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Direct Subunit-Dependent Multimodal 5-Hydroxytryptamine$_3$ Receptor Antagonism by Methadone

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ABSTRACT

Homomeric 5-hydroxytryptamine (5-HT)$_{3A}$ and heteromeric 5-HT$_{3AB}$ receptors mediate rapid excitatory responses to serotonin in the central and peripheral nervous systems. The alkaloid morphine, in addition to being a μ-opioid receptor agonist, is a potent competitive inhibitor of 5-HT$_3$ receptors. We examined whether methadone, an opioid often used to treat morphine dependence, also exhibited 5-HT$_3$ receptor antagonist properties. Racemic (*R*/*S*)-methadone inhibited currents mediated by human homomeric 5-HT$_{3A}$ receptors (IC_{50} = 14.1 ± 2.5 μM). Incorporation of the 5-HT$_{3B}$ subunit into heteromeric 5-HT$_{3AB}$ receptors reduced the potency of inhibition by (*R*/*S*)-methadone (IC_{50} = 41.1 ± 0.9 μM). (*R*/*S*)-Methadone also increased apparent desensitization of both 5-HT$_3$ receptor subtypes. The inhibition of the 5-HT$_{3A}$ receptor was competitive; however, incorporation of the 5-HT$_{3B}$ subunit caused the appearance of inhibition that was insurmountable by 5-HT. In the absence of rapid desensitization, when dopamine was used as an agonist of 5-HT$_{3AB}$ receptors, the inhibition by (*R*/*S*)-methadone was voltage-dependent. The antagonist and desensitization-enhancing effects of (*R*/*S*)-methadone were shared by pure (*R*)- and (*S*)-methadone enantiomers, which had similar actions on 5-HT-evoked currents mediated by 5-HT$_3$ receptors. However, (*R*)-methadone exhibited a larger voltage-dependent inhibition of dopamine-evoked currents mediated by 5-HT$_{3AB}$ receptors than did (*S*)-methadone. Inhibition of 5-HT$_{3A}$ receptors by (*R*/*S*)-methadone was not influenced by voltage. Thus, methadone displays multimodal subunit-dependent antagonism of 5-HT$_3$ receptors.

The 5-hydroxytryptamine (5-HT) type 3 receptor is a ligand-gated cation channel that mediates rapid serotonergic excitatory synaptic transmission (Sugita et al., 1992). It contains binding sites for 5-HT and several allosteric modulators. The 5-HT$_3$ receptor is a member of the Cys-loop superfamily of pentameric receptors, which also includes the nicotinic acetylcholine, γ-aminobutyric acid, and glycine receptors, and the Zn^{2+}-activated ion channel (Barnes et al., 2009). The 5-HT$_{3A}$ subunit forms homomeric receptors and can also combine into heteromeric receptors with the 5-HT$_{3B}$ subunit, which is by contrast unable to form homomeric receptors (Davies et al., 1999). Coexpression of the 5-HT$_{3A}$ subunit with the 5-HT$_{3B}$ subunit confers unique properties (Davies et al., 1999; Peters et al., 2005). Genes encoding 5-HT$_{3C}$, 5-HT$_{3D}$, and 5-HT$_{3E}$ subunits have also been cloned; however, their functional significance is poorly understood (Niesler et al., 2003).

5-HT$_3$ receptors participate in nausea and vomiting, nociception, gastrointestinal motility, and reward (Allan et al., 2001; Galligan, 2002; Thompson and Lummis, 2006). Many therapeutic drugs structurally distinct from 5-HT affect 5-HT$_3$ receptor function. These include competitive antagonists such as the "setrons" (including ondansetron); the nicotinic drugs curare (Peters et al., 1990), epibatidine, and mecamylamine (Drisdel et al., 2007); cannabinoids (Barann et al., 2002); and some opioids (Fan, 1995; Wittmann et al., 2006). 5-HT$_3$ receptor antagonists are used to treat nausea and vomiting and, to a lesser extent, irritable bowel syndrome (Galligan, 2002). Ondansetron is also effective in the treatment of early onset alcoholism (Kranzler et al., 2003) and seems to aid detoxification of heroin-dependent individuals (Ye et al., 2001).

The alkaloid morphine, the principal active metabolite of heroin, has been known for more than 50 years to have inhibitory effects on specific serotonin receptor subtypes such as those located in the guinea pig ileum (Gaddum and Picarelli, 1957). Morphine-sensitive, so called 5-HTM receptors were later renamed 5-HT$_3$ receptors (Bradley et al., 1986). Morphine directly and competitively inhibits 5-HT$_3$ receptors at low concentrations (Fan, 1995; Wittmann et al., 2006).

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; HEK, human embryonic kidney; hERG, human *ether-à-go-go*-related gene; ANOVA, analysis of variance; NMDA, *N*-methyl-D-aspartate; Meth, methadone.

We investigated whether the 5-HT$_3$ receptor-inhibiting properties of morphine were shared by methadone, an opioid frequently used to treat morphine dependence. Methadone is a chiral molecule and as such exists as either an *R*- or *S*-enantiomer. Compared with (*S*)-methadone, (*R*)-methadone binds preferentially to the μ-opioid receptor (Kristensen et al., 1995) and exhibits more potent inhibitory effects at the NMDA subtype of the glutamate receptor (Callahan et al., 2004). By contrast, compared with (*R*)-methadone, (*S*)-methadone more potently inhibits cardiac hERG K^+ channels (Eap et al., 2007). We tested (*R*)- and (*S*)-methadone to determine whether the modulatory effects of (*R*/*S*)-methadone on 5-HT$_3$ receptors are enantiomer-specific.

Materials and Methods

Cell Culture and Transfection. Human embryonic kidney (HEK) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin in a humid atmosphere of 5% CO_2. HEK cells were transfected by calcium phosphate precipitation with cDNA encoding the human 5-HT$_{3A}$ subunit either alone or in combination with the human 5-HT$_{3B}$ subunit cDNA in the pCDM8 vector at a cDNA ratio of 1:1, as described previously (Davies et al., 1999). All tissue culture reagents were from Invitrogen (Carlsbad, CA). Cells were used 48 to 96 h after transfection for electrophysiological experiments.

Electrophysiological Recording. The whole-cell configuration of the patch-clamp technique was used to record currents from HEK cells expressing recombinant receptors. The electrode solution contained140 mM CsCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.1 mM EGTA, and 10 mM HEPES (pH 7.4 with CsOH). The extracellular solution contained 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose (pH 7.4 with NaOH). Unless otherwise stated, cells were voltage-clamped at an electrode potential of −60 mV. In experiments investigating the voltage dependence of inhibition by methadone, the voltage was adjusted between −60 and +60 mV (in 20-mV increments). No correction was made for the compensation for liquid junction potential. 5-HT$_3$ receptors were activated by locally applying 5-HT or dopamine to the cell by pressure (10 psi) ejection (Picospritzer II; General Valve, Fairfield, NJ). The recording chamber was continuously perfused with extracellular solution (5 ml/min). Methadone was diluted from frozen stocks into the extracellular solution on the day of recording. During experiments examining the concentration dependence of inhibition of 5-HT$_3$ receptors, methadone was bath-applied, whereas 5-HT (30 μM) was applied every 60 s for 100 ms from a micropipette positioned ~50 μm from the cell. When investigating the concentration dependence of 5-HT and desensitization in the absence and presence of methadone, 5-HT alone or 5-HT plus methadone was applied for 1 s from the micropipette, as described previously (Adodra and Hales, 1995). A period of at least 120 s elapsed between each application to allow recovery from desensitization. The ratio of 5-HT-evoked current amplitudes recorded at −60 and 60 mV was established before applying methadone to an HEK cell transfected with 5-HT$_{3A}$ and 5-HT$_{3B}$ subunit cDNAs. A 5-HT-evoked 60/-60 mV ratio of ~1 (compared with ~0.5 for 5-HT$_{3A}$ receptors) was used as an indication of successful 5-HT$_{3B}$ subunit incorporation into heteromeric 5-HT$_{3AB}$ receptors (e.g., Fig. 5). Currents were recorded using an Axopatch 200B amplifier, low-pass filtered at 2 KHz, digitized at 10 KHz using a Digidata 1320A interface, and acquired using pCLAMP8 software (all from Molecular Devices, Sunnyvale, CA) on to the hard drive of a personal computer for off-line analysis. All experiments were performed at room temperature.

