests that there are differences between human and mouse receptors, and this is further demonstrated by the presence of constitutively active 13′ mutants in their study and the absence of such effects at any of the mutants studied here. In contrast to the homo-

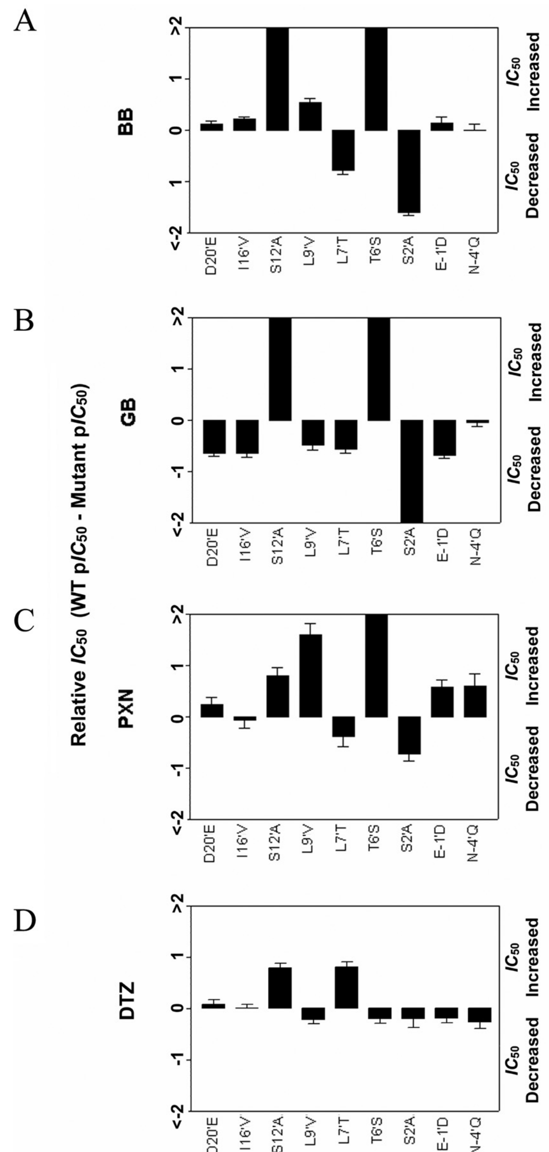

Fig. 3. Inhibition parameters of BB (A), GB (B), DTZ (C), and PXN (D) at wild-type and mutant $5\text{-}HT_3$ receptors. The difference between wild-type and mutant pIC_{50} values are shown for each of the compounds (mean ± S.E.D.). *n* can be found in Tables 2 to 5.

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meric receptors, K_B (149 μM) and K_i (183 μM) values were similar at 5-HT_3AB receptors, suggesting that inhibition of the functional response in these receptors can be accounted for entirely by competitive inhibition. For PXN, it

TABLE 3
Ginkgolide B inhibition parameters at wild-type and mutant 5-HT_3 receptors
Values are presented as mean ± S.E.M.

Receptor	pIC_{50}	IC_{50}	Hill Slope	n
		μM		
5-HT_3A	3.14 ± 0.05	727	1.10 ± 0.13	4
D20′E	3.78 ± 0.05*	170	1.49 ± 0.23	4
I16′V	3.79 ± 0.07*	160	1.18 ± 0.15	5
S12′A	N.I.*	N.I.	N.I.	4
L9′V	3.63 ± 0.08*	240	0.79 ± 0.12	5
L7′T	3.71 ± 0.07*	210	1.46 ± 0.29	4
T6′S	N.I.*	N.I.	N.I.	3
S2′A	5.24 ± 0.08*	5.8	0.75 ± 0.10	5
E−1′D	3.82 ± 0.04*	150	1.07	4
N−4′Q	3.18 ± 0.08	660	0.87 ± 0.08	5
5-HT_3AB[a]	2.41 ± 0.22*	3900	0.52 ± 0.14	4

N.I., no inhibition at 1 mM.
[a] Values taken from Thompson et al. (2011) were recorded at the same time as the current results and can be directly compared.
*$P < 0.05$, significantly different from wild type (ANOVA with Dunnett's post test).

TABLE 4
Picrotoxinin inhibition parameters at wild-type and mutant 5-HT_3 receptors
Values are presented as mean ± S.E.M.

