Further characterization of the putative 5-HT receptor which mediates blockade of neurogenic plasma extravasation in rat dura mater

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1 We describe the effects of pretreatment with 5-hydroxytryptamine (5-HT) receptor agonists and antagonists on neurogenically-mediated plasma protein extravasation ($[^{125}\text{I}]$ -albumin) in rat dura mater and in extracranial tissues (temporalis muscle fascia, conjunctiva, eyelid and lip) induced by electrical stimulation of the right trigeminal ganglion.

2 Leakage of $[^{125}\text{I}]$ -bovine serum albumin from blood vessels in dura mater following high intensity stimulation (1.2 mA, 5 ms, 5 Hz for 5 min) was significantly reduced by the intravenous administration of drugs active at 5-HT receptors with some selectivity for the 5-HT₁ receptor subtypes: 5-carboxamidotryptamine (5-CT) (threshold dose, 1 ng kg⁻¹); 5-benzyloxytryptamine (5-BT) (10, 30 or 100 µg kg⁻¹); 8-hydroxydipropylaminotetralin (8-OH-DPAT) (300 µg kg⁻¹); and as previously reported, sumatriptan (100 µg kg⁻¹), dihydroergotamine (DHE) (50 µg kg⁻¹), ergotamine tartrate (100 µg kg⁻¹) and chronically administered methysergide (1 mg kg⁻¹).

3 The putative 5-HT receptor antagonist, metergoline 100 µg kg⁻¹, inhibited partially the effect of sumatriptan in dura mater providing additional evidence for a 5-HT₁ receptor subtype-mediated mechanism, although it was not effective against 5-CT (1 ng kg⁻¹). Methiothepin (300 µg kg⁻¹) did not affect the response to sumatriptan. When administered at high concentrations (1 mg kg⁻¹) methiothepin and metergoline decreased plasma protein extravasation in rat dura mater.

4 Pretreatment with the 5-HT₂ receptor antagonists pizotifen, 300 µg kg⁻¹, or ketanserin, 300 µg kg⁻¹, or the 5-HT₃ receptor antagonists MDL 72222, 300 µg kg⁻¹, or ICS 205-930, 300 µg kg⁻¹, did not affect plasma protein leakage following electrical trigeminal stimulation. Blockade by sumatriptan of plasma protein extravasation was not inhibited by pizotifen (300 µg kg⁻¹) or MDL 72222 (300 µg kg⁻¹).

5 The 5-HT receptor(s) mediating this response were present only on intracranial tissues innervated by the trigeminal nerve; plasma protein extravasation in extracranial tissues was not blocked by pretreatment with the equivalent or higher concentrations of the above drugs following low intensity trigeminal stimulation (0.1 mA, 5 ms, 5 Hz).

6 The putative 5-HT receptor(s) mediating this response were not present on sympathetic fibres innervating dura mater since unilateral removal of the superior cervical ganglion did not prevent the development of plasma protein extravasation nor did it affect the blockade by sumatriptan 100 µg kg⁻¹.

7 The above pharmacological data suggest that intracranial vessels possess 5-HT receptor(s) which are coupled to inhibition of neurogenically-mediated plasma protein extravasation. These receptors cannot be detected on extracranial cephalic blood vessels innervated by the trigeminal nerve, although available evidence strongly suggests that the 5-HT receptors reside on perivascular trigeminal nerve fibres. The rank order of effective doses (threshold concentrations; 5-CT < 5-BT < DHE < sumatriptan < 8-OHDPAT) is most consistent with a 5-HT_{1B}- or 5-HT_{1D}-mediated response, among the known 5-HT₁ family of receptors. However, the lack of effect of methiothepin against the actions of sumatriptan, or metergoline against the effects of 5-CT suggest important differences and the possibility that a previously unrecognized 5-HT receptor(s) is involved in this response.

Keywords: Neurogenic inflammation; 5-HT receptors; sumatriptan; dihydroergotamine; dura mater

Introduction

Neurogenic inflammation (vasodilatation and plasma protein extravasation) may be important to the pathophysiology of migraine headaches and to the action of antimigraine drugs (Chapman *et al.*, 1961; Dalessio, 1974; Moskowitz *et al.*, 1979; Fozard, 1982; Hardebo, 1984; Moskowitz, 1984; Saito *et al.*, 1988). Neurogenic plasma extravasation develops in dura mater and extracranial cephalic tissues following electrical trigeminal stimulation or systemic capsaicin administration and can be detected by leakage of albumin tracer (Markowitz *et al.*, 1987). Extravasation appears to be mediated in part by released neuropeptides (e.g., substance P, neurokinin A, and

calcitonin gene-related peptide) from perivascular sensory fibres (Saria *et al.*, 1985). In the dura mater there is ultrastructural evidence for mast cell degranulation, platelet accumulation, and increase in the number of endothelial vesicles specifically in postcapillary venules following electrical stimulation (Buzzi *et al.*, 1990; Dimitriadou *et al.*, 1990). Protein leakage does not develop in the dura mater after pretreatment with clinically relevant doses of the antimigraine drugs sumatriptan, and the ergot alkaloids ergotamine tartrate, dihydroergotamine and chronic methysergide (Saito *et al.*, 1988; Buzzi & Moskowitz, 1990). Furthermore, the ultrastructural changes are blocked by sumatriptan and dihydroergotamine (Buzzi *et al.*, 1990).