Data Analysis. The peak amplitudes of agonist-activated currents were measured using pCLAMP8 software. Systematic effects of 5-HT-evoked current rundown were corrected using regression analysis, normalizing current amplitudes to that evoked by 100 μM 5-HT. Concentration-response relationships were fitted with a modified logistic function to determine EC_{50}, IC_{50}, and Hill slope values, as described previously (Adodra and Hales, 1995). We used the method of Lew and Angus (1995) to investigate whether (*R*/*S*)-methadone had competitive or noncompetitive inhibitory effects on 5-HT$_3$ receptors. The inhibition of 5-HT$_{3AB}$ receptors by (*R*/*S*)-methadone exhibited a component that was insurmountable by 5-HT, thus precluding calculation of binding affinity. The pEC_{50} values for 5-HT [determined in the absence and presence of differing concentrations of (*R*/*S*)-methadone] were plotted against (*R*/*S*)-methadone concentration. Data points were fitted with the following equation:

$$pEC_{50} = c - \log([B] + 10^{-pK_B}) \quad (1)$$

where [B] is the concentration of (*R*/*S*)-methadone and c is a fitting constant. As recommended by Lew and Angus (1995), we also fitted the plots of 5-HT pEC_{50} values versus [(*R*/*S*)-methadone] with formulae that allow deviations equivalent, when using Schild analysis, to either nonlinearity:

$$pEC_{50} = c - \log([B](1 + n[B]/10^{-pK_B})) + 10^{-pK_B} \quad (2)$$

or a nonunity slope:

$$pEC_{50} = c - \log([B]^n + 10^{-pK_B}) \quad (3)$$

Whether the interaction was competitive was then determined by comparisons of the goodness of fit. Fitting the data with eqs. 2 and 3 failed to significantly improve the fidelity of the fits (established using the F-test) achieved using eq. 1. A Clarke plot was generated

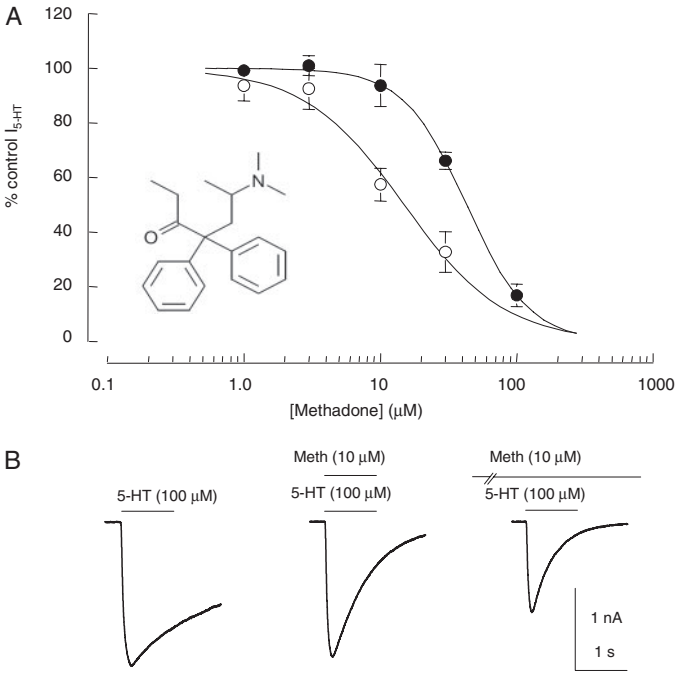

Fig. 1. Concentration-dependent inhibition of 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptors by (*R*/*S*)-methadone. Inhibition of currents mediated by 5-HT$_3$ receptors activated by 5-HT (30 μM) in the absence and presence of (*R*/*S*)-methadone bath-applied before and during local 5-HT (30 μM) application. A, concentration-response relationship for (*R*/*S*)-methadone as an inhibitor of 5-HT$_{3A}$ (open symbols) and 5-HT$_{3AB}$ receptors (closed symbols). IC_{50} values, generated by logistic fits to the data points, were 14.1 ± 2.5 and 41.1 ± 0.9 μM, respectively. Data are expressed as percentage of control 5-HT (30 μM)-evoked current amplitude. Vertical lines are ± S.E.M. B, 5-HT-evoked currents mediated by 5-HT$_{3A}$ receptors recorded from the same cell. Left, 5-HT (100 μM) was applied for 1 s in the absence of (*R*/*S*)-methadone (Meth). Middle, 5-HT (100 μM) was applied for 1 s with (*R*/*S*)-methadone (10 μM). Right, (*R*/*S*)-methadone (10 μM) was preapplied for 5 min before its coapplication with 5-HT (100 μM). Preapplication of (*R*/*S*)-methadone enhanced apparent desensitization and caused a reduction in peak current amplitude.

