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Blockade of 5-HT_3 receptor-mediated currents in dissociated frog sensory neurones by benzoxazine derivative, Y-25130

[1] Takashi Yakushiji & *Norio Akaike

Research Laboratories, Yoshitomi Pharmaceutical Industries Ltd., Yoshitomi 871, and *Department of Neurophysiology, Tohoku University School of Medicine, Sendai 980, Japan

1 The effect of Y-25130, ((±)-N-(1-azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1,4-benzoxazine-8-carboxamide hydrochloride), a high affinity 5-hydroxytryptamine$_3$ (5-HT_3) receptor ligand, was examined on the 5-HT-induced response in dissociated frog dorsal root ganglion (DRG) neurones by use of the extremely rapid concentration-jump ('concentration-clamp') and the conventional whole-cell patch-clamp techniques.

2 5-HT induced a rapid transient inward current associated with an increase in membrane conductance at a holding potential of -70 mV. The current amplitude increased sigmoidally as 5-HT concentration increased. The half-maximum value (K_a) and the Hill coefficient estimated from the concentration-response curve were 1.7×10^{-5} M and 1.7, respectively.

3 The current-voltage ($I-V$) relationship of 5-HT-induced current ($I_{5\text{-HT}}$) showed inward rectification at potentials more positive than -40 mV. The reversal potential ($E_{5\text{-HT}}$) was -11 mV. The $E_{5\text{-HT}}$ value was unaffected by total replacement of intracellular K^+ by Cs^+, indicating that the 5-HT-gated channels might be large cation channels.

4 Both the activation and inactivation phases of $I_{5\text{-HT}}$ were single exponentials. The time constants of activation and inactivation (τ_a and τ_i) decreased with increasing 5-HT concentration.

5 The 5-HT response was mimicked by a selective 5-HT_3 receptor agonist, 2-methyl-5-HT, but the maximum response induced was approximately 25% that of 5-HT. The 5-HT response was reversibly antagonized by the 5-HT_3 receptor antagonists, ICS 205-930, metoclopramide and Y-25130, but not by a 5-HT_{1A} receptor antagonist, spiperone, and a 5-HT_2 receptor antagonist, ketanserin. The half-inhibition concentrations (IC_{50}) were 4.9×10^{-10} M for Y-25130, 4.8×10^{-10} M for ICS 205-930 and 8.6×10^{-9} M for metoclopramide.

6 Y-25130 (5×10^{-10} M) caused a rightward shift of the concentration-response curve for 5-HT while decreasing the maximum response.

7 The results suggest that Y-25130 is a potent antagonist of the 5-HT_3 receptor-channel complex.

Keywords: Frog dorsal root ganglion neurone; 5-HT_3 receptor-mediated response; benzoxazine derivative; Y-25130

Introduction

5-Hydroxytryptamine (5-HT) induces a rapid depolarization accompanied by conductance increase in sensory neurones (Todorovic & Anderson, 1990) and sympathetic and parasympathetic neurones (Higashi, 1977; Akasu *et al.*, 1987; Wallis & Dun, 1988). In cultured neuroblastoma cells and cultured neurones, voltage-clamp studies demonstrated that 5-HT-induced transient current is mediated by 5-HT_3 receptors (Neijt *et al.*, 1988a; Yakel & Jackson, 1988; Derkach *et al.*, 1989; Lambert *et al.*, 1989). These transient depolarizing responses and/or inward currents evoked by 5-HT were inhibited by selective 5-HT_3 receptor antagonists, such as ICS 205-930 (Neijt *et al.*, 1988a; Derkach *et al.*, 1989; Robertson & Bevan, 1991) and GR38032F (Lambert *et al.*, 1989), at picomolar concentrations and mimicked by a selective 5-HT_3 receptor agonist, 2-methyl-5-HT. Since 5-HT_3 receptor-induced responses characteristically exhibit rapid activation and subsequent inactivation, a rapid application technique is necessary to study the kinetics and pharmacological properties of 5-HT_3 receptor-induced responses. Recently, an extremely rapid concentration-jump method, termed the 'concentration-clamp' technique was developed (Akaike *et al.*, 1986; Inoue *et al.*, 1986). This technique combines intracellular perfusion (Akaike *et al.*, 1978; Hattori *et al.*, 1984) and rapid exchange (within 2 ms) of external solution (Krishtal *et al.*, 1983), using single-electrode voltage-clamp, and allows detailed analysis of the kinetics of ligand-gated ionic currents in various neurones. In the present study, we have investigated the physiological and pharmacological properties of the 5-HT-induced current in dissociated frog dorsal root ganglion (DRG) neurones by the use of the 'concentration-clamp' technique. In addition, the blocking action of 5-HT_3 receptor-induced current by a newly synthesized benzoxazine derivative, Y-25130, which is effective against emesis induced by cytotoxic drugs or total body x-radiation (Fukuda *et al.*, 1991), was also studied.

