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Mediation by 5-HT_3 receptors of an excitatory effect of 5-HT on dorsal vagal preganglionic neurones in anaesthetized rats: an ionophoretic study

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1 Extracellular recordings were made from 141 vagal preganglionic neurones in the dorsal vagal nucleus (DVN). The effects of ionophoretic administration of 5-hydroxytryptamine (5-HT), the 5-HT_3 receptor agonist, phenylbiguanide (PBG) and the antagonists, granisetron and tropisetron (ICS 205–930) on these vagal preganglionic neurones were studied in pentobarbitone sodium anaesthetized rats.

2 Ionophoretic application of 5-HT at low currents (< 10 nA) increased the activity in 46 (73%) of 63 neurones tested. Application of granisetron (5–20 nA) or tropisetron (5–20 nA) abolished or attenuated the 5-HT excitatory responses in 8 out of 11 and 5 out of 5 neurones respectively. At the currents used, neither antagonist had any effect on baseline firing rate.

3 Ionophoresis of the selective 5-HT_3 receptor agonist, phenylbiguanide (0–40 nA) excited 54 (82%) of the 66 vagal neurones tested, whilst the remaining 12 neurones were unaffected.

4 Granisetron applied either ionophoretically (8/11) or intravenously (3/3), abolished or attenuated the excitations evoked by PBG. Similarly, tropisetron administered either ionophoretically (2/3) or intravenously (2/2), attenuated the PBG excitation. In contrast, the PBG excitations were unaffected by the 5-HT_2 receptor antagonist, cinanserin (2/2), and the selective 5-HT_{1A} receptor antagonist, WAY-100802 (6/6).

5 In conclusion, excitation of vagal preganglionic neurones evoked by ionophoretic application of 5-HT is mediated in part by 5-HT_3 receptors and activation of 5-HT_3 receptors on and/or in the vicinity of vagal motoneurones causes excitation of these neurones.

Keywords: 5-Hydroxytryptamine; dorsal vagal nucleus; 5-HT_3 receptor; phenylbiguanide

Introduction

The dorsal vagal nucleus (DVN) is a major site of vagal preganglionic motoneurones (Nosaka *et al.*, 1979; 1982; Kalia, 1981; Jones *et al.*, 1995). Immunohistochemical studies have demonstrated that the dorsal vagal nucleus is densely innervated by 5-hydroxytryptamine (5-HT) containing terminals (Steinbusch, 1981; Izzo *et al.*, 1993; Sykes *et al.*, 1994). This innervation comprises fibres originating in the midline raphe nuclei and parapyramidal nucleus (Haxhiu *et al.*, 1993) and from vagal afferents (Sykes *et al.*, 1994). Data from *in vivo* studies indicate that activation of 5-HT_{1A} receptors increases the excitability of cardiac vagal motoneurones (Ramage & Fozard, 1987; Bogle *et al.*, 1990; Sporton *et al.*, 1991; Chitravanshi & Calaresu, 1992; Futuro-Neto *et al.*, 1993; Dando *et al.*, 1994; McCall *et al.*, 1994; Shepheard *et al.*, 1994) and pulmonary vagal preganglionic neurones (Bootle *et al.*, 1996). However, the excitation of preganglionic vagal neurones in the DVN of the rat by ionophoretic application of 5-HT *in vivo* (Wang *et al.*, 1995b) was only partially attenuated by the ionophoretic application of 5-HT_{1A} receptor antagonists. Since autoradiographic binding studies have localized 5-HT_3 binding sites in the dorsal vagal nucleus (see Pratt *et al.*, 1990; Steward *et al.*, 1993; Leslie *et al.*, 1994), the aim of the present experiments was to test whether the excitatory effect of 5-HT on DVN neurones was mediated in part by the activation of 5-HT_3 receptors. Consequently, 5-HT and a selective 5-HT_3 receptor agonist, phenylbiguanide (PBG), were applied by ionophoresis onto antidromically identified dorsal vagal preganglionic neurones. The influence of the following selective 5-HT receptor antagonists on the responses evoked by PBG was also examined: (i) the 5-HT_3 receptor antagonists, tropisetron (ICS 205–930; Richardson *et al.*, 1985) and granisetron (Sanger & Nelson, 1989); (ii) the 5-HT_2 receptor antagonist, cinanserin (Rubin *et al.*, 1964; Hoyer & Fozard, 1991) and (iii) the 5-HT_{1A} receptor antagonist, WAY-100802 (Ramage & Mirtsou-Fidani, 1995). A preliminary account of some of these data has been published (Wang *et al.*, 1996).