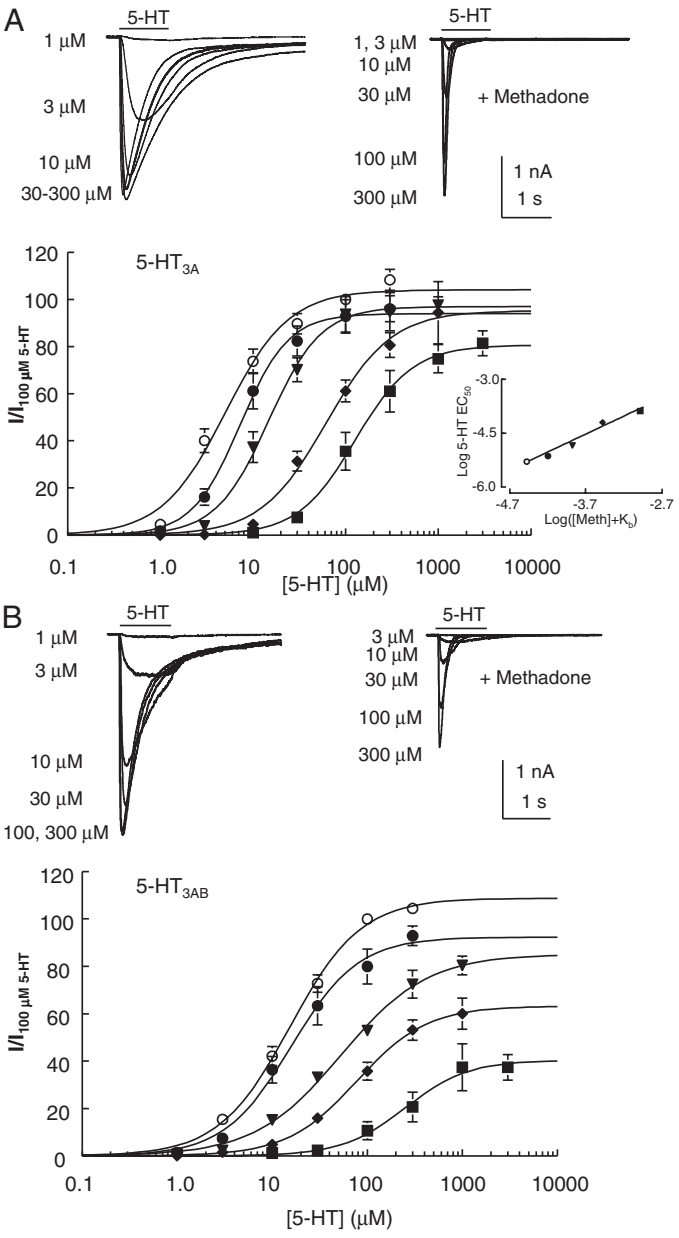

Fig. 2. Competitive and insurmountable antagonism of 5-HT_3 receptors by (*R*/*S*)-methadone. A, superimposed traces are exemplar 5-HT (1–300 μM)-evoked currents recorded, from the same HEK cell expressing 5-HT_{3A} receptors, in the absence (left) and presence (right) of (*R*/*S*)-methadone (300 μM) applied simultaneously with 5-HT. The graph depicts mean current amplitudes activated by 5-HT in the presence of (*R*/*S*)-methadone expressed as a percentage of control 5-HT (100 μM)-evoked current amplitudes recorded from each cell. Open and closed symbols represent 5-HT-evoked current amplitudes recorded in the absence and presence of (*R*/*S*)-methadone, respectively. The 5-HT concentration-response relationship was shifted to the right by increasing concentrations of (*R*/*S*)-methadone: 30 (●), 55 (▼), 300 (♦), and 1000 (■) μM. Vertical lines represent ± S.E.M. 5-HT EC_{50}, maximum current, and Hill slope values for the activation of 5-HT_{3A} receptors in the absence and presence of (*R*/*S*)-methadone were determined from logistic fits to the data points (Table 1). The inset graph is a Clark plot of the 5-HT EC_{50} values (log_{10}) versus log ([methadone] + K_b), where K_b is the apparent binding affinity calculated as described under *Materials and Methods* (Lew and Angus, 1995). The straight line is the idealized relationship between 5-HT EC_{50} value and [methadone], with the calculated K_b value (see *Results*). B, superimposed traces are exemplar 5-HT-evoked currents recorded from an HEK cell expressing 5-HT_{3AB} receptors in the absence (left) and presence (right) of (*R*/*S*)-methadone (300 μM) applied simultaneously with 5-HT. The graph represents mean 5-HT-evoked current amplitudes recorded in the presence of (*R*/*S*)-methadone expressed as a percentage of control 5-HT (100 μM)-evoked current amplitudes recorded from each cell. 5-HT EC_{50}, maximum current, and Hill slope values for the activation of 5-HT_{3AB} receptors by 5-HT in the presence of (*R*/*S*)-methadone were determined from the logistic fits (Table 1). Vertical lines represent ± S.E.M.

to illustrate the predicted relationship between the 5-HT EC_{50} values and the concentration of alkaloid, using the value of K_b generated by eq. 1. This approach demonstrated that (*R*/*S*)-methadone caused competitive inhibition of the 5-HT_{3A} receptor.

Results

Concentration-Dependent Inhibition of 5-HT_{3A} Receptors by (*R*/*S*)-Methadone. In addition to its classic interaction with the μ-opioid receptor, morphine directly and competitively inhibits 5-HT_3 receptors (Fan, 1995; Wittmann et al., 2006). We examined whether this property was shared by methadone. We used the whole-cell patch-clamp technique to record currents from voltage-clamped HEK cells transiently expressing human 5-HT_{3A} receptors. 5-HT (30 μM), applied transiently to cells clamped at −60 mV, activated inward currents with a mean peak amplitude of 4.0 ± 0.43 nA (n = 20). Bath-applied racemic (*R*/*S*)-methadone hydrochloride inhibited 5-HT-evoked currents in a concentration-dependent manner (Fig. 1A). We fitted the concentration-response relationship for (*R*/*S*)-methadone using the logistic equation, yielding an IC_{50} value of 14.1 ± 2.5 μM.

Lack of Agonist Action of (*R*/*S*)-Methadone on the 5-HT_{3A} Receptor. A previous study demonstrated that the alkaloid apomorphine acts as a weak partial agonist at 5-HT_3 receptors (van Hooft and Vijverberg, 1998). In keeping with this action, when applied simultaneously with 5-HT, apomorphine competitively inhibits 5-HT-evoked currents. We therefore examined the possibility that (*R*/*S*)-methadone (300 μM) is a partial agonist by locally administering the opioid alkaloid to HEK cells expressing recombinant 5-HT_{3A} receptors by pressure application. Five cells tested that responded robustly to 5-HT (30 μM) failed to exhibit currents in response to (*R*/*S*)-methadone application (data not shown). Therefore, methadone is a 5-HT_3 receptor antagonist that lacks efficacy as an agonist at concentrations that cause near-maximal inhibition of 5-HT_{3A} receptor-mediated currents (Fig. 1A).

Inhibition of Heteromeric 5-HT_{3AB} Receptors by (*R*/*S*)-Methadone. When expressed with the 5-HT_{3B} subunit the 5-HT_{3A} subunit forms heteromeric receptors with characteristic functional properties (Davies et al., 1999; Das and Dillon, 2005). For example, heteromeric 5-HT_{3AB} receptors are less sensitive to inhibition by the plant alkaloids curare and picrotoxin than are homomeric 5-HT_{3A} receptors. We tested the effects of (*R*/*S*)-methadone on 5-HT (30 μM)-activated currents mediated by heteromeric 5-HT_{3AB} receptors. Application of 5-HT to HEK cells transiently transfected with cDNAs encoding 5-HT_{3A} and 5-HT_{3B} subunits activated inward currents recorded at a holding potential of −60 mV. Bath application of (*R*/*S*)-methadone (Fig. 1A) caused concentration-dependent inhibition of 5-HT-activated currents mediated by heteromeric 5-HT_{3AB} receptors. We fitted the concentration-response relationship with the logistic equation, yielding an estimate of the IC_{50} value (41.1 ± 0.9 μM). (*R*/*S*)-Methadone was significantly ($p < 0.001$; Student's t test) less potent as an inhibitor of 5-HT_{3AB} receptors com-

pared with 5-HT$_{3A}$ receptors. The Hill slope values (1.2 ± 0.2 and 1.9 ± 0.1) for the inhibition by (*R*/*S*)-methadone of 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptors, respectively, also differed significantly ($p < 0.001$; Student's *t* test).

Competitive Antagonism of 5-HT$_3$ Receptors by (*R*/*S*)-Methadone. Previous studies demonstrate that morphine competitively inhibits 5-HT$_3$ receptor-mediated currents (Fan, 1995; Wittmann et al., 2006). We examined the nature of 5-HT$_3$ receptor antagonism by (*R*/*S*)-methadone of currents mediated by 5-HT$_{3A}$ receptors. Experiments examining the concentration dependence of the inhibition of 5-HT-evoked currents revealed that (*R*/*S*)-methadone caused an increase in the apparent desensitization of 5-HT$_{3A}$ receptors. Rapid apparent desensitization of 5-HT-evoked currents after preapplication of (*R*/*S*)-methadone probably compromised our ability to measure the peak 5-HT-evoked current amplitude (Fig. 1B, right). Apparent desensitization was enhanced to a lesser extent when 5-HT and (*R*/*S*)-methadone were applied simultaneously and the reduction in peak current amplitude was negligible (Fig. 1B, middle). Therefore, to minimize desensitization, we applied 5-HT and (*R*/*S*)-methadone simultaneously in subsequent experiments examining their competitive interactions.

5-HT (1–1000 μM) caused a concentration-dependent activation of recombinant 5-HT$_{3A}$ receptors expressed in HEK cells (Fig. 2A; Table 1). (*R*/*S*)-Methadone (30–1000 μM) applied simultaneously with 5-HT, caused concentration-dependent dextral shifts of the 5-HT (1–1000 μM) concentration-response relationships of 5-HT$_{3A}$ receptors (Fig. 2A), reducing the apparent potency of 5-HT (Table 1). Increasing the concentration of 5-HT overcame most of the inhibition by (*R*/*S*)-methadone even when high concentrations of (*R*/*S*)-methadone were used (Fig. 2A). Only at the highest (*R*/*S*)-methadone concentration (1 mM) tested was there a small reduction in the maximal efficacy of 5-HT (Table 1).