Receptor	pIC_{50}	IC_{50}	Hill Slope	n
		μM		
5-HT_3A	4.97 ± 0.12	11	0.68 ± 0.12	13
D20′E	4.73 ± 0.06	18	0.78 ± 0.07	4
I16′V	5.03 ± 0.11	9.3	0.70 ± 0.11	5
S12′A	4.18 ± 0.11*	66	0.69 ± 0.09	5
L9′V	3.38 ± 0.20*	420	0.91 ± 0.25	3
L7′T	5.36 ± 0.15	4.4	0.51 ± 0.09	8
T6′S	N.I.*	N.I.	N.I.	4
S2′A	5.70 ± 0.07*	2.0	0.61 ± 0.06	4
E−1′D	4.40 ± 0.06*	40	1.00 ± 0.11	5
N−4′Q	4.37 ± 0.21*	43	0.80 ± 0.22	4
5-HT_3AB[a]	4.20 ± 0.11*	62	0.68 ± 0.09	4

N.I., no inhibition at 1 mM.
[a] Values taken from Thompson et al. (2011) were recorded at the same time as the current results and can be directly compared.
*$P < 0.05$, significantly different from wild type and (ANOVA with Dunnett's post test).

TABLE 5
Diltiazem inhibition parameters at wild-type and mutant 5-HT_3 receptors
Values are presented as mean ± S.E.M.

Receptor	pIC_{50}	IC_{50}	Hill Slope	n
		μM		
5-HT_3A	4.67 ± 0.07	21	0.84 ± 0.11	7
D20′E	4.59 ± 0.05	26	1.4 ± 0.20	4
I16′V	4.67 ± 0.07	21	0.99 ± 0.17	5
S12′A	3.82 ± 0.05*	150	1.64 ± 0.33	4
L9′V	4.91 ± 0.09	12	0.86 ± 0.15	4
L7′T	3.80 ± 0.06*	160	0.83 ± 0.08	5
T6′S	4.89 ± 0.07	13	0.96 ± 0.15	7
S2′A	4.89 ± 0.17	13	0.60 ± 0.15	5
E−1′D	4.88 ± 0.07	13	1.01 ± 0.17	3
N−4′Q	4.96 ± 0.12	11	1.08 ± 0.14	3
5-HT_3AB	3.52 ± 0.10*	300	0.79 ± 0.11	5

*$P < 0.05$, significantly different from wild type (ANOVA with Dunnett's post test).