Methods

Preparation

The experimental methods used were as described previously (Hattori *et al.*, 1984; Ishizuka *et al.*, 1984; Akaike *et al.*, 1986). In brief, isolated lumbar dorsal root ganglia of bullfrog (*Rana catesbiana*) were digested in normal Ringer solution containing 0.3% collagenase and 0.05% trypsin at pH 7.4 for 15 to 20 min at 37°C. During the enzyme treatment, the preparation was gently agitated by bubbling the bathing medium with 95% O_2 plus 5% CO_2. Then, neurones were mechanically dissociated from the ganglia with finely polished pins under a binocular microscope. The isolated neurones were stored in a solution containing equal amounts of Ringer solution and Eagle's minimum essential medium (Nissui, Tokyo) at room temperature for a minimum of 3 h.

[1] Author for correspondence.

Solutions

Dissociated DRG neurones were perfused with external and internal solutions, the ionic compositions of which were (in mM): external, NaCl 115, KCl 2.5, $CaCl_2$ 2, glucose 5 and N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid 10 (HEPES); internal, NaCl 5, KCl 55, K-aspartate 70, ethylene glycol-*bis*-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 1 and HEPES 10. Current-voltage ($I-V$) relationships were determined in modified external and internal solutions in which K^+ was replaced with equimolar Cs^+. The pH of external and internal solutions was adjusted to 7.4 and 7.2, respectively, with tris (hydroxymethyl) aminomethane-base (Tris-OH).

Rapid drug application using a concentration-clamp technique

The concentration-clamp technique was used for rapid application of external test solutions within 2 ms (Akaike *et al.*, 1986). The cell-attached tip of the suction-pipette was inserted into a plastic tube through a hole (500 μm diameter). The lower end of the tube could be exposed directly to the external test solution by moving up and down a stage on which dishes containing the drug were placed. A negative pressure (about − 30 cmHg) was applied to the upper end of the tube. The exchange speed and amount of external solution were controlled by adjusting both the negative pressure and the opening speed of the electromagnetic valve driven by 24 V d.c. The power supply was switched on for the desired duration by a stimulator (Nihon Kohden, SEN-7103).

Electrical measurements

Membrane potential was measured through an Ag-AgCl wire in a Ringer-agar plug mounted on a suction-pipette holder. The reference electrode was also an Ag-AgCl wire connected to the bathing medium through a Ringer-agar plug mounted on a rapid solution-change tube. The resistance between the suction pipette (tip diameter 7 μm) and the reference electrode in Ringer solution was 200–300 kΩ. Both electrodes were connected to a voltage-clamp circuit for single-electrode recording (Ishizuka *et al.*, 1984). Both current and voltage were monitored on a digital storage oscilloscope (National, VP-5730A) and simultaneously recorded on a pen recorder (Graftic, SR-6335). Data were also stored on an FM data recorder (Teac, MR30) for computer analysis.

Drugs

Collagenase and metoclopramide hydrochloride (Sigma), trypsin (Difco) and 5-hydroxytryptamine creatinine sulphate (Merck) were purchased. Y-25130 (Figure 1), ICS 205-930 ((3α-tropanyl)-1*H*-indole-3-carboxylic acid ester), ketanserin hydrochloride, spiperone and 2-methyl-5-HT hydrobromide were synthesized by our research laboratories. Y-25130, ICS 205-930, metoclopramide, ketanserin and spiperone were initially dissolved in dimethyl sulphoxide (DMSO) and diluted with the external test solution just before use. DMSO at final concentrations (0.2% or less) did not affect the 5-HT response. All experiments were carried out at room temperature (20–24°C).