We used the method of Lew and Angus (1995) to evaluate the nature of (*R*/*S*)-methadone's inhibition of the 5-HT$_{3A}$ receptor. Plots of (*R*/*S*)-methadone concentration versus 5-HT pEC$_{50}$ values were well fitted by eq. 1 (see *Materials and Methods*), and the fidelity of the fit was not improved significantly by modifications incorporated into either eqs. 2 or 3 (data not shown). The results of this analysis are illustrated in the Clark plot (Fig. 2A). The line represents the predicted relationship between the 5-HT EC$_{50}$ values and the concentration of (*R*/*S*)-methadone, with the value of K_b (34.2 μM) generated by eq. 1. This K_b value reflects the affinity of (*R*/*S*)-methadone when it is simultaneously applied with 5-HT. Under these conditions, there is effectively a "race" for occupation of the agonist binding site. Binding affinity seems to be somewhat enhanced by applying (*R*/*S*)-methadone before 5-HT$_{3A}$ receptor activation as evidenced from the IC$_{50}$ value for (*R*/*S*)-methadone of 14.1 ± 2.5 μM.

(*R*/*S*)-Methadone (30–300 μM) also caused concentration-dependent inhibition of 5-HT (1–1000 μM)-evoked currents recorded from HEK cells expressing recombinant 5-HT$_{3AB}$ receptors (Fig. 2B). Consistent with previous reports (Davies et al., 1999; Stewart et al., 2003), 5-HT$_{3AB}$ receptors were less potently activated by 5-HT (Table 1) and desensitized more rapidly than 5-HT$_{3A}$ receptors (Fig. 2B). (*R*/*S*)-Methadone induced dextral shifts of the 5-HT concentration-response relationships mediated by 5-HT$_{3AB}$ receptors (Fig. 2B; Table 1). These data also reveal that all concentrations of (*R*/*S*)-methadone that inhibited 5-HT-evoked current amplitudes caused a component of inhibition that could not be surmounted by increasing the concentration of 5-HT (Fig. 2B; Table 1). The presence of an insurmountable component to the inhibition of 5-HT$_{3AB}$ receptors precludes the determination of a binding affinity for (*R*/*S*)-methadone. The insurmountable block of 5-HT$_{3AB}$ receptors by (*R*/*S*)-methadone could represent either noncompetitive or uncompetitive antagonism. The term uncompetitive antagonism describes inhibition that occurs less effectively at low agonist concentrations compared with high agonist concentrations. Such an effect of methadone would probably cause a systematic change in the Hill coefficients for the 5-HT concentration-response relationships. Because this did not occur, the inhibition by methadone does not seem to be uncompetitive (Table 1).

***R*- and *S*-Enantiomers Cause Similar Shifts of the 5-HT Concentration-Response Relationship.** Methadone is a chiral molecule and as such exists in two isomeric forms (Fig. 3A). Methadone used thus far in this study was the racemic mixture of *R*- and *S*-enantiomers. (*R*)-Methadone binds preferentially to the μ-opioid receptor (Kristensen et al., 1995). By contrast, (*S*)-methadone exerts the most potent inhibitory effect on cardiac hERG K^+ channels (Eap et al., 2007). We compared the abilities of pure (*R*)- and (*S*)-methadone to reduce the potency of 5-HT at 5-HT$_{3A}$ (Fig. 3B) and 5-HT$_{3AB}$ (Fig. 3C) receptors. There was no difference between the 5-HT concentration-response relationships of either 5-HT$_{3A}$ or 5-HT$_{3AB}$ receptors recorded in the presence of 100 μM (*R*)- or (*S*)-methadone, suggesting that competitive antagonism by methadone of 5-HT$_3$ receptors is not stereoisomer-specific (Fig. 3, B and C).

Methadone Increases Apparent Desensitization of 5-HT$_3$ Receptors. An increased rate of 5-HT-evoked current

TABLE 1

Parameters of 5-HT concentration-response relationships in the presence and absence of (*R*/*S*)-methadone

5-HT concentration-response relationships in the presence and absence of (*R*/*S*)-methadone at the concentrations indicated. Current amplitudes were normalized to those recorded from the same cells activated by 5-HT (100 μM) in the absence of (*R*/*S*)-methadone. Concentration-response relationships were fitted with a logistic equation (Fig. 2), yielding the parameters provided in this table.

	5-HT$_{3A}$ Receptor			5-HT$_{3AB}$ Receptor		
	EC$_{50}$	I_{max}	Hill Slope	EC$_{50}$	I_{max}	Hill Slope
	μM	%		*μM*	%	
5-HT alone	5.0 ± 1.0	104 ± 5	1.3 ± 0.3	15.3 ± 1.4	109 ± 3	1.2 ± 0.1
5-HT + methadone (30 μM)	7.3 ± 0.5*	94.0 ± 2.1	1.7 ± 0.2	15.8 ± 2.4	92.4 ± 4.4*	1.2 ± 0.2
5-HT + methadone (100 μM)	14.8 ± 1.1*	97.0 ± 2.0	1.5 ± 0.2	53.1 ± 7.4*	85.2 ± 3.4*	1.0 ± 0.1
5-HT + methadone (300 μM)	61.6 ± 8.8*	95.2 ± 4.5	1.2 ± 0.2	77.8 ± 2.7*	63.2 ± 0.8*	1.2 ± 0.04
5-HT + methadone (1000 μM)	127 ± 11*	80.6 ± 2.2*	1.4 ± 0.1	249 ± 52*	40.4 ± 3.0*	1.3 ± 0.3

* Significantly different from equivalent 5-HT alone value, $p < 0.01$, ANOVA, post hoc Dunnett's test.

desensitization is a well documented effect of incorporation of the 5-HT_{3B} subunit (Dubin et al., 1999; Stewart et al., 2003). Inspection of 5-HT_{3A} and 5-HT_{3AB} receptor-mediated currents (Figs. 2 and 4) confirms these previous findings. After 1 s, 5-HT (100 μM)-evoked currents mediated by 5-HT_{3A} and 5-HT_{3AB} receptors declined from initial peak amplitudes by 32 $\pm$ 5% (n = 18) and 80 $\pm$ 3% (n = 21), respectively. (*R*/*S*)-Methadone caused a striking increase in the apparent desensitization of currents mediated by both 5-HT_{3A} (Figs. 1B and 4A) and 5-HT_{3AB} receptors (Fig. 4B). After 1 s, 5-HT (100 μM)-evoked currents mediated by 5-HT_{3A} and 5-HT_{3AB} receptors in the presence of (*R*/*S*)-methadone (100 μM) had declined by 99 $\pm$ 0.1% (n = 3) and 99 $\pm$ 0.2% (n = 4), respectively. Similar increases in apparent desensitization were observed for both (*R*)- and (*S*)-methadone (Fig. 4). The time courses of currents mediated by 5-HT_3 receptors in the presence of (*R*/*S*)-, (*R*)-, or (*S*)-methadone were indistinguishable (n = 4). These results suggest that increased apparent desensitization of 5-HT_3 receptors by methadone is not stereoisomer-specific.