Methods

Experiments were performed on 53 male Sprague-Dawley rats (330–460 g body weight) anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.). Depth of anaesthesia was assessed by monitoring the stability of the arterial blood pressure and heart rate and the cardiovascular responses to pinching the paws. Anaesthesia was supplemented when necessary. A tracheotomy was performed low in the neck and a femoral artery and vein cannulated for measurement of blood pressure and administration of supplemental anaesthetics and drugs. Tracheal and arterial blood pressures were measured with pressure transducers (Statham P23Db). Animals were ventilated with oxygen-enriched room air using a positive pressure ventilator (Harvard rodent ventilator, model 683) with 1 cmH_2O positive end-expiratory pressure. End-tidal CO_2 was continuously measured with a fast response CO_2 meter (ADC Ltd., Model FM1) and maintained at 4%. Arterial blood samples were regularly taken and blood gases and pH monitored with a Corning pH/blood gas analyser (Model 238). Blood gases were maintained in the following ranges: P_{O_2} 90–130 mmHg, P_{CO_2} 40–50 mmHg and pH 7.3–7.4 by slow i.v. infusions of sodium bicarbonate (1.0 M) or adjustments of the respiratory pump.

Animals were fixed in a stereotaxic frame and the right vagus nerve dissected free from the sympathetic trunk and

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placed on bipolar silver electrodes for electrical stimulation (1 Hz, 2–5 V, 1.0 ms) with a digital programmer (Master 8, AMPI) and isolated stimulator (Digitimer DS2). To expose the caudal brainstem in the region of the DVN the nuchal muscles were removed from the back of the neck, the occipital bone opened and the dura overlying the brainstem cut and reflected laterally. To access the DVN, in some experiments it was necessary to retract the cerebellum rostrally.

Protocol

Extracellular recordings were made from brainstem neurones with 5- or 7-barrelled microelectrodes (tip diameter 3–5 μm) made from borosilicate glass (Clarke Electromedical, GC 150F–10). The recording barrel contained 4 M sodium chloride, and the other barrels contained Pontamine sky blue dye, DL-homocysteic acid (DLH), 5-HT, and a selection of 5-HT