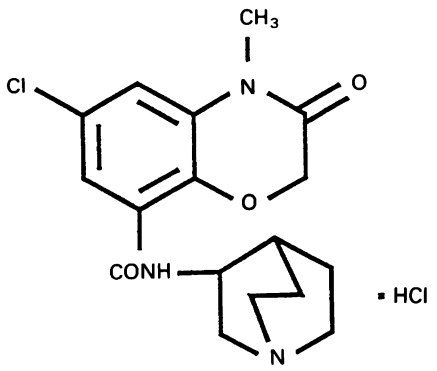

Figure 1 Chemical structure of Y-25130, ((±)-N-(1-azabicyclo [2.2.2] oct-3-yl) -6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1, 4-benzoxazine-8-carboxamide hydrochloride).

Results

Concentration-dependence of 5-HT-induced currents ($I_{5\text{-}HT}$)

5-HT (2×10^{-5} M) elicited a transient depolarization under current-clamp conditions. The depolarizing response was accompanied by an apparent decrease in membrane input resistance (Figure 2a). Figure 2b (inset) shows the 5-HT-induced inward currents at a holding potential (V_H) of − 70 mV under voltage-clamp, which underlies the 5-HT-induced depolarization under current-clamp. In 32% of DRG neurones examined ($n = 172$), 5-HT elicited a transient inward current at a V_H of − 70 mV. The remaining 68% of the neurones sampled had no discernible response to 5-HT. The 5-HT-induced currents ($I_{5\text{-}HT}$) activated rapidly to peak amplitude and then completely inactivated within 2 s at all

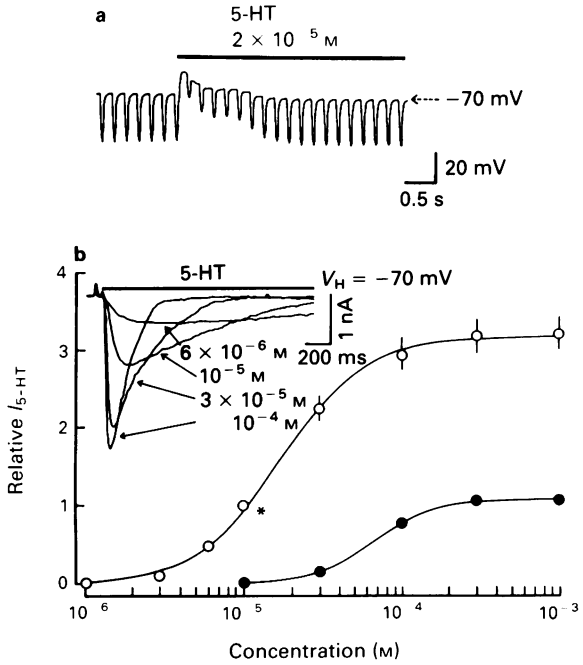

Figure 2 Response of frog DRG neurone to 5-hydroxytryptamine (5-HT). (a), 5-HT-induced depolarization under current-clamp. 5-HT was applied for the period shown by a solid line above the voltage trace. The response was accompanied by an apparent increase in membrane conductance, this being reflected in a decrease in amplitude of the electrotonic potentials. The hyperpolarizing current pulses (0.5 nA, 50 ms) were applied through the pipette at 5 Hz. (b) Inset: transient inward currents induced by 5-HT at various concentrations under voltage-clamp. The superimposed recordings of 5-HT-induced current ($I_{5\text{-}HT}$) were obtained from the same neurone. V_H was − 70 mV. With increasing 5-HT concentration the peak amplitude of the $I_{5\text{-}HT}$ increased and the kinetics became more rapid. (b) Concentration-response curves for 5-HT (○) and 2-methyl-5-HT (●) at a V_H of − 70 mV. All responses were normalized to the peak current amplitude (*) evoked by 10^{-5} M 5-HT alone. Theoretical curves were drawn using K_a (1.7×10^{-5} M) and n (1.7) for 5-HT, and K_a (6.6×10^{-5} M) and n (2.2) for 2-methyl-5-HT. Each point is the mean of values from 4–6 neurones and bars indicate ± s.e.mean.