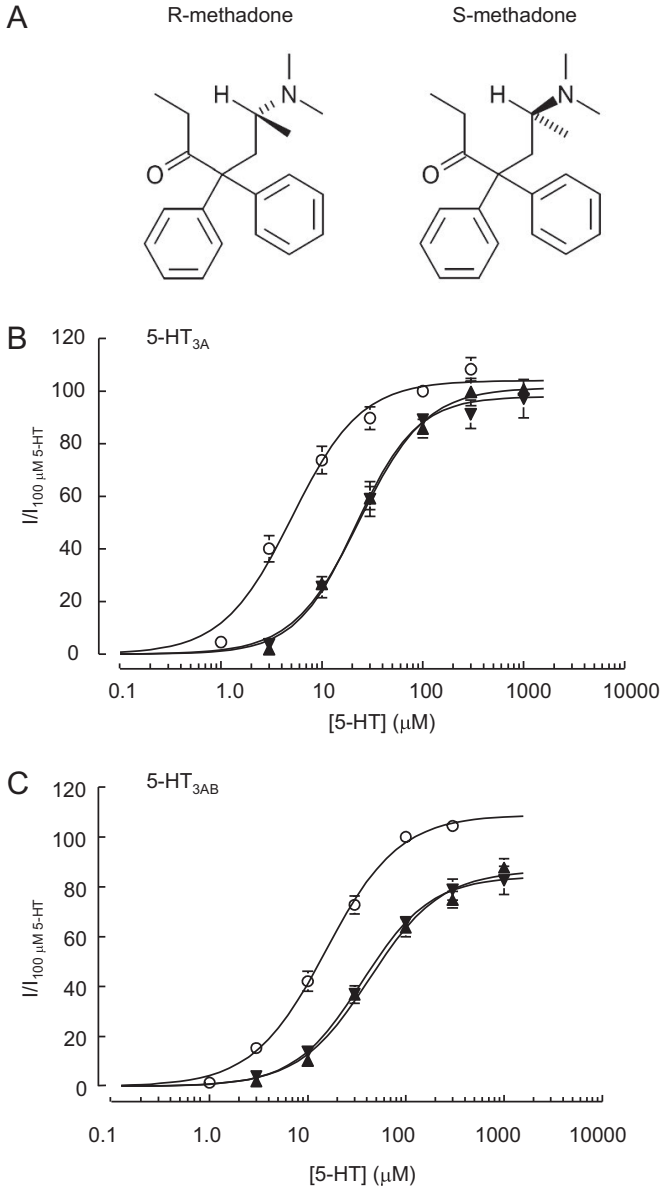

Fig. 3. Antagonism of 5-HT_3 receptors by (*R*)- and (*S*)-methadone. A, structural diagrams of (*R*)- and (*S*)-methadone illustrate the chiral carbon responsible for the stereoisomerization. The graphs illustrate 5-HT concentration-response relationships for recombinant 5-HT_{3A} (B) and 5-HT_{3AB} (C) receptors, evaluated in the absence (○) or presence of either (*R*)-methadone (▲) or (*S*)-methadone (▼) (100 μM). (*R*)- and (*S*)-Methadone caused similar reductions in the potency of 5-HT.

Voltage-Dependent Inhibition of 5-HT_{3AB} Receptors by Methadone. The apparent reduction by (*R*/*S*)-methadone of the efficacy of 5-HT as an activator of 5-HT_{3AB} receptors could be caused by enhanced desensitization. Indeed, currents rapidly decay in the presence of high concentrations of (*R*/*S*)-methadone and 5-HT potentially compromising measurements of peak current amplitude (Fig. 4). This is particularly likely in the case of the 5-HT_{3AB} receptor, which desensitizes rapidly even in the absence of (*R*/*S*)-methadone. However, in addition to its ability to increase desensitization, (*R*/*S*)-methadone may also reduce efficacy of 5-HT by exerting a negative allosteric effect and/or a direct channel block. The latter can be identified by the presence of voltage-dependent inhibition. Therefore we examined the current-voltage relationship of currents mediated by 5-HT_{3A} and 5-HT_{3AB} receptors in the presence and absence of (*R*/*S*)-methadone. (*R*/*S*)-Methadone was bath-applied at approximately similarly effective concentrations in experiments examining 5-HT_{3A} and 5-HT_{3AB} receptors (30 and 100 μM, respectively). 5-HT (30 μM)-evoked currents mediated by 5-HT_{3A} receptors were inhibited by (*R*/*S*)-methadone (30 μM) at potentials between −60 and 60 mV (Fig. 5, A and B). The 5-HT current-voltage relationship exhibited characteristic inward rectification both in the absence and presence of (*R*/*S*)-methadone (Fig. 5B). There was no significant ($p > 0.05$, ANOVA) change in the inhibition of the peak current amplitude by (*R*/*S*)-methadone at each potential (Fig. 6).

As reported previously (Davies et al., 1999), incorporation of the human 5-HT_{3B} subunit caused the 5-HT-evoked current-voltage relationship to become linear (Fig. 5, C and D). It is noteworthy that the presence of (*R*/*S*)-methadone (100 μM) caused the appearance of marked outward rectification (Fig. 5, C and D), with more inhibition of peak current amplitude at negative potentials compared with the equivalent positive potentials (Fig. 6).

It is possible that the voltage-dependence of the inhibition of 5-HT_{3AB} receptors by (*R*/*S*)-methadone results from an effect of voltage on desensitization. To address this possibility, we simultaneously applied 5-HT (100 μM) alone and with (*R*/*S*)-methadone (100 μM) and investigated the voltage dependence of inhibition and desensitization. Currents recorded from the same cell, activated by 5-HT (100 μM) at −60 and 60 mV exhibited similar kinetics (Fig. 7A). At a holding potential of −60 mV, (*R*/*S*)-methadone speeded up apparent desensitization as demonstrated previously (Fig. 4). The effect of (*R*/*S*)-methadone on the rate of current decay was most marked at −60 mV (Fig. 7A). Likewise, when (*R*/*S*)-methadone and 5-HT were applied simultaneously, the current amplitude was significantly larger at 60 than at −60 mV (Fig. 7B). These data could either be explained by a voltage-dependent increase in 5-HT_{3AB} receptor desensitization by (*R*/*S*)-methadone or alternatively the apparent desensitization in the presence of (*R*/*S*)-methadone could seem faster at −60 mV because of open channel block.

We attempted to reduce desensitization and test whether this diminished the voltage-dependent blockade when (*R*/*S*)-

methadone was applied simultaneously with 5-HT. 5-Hydroxyindole attenuates desensitization of 5-HT$_3$ receptors (Kooyman et al., 1993). However, as reported previously (Hu and Peoples, 2008), 5-hydoxyindole (10 mM) had little effect on desensitization of 5-HT-evoked currents mediated by 5-HT$_{3AB}$ receptors relative to 5-HT$_{3A}$ receptors (data not shown). Therefore, we adopted an alternative strategy using the partial agonist dopamine to activate slowly desensitizing currents. Dopamine (3 mM) activated 9.8 ± 3.2% (n = 6) of the current amplitude evoked by 5-HT (100 μM) when applied to cells expressing 5-HT$_{3AB}$ receptors. Administration of 10 mM dopamine failed to increase the 5-HT$_{3AB}$ receptor-mediated current amplitude (n = 6; data not shown), demonstrating that at 3 mM, dopamine had reached its maximal efficacy. Dopamine-evoked currents exhibited little desensitization after 1 s (Fig. 7C). At −60 mV 82 ± 3% (n = 4) of the dopamine-evoked current remained after 1 s of its application to 5-HT$_{3AB}$ receptors. By contrast, after 1 s of application

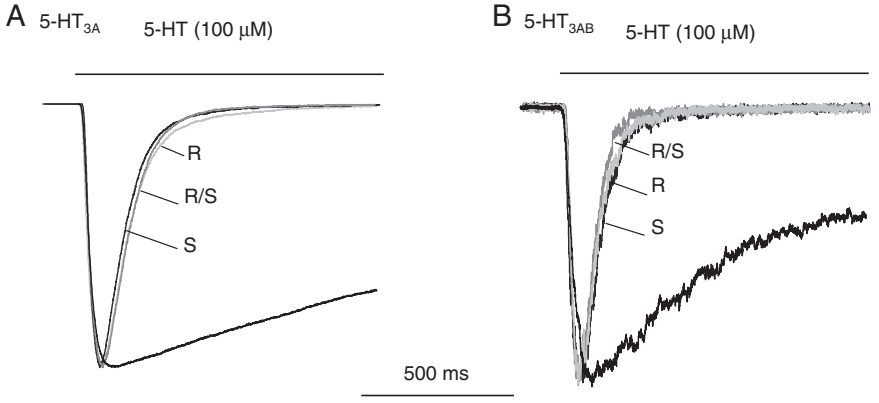

Fig. 4. (R)- and (S)-Methadone increase 5-HT$_3$ receptor apparent desensitization. Superimposed and normalized 5-HT (100 μM)-evoked currents recorded from cells expressing either 5-HT$_{3A}$ (A) or 5-HT$_{3AB}$ (B) receptors. In each case, currents were recorded from the same HEK cell in the absence and presence of (R/S)-, (R)-, or (S)-methadone (100 μM).

