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Involvement of 5-HT_{1B} and 5-HT_{1D} receptors in sumatriptan mediated vasocontractile response in rabbit common carotid artery

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1 In this study we examined the involvement of 5-HT_{1B} and 5-HT_{1D} receptors in the vasocontractile response induced by 5-$HT_{1B/D}$-receptor agonist sumatriptan in rabbit common carotid artery (CCA).

2 Immunoblotting experiments using specific antisera against 5-HT_{1B} or 5-HT_{1D} receptors revealed the presence of one weak (at 93 kD for 5-HT_{1B} or at 105 kD for 5-HT_{1D}) and one strong band (at 46 kD for 5-HT_{1B} or at 52 kD for 5-HT_{1D}) in CCA.

3 Sumatriptan-mediated vasocontractile response was antagonized by SB216641 with an apparent pKb value of 8.6, which was consistent with its affinity for 5-HT_{1B} receptor. Antagonism by BRL15572 was weak and calculated apparent pKb (6.0) value was consistent with its affinity for 5-HT_{1B} subtype (but not for 5-HT_{1D} subtype). This result indicates insignificant or no involvement of 5-HT_{1D} receptor in the vasocontractile response.

4 The vasocontractile response induced by sumatriptan was highly sensitive to pertussis toxin treatment of CCA. Nicardipine, a calcium channel blocker, also potently antagonized vasocontractile response induced by sumatriptan.

5 5-HT, but not sumatriptan, stimulated inositol phosphate accumulation in CCA.

6 These results indicate that stimulation of 5-HT_{1B} subtype activate a pertussis toxin (PTX) sensitive G protein (Go/Gi) and mediate vasocontraction, in which L-type voltage dependent calcium channels are involved.

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Keywords: Sumatriptan; vascular contractions; serotonin; inositol phosphate accumulation; cyclic AMP; voltage-dependent calcium channels; Gi/o proteins; 5-HT_{1B} receptors; 5-HT_{1D} receptors; rabbit common carotid artery

Abbreviations: CCA, common carotid artery; ECL, enhanced chemoluminescence; IP, inositol phosphate; PBS, phosphate buffered saline; PLC, phospholipase C; PTX, pertussis toxin

Introduction

Serotonergic agonists produce vasocontraction by activating 5-HT2 and 5-$HT_{1B/D}$ receptors. The 5-HT2 receptor class comprises three subtypes, 5-HT2A, 5-HT2B and 5-HT2C. These receptors couple preferentially to Gq/G11 protein to increase the hydrolysis of phosphatidyl inositol and elevate intracellular calcium. The main subtype involved in vascular smooth muscle contraction has been reported to be 5-HT2A receptor (Hoyer *et al*., 1994).

Among the 5-HT1 receptors, the 5-$HT_{1B/D}$ subtypes have been attributed to the vasocontractile response to serotonergic agonists. Some studies have shown that mainly 5-HT_{1B} receptors mediate vasocontractile response to 5-$HT_{1B/D}$ receptor agonists. (Hamel *et al*., 1993; Ullmer *et al*., 1995; Hoyer *et al*., 1994; Sgard *et al*., 1996; Nilsson *et al*., 1999; Geerts *et al*., 2000).

The pharmacology of 5-HT_{1B} and 5-HT_{1D} receptors is very similar. Several experimental results, especially those that have been obtained in recombinant experimental systems, have shown that they are both coupled to Gi/Go proteins, and inhibit cyclic AMP formation, stimulate phospholipase C (PLC), increase inositol phosphates and intracellular calcium (Zgombick *et al*., 1993; Dickenson & Hill, 1998; Wurch *et al*., 1997). However, exact signalling pathway(s) that couples the receptor stimulation to the vasocontraction has not been fully identified. Sumner *et al*. (1992) have suggested that contractile responses of dog isolated saphenous vein, which is mediated by 5-HT1-like receptors, are associated with both an influx of extracellular calcium ions and an inhibition of adenylate cyclase.