concentrations used, indicating receptor desensitization. In the present study, 5-HT was applied every 3 min, at which interval a constant 5-HT response could be repeatedly evoked for 1 h or more. Figure 2b shows the peak amplitude of $I_{5\text{-HT}}$ plotted as a function of 5-HT concentration. In the figure all $I_{5\text{-HT}}$ were normalized to the peak current amplitude induced by 10^{-5} M 5-HT (*). The current amplitude increased in a sigmoidal fashion as the 5-HT concentration increased. The threshold concentration of 5-HT was 3×10^{-6} M, and the half-maximum concentration (K_a) was 1.7×10^{-5} M. A nearly maximum response was observed at 3×10^{-4} M. The concentration-response relationship for $I_{5\text{-HT}}$ was in accordance with the conventional expression:

$$I = I_{max} \frac{C^n}{C^n + K_a^n} \quad (1)$$

where I is the observed $I_{5\text{-HT}}$, I_{max} is the maximum current, C is the 5-HT concentration, K_a is the 5-HT concentration that evokes the half-maximal response, and n is the Hill coefficient. When a continuous line was drawn according to equation (1) with n (1.7) and K_a (1.7×10^{-5} M), all experimental points fitted the theoretical curve well. Although a selective 5-HT$_3$ receptor agonist, 2-methyl-5-HT, elicited similar inward currents, maximum current amplitude was approximately 25% of that obtained with 5-HT, indicating that 2-methyl-5-HT is a partial agonist at this 5-HT receptor. The K_a value and the Hill coefficient for 2-methyl-5-HT were 6.6×10^{-5} M and 2.2, respectively.

Voltage-dependence of $I_{5\text{-HT}}$

As seen in Figure 3, the current-voltage (I–V) relationship for $I_{5\text{-HT}}$ showed inward rectification at membrane potentials more positive than – 40 mV, indicating a voltage-dependency of the 5-HT response. The reversal potential for 5-HT ($E_{5\text{-HT}}$) estimated from the I–V relationship was – 11 mV, which was close to the reversal potential (0 mV) of a non-selective large cation channel (E_{Na+K} or E_{Na+Cs}) calculated from the Nernst values for an external solution containing 115 mM Na^+ and 2.5 mM K^+ or Cs^+ and an internal solution of 5 mM Na^+ and 125 mM K^+ or Cs^+. The results indicate that the 5-HT-gated channels are almost equally permeable to Na^+, K^+ and Cs^+. In addition, $E_{5\text{-HT}}$ was not affected by changing external Ca^{2+} and Cl^- concentrations (data not shown).

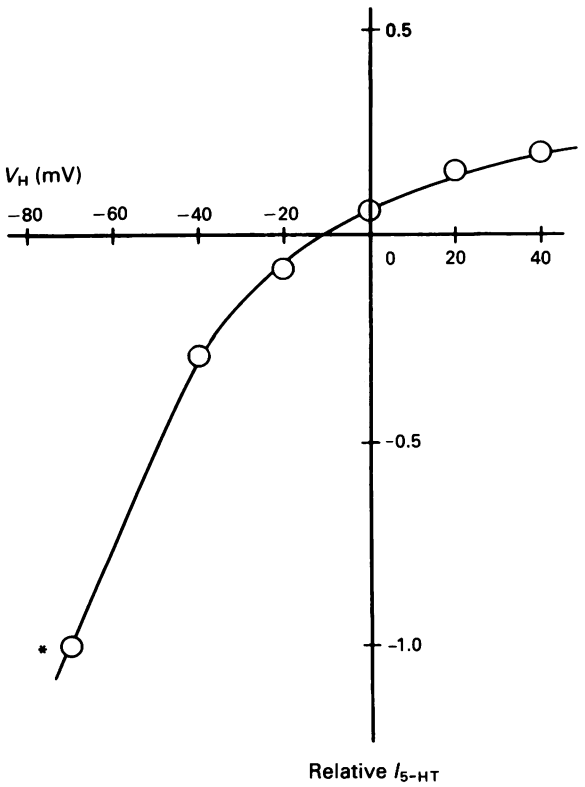

Figure 3 Current-voltage relationship of 5-hydroxytryptamine (5-HT)-induced currents ($I_{5\text{-HT}}$) in a neurone. All responses were normalized to the peak current amplitude (*) evoked by 2×10^{-5} M 5-HT alone at a V_H of – 70 mV. The equilibrium potential for 5-HT ($E_{5\text{-HT}}$) was – 11 mV. Similar results were obtained from another two neurones.