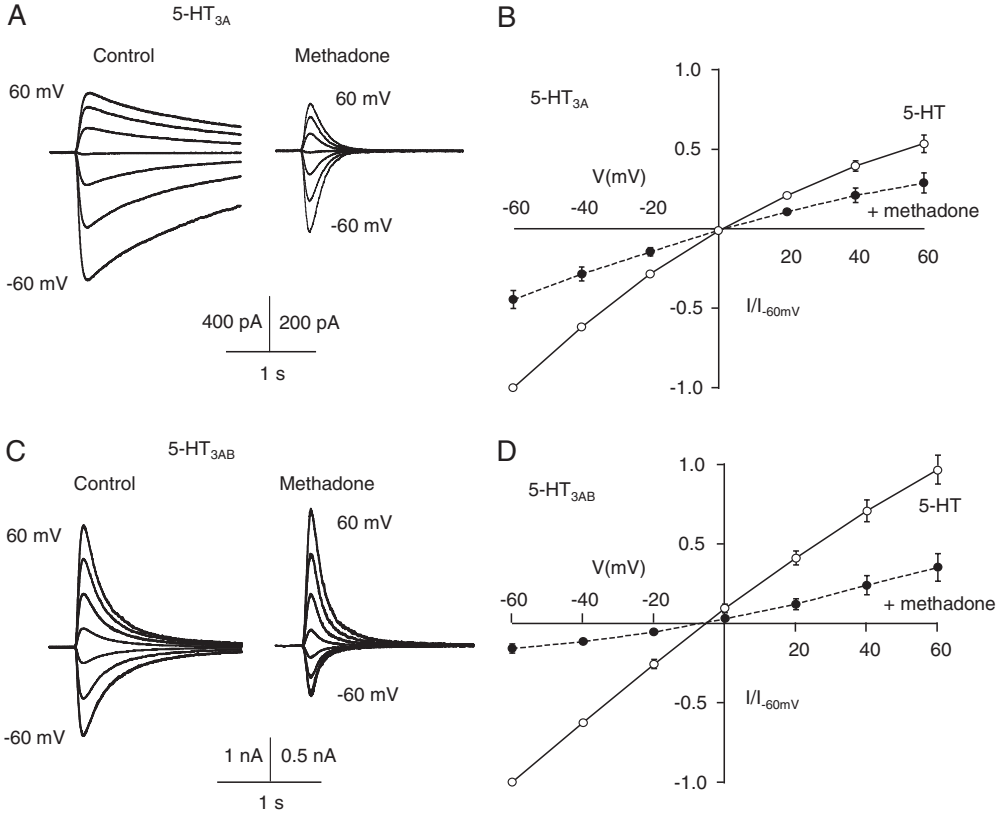

Fig. 5. (R/S)-Methadone affects 5-HT-evoked current-voltage relationships. A, superimposed, leak subtracted 5-HT (30 μM)-evoked currents recorded from an HEK cell expressing 5-HT$_{3A}$ receptors in the absence (left) and presence (right) of (R/S)-methadone (30 μM). (R/S)-Methadone was bath-applied before and during local 5-HT application. The same cell was used to generate all traces at holding potentials between −60 and 60 mV (20-mV increments). Note that 5-HT-evoked currents, recorded in the absence and presence of (R/S)-methadone, are displayed using different scale bars. B, 5-HT current-voltage relationships recorded in the presence and absence of (R/S)-methadone from cells expressing 5-HT$_{3A}$ receptors. Current amplitudes were normalized to 5-HT-evoked current amplitudes recorded from the same cell at −60 mV. Data points are averages of four recordings and vertical lines represent ± S.E.M. C, superimposed, leak subtracted 5-HT (30 μM)-evoked currents recorded from an HEK cell expressing 5-HT$_{3AB}$ receptors in the absence (left) and presence (right) of (R/S)-methadone (100 μM). The same cell was used to generate all traces at holding potentials between −60 and 60 mV (20-mV increments). Note that 5-HT-evoked currents, recorded in the absence and presence of (R/S)-methadone, are displayed using different scale bars. D, 5-HT current-voltage relationships recorded in the presence and absence of (R/S)-methadone. Current amplitudes were normalized to 5-HT current amplitudes recorded from the same cell at −60 mV. Data points are averages of five recordings and vertical lines represent ± S.E.M.

of 5-HT (100 μM) to the same cells only 12 ± 3% (n = 4) of current remained. (*R*/*S*)-Methadone (100 μM) reduced the peak amplitude of dopamine-evoked currents mediated by 5-HT_{3AB} receptors at −60 and 60 mV by 44 ± 5 and 27 ± 3% (n = 5), respectively (Fig. 7D). The inhibition was significantly ($p < 0.05$) reduced at a holding potential of 60 mV.

We compared the voltage-dependent blockade of dopamine-evoked currents by (*R*)- and (*S*)-methadone (100 μM). The inhibition of dopamine-evoked currents by (*R*)-methadone at −60 mV was 48 ± 6% (n = 5). Inhibition by (*R*)-methadone was reduced to 32 ± 4% at 60 mV (Fig. 7D). By contrast (*S*)-methadone caused a smaller inhibition of dopamine-evoked currents (26 ± 4%; n = 5) than did either (*R*/*S*)-or (*R*)-methadone (Fig. 7C). This weaker inhibition was essentially reversed (3.1 ± 2.7%) by a holding potential of 60 mV.

Taken together, these recordings of dopamine-evoked currents demonstrate that there is a voltage-dependent component to the inhibition of 5-HT_{3AB} receptors that is present despite diminution of receptor desensitization and therefore represents open channel blockade. The noncompetitive block by methadone is influenced by the identity of its stereoisomer, with (*R*)-methadone causing a stronger block than (*S*)-methadone.

Discussion

The opioid alkaloid methadone inhibited 5-HT-evoked currents mediated by homomeric 5-HT_{3A} receptors in a concentration-dependent manner. Increasing concentrations of 5-HT surmounted the inhibitory effect of (*R*/*S*)-methadone. The inhibition was predominantly competitive; increasing concentrations of (*R*/*S*)-methadone caused a linear dextral shift in the 5-HT concentration-response relationship. The incorporation of the 5-HT_{3B} subunit reduced the potency of inhibition by (*R*/*S*)-methadone and caused the appearance of a component of antagonism that could not be overcome by 5-HT. Methadone also increased 5-HT_{3A} and 5-HT_{3AB} receptor apparent desensitization. The insurmountable inhibition of 5-HT_{3AB} receptors by (*R*/*S*)-methadone was influenced by voltage. Inhibition was significantly larger at negative voltages than corresponding positive voltages. By contrast, the inhibition of 5-HT_{3A} receptors by (*R*/*S*)-methadone was independent of voltage.