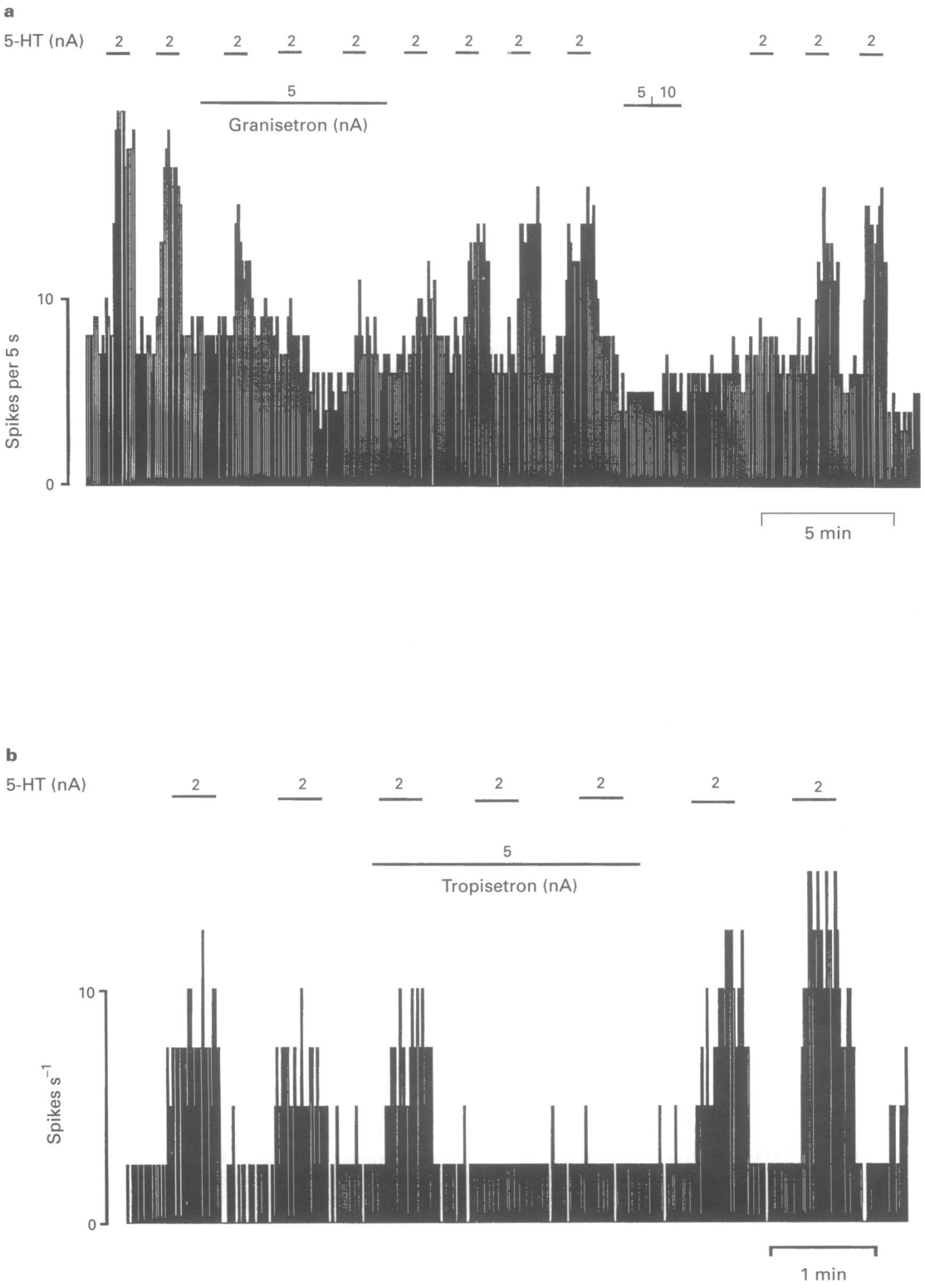

Figure 1 Effects of granisetron and tropisetron on the excitatory effects of 5-HT applied ionophoretically to vagal preganglionic neurones with the stated ejection currents during the time shown by the bars. (a) Shows a continuous ratemeter record during pulse application of 5-HT before, during and following ionophoretic application of granisetron. (b) Shows a continuous ratemeter record during pulse application of 5-HT before, during and following ionophoretic application of tropisetron.

receptor agonists and antagonists. Between the drug ejection periods, a retaining current of 10–20 nA was applied to each drug barrel. Neuronal recordings were amplified × 5000 (Dagan 2400) and filtered (0.1–3 kHz). Vagal preganglionic neurones in the DVN were identified by standard criteria of antidromic activation, including constant latency of the responses to electrical stimulation of the cervical vagus nerve and collision of the antidromically-evoked response with appropriately timed ongoing activity (Wang *et al.*, 1995b). The characteristics of the ongoing activity of these neurones, their synaptic inputs and location have been described previously (Nosaka *et al.*, 1982; Jones *et al.*, 1995; Wang *et al.*, 1995b).

Drugs were applied in the vicinity of identified neurones by ionophoresis (Neurophore, Medical Systems). Neurones with no ongoing activity were induced to fire by application of low currents (0–20 nA) of the excitant amino acid DLH. When the neuronal firing rate was steady, the effects of agonist and/or antagonist drugs given alone and/or together were then tested. In all experiments, possible current artefacts were overcome using the automatic current balancing available on the Neurophore. In some experiments, the possibility of current and/or pH artefacts were tested directly by passing current through saline of the same pH as the ejected drug. A significant artefact was never seen with this test.