The pharmacological data suggest that a 5-HT receptor subtype mediates this response. To define more clearly the

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5-HT receptor which mediates the blockade of neurogenic plasma extravasation, we examined extravasation in the dura mater in the presence of drugs active at a variety of 5-HT subtypes. The possibility that the 5-HT receptor mediating this response resides on sympathetic fibres innervating blood vessels of the dura mater was also examined.

Methods

Electrical trigeminal stimulation and tissue dissection

Male Sprague-Dawley rats (150–200 g, Charles River Laboratories, Wilmington, Massachusetts, U.S.A.) were housed under diurnal lighting conditions and allowed food and water *ad libitum*. Anaesthetized rats (pentobarbitone, 60 mg kg⁻¹, i.p.) were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) and the skull was exposed by a midline incision. Symmetrical burr holes were drilled 2.5–2.8 mm laterally and 3.2 mm posteriorly to bregma for electrode placement. [¹²⁵I]-bovine serum albumin 70 µCi kg⁻¹ was then injected into the left femoral vein as a bolus. No untoward effects of this protein were noted on blood pressure or body temperature in selected animals. After 5 min stainless steel bipolar electrodes, 5 mm shaft (Rhodes medical Instruments, Woodland Hills, CA) were lowered into the trigeminal ganglia to a depth of 9.2 mm from the dura mater. The incisor bar was set at –2.5 mm from the horizontal. To examine the extravasation response in dura mater, the right trigeminal ganglion was stimulated for either 5 min (1.2 mA, 5 ms duration, 5 Hz), or when indicated, for 3 min using identical parameters. To examine the response in facial tissues, the right trigeminal ganglion was stimulated for 1 or 2 min (0.1 mA, 5 ms, 5 Hz) (Microprocessor Stimulator Microstim III, Linear Iso III, World Precision Instruments, San Carlos, CA, U.S.A.; Hitachi Densi Oscilloscope, Tokyo). After the stimulation period, the animals were promptly perfused with saline via the left cardiac ventricle for 2 min at a constant pressure of 100 mmHg (approximately 100 ml) in order to remove completely iodinated albumin from blood vessels. The anaesthetized animals died during this procedure. The skull was then opened, the brain removed and the cranial cavity thoroughly rinsed with saline. Dura mater was dissected bilaterally as previously described (Markowitz *et al.*, 1987). The large dural sinuses, the region of dura through which the electrodes penetrated, and the dura covering the trigeminal ganglion were excluded from analysis. Fascia covering the temporalis muscle (approximately 3.5 mg wet weight), conjunctiva, eyelid and lip were dissected on both sides and levels of radioactivity determined (Micromedex Systems Inc., Huntsville, AL, U.S.A.).

Superior cervical ganglionectomy

In anaesthetized (pentobarbitone, 60 mg kg⁻¹) Sprague-Dawley rats (100–125 g) the cervical region was opened with an anterior midline incision. The underlying muscles were separated in order to expose the common carotid artery. The artery on the right side only was isolated with a 3-0 silk surgical suture and gently lifted aside at its bifurcation to visualize the ipsilateral superior cervical ganglion. Once the ganglion was exposed, it was totally removed with forceps. In a separate group of animals the common carotid artery was exposed and lifted as described above, and the superior cervical ganglion visualized but not removed (sham). Animals were allowed to recover from anaesthesia under a warming lamp, and then returned to the animal facilities where they were kept for twelve days before electrical stimulation of the right trigeminal ganglion was performed. Ptosis on the ipsilateral side of the above surgical procedure confirmed the success of the surgery.

Protocol

Drugs were administered i.v. 10 min before electrical trigeminal stimulation and 5 min before [¹²⁵I]-albumin to test their ability to block the extravasation response (i.e., to behave as agonists): sumatriptan 100 or 300 µg kg⁻¹; 8-OH-DPAT 100 or 300 µg kg⁻¹; 5-CT 0.1, 1, 10, 30, 100 and 300 ng kg⁻¹; metergoline 100, or 500 µg kg⁻¹ or 1 mg kg⁻¹; methiothepin 0.3, 1 mg kg⁻¹; 5-BT 10, 30, 100 or 300 µg kg⁻¹; pizotifen 300 µg kg⁻¹; ketanserin 300 µg kg⁻¹; ICS 205-930 300 µg kg⁻¹; MDL 72222 300 µg kg⁻¹.

Some compounds were also tested as antagonists of sumatriptan. Metergoline 100 µg kg⁻¹, 5-BT 30 µg kg⁻¹, methiothepin 300 µg kg⁻¹, pizotifen 300 µg kg⁻¹, or MDL 72222 300 µg kg⁻¹ were administered 20 min before electrical trigeminal stimulation and 10 min before sumatriptan (100 µg kg⁻¹). Metergoline (100 or 300 µg kg⁻¹) was also given 10 min before 5-CT (1 or 300 ng kg⁻¹). Animals were perfused immediately after stimulation and treated as described above.

Twelve days after the right superior cervical ganglionectomy or sham surgery, operated animals were subjected to electrical stimulation of the right trigeminal ganglion as described above. Sumatriptan 100 µg kg⁻¹ or vehicle was administered 10 min before electrical stimulation.

Intra-arterial blood pressure recording

A 22 gauge catheter was placed in the left femoral artery, and mean arterial blood pressure (MABP) recorded (Winston Electronics Co., Millbrae, CA) in selected animals, starting 5 min before drug or vehicle administration and continuing until the end of the electrical trigeminal stimulation.