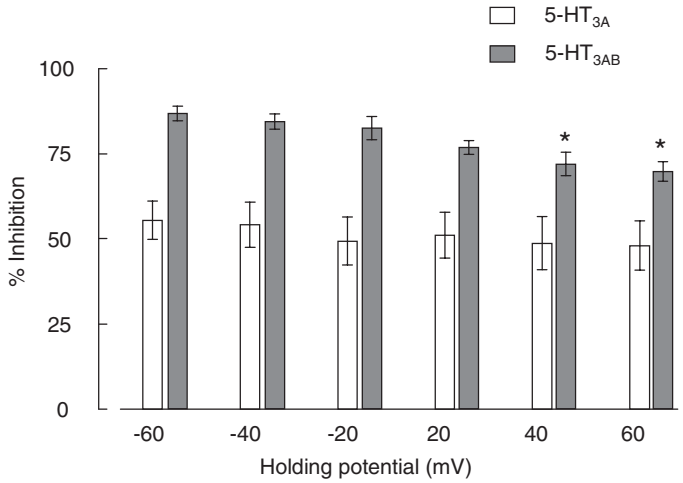

Fig. 6. Voltage-dependent inhibition of 5-HT_{3AB} receptors by (*R*/*S*)-methadone. The graph depicts the effect of voltage on inhibition by (*R*/*S*)-methadone (30 and 100 μM) of 5-HT-evoked currents mediated by 5-HT_{3A} and 5-HT_{3AB} receptors, open and gray bars, respectively. (*R*/*S*)-Methadone was bath-applied before and during local 5-HT (30 μM) application (Fig. 5). Bars are mean inhibitions recorded from at least four cells in each case; vertical lines represent ± S.E.M. Asterisks indicate that inhibitions of currents mediated by 5-HT_{3AB} receptors, at 40 and 60 mV, were significantly smaller than those at equivalent negative holding potentials, $p < 0.05$ and 0.01, respectively.

The voltage-dependent nature of the insurmountable inhibition of 5-HT_{3AB} receptors by (*R*/*S*)-methadone could reflect attenuation of desensitization at positive voltages or relief from open channel block of outward currents. There was no effect of voltage on the rate of desensitization of 5-HT-evoked currents mediated by 5-HT_{3AB} receptors in the absence of (*R*/*S*)-methadone. However, apparent desensitization in the presence of (*R*/*S*)-methadone was faster at −60 mV compared with 60 mV. A voltage-dependent open channel block by methadone could contribute to the apparent desensitization of 5-HT-evoked currents mediated by 5-HT_{3AB} receptors. To investigate this further, we used dopamine, a partial agonist of 5-HT_3 receptors. Maximal dopamine-evoked current mediated by 5-HT_{3AB} receptors exhibited little desensitization. In the presence of (*R*/*S*)-methadone, dopamine-evoked currents also exhibited slower apparent desensitization than did 5-HT-evoked currents. Nevertheless, (*R*/*S*)-methadone caused voltage-dependent inhibition of dopamine-evoked currents mediated by 5-HT_{3AB} receptors. These data suggest that the voltage-dependent inhibition of currents mediated by 5-HT_{3AB} receptors is independent of desensitization. Instead, it is likely that the voltage-dependent inhibition reflects a block by (*R*/*S*)-methadone within the channel of heteromeric 5-HT_{3AB} receptors, an action that is lacking from homomeric 5-HT_{3A} receptors. Compared with other opioid alkaloids, methadone has a high pK_a value (~9.3) and is therefore predominantly cationic at physiological pH (Carr and Cousins, 1998) and, as such, it may most effectively access the channel of the 5-HT_{3AB} receptor at negative membrane potentials.

Methadone can exist as either *R*- or *S*-enantiomers. (*R*)-Methadone binds to μ-opioid receptors with higher affinity than (*S*)-methadone. We used pure (*R*)- and (*S*)-methadone to examine whether competitive inhibition, increased desensitization, voltage-dependent inhibition of 5-HT_3 receptors, or a combination are also stereoselective actions. The shifts by (*R*)- and (*S*)-methadone of the 5-HT concentration-response relationships for both 5-HT_{3A} and 5-HT_{3AB} receptors were similar, as were the levels of apparent desensitization that the isomers induced. Nor was there an apparent selectivity of methadone enantiomers for the insurmountable inhibition of 5-HT-evoked currents mediated by 5-HT_{3AB} receptors. However, when dopamine was used as an agonist to minimize desensitization, the voltage-dependent inhibition by (*R*)-methadone of 5-HT_{3AB} receptors was significantly greater than was the equivalent inhibition by (*S*)-methadone. Taken together, our findings suggest that (*R*)-methadone has a stronger interaction than (*S*)-methadone with a binding site on the 5-HT_{3B} subunit located within the second transmembrane domain lining the channel pore. Future studies will be required to identify the residues involved.

Despite the lack of voltage-dependent inhibition of homomeric 5-HT_{3A} receptors, (*R*/*S*)-methadone in the low micromolar concentration range caused increased desensitization. This effect was accentuated by preapplication of (*R*/*S*)-methadone and may contribute to the clinical actions of the drug.

The increased rate of desensitization makes measurement of equilibrium binding affinity problematic. The IC_{50} value for inhibition of 5-HT-evoked currents, determined by preapplying (*R*/*S*)-methadone, was somewhat lower than the calculated binding affinity derived from shifts of the 5-HT concentration-response relationships by simultaneously applied (*R*/*S*)-methadone. It is possible that greater desensitization, induced by prolonged application, may increase (*R*/*S*)-methadone's affinity for the 5-HT$_{3A}$ receptor. Alternatively (*R*/*S*)-methadone may not reach equilibrium-binding conditions when applied simultaneously with 5-HT. The route to the binding site is probably tortuous and 5-HT may win the race to access its site. However, the explanation that we favor is that preapplication induces greater desensitization, which compromises the ability to resolve the peak current amplitude when (*R*/*S*)-methadone is preapplied. Under these conditions, the IC_{50} value reflects the affinity of (*R*/*S*)-methadone for its site of desensitization and the 5-HT binding site. Our data suggest that these two sites are distinct. First, (*R*/*S*)-methadone does not act as a partial agonist and therefore would be unlikely to increase desensitization through occupancy of the agonist binding site. Second, (*R*/*S*)-methadone causes increased 5-HT$_3$ receptor desensitization even in the presence of saturating concentrations of 5-HT, which would completely displace (*R*/*S*)-methadone from the agonist binding site. Therefore, we propose that there are at least three binding sites for (*R*/*S*)-methadone on 5-HT$_3$ receptors: one site that overlaps with the agonist binding site and accounts for the observed competitive antagonism; a second that is outside the agonist binding site, occupancy of which enhances desensitization; and a third located within the channel pore of the heteromeric 5-HT$_{3AB}$ receptor, which is responsible for voltage-dependent blockade by (*R*/*S*)-methadone.