In 16 experiments recording sites were marked by ionophoretic deposition of Pontamine sky blue dye. At the end of these experiments the brain was removed and fixed in 10% formal saline. Frozen sections (80 μm) were cut, the marked sites visualized and mapped onto standard sections of the brain (Paxinos & Watson, 1986).

Analysis of data

Arterial blood pressure, tracheal pressure and neuronal activity were recorded on video tape via a digital interface (Instrutech, VR-100B). Off-line analysis of the recorded data was made with commercially available software (CED Spike 2 and Signal Averager) on a computer (Viglen 486 DX2 66) accessed via an A–D interface (CED 1401plus). Single unit activity was discriminated on a window discriminator (Digitimer D130) and displayed as a rate histogram. Drugs were classed as evoking excitation or inhibition if, during the ejection period, activity was increased or decreased by at least 20% of baseline.

Drugs and solutions

The following drugs were freshly dissolved in 1 mM saline and their pH adjusted by addition of drops of either 0.1 M HCl or 0.1 M NaOH:- 5-hydroxytryptamine maleate (5-HT; 50 mM, pH 5), phenylbiguanide (PBG, 10 mM, pH 10.6), 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 20 mM, pH 4), tropisetron (10 mM, pH 4), cinanserin (50 mM, pH 4), all from Research Biochemicals., Semat Technical Ltd, St. Albans, U.K., granisetron (10 mM, pH 4; a gift from SB, Harlow, U.K.); R-2,3,4,5,6,7-hexahydro-1(4-(1-(4-(2-methoxyphenyl)-piperazinyl))-2-pyridyl)butanoyl-1H-azepine HCl (WAY-100802; 10 mM, pH 4; a gift from Wyeth Research, Maidenhead, U.K.). Pontamine sky blue dye (20 mg ml^{-1}; BDH, Poole) was dissolved in 0.5 M sodium acetate and DL-homocysteic acid (DLH, 100 mM, pH 8.5; Sigma, Poole, UK.) in distilled water.

Results

A total of 141 antidromically identified dorsal vagal preganglionic neurones were recorded and their calculated axonal conduction velocities were all in the C-fibre range as previously reported (Wang *et al.*, 1995b). The Pontamine sky blue marked recording sites in 16 animals were all successfully recovered histologically and found to be located within the area of the dorsal vagal nucleus.