Data analysis

Three to five animals were studied in each group and each experiment was repeated at least once on separate days. Plasma [¹²⁵I]-bovine serum albumin extravasation is expressed as the ratio: c.p.m. mg⁻¹ wet wt. (stimulated side)/c.p.m. mg⁻¹ wet wt. (unstimulated side). Results are shown as mean ± s.e.mean. Student's *t* test was used for statistical analysis between drug- and vehicle-treated groups. Blood pressure data were analyzed by ANOVA. Probability values (*P*) of less than 0.05 were considered significant.

Drugs

The following chemicals and drugs were used: [¹²⁵I]-bovine serum albumin (New England Nuclear, Boston, MA), capsaicin (Polyscience Inc., Wilmington, Pennsylvania), 5-HT (Sigma Chemical Co., St Louis, Missouri), sumatriptan [3-(2-dimethylaminoethoxy)-1H-indole-5-(N-methyl)methane sulphamide], 5-carboxamidotryptamine (5-CT) (Glaxo, Ware, Hertfordshire, United Kingdom), 8-hydroxydipropylaminotetralin (8-OH-DPAT) (Research Biochemical Inc., Natick, Massachusetts), 5-benzoyloxytryptamine (5-BT) (Aldrich), methiothepin maleate (Roche), metergoline (Farmitalia), ketanserin (Janssen), pizotifen, ICS 205-930 (Sandoz), 1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate hydrochloride (MDL 72222) (Merrell Dow).

[¹²⁵I]-bovine serum albumin was diluted in saline; capsaicin was solubilized in saline:ethanol:Tween 80 8:1:1; 5-HT and sumatriptan were dissolved in saline 0.9%; 5-CT and MDL 72222 in distilled water; methiothepin, pizotifen, and ICS 205-930 in dimethylsulphoxide:saline 1:9; 5-BT in glacial acetic acid:distilled water 1:30; ketanserin and metergoline in dimethylsulphoxide. All the above were injected intravenously in a volume of 1 ml kg⁻¹, except metergoline and ketanserin which were given in a volume of 0.3 ml kg⁻¹. The same volume of vehicle was administered to the control group in each experiment.

Results

Mean arterial blood pressure

Baseline MABP ranged from 83 to 102 mmHg in pentobarbitone-anaesthetized animals. A 10–20 mmHg increase was observed during the electrical stimulation of the right trigeminal ganglion in all animals although it did not reach statistical significance in drug-treated animals. Baseline MABP after superior cervical ganglionectomy did not differ from baseline values in normal animals. Significant decreases in MABP were observed immediately following 5-CT, 8-OH-DPAT or ketanserin administration at the indicated doses. However MABP returned to baseline values within 2–8 min, and was at baseline prior to electrical trigeminal ganglion stimulation (Table 1).

Electrical trigeminal stimulation increases plasma protein extravasation in dura mater and extracranial tissues

High intensity stimulation (1.2 mA, 5 ms, 5 Hz, 5 min) In vehicle-treated animals, levels of iodinated albumin on the unstimulated side were 14 ± 2 , 54 ± 9 , and 36 ± 11 c.p.m.mg⁻¹ wet wt. in dura mater, conjunctiva and temporalis muscle fascia, respectively. Unilateral electrical stimulation of the trigeminal ganglion increased leakage of iodinated albumin on the ipsilateral side to 26 ± 2 c.p.m.mg⁻¹ wet wt. in dura mater, 293 ± 14 c.p.m.mg⁻¹ wet wt. in conjunctiva, and 87 ± 19 c.p.m.mg⁻¹ wet wt. in fascia. The ratio between stimulated and non-stimulated sides ranged from 1.4 ± 0.1 to 2.2 ± 0.2 in dura mater (70 experiments, $n = 400$). Larger ratios with greater variances were observed in extracranial tissues at this current intensity and ranged from 2.3 ± 0.3 to 4.8 ± 1.0 in conjunctiva (13 experiments, $n = 52$), and from 1.3 ± 0.1 to 2.8 ± 0.3 in fascia (9 experiments, $n = 36$) (Table 2).

Low intensity stimulation (0.1 mA, 5 ms, 5 Hz, 3 min) A significant increase in iodinated albumin was observed in extracranial tissues (conjunctiva, eyelid and lip) but not dura mater.

The ratio between the two sides in 4 separate experiments ranged from 1.5 ± 0.1 to 1.9 ± 0.4 in conjunctiva, 1.2 ± 0.1 to 1.8 ± 0.2 in the eyelid, and 1.5 ± 0.2 to 2.0 ± 0.2 in lip in vehicle-treated animals. Because these ratios most closely approximated the response in dura mater at higher currents, 0.1 mA for 3 min was used in subsequent experiments to determine whether sumatriptan could block extravasation in extracranial tissues.

5-HT receptors and the neurogenic inflammatory response in dura mater

Plasma protein leakage in dura mater did not differ on the unstimulated side between vehicle- and drug-treated animals. The data (c.p.m.mg⁻¹ wet wt.) contralateral to stimulation following 5-CT administration (1, 100 ng kg⁻¹) was 7.1 ± 0.4 ($n = 32$) versus 8.3 ± 0.6 (control, $n = 32$); 12.1 ± 1.1 (5-BT, 10–300 µg kg⁻¹, $n = 29$) versus 12.8 ± 0.9 ($n = 25$, control); 11.1 ± 1.2 (8-OH-DPAT 100,300 µg kg⁻¹, $n = 20$) versus 10.9 ± 1.2 ($n = 15$, control); 10.3 ± 1.0 (sumatriptan 30–300 µg kg⁻¹, $n = 28$) versus 10.2 ± 0.9 ($n = 21$; control). Data on the unstimulated side from conjunctiva and fascia also showed no differences between vehicle- and drug-treated animals (data not shown).

