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ACCELERATED COMMUNICATION

Expression of mRNA for the Serotonin 5-Hydroxytryptamine$_{1D\beta}$ Receptor Subtype in Human and Bovine Cerebral Arteries

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SUMMARY

Serotonin [5-hydroxytryptamine (5-HT)] has been implicated in the pathophysiology of migraine, and the clinical efficacy of the 5-HT$_{1B}$/5-HT$_{1D}$ receptor agonist sumatriptan points to neural and/or vascular 5-HT$_{1D}$ receptors as relevant targets in migraine therapy. We characterized the human and/or bovine 5-HT$_{1D}$ receptor subtype in cerebral blood vessels pharmacologically by correlation analysis and molecularly by Northern blot hybridization of cerebrovascular RNA extracts. Pharmacological analysis showed that sumatriptan was less potent than 5-HT in inducing contraction in freshly isolated human cerebral arteries and revealed an overall pharmacological profile positively and significantly correlated with that published for the 5-HT$_{1D\alpha}$ ($r = 0.746$, $p = 0.021$) and 5-HT$_{1D\beta}$ ($r = 0.942$, $p = 0.0001$) cloned human receptor subtypes. These results are suggestive of a contractile 5-HT$_{1D\beta}$ receptor subtype but are not conclusive. However, Northern blots revealed the presence of mRNA transcripts for the 5-HT$_{1D\beta}$ subtype, but not the 5-HT$_{1D\alpha}$ subtype, in bovine (~2.2 kilobases) and human (~4.5 kilobases) cerebral blood vessels. Expression of either subtype could not be detected in intraparenchymal microvessels or capillaries isolated from bovine or human cerebral cortex. These results clearly indicate that the beneficial effect of sumatriptan in migraine attack, if vascularly related, is mediated by contractile 5-HT$_{1D\beta}$ receptors most likely located on cerebral blood vessels at the surface of the brain. This study points to the 5-HT$_{1D\beta}$ receptor subtype as the putative cerebrovascular target for migraine therapeutic agents.

Several lines of evidence implicate 5-HT (serotonin) in the pathophysiology of migraine (1). The most striking is the clinical efficacy of the 5-HT$_1$ receptor agonist sumatriptan in aborting migraine and cluster headaches (2). Sumatriptan has been shown to interact preferentially with 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors (3, 4), and both vascular and neural 5-HT$_{1D}$ receptor-mediated mechanisms have been proposed for the action of sumatriptan in migraine relief (5, 6). Contractile postsynaptic 5-HT$_{1D}$-like receptors have been characterized pharmacologically in human cerebral blood vessels (7), and inhibitory, presynaptic 5-HT$_{1B}$ (rat) (8) or 5-HT$_{1D}$-like (guinea pig) (9) heteroreceptors are found on dural trigeminovascular afferents. It has been hypothesized that trigeminal-induced neurogenic inflammation (which results in plasma protein extravasation and vasodilatation) is the triggering factor of migraine-associated pain (10). The beneficial effect of sumatriptan in migraine therapy could thus be related to its interaction with the 5-HT$_{1D}$ receptors that mediate the blockade of neuropeptide release from dural trigeminovascular sensory fibers (5, 6). Alternatively, sumatriptan could activate the contractile 5-HT$_{1D}$ receptors on cerebral arteries, the dilatation of which might be crucial in migraine manifestation (1). It is also possible that both mechanisms contribute to the efficacy of sumatriptan in migraine treatment.

Although the vascular and neural receptors both correlate with 5-HT$_{1D}$ pharmacology, two genes have now been identified that encode two variants of the pharmacological 5-HT$_{1D}$ receptor (11). These two receptor subtypes have been defined as 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ (12–15), the latter being the human homologue of the rodent 5-HT$_{1B}$ receptor (15–18). The extreme degree of pharmacological similarity between the two subtypes, as assessed in cell lines expressing the individual gene products, makes them almost indistinguishable based on pharmacology alone (16, 19). It is thus possible that the vascular and neural 5-HT$_{1D}$ receptors correspond to two different molecular entities, both activated by sumatriptan.