Effects of 5-HT$_3$ receptor antagonists on 5-HT excitation of vagal preganglionic neurones

Ionophoretic application of 5-HT at low ejection currents (0–10 nA) predominantly evoked excitatory responses, increasing firing rate in 46 out of 63 dorsal vagal preganglionic neurones (Table 1A) in agreement with a previous report (Wang *et al.*, 1995b). Ionophoretic application of granisetron (5–20 nA) abolished the 5-HT-evoked excitation in 6 out of the 11 neurones tested and attenuated this excitation in another 2 neurones (Figure 1a); it failed to have any effect on the remaining 3 (Table 1B). Similarly, tropisetron (5–20 nA) abolished the 5-HT-evoked excitations in 4 neurones and attenuated the excitation in the other neurone tested (Figures 1b, 4b and Table 1B). At the currents used to attenuate or abolish this 5-HT evoked excitation, neither granisetron (Figure 1a) nor tropisetron (Figure 1b) significantly changed the baseline firing rate of these neurones.

Effects of the 5-HT$_3$ receptor agonist, phenylbiguanide, on dorsal vagal preganglionic neurones

Ionophoretic application of phenylbiguanide (PBG, 0–40 nA) excited 54 out of 66 neurones tested (Table 1A). This excitatory response was current-dependent, neural firing rate increasing when the ejection current was raised (Figure 2a). The response to PBG was rapid, the firing rate of the neurones quickly increasing when PBG was applied, and following removal of the ejection current the firing rate gradually returned to baseline. The other 12 neurones tested with PBG did not respond. In 5 neurones, when a submaximal PBG ejection current was prolonged for up to 5 min, the excitation of the tested neurones was maintained. There was no indication of desensitization.

Effect of 5-HT receptor antagonists on the excitatory effect of PBG

In the 11 neurones tested, PBG excitations were abolished ($n=5$) or attenuated ($n=3$) by ionophoretic application of granisetron (5–20 nA; Figures 2b, 4a, Table 1B). Tropisetron (5–20 nA) similarly attenuated the PBG-evoked excitation in

Table 1A Effects of 5-HT and the 5-HT$_3$ receptor agonist PBG on the dorsal vagal preganglionic neurones

	Excitation	*Inhibition*	*No effect*
5-HT (0–10nA)	46/63 (73%)	0/63	17/63 (27%)
PBG (0–40nA)	54/66 (82%)	0/66	12/66 (18%)

B Effects of ionophoretic application of granisetron and tropisetron on the excitations produced by 5-HT and PBG

	Granisetron			*Tropisetron*		
	Abolition	*Attenuation*	*No interaction*	*Abolition*	*Attenuation*	*No interaction*
5-HT	6/11	2/11	3/11	4/5	1/5	0/5
PBG	5/11	3/11	3/11	0/3	2/3	1/3

2 of the 3 dorsal vagal preganglionic neurones tested (Table 1B). In addition intravenous administration of a single dose of granisetron (0.8–1.5 mg kg^{-1}, $n=3$) or tropisetron (0.8–1.2 mg kg^{-1}, $n=2$) effectively blocked the excitation evoked by ionophoretic application of PBG (Figure 3).

In contrast, PBG excitations were unaffected by cinanserin (5–20 nA) in 2 of 2 neurones (Figure 2b) or WAY-100802 (5–20 nA) in all 6 neurones tested (Figure 3a). However, at the currents used (5–20 nA) WAY-100802 did attenuate the excitatory actions of 8-OH-DPAT (10–30 nA) in 2 vagal neurones tested (data not shown).

Effect of granisetron and tropisetron on the DLH-evoked excitation of DVN neurones

The DLH-evoked excitation was unaffected ($n=6$), potentiated ($n=2$) or inhibited ($n=2$) by granisetron in 10 dorsal vagal preganglionic neurones tested. In 8 of these 10 neurones (excluding the 2 in which granisetron inhibited the excitatory effect of DLH), granisetron attenuated either the PBG excitation ($n=5$) or the 5-HT excitation ($n=3$) but not the excitation evoked by the excitant amino acid, DLH (5–15 nA; Figure 4a). Similarly, tropisetron had no effect on the DLH-evoked excitation in 2 out of 3 neurones, but attenuated the 5-HT excitation in these 2 neurones (Figure 4b).