Putative 5-HT₁ receptor agonists

5-Carboxamidotryptamine 5-CT reduced the ratio of c.p.m.mg⁻¹ wet wt. (stimulated side)/c.p.m.mg⁻¹ wet wt. (unstimulated side) in doses as low as 1 ng kg⁻¹ [1.9 ± 0.1 ($n = 20$) in vehicle-treated animals vs 1.3 ± 0.1 ($n = 22$), $P < 0.001$]. Ten, 30, 100, 300 ng kg⁻¹ reduced the ratio by 85, 55, 50 and 67%, respectively. The lowest tested dose (0.1 ng kg⁻¹) decreased the ratio by 30% but this decrease did not reach statistical significance (Figure 1).

8-Hydroxydipropylaminotetralin 8-OH-DPAT 300 µg kg⁻¹ decreased the extravasation [1.7 ± 0.1 ($n = 15$) in vehicle-treated animals vs 1.4 ± 0.1 ($n = 20$), $P < 0.05$], whereas 100 µg kg⁻¹ did not.

5-Benzoyloxytryptamine 5-BT 10, 30 or 100 µg kg⁻¹ administration decreased extravasation in dura mater by 42, 75,

Table 1 Mean arterial blood pressure responses (mmHg) to drug administration and/or electrical trigeminal stimulation in normal rats or following superior cervical ganglionectomy

Treatment (n)	Baseline	0 ^a	Time (min)		
			5	10 ^b	15
Vehicle (3)	96 ± 8	97 ± 4	98 ± 4	95 ± 4	110 ± 5*
5-CT (100 ng kg ⁻¹) (4)	86 ± 2	92 ± 5	91 ± 4	92 ± 5	100 ± 5
8-OH-DPAT (300 µg kg ⁻¹) (5)	87 ± 3	86 ± 3	94 ± 4	88 ± 5	98 ± 3
Ketanserin (300 µg kg ⁻¹) (3)	88 ± 3	87 ± 2	93 ± 6	92 ± 4	97 ± 2
Methiothepin (1 mg kg ⁻¹) (3)	97 ± 3	99 ± 1	94 ± 2	92 ± 3	108 ± 5
Metergoline (1 mg kg ⁻¹) (3)	92 ± 5	91 ± 5	95 ± 1	91 ± 3	101 ± 8
5-BT (100 µg kg ⁻¹) (3)	97 ± 4	99 ± 3	101 ± 2	101 ± 1	—
5-BT (30 µg kg ⁻¹) (3)	95 ± 3	95 ± 3	97 ± 3	100 ± 3	—
Ganglionectomy (3)	96 ± 4	98 ± 2	98 ± 1	99 ± 3	110 ± 1*

Mean arterial blood pressure (MABP) was recorded via the left femoral artery in anaesthetized (pentobarbitone, 60 mg kg⁻¹, i.p.) rats placed in a stereotaxic frame with electrodes in the trigeminal ganglia. After steady baseline values were obtained, drugs or vehicle were administered i.v. at time 0 (a) at the indicated doses. Electrical stimulation of the trigeminal ganglion (1.2 mA, 5 ms, 5 Hz, 5 min) was begun at 10 min (b) and MABP recorded until the end of unilateral electrical stimulation. A slight increase was noted in all groups, which reached statistical significance in vehicle-treated animals and in sympathectomized animals. Transient (2–4 min) and significant decreases in MABP were observed following 5-CT, 8-OH-DPAT, or ketanserin administration (not shown). Five and 10 min after drug or vehicle injection however, MABP was not significantly different from baseline. In those groups receiving 5-BT, MABP was not recorded at 15 min. Data are expressed as mean ± s.e.mean. * $P < 0.05$ as compared to values at 10 min. For abbreviations, see text.

Table 2 Plasma protein extravasation in dura mater and extracranial tissues following pretreatment with drugs that bind to 5-HT receptors

<i>Dura mater</i>			
<i>Vehicle (n)</i>	<i>Drug</i>	<i>(n)</i>	
1.6 ± 0.1 (9)	1.7 ± 0.2	(11)	Ketanserin (300 µg kg ⁻¹)
1.9 ± 0.2 (6)	1.6 ± 0.1	(7)	Pizotifen (300 µg kg ⁻¹)
1.7 ± 0.1 (4)	1.8 ± 0.1	(6)	ICS 205-930 (300 µg kg ⁻¹)
1.9 ± 0.2 (10)	1.9 ± 0.2	(18)	MDL 72222 (300 µg kg ⁻¹)
<i>Conjunctiva</i>			
<i>Vehicle (n)</i>	<i>Drug</i>	<i>(n)</i>	
2.6 ± 0.4 (8)	3.9 ± 0.4	(8)	Methiothepin (1 mg kg ⁻¹)
2.6 ± 0.3 (4)	2.2 ± 0.2	(4)	5-BT (300 µg kg ⁻¹)
3.2 ± 0.6 (7)	2.5 ± 0.2	(8)	5-BT (100 µg kg ⁻¹)
2.3 ± 0.3 (4)	2.7 ± 0.5	(5)	5-BT (30 µg kg ⁻¹)
4.8 ± 1.0 (8)	3.8 ± 0.7	(9)	Ketanserin (300 µg kg ⁻¹)
4.2 ± 1.7 (3)	5.0 ± 1.5	(5)	Pizotifen (300 µg kg ⁻¹)
4.8 ± 0.6 (12)	6.4 ± 1.0	(11)	MDL 72222 (300 µg kg ⁻¹)
<i>(n)</i>	<i>Conjunctiva</i>	<i>Eyelid</i>	<i>Lip</i>
Vehicle (14)	1.9 ± 0.1	1.6 ± 0.1	1.7 ± 0.2
Sumatriptan			
100 µg kg ⁻¹ (16)	1.7 ± 0.2	1.7 ± 0.2	1.5 ± 0.2
Vehicle (9)	1.6 ± 0.1	1.4 ± 0.1	1.6 ± 0.1
Sumatriptan			
300 µg kg ⁻¹ (8)	1.4 ± 0.2	1.3 ± 0.1	1.5 ± 0.1