To further characterize the molecular target(s) for sumatriptan in migraine therapy, we studied the detailed pharmacology

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; kb, kilobase(s); CAP, capillary; MV, microvessel; 8-OH-DPAT, 8-hydroxy-2-di-*N*-propylaminotetralin.

of the contractile cerebrovascular 5-HT$_{1D}$ receptor and the expression of 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor subtypes in cerebral arteries and intracortical blood vessels from bovine and humans, two species known to contain cerebrovascular 5-HT$_{1D}$-like receptors (7).[1] The data show much better correlation of the human cerebrovascular receptor with the 5-HT$_{1D\beta}$ subtype, as well as the presence of mRNA transcripts for 5-HT$_{1D\beta}$ but not 5-HT$_{1D\alpha}$ receptors in bovine and human cerebral arteries. These results clearly indicate that the contractile response to sumatriptan in this tissue is mediated by 5-HT$_{1D\beta}$ receptors, and they point to this subtype as a putative target for antimigraine therapy.

Materials and Methods

Functional assays in human cerebral blood vessels. Human pial arteries corresponding to a temporal ramification of the middle cerebral artery (outside diameter, ≈1 mm) were obtained from epileptic patients undergoing temporal lobe surgery. The pia-arachnoid membrane was removed from the cortical surface and the vessels were dissected in ice-cold Krebs Ringer buffer (in mM: NaCl, 118; KCl, 4.5; $MgSO_4 \cdot 7\ H_2O$, 1.0; KH_2PO_4, 1.0; $NaHCO_3$, 25; $CaCl_2 \cdot 2H_2O$, 2.5; glucose, 6.0). They were then used for isometric measurement of changes in smooth muscle tension, as described previously by us (7), using a force displacement transducer (Grass FT 103D) and a Grass polygraph (model 7E) coupled to a computer for automatic data acquisition and analysis. Potencies of 5-HT and sumatriptan were established by determination of pD_2 values (−log of the molar concentration of agonist that produces 50% of the maximal response, −log EC_{50}), calculated mathematically as:

$$pD_2 = -\log[A] - \log\left[\frac{E_{A_{max}}}{E_A} - 1\right]$$

where $E_{A_{max}}$ is the maximal contraction induced by agonist A and E_A is the contractile response to a given concentration of agonist [A] (20).

Correlation analysis. All results are given as means ± standard errors. Linear regression lines and correlation coefficients were calculated to detect and quantify any correlation between potencies of 5-HT receptor agonists and antagonists in human vessels and those published (11–14, 18, 21) for these compounds with the cloned human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor subtypes stably expressed in cell lines. For all 5-HT receptor analogues except sumatriptan, which was tested in the present study, the cerebrovascular values used in the correlation analyses were taken from the work of Hamel and Bouchard (7), based on the identical potency of 5-HT in human vessels obtained postmortem (pD_2 = 7.61 ± 0.08; 33 vessel segments) (7) and at surgery (pD_2 = 7.59 ± 0.08; 15 vessel segments) (this study).

Isolation of human and bovine MVs and CAPs. Bovine brains (obtained fresh from a local slaughterhouse) and human brains (postmortem delay, 4–8 hr; obtained from the Douglas Hospital Brain Bank, Verdun, Québec, Canada) were used for isolation of microvascular fractions from the cerebral cortex according to procedures modified from those described originally (22). The vascular fractions were obtained by consecutive centrifugations on 15% dextran in 50 mM phosphate-buffered saline and sieving through 150-μm (MVs) or 50-μm (CAPs) nylon mesh. MVs and CAPs were blotted onto slides and stained with cresyl violet, and their purity was assessed by light microscopy. In addition, activities of the endothelial marker enzymes alkaline phosphatase and γ-glutamyl transpeptidase were determined by spectrophotometry as described previously (22, 23). These were found to be highly enriched, compared with cortical tissues (alkaline phosphatase, 6-fold and 14-fold in MVs and 5-fold and 20-fold in CAPs in bovine and human tissues, respectively; γ-glutamyl transpeptidase, 26-fold and 19-fold in MVs and 28-fold and 27-fold in CAPs in bovine and human tissues, respectively.