Discussion

Ionophoresis of the selective 5-HT$_3$ receptor antagonists, granisetron (Sanger & Nelson, 1989) or tropisetron (Richardson *et al.*, 1985) produced a significant blockade of the excitatory effects of both 5-HT and PBG on identified vagal motoneurones in the dorsal vagal nucleus (DVN). The failure of both 5-HT$_2$ and 5-HT$_{1A}$ receptor antagonists to modify this excitatory action of PBG at the same current ranges which

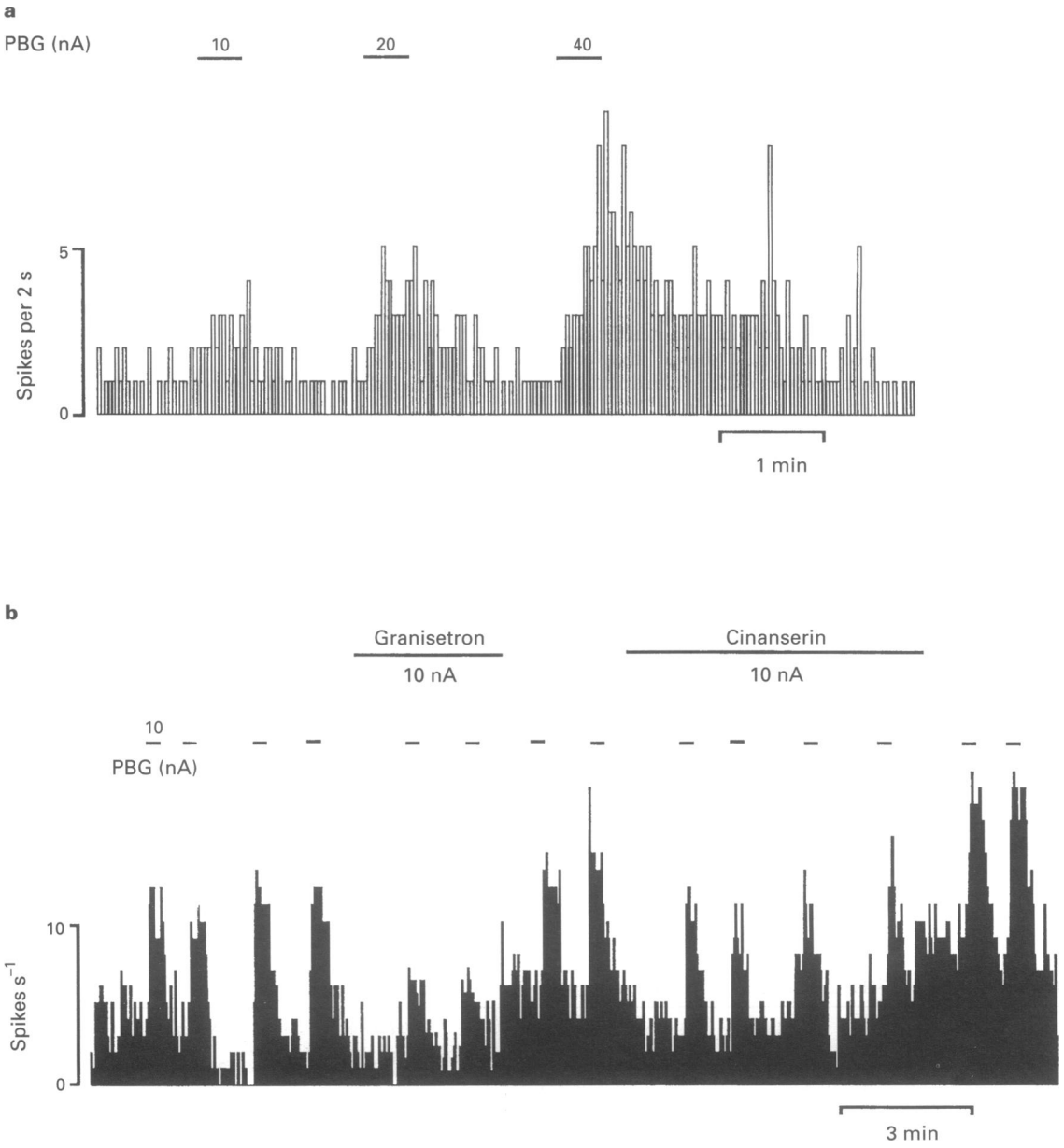

Figure 2 Ratemeter records of vagal preganglionic neurone activity during application of the 5-HT$_3$ receptor agonist, PBG with the stated ionophoretic ejection currents at the time shown by the bars. (a) A continuous ratemeter record showing the increase of activity produced during application of PBG. Note the current-dependent excitation of this neurone to PBG application. (b) A continuous ratemeter record showing that the excitation produced by application of PBG was attenuated by ionophoresis of granisetron but not by cinanserin (note that despite cinanserin reducing the baseline firing rate, the excitation evoked by PBG during cinanserin was similar to the control response).

inhibit 5-HT-evoked excitation (Wang *et al.*, 1995a) and block the excitatory effects of 8-OH-DPAT (present experiments), supports the view that PBG is activating selectively 5-HT$_3$ receptors to cause excitation of these neurones. It is unlikely that granisetron and tropisetron produced their suppression of neuronal firing by a local anaesthetic action since they antagonized the effects of 5-HT and PBG at low ejection currents (5–20 nA), whereas local anaesthetic action has been observed only at higher currents (>30 nA) (Ashby *et al.*, 1991). In addition, they reduced the PBG and 5-HT-evoked excitations without altering those evoked by DLH. Overall, these combined observations demonstrate for the first time *in vivo* that 5-HT$_3$ receptor antagonists attenuate, and that a 5-HT$_3$ receptor agonist mimics, the excitatory effect of 5-HT on identified vagal motoneurones in the dorsal vagal nucleus (DVN) by a selective action on 5-HT$_3$ receptors.