Drugs or vehicle were administered i.v. at the indicated doses 10 min before electrical trigeminal stimulation (1.2 mA, 5 min) and 5 min before [¹²⁵I]-albumin. Animals were perfused and dura mater and conjunctiva dissected on each side as described in Methods. In other experiments involving the use of sumatriptan, animals were stimulated at a lower current intensity (0.1 mA, 3 min) and conjunctiva, eyelid and lip harvested on each side. Data represent the mean ± s.e.mean of the ratio:

$$\frac{\text{c.p.m. mg}^{-1} \text{ wet wt. (stimulated side)}}{\text{c.p.m. mg}^{-1} \text{ wet wt. (unstimulated side)}}$$

The extravasation of plasma proteins was reduced within extracranial tissues at the lower current intensity, although sumatriptan did not attenuate this response.

Figure 1 5-Carboxamidotryptamine (5-CT) significantly reduced plasma protein extravasation ([¹²⁵I]-albumin) in rat dura mater following electrical stimulation of the right trigeminal ganglion. Animals were injected with 0.1 (*n* = 9), 1 (*n* = 22), 10 (*n* = 7), 30 (*n* = 13), 100 (*n* = 10), or 300 (*n* = 12) ng kg⁻¹ i.v. of 5-CT 10 min before stimulation (1.2 mA, 5 Hz, 5 ms) and 5 min before [¹²⁵I]-albumin injection. After 5 min of stimulation, animals were perfused and dura dissected as described in Methods. 5-CT blocked plasma extravasation at a dose as low as 1 ng kg⁻¹. 5-CT did not change the level of radioactivity (c.p.m. mg⁻¹ wet wt.) on the unstimulated side as compared to the vehicle-treated animals. The same number of vehicle-treated animals (open columns) was studied for each 5-CT-treated group (closed columns). Data represent mean (s.e.mean shown by vertical bars) of the ratio:

$$\frac{\text{c.p.m. mg}^{-1} \text{ wet wt. (stimulated side)}}{\text{c.p.m. mg}^{-1} \text{ wet wt. (unstimulated side)}}$$

** *P* < 0.01; *** *P* < 0.001 as compared to vehicle-treated group.

100%, respectively; 300 µg kg⁻¹ did not reach statistical significance (Figure 2). 5-BT did not exhibit any effect in conjunctiva (Table 2).

Sumatriptan High intensity stimulation: Pretreatment with sumatriptan (100 µg kg⁻¹) decreased the ratio in dura mater [1.8 ± 0.1 (*n* = 24) in vehicle-treated animals vs 1.1 ± 0.0 (*n* = 23) in sumatriptan-treated group, *P* < 0.001].

Low intensity stimulation: The administration of either 100 or 300 µg kg⁻¹ did not block the response to electrical stimulation in extracranial tissues. (Table 2).

Putative 5-HT₁ receptor antagonists Pretreatment with metergoline or methiothepin at high concentrations (1 mg kg⁻¹) significantly decreased the ratio in dura mater [1.9 ± 0.1 (*n* = 9; control) vs 1.2 ± 0.1 (*n* = 9) (*P* < 0.001) and 1.8 ± 0.1 (*n* = 7; control) vs 1.2 ± 0.1 (*n* = 7) (*P* < 0.001), respectively]. Metergoline (100, 500 µg kg⁻¹) or methiothepin (300 µg kg⁻¹) were without effect.

5-HT₂ and 5-HT₃ receptor antagonists Plasma protein extravasation in dura mater following trigeminal stimulation was not inhibited by pretreatment with ketanserin, pizotifen, MDL 72222, or ICS 205-930 (Table 2). Ketanserin, pizotifen or MDL 72222 pretreatment did not affect plasma extravasation in conjunctiva (Table 2).

Pretreatment with 5-HT antagonists prior to sumatriptan or 5-CT administration The effect of sumatriptan in dura mater was partially antagonized by metergoline, 100 µg kg⁻¹: vehicle-treated 1.8 ± 0.1 (*n* = 10); sumatriptan-treated 1.1 ± 0.1 (*n* = 9, *P* < 0.001 as compared to the vehicle group);

Figure 2 5-Benzoyloxytryptamine (5-BT) significantly decreased plasma extravasation in dura mater. 5-BT 10 ($n = 4$), 30 ($n = 14$), 100 ($n = 7$), or 300 ($n = 4$) $\mu\text{g kg}^{-1}$ were administered i.v. 10 min before electrical stimulation of the trigeminal ganglion (1.2 mA, 5 ms, 5 Hz) and 5 min before iodinated albumin injection. After stimulation (5 min), animals were perfused and dura dissected as described in Methods. 5-BT did not change the level of radioactivity on the unstimulated side as compared to vehicle-treated animals. A significant decrease of the stimulated side/unstimulated side ratio was observed after pretreatment with 5-BT 100, 30, or 10 $\mu\text{g kg}^{-1}$. A small, not significant decrease was also observed after treatment with 300 $\mu\text{g kg}^{-1}$. The number of vehicle-treated animals (open columns) in each group differed by less than 10% from those given 5-BT (closed columns). Data are expressed as in Figure 1. * $P < 0.05$; ** $P < 0.01$ as compared to the vehicle group.

metergoline + sumatriptan 1.6 ± 0.1 ($n = 10$, $P < 0.05$ as compared to the sumatriptan group) (Figure 3).