Cloning and sequence determination of 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor genes. To amplify the full length 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor genes, two sets of primers were designed according to published data (12, 14) (set 1, 5-HT$_{1D\alpha}$: 1Dαf, 5′GGGGTTTGAATTCATGTCCCACTGAACCAGTC-3′; 1Dαr, GGGGTTTGTCGACCTAGGAGGCCTTCCGGAAA-3′; set 2, 5-HT$_{1D\beta}$: 1Dβf, 5′-GGTTTGATATCATGGAGGAACCGGGTGCTC-3′; 1Dβr, GGGGTTTCTAGATCAACTTGTGCACTTAAAACGTA-3′). These two sets of primers were used to amplify the 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor genes from human genomic DNA (CLONTECH) by PCR. The PCR was performed with *Thermus aquaticus* DNA polymerase (Promega) under the following conditions: 20 sec at 94°, 30 sec at 50°, and 1 min at 72° for 28 cycles, followed by one cycle of 10 min at 72°. The resultant PCR products were subcloned into the pBluescript plasmid (Stratagene). The identity of the clones was confirmed by restriction analysis and sequencing.

Northern blot hybridization. Total cellular RNA was extracted in 4 M guanidinium thiocyanate (24) from bovine and human brain tissues (cerebral cortex and/or caudate nucleus), pial vessels, and intracortical MVs and CAPs (see above). Total RNA (10 μg) was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, and permanently bound by UV irradiation using a Stratalinker (Stratagene). The membranes were hybridized (42°, 16 hr) with 1.1-kb PCR radioactively labeled DNA fragments encoding the entire 5-HT$_{1D\alpha}$ or 5-HT$_{1D\beta}$ receptor gene (see below), in a hybridization buffer containing 50% formamide, 5× SSC, 1× Denhardt's, 0.1% SDS, and 100 μg/ml salmon sperm DNA. The membranes were then washed with 2× SSC/0.5% SDS for 30 min at room temperature, followed by a 30-min wash at 42° in 1× SSC/0.5% SDS and finally 30 min in 0.2× SSC/0.5% SDS at 65°. The membranes were exposed to Kodak XAR-5 film for 6 days at −80°, with intensifying screens.

^{32}P-labeled DNA probes by PCR. Two pairs of primers flanking the coding region of 5-HT$_{1D\alpha}$ (1Dαf, 5′-ATGTCCCACTGAACCAGTC-3′; 1Dαr, 5′-TAGGAGGCCTTCCGGAAA-3′) and 5-HT$_{1D\beta}$ (1Dβf, 5′-ATGGAGGAACCGGGTGCTC-3′; 1Dβr, 5′-TCAACTTGTGCACTTAAAACG-3′) were used to amplify the 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ fragments separately from positive subclones. The PCR was performed with *Thermus aquaticus* DNA polymerase (Promega) in a volume of 20 μl of PCR mixture containing 2 nM dCTP and 100 μCi of [^{32}P]dCTP. A 50% incorporation was obtained after 17 cycles of 1 min at 94°, 2 min at 55°, and 3 min at 72°. These amplified probes were confirmed by cross-hybridization with the 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor genes and were purified on spin columns (Pharmacia); 1×10^6 cpm/ml probe was used to hybridize the membranes.

Results and Discussion

The results of the pharmacological analysis of 5-HT and sumatriptan with freshly isolated human cerebral arteries obtained at surgery are shown in Fig. 1. Cumulative concentrations of sumatriptan induced a dose-dependent constriction that compared well in intensity (0.38 ± 0.05 g) with that elicited by 5-HT (0.40 ± 0.05 g). The potency of sumatriptan in these vessels was, however, significantly lower than that of the natural transmitter (respective pD_2 values of 6.86 ± 0.08 and 7.59 ± 0.08, $n = 15$, $p < 0.001$), in agreement with previous studies of human basilar arteries obtained postmortem (25). Pooling of these cerebrovascular affinities for sumatriptan and 5-HT in fresh vessels with those obtained previously by us for other 5-HT receptor agonists and antagonists in human pial arteries (7) yielded a rank order of agonist potencies of 5-carboxamidotryptamine > 5-HT > sumatriptan = RU24969 > methysergide ≫ 8-OH-DPAT. This pharmacological profile compares well with that reported recently for the cloned and expressed

[1] E. Hamel, L. Grégoire, and B. Lau. 5-HT$_1$ receptors mediating contraction in bovine cerebral arteries: a model for human cerebrovascular "5-HT$_{1D\beta}$" receptors. *Eur. J. Pharmacol.*, in press.