The ability of the 5-HT$_3$ receptor antagonists to block 5-HT-induced excitation was found to be complete in the majority of the neurones tested. However, in some neurones the excitations were unaffected or only attenuated. Although this partial blockade could be the result of insufficient drug delivery by ionophoresis, this is unlikely since in the same animal using the same electrode and same ejecting current and period, granisetron abolished 5-HT-evoked excitations in one neurone but only slightly attenuated the excitation in another. This suggests that although 5-HT$_3$ receptors certainly can play a major role in mediating the excitatory action of 5-HT, the effects of 5-HT on DVN neurones may not be explained exclusively by an action on 5-HT$_3$ receptors. Indeed, it has been previously demonstrated that 5-HT$_{1A}$ (Wang *et al.*, 1995b) and

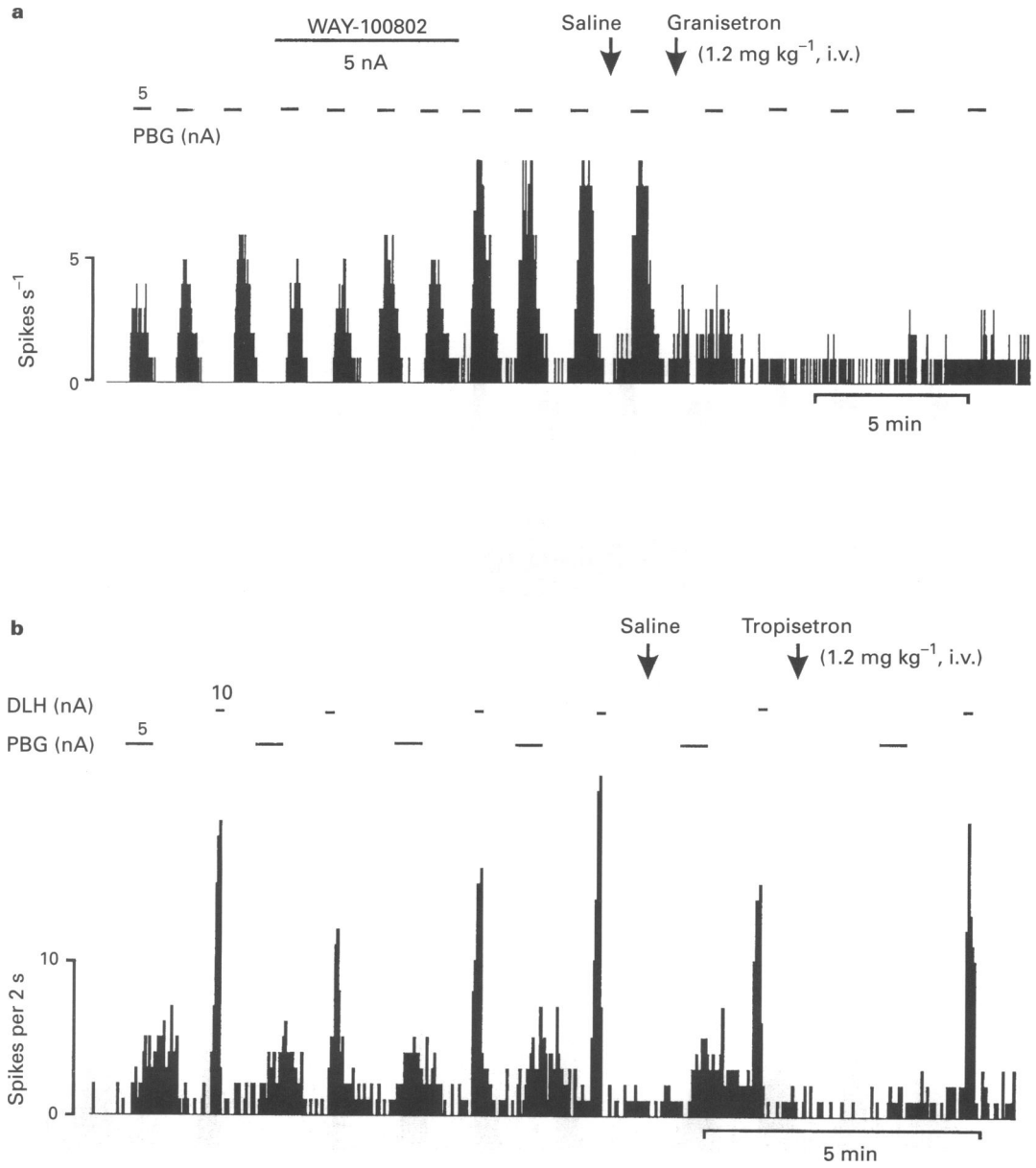

Figure 3 Effects of intravenous granisetron and tropisetron and ionophoretic WAY-100802 on the effects of PBG and DLH applied ionophoretically to vagal preganglionic neurones with the stated ejection currents at the time shown by the bars. (a) A continuous ratemeter record showing the increase of activity produced by pulse application of PBG before, during and following ionophoretic application of WAY-100802 and following single intravenous injections of granisetron and a similar volume of saline. The 'off-excitation' seen in this neurone is often observed following application of 5-HT$_{1A}$ antagonists and is not due to interactions with the PBG, the excitation being unchanged during the application of the antagonist. (b) A continuous ratemeter record showing the increase of activity produced by pulse application of PBG and DLH before and following single intravenous injections of tropisetron and a similar volume of saline.

5-HT$_2$ receptor antagonists (Wang *et al.*, 1995a) can also attenuate the excitatory action of 5-HT on DVN neurones, and autoradiographic studies have demonstrated 5-HT$_{1A}$, 5-HT$_2$ and 5-HT$_3$ binding sites in the dorsal vagal nucleus (Dashwood *et al.*, 1988; Manaker & Verderame, 1990; Pratt, *et al.*, 1990; Thor *et al.*, 1992; Steward *et al.*, 1993; Leslie *et al.*, 1994).

The reasons why vagal preganglionic neurones receive excitation through three 5-HT receptor subtypes is unclear. One possibility is that the type(s) of 5-HT receptors mediating the excitation depends on the function of the particular neurone i.e. in cardiac or gastrointestinal control. In this respect, microinjection of 5-HT into the rat DVN activates 5-HT$_{1A}$ receptors to cause cardiac slowing (Sporton *et al.*, 1991) and 5-HT$_2$ receptors to evoke increases in gastric motility and acid secretion (Yoneda & Tache, 1995). Similarly, in cats, IVth ventricular application of 8-OH-DPAT increased the excitability of cardiac but not other central parasympathetic motoneurones (Shepheard *et al.*, 1994). However, the effects of selective 5-HT receptor agonists and antagonists on functionally-identified preganglionic neurones has not yet been studied.

In vitro studies of visually identified neurones in the DVN of the neonatal rat have also demonstrated that 5-HT excitation can be attenuated by 5-HT$_3$ receptor antagonists (Albert & Brooks, 1995) and mimicked by application of the selective agonist 2-methyl-5-HT (Travagli *et al.*, 1992; Albert *et al.*, 1995). In contrast to the present data, however, Albert & Brooks (1995) suggest that 5-HT$_3$ receptor excitation of DVN neurones comprises only a small component relative to 5-HT$_2$ receptor-mediated excitation. This may reflect a developmental difference between adult animals (the present studies) and neonatal rats (from which slices were obtained). Alternatively, it may reflect differences in the neuronal location of different receptor subtypes, since ligands applied ionophoretically *in vivo* may access a different population of receptors to those applied by superfusion *in vitro*. Data from other *in vivo* studies