There are controversial studies about the presence of mRNA of 5-HT_{1D} receptors in vascular tissues. Some studies have failed to show the presence of mRNA of 5-HT_{1D} receptors in blood vessels (Ullmer *et al*., 1995; Sgard *et al*., 1996; Nilsson *et al*., 1999; Geerts *et al*., 2000), whereas some others have been able to show its presence in bovine, human cerebral and coronary arteries (Bouchelet *et al*., 1996; Ishida *et al*., 1999). Using two different antibodies in rabbit carotid artery preparations, we were able to detect two bands for 5-HT_{1D}-receptor protein in immunoblots, while Geerts *et al*. (2000) have failed to show the presence of mRNA of the 5-HT_{1D} receptor in the same preparation.

We therefore examined the presence of 5-HT_{1B} and 5-HT_{1D} receptors and the involvement of these subtypes in vasocontractile response induced by 5-$HT_{1B/D}$ receptor

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agonist sumatriptan in rabbit common carotid artery (CCA) as well as the role of signalling molecules such as G proteins, inositol phosphates and L-type voltage dependent calcium channels in this response.

Methods

Common carotid arteries were obtained from New Zealand albino rabbits (1.5–2.5 kg) that were exsanguinated under thiopental (35 mg kg^{-1} i.v.) anaesthesia. Surrounding tissues were removed and the vessels were placed in cold Krebs-Henseleit solution. The vessels were cut into rings (approximately 3 mm width) and the endothelium was removed by passing a cannula through the arterial lumen. Functional integrity of endothelium was tested by observing acetylcholine-mediated vasorelaxation. The rings were opened by a single cut and then fixed with stainless steel clips at both ends in organ baths of 5 ml volume containing oxygenated (5% CO_2, 95% O_2) and warmed (37°C) Krebs solution (pH = 7.4) with the following composition (in mM): NaCl 112, KCl 5, $NaHCO_3$ 25, NaH_2PO_4 1, $MgCl_2$ 0.5, $CaCl_2$ 2.5, glucose 11.5. Isometric contractions were measured using force-displacement transducers (Grass FT.03) and a general-purpose amplifier (MayCom, Ankara, Turkey) connected to a personal computer. All preparations were given an initial tension of 1–1.5 g and were allowed to equilibrate for 1 h by changing the bath buffer every 10 min. Following the equilibration period, the vessels were contracted by using 10 μM of 5-HT (which produces the maximal contractile response) and quickly washed three times. All subsequent responses were normalized with respect to this contraction. The preparations were then allowed to equilibrate for another 1 h at the above mentioned conditions. Concentration-response curves were obtained by using cumulatively increasing concentrations of agonists (in 1/2 Log steps) in the presence or absence of antagonists. Prazosin (100 nM) was added to the organ baths 20 min before obtaining sumatriptan or 5-HT responses, since our previous studies were shown that 5-HT or other serotonergic agonists may cause contraction *via* α_1-adrenoceptors in common carotid artery (Gurdal & Tulunay, 1992). All agents were tested in series separated by 1 h washout periods. Integrity of agonist responses in serial experiments was tested in parallel controls. Parameters of concentration-response curves were estimated by means of nonlinear regression of a three-parameter logistic function described by Kenakin (1984). Antagonists were added to the organ baths 30 min before the assay. None of the antagonists that were used here (i.e. prazosin, ketanserin, ritanserin, spiperone, GR127935, BRL15572, SB216641, nicardipine) affected the basal tension of the vessels when added at indicated concentrations. Considering the apparently insurmountable nature of antagonism, apparent pK_b values of the antagonists were determined by using the method described by Kenakin (1984), assuming that the origin of the observed insurmountability of the antagonism was the slow dissociation rate of the antagonists from the relevant receptors.

Incubation with pertussis toxin

The 3 mm rings were transferred to 25 cm^2 flasks containing 5–8 ml of Dulbecco's modified Eagle's medium with 250 u ml^{-1} penicillin/streptomycin and with or without 1 μg ml^{-1} pertussis toxin (Sigma) and placed in a 37°C incubator containing 5% CO_2, 95% air and incubated for 12 h. After incubation, rings were washed with oxygenated (5% CO_2, 95% O_2) and warmed (37°C) Krebs solution (pH = 7.4) and vasocontractile responses were measured as described above.