Kinetics of activation and inactivation of $I_{5\text{-HT}}$

The activation and inactivation phases of $I_{5\text{-HT}}$ at a V_H of – 70 mV each consisted of a single exponential. Figure 4 shows the activation and inactivation time constants (τ_a and τ_i) of the currents induced by 5-HT at various concentrations. Both τ_a and τ_i plotted as a function of 5-HT concentration decreased with increasing 5-HT concentration, indicating that the time constants have a definite concentration-dependence. However, τ_a and τ_i of $I_{5\text{-HT}}$ did not show any voltage-dependence (data not shown).

Pharmacological characteristics of $I_{5\text{-HT}}$

To identify the receptor specificity of $I_{5\text{-HT}}$, the effects of 5-HT$_{1A}$, 5-HT$_2$ and 5-HT$_3$ receptor antagonists were examined. The inhibitory effects of the antagonists developed time-dependently. The pretreatment time of the 5-HT$_3$ antagonists for 1 min was enough to produce a steady-state inhibition of the 5-HT$_3$ response because a much longer pretreatment time of 3 min made no difference to the inhibitory action of antagonists. In the following experiments, the preparations were pretreated for 1 min with each antagonist; thereafter simultaneous application of one of antagonists and 5-HT was made. The inward current induced by 2×10^{-5} M 5-HT was inhibited by the selective 5-HT$_3$ receptor antagonist (ICS 205-930) and the non-selective 5-HT$_3$ receptor antagonist (metoclopramide) with half inhibitory concentrations (IC_{50} values) of 4.8×10^{-10} M and 8.6×10^{-9} M, respectively. These inhibitions of $I_{5\text{-HT}}$ by ICS 205-930 and metoclopramide were completely reversible. Y-25130 also reversibly inhibited the $I_{5\text{-HT}}$ in a concentration-dependent manner. The IC_{50} value of Y-25130 was 4.9×10^{-10} M (Figure 5). Neither a 5-HT$_{1A}$ receptor antagonist (spiperone, 10^{-6} M) nor a selective 5-HT$_2$ receptor antagonist (ketanserin, 10^{-6} M) had any inhibitory effects on the $I_{5\text{-HT}}$ in frog DRG neurones. The results indicate that $I_{5\text{-HT}}$ is mediated by 5-HT$_3$ receptor and that Y-25130 is a potent antagonist at this receptor.

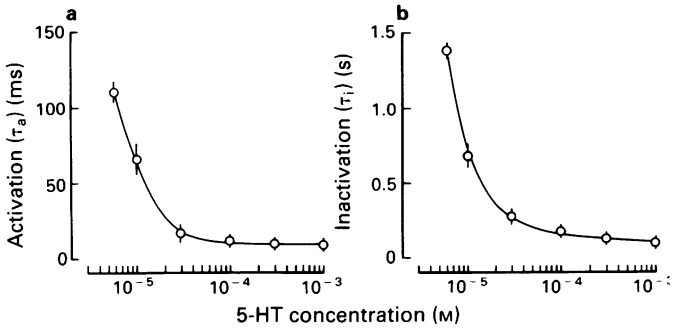

Figure 4 Concentration-dependence of activation and inactivation kinetics of 5-hydroxytryptamine (5-HT)-induced currents ($I_{5\text{-HT}}$) at a V_H of – 70 mV. The time constants of both activation (a) and inactivation (b) decreased with increasing concentration of 5-HT. Each point is the mean from 6 neurones and bars indicate ± s.e.mean.