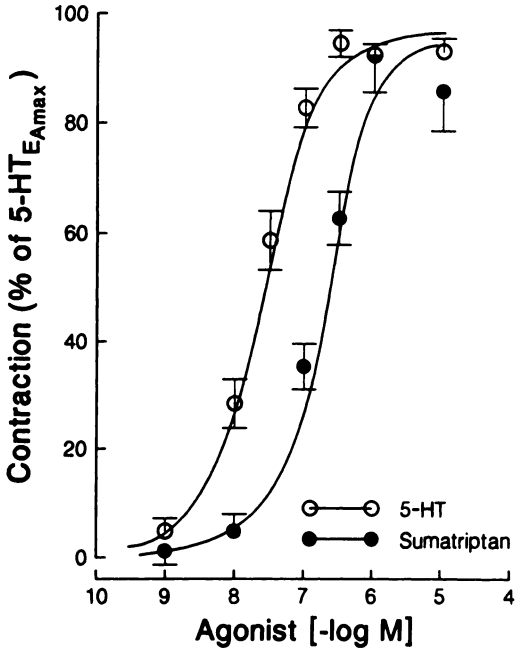

Fig. 1. Concentration-response curves for 5-HT and sumatriptan in human cerebral arteries (temporal ramifications of the middle cerebral artery) obtained at surgery. The contractile response was induced in arterial segments under resting tension. *Vertical bars*, standard error of 15 vessel segments for each compound.

human 5-HT$_{1D\beta}$ (5-HT$_{1B}$) receptor subtype (11–13, 18, 21). Similarly, this rank order of potency is reminiscent of that obtained for some of these compounds for adenylate cyclase inhibition of a cloned and transfected human 5-HT$_{1D}$ receptor (12), the sequence of which indicated its correspondence to the human 5-HT$_{1D\beta}$ subtype (19). However, upon correlation analyses of cerebrovascular agonist and antagonist potencies and those published for these compounds at the cloned human 5-HT$_{1D}$ receptor subtypes, positive and significant correlations were obtained with both the human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ (or 5-HT$_{1B}$) receptor subtypes (Fig. 2). Although the correlation was clearly better for the 5-HT$_{1D\beta}$ subtype (r = 0.942, p = 0.0001) than the 5-HT$_{1D\alpha}$ subtype (r = 0.746, p = 0.021), pharmacological criteria were not sufficient to discriminate the 5-HT$_{1D}$ receptor subtype with which sumatriptan interacts to constrict human cerebral arteries. This contrasts with the pharmacological characterization of the bovine cerebrovascular contractile receptor, where a significant correlation could be obtained for the 5-HT$_{1D\beta}$ subtype but not the 5-HT$_{1D\alpha}$ subtype.[1] However, neither bovine nor human cerebrovascular 5-HT receptors could be correlated with any other known 5-HT receptor subtypes, including the newly cloned 5-HT$_{1E}$ (26–28), 5-HT$_{1F}$ (29), and other 5-HT (30–32) receptor subtypes.