In addition to 5-HT$_3$ receptors, methadone directly interacts with several other ion channels, including hERG K^+ channels (Katchman et al., 2002; Eap et al., 2007), inwardly rectifying K^+ channels (Rodriguez-Martin et al., 2008), the NMDA subtype of the glutamate receptor (Ebert et al., 1995; Callahan et al., 2004), and the $\alpha3\beta4$ and $\alpha7$ nicotinic receptors (Xiao et al., 2001; Pakkanen et al., 2005). (*R*)-Methadone, the isomer that preferentially binds to μ-opioid receptors is also more potent than (*S*)-methadone as an inhibitor of NMDA receptors (Kristensen et al.,

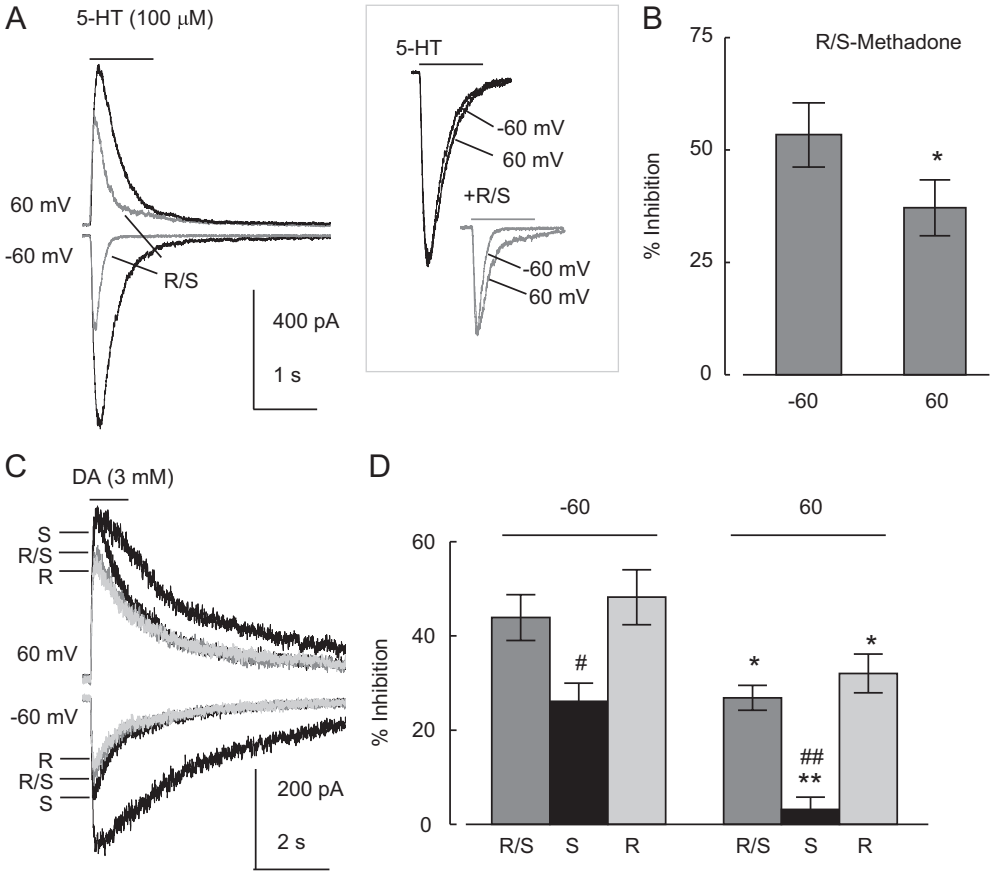

Fig. 7. Voltage-dependent inhibition by methadone of 5-HT$_{3AB}$ receptors is independent of desensitization. A, 5-HT$_{3AB}$ receptor-mediated currents evoked by 5-HT (100 μM) applied for 1 s either alone (in black) or simultaneously with (*R*/*S*)-methadone (100 μM; in gray) at holding potentials of −60 and 60 mV. Inset, currents recorded at −60 and 60 were normalized and superimposed to compare kinetics. The time course of desensitization of 5-HT-evoked currents recorded at both potentials in the absence of (*R*/*S*)-methadone (black traces) was similar. In the presence of (*R*/*S*)-methadone (gray traces), 5-HT-evoked currents decayed faster at −60 mV. B, percentage of inhibition at −60 and 60 mV ($n = 5$). The inhibition by (*R*/*S*)-methadone was significantly smaller at 60 mV than at −60 mV (*, $p < 0.05$; paired t test). Vertical lines are ± S.E.M. C, 5-HT$_{3AB}$ receptor-mediated currents evoked by dopamine (DA; 3 mM) applied for 1 s either alone (in black) or simultaneously with 100 μM (*R*/*S*)- (medium gray), (*R*)- (light gray), or (*S*)- (dark gray) methadone at holding potentials of −60 and 60 mV. D, percentage of inhibition at −60 and 60 mV ($n = 5$). The inhibitions by all three methadone formulations were significantly smaller at 60 mV than at −60 mV (*, $p < 0.05$; **, $p < 0.01$; paired t test). Furthermore, inhibition by (*S*)-methadone was significantly smaller than (*R*)-methadone at −60 mV (#, $p < 0.05$) and both (*R*/*S*)- and (*R*)-methadone at 60 mV (##, $p < 0.001$) as determined by ANOVA with post hoc Dunnett's test. Vertical lines are ± S.E.M.

1995; Callahan et al., 2004), whereas (*S*)-methadone has a higher potency than (*R*)-methadone as an inhibitor of hERG channels (Eap et al., 2007). In keeping with its preferential effect at μ-opioid and NMDA receptors, (*R*)-methadone caused a greater voltage-dependent inhibition of 5-HT_{3AB} receptors than did (*S*)-methadone.

Patients undergoing treatment for morphine dependence often receive high doses of methadone (Eap et al., 2007). Methadone has a long half-life and can reach micromolar concentrations equivalent to those that inhibit 5-HT_3 receptors, particularly in individuals who are slow metabolizers of the compound, raising the possibility that antagonism of 5-HT_3 receptors may be clinically relevant. 5-HT_3 receptors are distributed throughout the central and peripheral nervous systems, with dense expression in the dorsal vagal complex, an area that coordinates the vomiting reflex (Barnes et al., 2009). The antiemetic actions of 5-HT_3 receptor antagonists are likely to be mediated through this region of the brainstem. 5-HT_3 receptors are also expressed at lower levels elsewhere in the central nervous system, including the forebrain. In humans, there are 5-HT_3 binding sites in the caudate nucleus and putamen, two regions associated with drug craving (Harlan and Garcia, 1999; Thompson and Lummis, 2006). The use of in situ hybridization and immunohistochemistry demonstrated 5-HT_{3A} subunit expression in the brain. By contrast, the distribution of the 5-HT_{3B} subunit in the brain is somewhat controversial (van Hooft and Yakel, 2003). Some studies suggest that there is an absence of 5-HT_{3B} subunit transcript from the rodent brain (Morales and Wang, 2002), whereas others report labeling of rodent central neurons with antibodies to the 5-HT_{3B} subunit (Reeves and Lummis, 2006). 5-HT_{3B} subunit transcripts have consistently been detected in human brain tissue (Davies et al., 1999; Tzvetkov et al., 2007). However, there is a lack of functional studies implicating a role of the 5-HT_{3B} subunit in central neurons.

Recombinant studies of homomeric 5-HT_{3A} and heteromeric 5-HT_{3AB} receptors reveal that they have distinct functional properties. Homomeric 5-HT_{3A} receptors have a single channel conductance <1 pS and a similar permeability to Ca^{2+} and Na^{+} (Davies et al., 1999). By contrast, heteromeric 5-HT_{3AB} receptors are less permeable to divalent cations and have a single channel conductance of ~15 pS. Single channels mediated by 5-HT_3 receptors in enteric neurons have conductances similar to those of 5-HT_{3AB} receptors (Galligan, 2002). Therefore 5-HT_{3AB} receptors are located peripherally where they are likely to participate in the gastrointestinal effects of the setrons, which cause increased colonic transit time (Talley et al., 1990). Thus, antagonists with selectivity for homomeric 5-HT_{3A} receptors may have fewer peripheral side effects. Because (*R*/*S*)-methadone exhibits subunit selective inhibitory actions, modification of this opioid alkaloid structure may provide a strategy for designing new 5-HT_3 receptor antagonists that act either centrally or peripherally.

The list of drugs that have modulatory effects on 5-HT_3 receptors has expanded to include the alkaloid methadone. The complex multimodal actions of methadone on 5-HT_3 receptors reveals sites, distinct from the agonist binding site, through which alkaloids can affect desensitization and block the channel pore. The rich variety of alkaloids available for pharmacophore analysis provides probes for modeling the structure of competitive and noncompetitive antagonist binding sites in 5-HT_3 receptors.

Acknowledgments

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