Figure 6 shows the concentration-response curves for 5-HT with or without ICS 205-930, metoclopramide or Y-25130. The K_a values and Hill coefficients estimated from the 5-HT concentration-response curves were 1.7×10^{-5} M and 1.7 for control, 2.1×10^{-5} M and 1.2 in the presence of 5×10^{-10} M ICS 205-930, 3.8×10^{-5} M and 0.8 in the presence of 10^{-8} M metoclopramide, and 2.8×10^{-5} M and 1.0 in the presence of 5×10^{-10} M Y-25130, respectively. When all responses were normalized to the peak current amplitude induced by 10^{-5} M 5-HT (*), maximum response changed from 3.2 for control to 1.5, 2.2 and 1.8 in the presence of ICS 205-930, metoclopramide and Y-25130, respectively. Blockade of $I_{5\text{-HT}}$ by these compounds was apparently non-competitive, as indicated by the shift to the right of the concentration-response curves with decreasing maximum response.

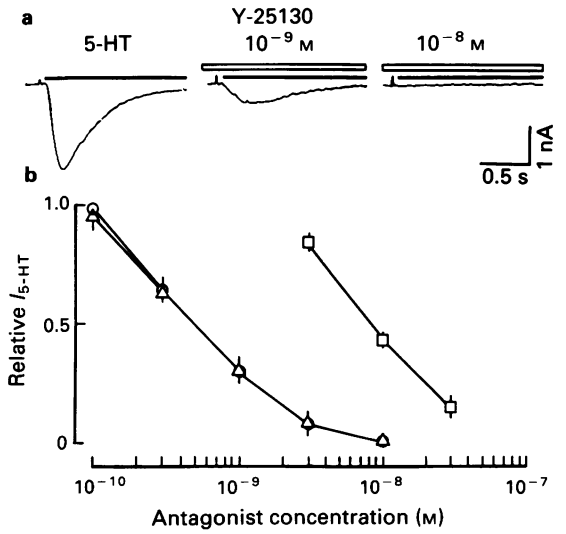

Figure 5 Effects of antagonists on 5-hydroxytryptamine (5-HT)-induced currents ($I_{5\text{-HT}}$) at a V_H of -70 mV. (a), Inhibition of $I_{5\text{-HT}}$ by Y-25130. (b), Dose-dependent inhibition of $I_{5\text{-HT}}$ by ICS 205-930 (Δ), metoclopramide (□) and Y-25130 (○). Each antagonist was applied for 1 min before the simultaneous application of 5-HT (2×10^{-5} M). All responses were normalized to the peak response evoked by 2×10^{-5} M 5-HT. Each point is the mean from 4 neurones and bars indicate ± s.e.mean.

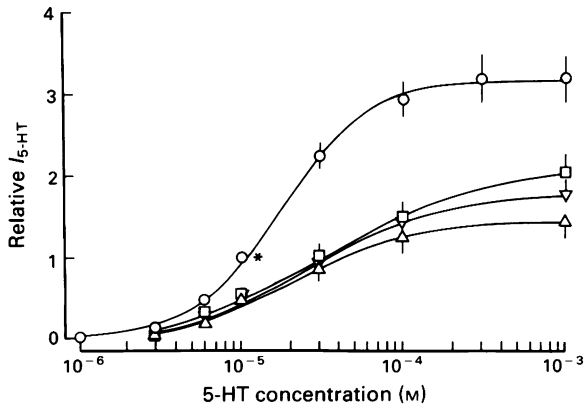

Figure 6 Concentration-response curves for 5-hydroxytryptamine (5-HT) in the presence of 5-HT_3 receptor antagonists. All responses were normalized to the peak response evoked by 10^{-5} M 5-HT (*). Each point is the mean from 4 neurones and bars indicate ± s.e. mean. V_H was -70 mV. Each antagonist was perfused for 1 min before application of 5-HT at various concentrations. Theoretical curves were drawn using K_a (1.7×10^{-5}M) and n (1.6) for control (○), K_a (2.2×10^{-5}M) and n (0.8) for 5×10^{-10}M ICS 205-930 (Δ), K_a (3.8×10^{-5}M) and n (1.2) for 10^{-8} M metoclopramide (□) and K_a (2.8×10^{-5} M) and n (1.0) for 5×10^{-10} M Y-25130 (▽).