Methiothepin 300 $\mu\text{g kg}^{-1}$ given 10 min before sumatriptan 100 $\mu\text{g kg}^{-1}$, did not antagonize the sumatriptan-induced blockade (Figure 3b). The administration of 5-BT 30 $\mu\text{g kg}^{-1}$ prior to sumatriptan added to the effect of sumatriptan: 2.2 ± 0.2 ($n = 3$; control), 1.4 ± 0.2 ($n = 3$; sumatriptan-treated group; $P < 0.05$ as compared to control), 1.0 ± 0.1 in sumatriptan + 5-BT-treated group ($n = 3$, $P < 0.05$ as compared to the vehicle group). Neither pizotifen (300 $\mu\text{g kg}^{-1}$) nor MDL 72222 (300 $\mu\text{g kg}^{-1}$) inhibited the effect of sumatriptan (Figure 3c,d).

The blockade achieved by 5-CT was not affected by metergoline pretreatment. When 5-CT was injected at 1 or 300 ng kg^{-1} , the ratio between the two sides decreased from

Figure 3 Metergoline, but not methiothepin, pizotifen or MDL 72222, antagonize blockade by sumatriptan of plasma protein extravasation in rat dura mater. (a) Metergoline (100 $\mu\text{g kg}^{-1}$) ($n = 10$), (b) methiothepin (300 $\mu\text{g kg}^{-1}$) ($n = 8$), (c) pizotifen (300 $\mu\text{g kg}^{-1}$) ($n = 4$) or (d) MDL 72222 (300 $\mu\text{g kg}^{-1}$) ($n = 4$) were administered i.v. 20 min before electrical trigeminal ganglion stimulation (1.2 mA, 5 min) and 10 min before sumatriptan (100 $\mu\text{g kg}^{-1}$, i.v.). Sumatriptan significantly reduced the stimulated side/unstimulated side ratio. Metergoline 100 $\mu\text{g kg}^{-1}$ partially inhibited this effect. The same number of animals were studied in vehicle- (open columns) and sumatriptan-treated (closed columns) groups; hatched columns: sumatriptan plus antagonist. Data are expressed as in Figure 1. * $P < 0.05$ and ** $P < 0.01$ as compared to vehicle-treated group. † $P < 0.05$ as compared to sumatriptan-treated group.

1.9 ± 0.1 (vehicle, $n = 16$) to 1.3 ± 0.1 ($n = 8$) or 1.2 ± 0.2 ($n = 4$), respectively, whereas after metergoline pretreatment at 100 $\mu\text{g kg}^{-1}$, the ratios were 1.2 ± 0.1 ($n = 8$) and 1.4 ± 0.1 ($n = 4$), respectively. When larger doses of metergoline (300 $\mu\text{g kg}^{-1}$) were administered prior to 5-CT at 1 or 300 ng kg^{-1} , the ratios between the two sides (1.0 ± 0.1 , $n = 4$ and 1.3 ± 0.1 , $n = 7$, respectively) did not differ from the response to 5-CT alone (1.3 ± 0.1 , $n = 4$, and 1.2 ± 0.1 , $n = 7$, respectively).

Sympathectomy and sumatriptan In rats previously subjected to unilateral superior cervical ganglionectomy, radioactivity (c.p.m. mg^{-1} wet wt.) on the unstimulated sides was similar to those in normal animals. However, a slight increase in the ratio was observed [2.4 ± 0.2 ($n = 16$) in superior cervical ganglionectomy vs 2.0 ± 0.1 ($n = 13$) in sham, $P < 0.05$]. Sumatriptan 100 $\mu\text{g kg}^{-1}$, decreased extravasation in both sympathectomized animals [2.4 ± 0.3 ($n = 10$) in vehicle-treated vs 1.4 ± 0.2 ($n = 8$), $P < 0.01$] and shams [1.9 ± 0.1 ($n = 8$) vs 1.4 ± 0.1 ($n = 8$), $P < 0.01$].

Discussion

5-HT_{1B}/5-HT_{1D} receptor binding sites

In the mid 1980's, two distinct but analogous subtypes of 5-HT₁ receptor were identified in various species (Heuring *et al.*, 1986). 5-HT_{1B} receptors are pharmacologically distinct from 5-HT_{1D} receptors, yet appear to mediate similar functions in different species. 5-HT_{1B} sites display < 10 nM affinity for 5-CT and moderate affinity for 5-BT, sumatriptan, methiothepin and metergoline (Table 3). The 5-HT_{1B} receptors are species specific (Hoyer & Middlemiss, 1989). 5-HT_{1B} binding sites have been demonstrated in rat and mouse brain and in a cell line derived from opossum kidney cells (Murphy & Bylund, 1988), but not in guinea-pig, cow, chicken, turtle, frog, or human tissues (Hoyer *et al.*, 1986; Heuring *et al.*, 1986). 5-HT_{1B} receptors also appear to exist outside the central nervous system in rats and mice (see Middlemiss & Hudson, 1990).