In an attempt to probe the molecular identity of the sumatriptan-sensitive receptor in cerebral blood vessels, total RNA was extracted from various human and bovine brain and cerebrovascular tissues. These extracts were used in Northern blot hybridization with PCR-labeled probes selective for the human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor subtypes. The choice of these two species was based on the reported pharmacological similarity between their brain 5-HT$_{1D}$ receptors (33) and our own data showing that 5-HT-induced contraction in bovine cerebral arteries is mediated by a receptor pharmacologically identical to that of human cerebral vessels.[1] Northern blot hybridization with the 5-HT$_{1D\alpha}$ probe revealed a single transcript of approximately 2.2 kb in extracts of bovine caudate nucleus and cerebral cortex but not of pial blood vessels (Fig. 3A). Similarly, one band (~4.5 kb) was present in human cerebral cortex but not in RNA extracts from pial vessels (Fig. 3B). When hybridization was performed with the 5-HT$_{1D\beta}$ probe, the presence of mRNA transcripts for this subtype was evident in both human and bovine cerebral tissues and pial vessels (Fig. 3). Although the possibility that other 5-HT receptor subtypes are expressed in cerebral blood vessels cannot be excluded, the vasocontractile property of sumatriptan clearly appears to be due to interaction with the 5-HT$_{1D\beta}$ receptor. Because it is expected that the coding sequences would be similar for bovine and human receptors, the apparent difference in size of 5-HT$_{1D\beta}$ transcripts may reflect differences in polyadenylation or the presence of more than one form of mRNA, with one being more highly expressed than the other depending on the species. In contrast to pial vessels, and despite good quality RNA being obtained from cortical MVs and CAPs, mRNA transcripts were not detected for either subtype of 5-HT$_{1D}$ receptor in bovine fractions or for the 5-HT$_{1D\beta}$ receptor in human microvascular fractions (data not shown).

An additional postulated target for sumatriptan in migraine therapy is the presynaptic 5-HT$_{1D}$ receptor on trigeminovascular afferents (5). These receptors have been pharmacologi-

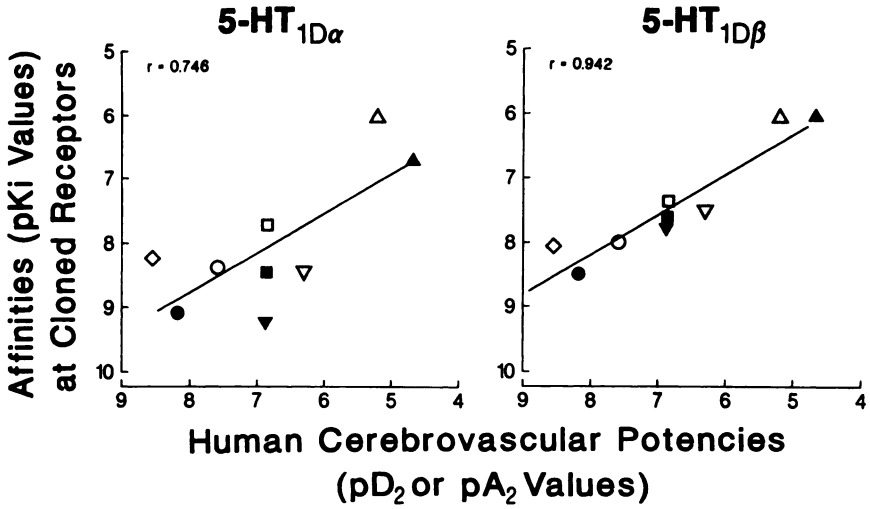

Fig. 2. Correlation analyses performed with agonist and antagonist cerebrovascular potencies (pD_2 and pA_2 values, respectively) in human pial arteries (this study and Ref. 7) and their published affinities (pK_i) at the cloned human 5-HT$_{1D\alpha}$ (11, 14) and 5-HT$_{1D\beta}$ (11–13, 18, 21) receptors. Details of the correlation analyses are as follows: 5-HT$_{1D\alpha}$, r = 0.746, p = 0.021, b = 0.615, and a = 3.86; 5-HT$_{1D\beta}$, r = 0.942, p = 0.0001, b = 0.632, and a = 3.144. The compounds used for the correlation are 5-HT (○), 5-carboxamidotryptamine (●), RU 24969 (□), sumatriptan (■), 2-CH$_3$-5-HT (△), 8-OH-DPAT (▲), methysergide (▽), metergoline (▼), and methiothepin (◇).