Discussion

The results indicate that $I_{5\text{-HT}}$ in frog DRG neurones is mediated by a 5-HT_3 receptor, based on the potent blockade of $I_{5\text{-HT}}$ by ICS 205-930 and metoclopramide and the mimetic action of the 5-HT_3 receptor agonist, 2-methyl-5-HT. Blockade of $I_{5\text{-HT}}$ by Y-25130 (IC_{50} 4.9×10^{-10} M) indicates that this new compound is also a potent antagonist at the 5-HT_3 receptor. In addition, the 5-HT_3 antagonists behaved in our experiments as non-competitive antagonists at the receptor. The possibility that the non-competitive action of the antagonists is open-channel blockade is conceivable. However, as shown in Figure 5a, this can be ruled out because the antagonists did not induce an increase in the rate of desensitization of the 5-HT_3 response. Radioligand binding studies on membranes prepared from NG108-15 cells indicated that the interactions of agonist and antagonist with [^{3}H]-ICS 205-930 recognition sites were competitive in nature, suggesting binding to the same recognition site (Neijt *et al.*, 1988b). Whether these discrepancies between electrophysiological and binding studies reflect subtle variations in the properties of 5-HT_3 receptors between cell types or simply differences in experimental protocol requires further investigation.

The concentration-response curve suggests that the 5-HT_3 receptor of frog DRG neurones has a K_a value of 1.7×10^{-5} M and a Hill coefficient of 1.7. In the present study, inactivation had a time constant of approximately 100 ms. With a drug application time of 2 ms, it is unlikely that there was significant response attenuation caused by desensitization. Therefore, the Hill coefficient of 1.7 for $I_{5\text{-HT}}$ suggests that the 5-HT_3 receptor of frog DRG neurones has at least two binding sites for the agonist. The maximum current evoked by the 5-HT_3 receptor agonist, 2-methyl-5-HT, was approximately 25% of that of 5-HT in the same neurone. The result indicates that 2-methyl-5-HT is a partial agonist at the 5-HT_3 receptor. Similar results were obtained for the 5-HT response in N1E-115 and NG108-15 cells (Yakel & Jackson, 1988; Sepulveda *et al.*, 1991).

As in NCB-20 cells (Lambert *et al.*, 1989), N1E-115 cells (Lambert *et al.*, 1989) and rat DRG neurones (Robertson & Bevan, 1991), the $I-V$ relationship for 5-HT in frog isolated DRG neurones also showed an inward rectification at potentials more positive than -40 mV. The $E_{5\text{-HT}}$ was close to the theoretical value calculated from extra- and intracellular Na^+ and K^+ or Cs^+ concentrations. These results suggest that $I_{5\text{-HT}}$ is generated by an increase in conductance to monovalent cations such as Na^+, K^+ and Cs^+ and that this channel could be classified as relatively non-selective, cation-specific pore. Such a cation selectivity of the 5-HT_3 receptor-channel complex was also reported for NG108-15 cells (Yakel *et al.*, 1990), NCB-20, N1E-115 cells (Lambert *et al.*, 1989) and rat DRG neurones (Robertson & Bevan, 1991).

In the present experiments, the activation and inactivation time constants (τ_a and τ_i) of $I_{5\text{-HT}}$ were single exponentials. The τ_a value is rapid enough to suggest that the 5-HT_3 receptor, unlike other 5-HT receptors, is directly coupled to an ion channel. The kinetics of $I_{5\text{-HT}}$ mediated by the 5-HT_3 receptor in frog DRG neurones were similar to those of responses induced by activation of the nicotinic acetylcholine receptor (cholinoceptor) in frog sympathetic ganglion neurones (Akaike *et al.*, 1989). Similarities include the reversal potential under standard ionic conditions, ion permeabilities, rapid activation and inactivation, sensitivity to curare (Higashi & Nishi, 1982; Akaike *et al.*, 1989; Peters *et al.*, 1990), and modulation of inactivation by forskolin (Seamon *et al.*, 1981; Yakel *et al.*, 1988).These numerous functional similarities between 5-HT_3 and nicotinic cholinoceptors may be explained by molecular biological evidence that the 5-HT_3 receptor gene is structurally related to the nicotinic cholinoceptor. A complementary DNA clone containing the coding sequence of the 5-HT_3 receptor-channel complex has been isolated by screening a neuroblastoma expression lib-

rary for funtional expression of 5-HT-induced currents in *Xenopus* oocytes (Maricq *et al.*, 1991). These studies indicate that the 5-HT$_3$ receptor exhibits sequence similarity to the α subunit of *Torpedo californica* nicotinic cholinoceptor.

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