In 1987, another distinct subtype of the 5-HT₁ receptor 'family' was identified in bovine brain membranes (Heuring & Peroutka, 1987). [³H]-5-HT binding in bovine caudate was examined in the presence of 100 nM 8-OH-DPAT and 100 nM mesulergine, a condition designed to displace over 90% of specific binding to 5-HT_{1A} and 5-HT_{1C} receptor sites (Heuring & Peroutka, 1987). However, a significant amount of [³H]-5-HT binding was still observed under such conditions. Moreover, the pharmacological characteristics of this site did not correlate with any known 5-HT receptor subtype. Therefore, this recognition site for [³H]-5-HT was designated the '5-HT_{1D}' binding site (Heuring & Peroutka, 1987). It has been suggested that the 5-HT_{1D} receptor is the receptor site responsible for the actions of ergot alkaloids and sumatriptan in migraine headaches (Hoyer *et al.*, 1989).

Among the 5-HT₁ receptor subtypes identified to date, the 5-HT_{1B}/5-HT_{1D} subtypes appear most relevant to neurogenic plasma extravasation in dura mater. Based on the threshold concentrations, the potency order among the tested drugs was 5-CT > 5-BT/dihydroergotamine (Saito *et al.*, 1988)/sumatriptan > 8-OH-DPAT, reflecting the potency order of the affinities for the 5-HT_{1B}/5-HT_{1D} receptors of these drugs (Table 3). (ID₅₀ values were difficult to calculate in this model because of the narrow range between threshold and maximum response.) The 5-HT_{1B}/5-HT_{1D} subtypes take on significance because the effect of sumatriptan can be blocked partially by metergoline (an antagonist with high affinity for the 5-HT_{1B}/5-HT_{1D} recognition site) and because sumatriptan and 5-BT bind with higher affinity to 5-HT_{1D} than 5-HT_{1A} receptors (Peroutka *et al.*, 1990) and are relatively inactive at the 5-HT_{1C} binding site. 5-HT₂ or 5-HT₃ receptor-mediated effects were ruled out because of the results following pretreatment with ketanserin, pizotifen, MDL 72222 and ICS 205-930,

Table 3 Drug potencies in inhibiting plasma protein extravasation in rat dura mater and drug affinities for 5-HT_{1B} and 5-HT_{1D} receptor binding sites in brain membranes

Drug	Effective dose for inhibition of plasma extravasation (nmol kg ⁻¹)	Drug affinities (nM)	
		K _i (5-HT _{1B})	K _i (5-HT _{1D})
5-CT	0.003–0.94	5.0	0.75
5-BT	33	40	40
DHE	85**	3.0	19
Sumatriptan	240	440	17
8-OH-DPAT	910	6,000	700
Methiothepin	> 750	43	36
Metergoline	> 1,200	26	4.2
Ketanserin	> 850*	1,900	> 10,000
Pizotifen	> 850*	> 10,000	2,800
ICS 205-930	> 850*	> 10,000	> 10,000
MDL 72222	> 850*	> 10,000	> 10,000

The plasma protein extravasation data shown are derived from the current study. Radioligand binding affinities were derived from Hoyer *et al.*, 1985; Heuring & Peroutka, 1987; Offord *et al.*, 1988; Schlicker *et al.*, 1989; Waeber *et al.*, 1988; Peroutka *et al.*, 1990 and Peroutka (unpublished observations). * Higher doses than 300 µg kg⁻¹ were not tested. ** Taken from Saito *et al.*, 1988. For abbreviations, see text.

and because both sumatriptan and 5-BT exhibit only weak activity at 5-HT₂ and 5-HT₃ receptors. Similarly, 5-HT_{1B}/5-HT_{1D} receptors probably mediate the blockade of extravasation by less specific drugs such as methysergide.

On the other hand, our data suggest that an additional, as yet unidentified subtype(s) of the 5-HT₁ receptor might be more involved in the inhibition of plasma leakage. Important differences from the predicted effects of these drugs at a known 5-HT₁ receptor subtype(s) include: (1) the high potency (1 ng kg⁻¹, threshold) of the non-selective agonist 5-CT, (2) the inability of metergoline to block the effects of 5-CT (metergoline, 100 or 300 µg kg⁻¹, given 10 min prior to 5-CT, 1 or 300 ng kg⁻¹, did not inhibit the effects of 5-CT in dura mater), (3) the partial inhibition of the action of sumatriptan by metergoline, (4) the inability of methiothepin pretreatment to affect inhibition by sumatriptan of plasma leakage, (5) the ability of the antagonists to promote plasma extravasation when administered alone, and (6) the lack of effect of 5-HT (see below) in this model. The development of more selective drugs to discriminate and identify other 5-HT₁ receptor subtypes might help to resolve some of these questions, especially since much of the discrepant data involves the use of 5-HT₁ antagonists.

Receptor localization

Several lines of experimental evidence support the conclusion that 5-HT heteroreceptors are located on neuropeptide-containing unmyelinated C fibre and that drug blockade of plasma leakage within dura mater is mediated by a prejunctional mechanism. Firstly, sumatriptan (100 or 300 µg kg⁻¹, i.v.) and dihydroergotamine (50 µg kg⁻¹, i.v. or 1 mg kg⁻¹, i.p.) are inactive when tested against concentrations of substance P or neurokinin A which cause plasma leakage that most closely approximate the response following electrical trigeminal stimulation or capsaicin administration (Saito *et al.*, 1988; Buzzi & Moskowitz, 1990). Secondly, dihydroergotamine (50 µg kg⁻¹) and to a lesser extent sumatriptan (300 µg kg⁻¹) attenuate the increases in immunoreactive calcitonin gene-related peptide within sagittal sinus blood (e.g., venous effluent) during electrical stimulation of the trigeminal ganglion (Buzzi *et al.*, 1991). Thirdly, sumatriptan and dihydroergotamine pretreatment block platelet aggregation and endothelial vesicle formation within post-capillary venules and mast cell secretion and degranulation in the dura mater (Buzzi *et al.*, 1990). It is difficult to reconcile these data with activation of a postjunctional receptor either on vascular smooth muscle, endothelial cells or sympathetic fibres. In fact, sympathetic fibres were ruled out as a possible site by direct experimentation reported here. Prejunctional receptors coupled to inhibition of neuropeptide release could mediate all the above changes. The identified putative 5-HT₁ heteroreceptor in dura