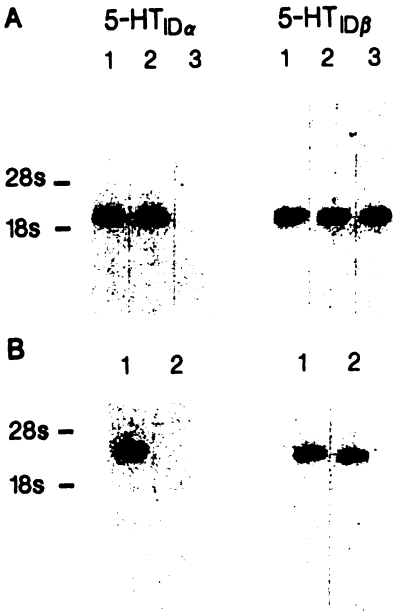

Fig. 3. Northern blot analysis of 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor transcripts in bovine (A) and human (B) cerebral tissues and pial vessels. Northern blot hybridization with the PCR-labeled 5-HT$_{1D\alpha}$ probe identified one band (~2.2 kb) in bovine caudate nucleus (*lane 1*) and cerebral cortex (*lane 2*) but not in cerebral blood vessels (*lane 3*) (A). Similarly, one band (~4.5 kb) was found in human cerebral cortex (*lane 1*) but not in cerebral vessels (*lane 2*) (B). In contrast, the 5-HT$_{1D\beta}$ receptor probe identified one band (~2.2 kb) in bovine cerebral tissues and blood vessels (A). One band (~4.5 kb) was present in both human cerebral cortex (*lane 1*) and pial blood vessels (*lane 2*) (B). These results clearly indicate that expression of the 5-HT$_{1D\beta}$ receptor in human and bovine pial vessels is tissue specific, because no 5-HT$_{1D\alpha}$ receptor mRNA was expressed in these vascular tissues.

cally characterized as 5-HT$_{1B}$ (8) or 5-HT$_{1D}$-like (9) in rat and guinea pig dura mater, respectively, but their human counterpart has yet to be identified. Recently, the expression of 5-HT$_{1B}$ receptors has been shown in rat trigeminal ganglion cells by *in situ* hybridization (34). Based on the fact that the rat 5-HT$_{1B}$ receptor is the species homologue of the human 5-HT$_{1D\beta}$ subtype (16–18), it was hypothesized that human trigeminal ganglia would be endowed with 5-HT$_{1D\beta}$ receptors (34). Such a suggestion would mean that both neural and vascular putative targets for sumatriptan would correspond to the same molecular entity. This possibility is speculative at present but clearly underlines the need to identify the putative 5-HT$_{1D}$ receptor subtype in the human trigeminal ganglion.

Whether the presynaptic 5-HT$_{1D}$ receptors on trigeminovascular afferents and the postsynaptic contractile 5-HT$_{1D\beta}$ (human 5-HT$_{1B}$) receptors are both necessary therapeutic targets for sumatriptan remains to be established. In the event that neural and vascular 5-HT$_{1D}$ receptors are different subtypes, the poor selectivity of sumatriptan could be clinically advantageous in migraine treatment. It is also possible, however, that sumatriptan-associated side effects such as chest pain could reflect the activation of 5-HT$_{1D}$-like receptors described in systemic vessels (35).

In conclusion, our results show that the 5-HT$_{1D\beta}$ receptor subtype is expressed in cerebral arteries located outside the brain parenchyma but not (or only at very low and undetectable levels) in intracortical blood vessels. The molecular identification of a cerebrovascular 5-HT$_{1D\beta}$ receptor subtype was corroborated by pharmacological analysis in bovine[1] but not human cerebral arteries in which an unequivocal discrimination between 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors could not be achieved pharmacologically. Taken together, these results indicate that the beneficial effect of sumatriptan in migraine attack, if vascularly related, is likely to occur at the level of cerebral blood vessels located at the surface of the brain. This statement is supported by the documented induction of cerebral vasoconstriction (36) and increase in blood flow velocity (37) in large cerebral arteries from migraine sufferers treated with sumatriptan. These observations also suggest that the drug can reach one of its putative therapeutic targets, the cerebral smooth muscle vasocontractile 5-HT receptor. The present findings underscore the importance of the 5-HT$_{1D\beta}$ receptor subtype in migraine therapy but also emphasize the urgent need for molecular identification of the human trigeminal ganglion 5-HT receptor subtype, the other postulated site of action for sumatriptan. Although such studies are hampered by the limited availability of this tissue, they represent a critical step in the final assessment of the real functional and molecular targets for migraine therapeutic agents.

Acknowledgments

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