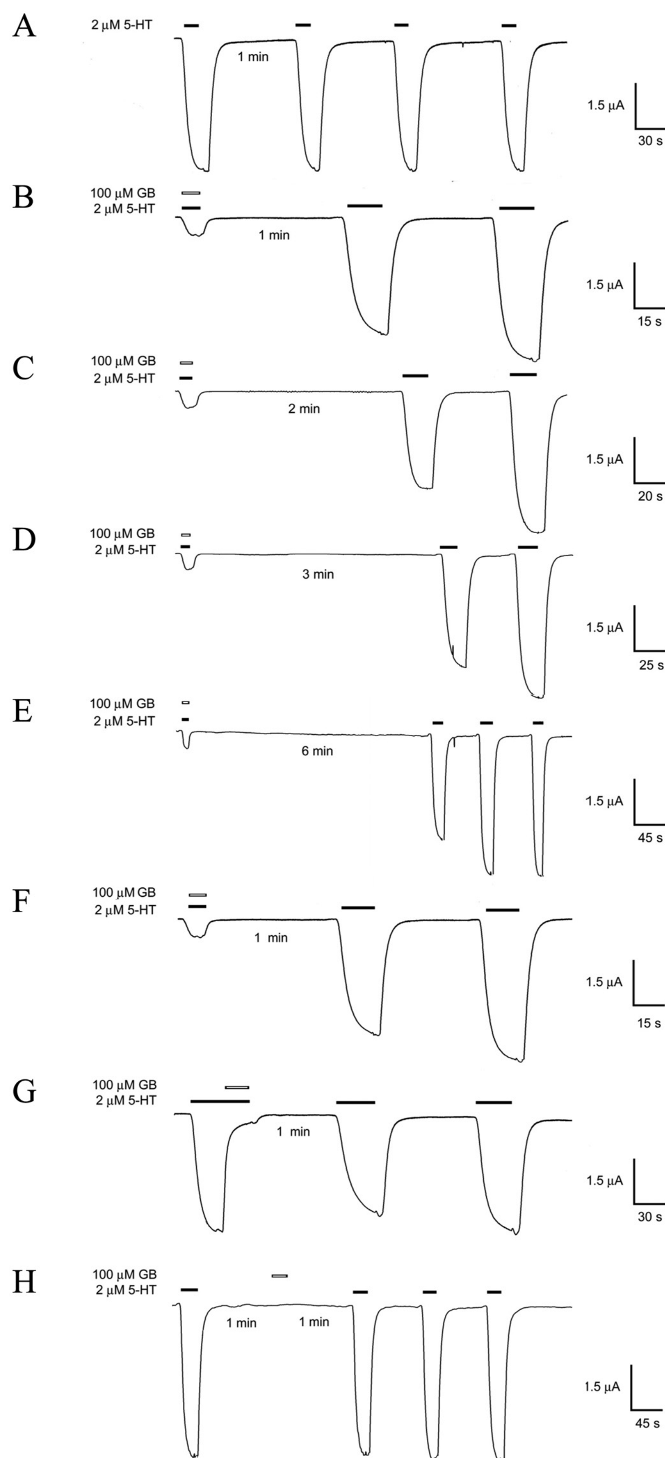

Fig. 4. GB is trapped in the closed state of the S2′A mutant receptor. Applications of EC_{50} 5-HT at 1-min intervals have the same current amplitude (A). After coapplication of GB and 5-HT, the next 5-HT response is reduced in amplitude, and only returns to its predrug amplitude with a second application of agonist (B). This effect is independent of the recovery period between applications (B–E) and is only present when GB is applied in the open state of the receptor (F–H). Traces are representative of eight similar experiments performed on different oocytes. Ligand trap was not seen for BB, DTZ, or PXN.

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has been suggested that allosteric effects on the agonist binding site of GABA receptors account for some of inhibitory effects of this compound (Smart and Constanti, 1986), but this is unlikely for DTZ, because the potency with which it displaced radioligand binding was similar at wild-type, L7′T, and S12′A mutant receptors. As all the residue positions studied here differ between A and B subunits, all could potentially disrupt binding in the channel. However, only L7′T and S12′A containing-receptors significantly altered DTZ potency, and K_B values (L7′T = 176 μM; S12′A = 182 μM) were close to the K_i for wild-type receptors (5-HT$_3$A = 171 μM; 5-HT$_3$AB = 183 μM). We therefore conclude that the differences in DTZ efficacy at homomeric and heteromeric receptors can be accounted for by differences in their channel properties. This interpretation is also consistent with the long-established finding that these two receptor types have identical binding site pharmacologies, but different noncompetitive pharmacologies (Brady et al., 2001; Thompson et al., 2007).

In summary, we have shown that BB, GB, and PXN inhibit 5-HT$_3$ receptor function by interacting with the 6′ and (to a lesser extent) the 2′ residue in the receptor channel, consistent with their sites of action being conserved across the Cys-loop family. For BB and GB, the 12′ residue was identified as a new site of interaction and may represent another binding region or may simply indicate there is an interaction with the compounds as they descend into the channel. In contrast, DTZ inhibition of the 5-HT$_3$ receptor contains both competitive (homomeric and heteromeric) and noncompetitive behaviors, the later of which can be attributed to the 7′ region of channel. The data are consistent with the binding sites shown on the cartoon in Fig. 5.

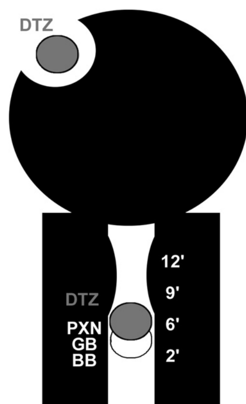

Fig. 5. A cartoon of potential binding regions for BB, DTZ, GB, and PXN at the 5-HT$_3$ receptor. The 12′ residue is located in the upper region of the channel (see Fig. 1), and its substitution reduces the potency of all four of the ligands tested. Given its location, we speculate that it may affect the passage of compounds as they pass to their binding sites lower down. The 6′ residue is particularly critical for BB, GB, and PXN binding as substitution abolishes inhibition (open circle). At S2′A mutant receptors, the potencies of BB, GB, and PXN were all increased, which may be the result of the smaller Ala substitute, allowing the ligands to adopt a more energetically favorable position. DTZ potency is altered by mutation at the 7′ position (gray circles) suggesting it binds here, in addition to acting at the agonist binding site.

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Authorship Contributions

Participated in study design: Thompson and Lummis.
Contributed experiments: Thompson.
Contributed materials: Duke.
Performed data analysis: Thompson and Lummis.
Wrote or contributed to the writing of the manuscript: Thompson, Duke, and Lummis.
Other: Lummis acquired funding for this project.

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Address correspondence to: Dr. Sarah C. R. Lummis, Department of Biochemistry, Tennis Court Road, Cambridge CB2 1QW, UK. E-mail: sl120@cam.ac.uk

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