mater may be inhibiting calcium currents, as suggested by the observations that voltage-sensitive Ca²⁺ channels (dihydropyridine-sensitive) mediate substance P release from cultured rat primary sensory neurons (Perney *et al.*, 1986).

The natural ligand for the putative 5-HT_{1B}/5-HT_{1D} receptor identified on trigeminal perivascular fibres is most probably 5-HT. However, 5-HT itself is relatively inactive in the model studied here. In fact, pretreatment with 5-HT does not cause plasma extravasation after intravenous administration, except at very large concentrations (1 µmol kg⁻¹) (Markowitz *et al.*, 1987), nor does it block the extravasation response following electrical stimulation or capsaicin administration. In one experiment (*n* = 3 per group), the response to capsaicin (149% ± 0.5 versus 100% ± 0.5 vehicle) was unaffected by 5-HT pretreatment (0.1 µmol kg⁻¹; 150% ± 12).

The possibility that blockade of neurogenically-mediated extravasation is due to decreases in surface area and perfusion pressure secondary to vasoconstriction (e.g., postjunctional mechanism) deserves consideration. Sumatriptan constricts isolated canine saphenous vein (Humphrey *et al.*, 1988), canine and primate cerebral vessels (Connor *et al.*, 1989), canine carotid arterial circulation (Feniuk *et al.*, 1989). Ergotamine constricts isolated extracranial arteries (Muller-Schweinitzer & Weidmann, 1978). *In vivo*, methysergide selectively constricts the carotid vascular bed (Saxena, 1974). Sumatriptan (Perren *et al.*, 1989), ergotamine (Bom *et al.*, 1989a), 5-CT (Saxena & Verdouw, 1985) and 8-OH-DPAT (Bom *et al.*, 1989b) constrict arteriovenous shunts, although the effects of ergotamine may not be mediated by 5-HT₁-like or 5-HT₂ receptors (Bom *et al.*, 1989a). If closing arteriovenous shunts decreases vascular permeability following trigeminal stimulation, than leakage induced by intravenously administered neuropeptides (substance P or neurokinin A) should have been blocked as well. Constriction of arterioles (such as with phenylephrine) does not block neurogenic plasma extravasation nor does it reduce the increase in levels of calcitonin gene-related peptide in the sagittal sinus accompanying electrical stimulation (Saito *et al.*, 1988; Buzzi *et al.*, 1990). Constriction of extracranial cephalic blood vessels follows ergotamine administration but neurogenic plasma extravasation is not blocked in these tissues. Finally, it would be difficult to conceive how a vasoconstrictor drug action alone would explain the blockade of neuropeptide increases in sagittal sinus, platelet accumulation and mast cell degranulation accompanying trigeminal stimulation (Buzzi *et al.*, 1990; 1991).

Clinical relevance

Perivascular inflammation may play an important role in the pathogenesis of headache. Its development in cephalic tissues

has been documented in man (Sweet & Wepsic, 1974; Drummond *et al.*, 1983; Goadsby *et al.*, 1988) and experimental animals (Jancso-Gabor & Szolcsanyi, 1972; Couture & Cuello, 1984; Goadsby *et al.*, 1988). Both neurogenic plasma extravasation and headache can be blocked by aspirin and in selected cases, by indomethacin pretreatment (Buzzi *et al.*, 1989). A central role in migraine pathogenesis has been proposed for dural vessels (Penfield & McNaughton, 1940). Dural vessels are among the few pain-sensitive intracranial tissues (Ray & Wolff, 1940); they contain structures important to the development of neurogenic inflammation, namely fenestrated capillary venules surrounded by a network of neuropeptide-containing unmyelinated C-fibres and mast cells (McNaughton, 1938; Andres *et al.*, 1987). Platelet aggregation, mast cell degranulation, and fluid accumulation in the perivascular space of postcapillary venules attendant on electrical trigeminal stimulation may promote sensitization of polymodal nociceptors and sustain the process of sterile inflammation (Cline *et al.*, 1989). It is possible that the consequences of neurogenic inflammation such as local fluid accumulation, sensitization of polymodal nociceptors and release of proin-

flammatory agents underlie and sustain the pain of headache. The ability of putative 5-HT₁ receptor agonists which lack inherent analgesic properties, to prevent neurogenic plasma extravasation in the dura mater, not only suggests an action directly on primary afferent fibres, but in itself adds further weight to the notion that dural inflammation may be relevant to headache pathogenesis. Peripheral (dural) mechanisms are further emphasized by the observation that sumatriptan is hydrophilic and penetrates the blood brain barrier very poorly, if at all (P.P.A. Humphrey, personal communication). For the above reasons vasoconstriction must diminish in importance as a mechanism to explain the analgesic action of these drugs in headache patients. Rather, the data favour the importance of neurogenic inflammation in the pathogenesis of migraine, and the unmyelinated C-fibre as the site of the putative 5-HT receptor subtype.

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