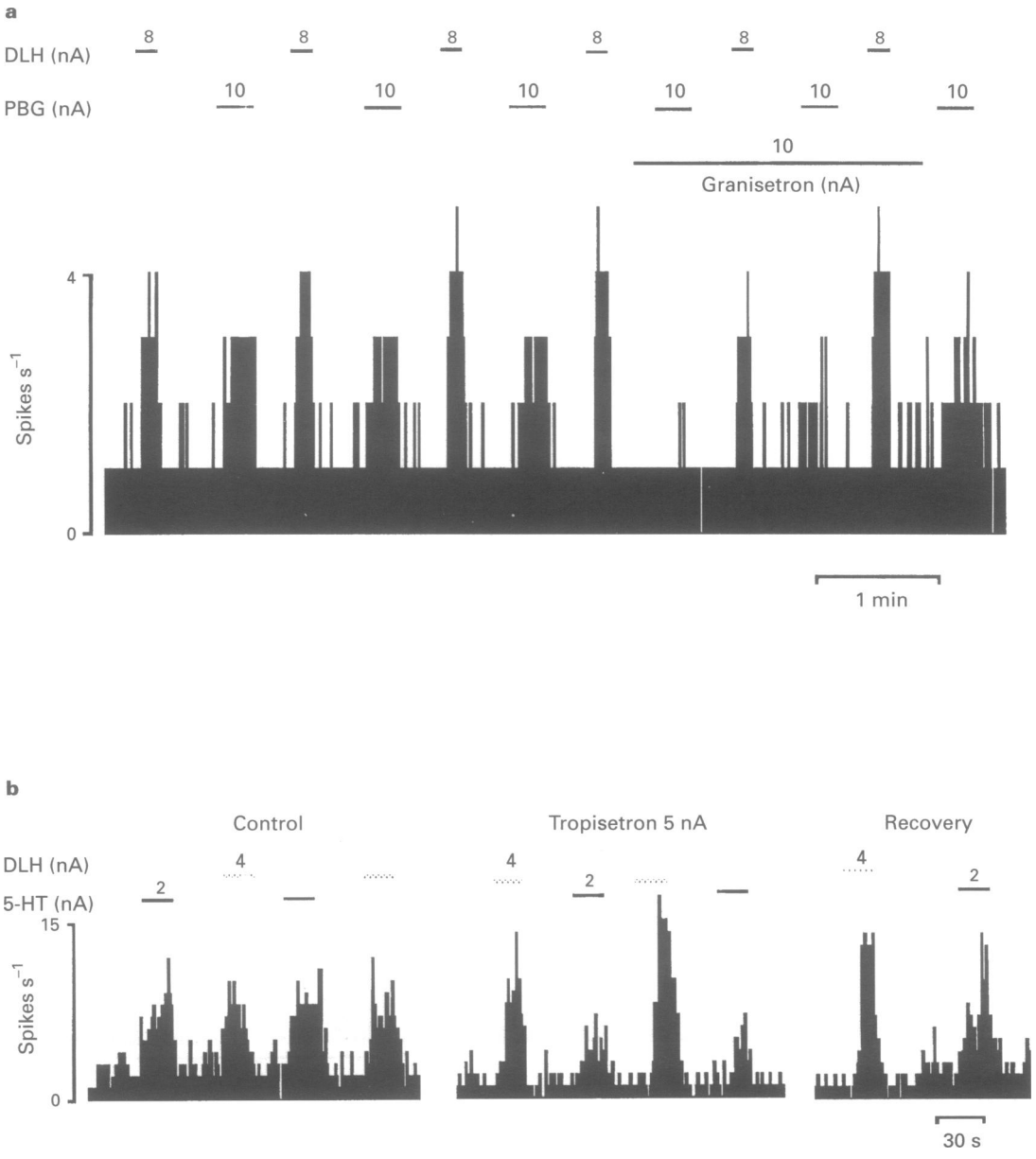

Figure 4 (a) Effects of granisetron on the excitatory effects of PBG and DLH applied ionophoretically to a vagal preganglionic neurone with the stated ejection currents at the time shown by the bars. The trace shows a continuous ratemeter record during pulse application of PBG and DLH before, during and following ionophoretic application of granisetron. (b) Effects of tropisetron on the excitatory effects of 5-HT and DLH applied ionophoretically to a vagal preganglionic neurone with the stated ejection currents at the time shown by the bars. The traces show ratemeter records during pulse application of PBG and DLH before (control), during (tropisetron) and following (recovery) ionophoretic application of tropisetron.

also support a functional role for 5-HT$_3$ receptor-mediated excitation of vagal motoneurones. Administration of the 5-HT$_3$ receptor antagonist, granisetron (i.c.) effectively attenuated the vagal bradycardias evoked by stimulation of the upper airway with smoke in rabbits (Dando *et al.*, 1995) or by stimulation of cardio-pulmonary C-fibre afferents in rats (Pires *et al.*, 1995).

Electrophysiological studies indicate the existence of both pre- and postsynaptic central 5-HT$_3$ receptors regulating neurotransmitter release and mediating rapid ionotropic neurotransmission, respectively (see Peters *et al.*, 1992). The 5-HT$_3$ receptor agonist, 2-methyl-5-HT, produced depolarization and/or increased spontaneous postsynaptic potentials when applied to neurones in the nucleus tractus solitarius (Glaum *et al.*, 1992) or the dorsal vagal nucleus (Albert *et al.*, 1995) *in vitro*, indicating that responses may be mediated by either pre- or postsynaptically-located 5-HT$_3$ receptors. In the present experiments, the fast onset time and size of response observed with both 5-HT and PBG might suggest a postsynaptic action and would be consistent with 5-HT$_3$ receptors opening a ligand-gated channel (see Peters *et al.*, 1992; Jackson & Yakel, 1995). However, in the rat medial prefrontal cortex, the only other area in which 5-HT$_3$ receptors have been studied *in vivo* using ionophoresis, activation of these receptors caused inhibition of neuronal activity (Ashby *et al.*, 1991) via a metabotropic mechanism (Ashby *et al.*, 1994). The present *in vivo* excitation mediated by 5-HT$_3$ receptors requires further investigation with intracellular techniques to determine whether this is due to activation of receptors located pre- and/or postsynaptically.

In conclusion, we have shown that 5-HT$_3$ receptor agonists applied ionophoretically produce an excitatory effect on identified dorsal vagal motoneurones. 5-HT$_3$ antagonists could selectively attenuate these responses and the excitation evoked by 5-HT itself. These results support the view that 5-HT$_3$ receptors in the dorsal vagal nucleus play an important role in mediating an excitatory action of 5-HT.

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