We tested the vasocontractile response to agonists before and after the incubation in pertussis toxin untreated rings as a control experiment. We did not observe any change in the potencies or maximal responses before or after the incubation (data not shown).

Membrane preparation

After removing surrounding tissues of the vessels, they were homogenized with use of a motor-driven glass to glass homogenizer in cold 50 mM Tris-HCl buffer containing 0.2 mM phenylmethylsulphonyl fluoride, 5 μg ml^{-1} aprotinin, 5 mM EDTA, pH 7.4 and centrifuged at $500 \times g$ for 10 min at 4°C. The supernatant was centrifuged ($40{,}000 \times g$) for 30 min at 4°C. The pellet was suspended in 50 mM Tris-HCl pH 7.4 buffer containing 1 mM EDTA and protein amount was measured by the method of (Bradford, 1976).

Immunoblotting

Plasma membranes (30 μg protein) were subjected to 10% SDS–PAGE and then transferred electrophoretically to nitrocellulose membrane (Laemmli, 1970). Immunoblotting was performed using antiserum against 5-HT_{1B} (SR-1B (C-19), Santa Cruz, CA, U.S.A.) or 5-HT_{1D} receptors (SR-1D (R-20) and SR1D (C-18), Santa Cruz, CA, U.S.A.) (dilutions 1 : 5000) and enhanced chemoluminescence (ECL). Briefly, nitrocellulose membranes were incubated overnight at 4°C in PBS (phosphate buffered saline: 20 mM NaH_2PO_4-Na_2HPO_4 (pH 7.6) containing 154 mM NaCl, 3% bovine serum albumin and 8% nonfat dry milk). Blots were washed several times with PBS containing 0.1% Tween, then incubated with antiserum at room temperature for 1–2 h by shaking. Blots were then washed several times with PBS, incubated with horseradish peroxidase-labelled anti-goat IgG (Santa Cruz, CA, U.S.A.) (dilutions $1 : 10{,}000^{-1}$–$1 : 12{,}000^{-1}$) for 1 h at room temperature. Blots were washed several times with PBS and then incubated with ECL Western blotting reagent (Amersham, Vienna, Austria) for 1 min and exposed to X-ray film for 45–90 s. We also used blocking peptide for the antiserum against 5-HT_{1D} receptors (SR1D (C-18) Santa Cruz, CA, U.S.A.). SR1D (C-18) antiserum (dilution $1 : 5000^{-1}$) was incubated with blocking peptide (4–10 μg ml^{-1}) for 2 h at room temperature and then immunoblotting was performed.

Inositol phosphate accumulation

Inositol phosphate accumulation was measured as described previously (Kendall & Hill, 1990; Gurdal *et al*., 1995). Briefly, surrounding tissues were removed and the vessels were placed in cold Krebs-Henseleit solution. The vessels were cut into rings (approximately 3 mm width) and the endothelium was removed by passing a cannula through the arterial lumen. The 3 mm rings were transferred to 25 cm^2 flasks containing

5–8 ml of Dulbecco's modified Eagle's medium with 250 u ml^{-1} penicillin/streptomycin, 10 μCi ml^{-1} of [^{3}H]myo-inositol (19 Ci $mmol^{-1}$, Amersham, Vienna, Austria) and placed in a 37°C incubator containing 95% air, 5% CO_2 and incubated for 12–16 h. Labelled rings were washed 4–6 times with oxygenated HEPES buffer of the following composition (mM) NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 10, HEPES 15, pH 7.4 at 37°C and placed in individual tubes and equilibrated for 30 min with or without antagonist. LiCl was added in the tubes (final 10 mM) then rings were incubated with an agonist for 30 min. Termination of reaction was carried out by removing of buffer and adding 300 μl of ice-cold 15% trichloroacetic acid. Tubes were then left on ice for 60 min. The tubes were then centrifuged ($1500 \times g$ 10 min) and supernatant were added to 125 μl of 10 mM EDTA in 1.5 ml microcentrifuge tubes, followed by 500 μl of 1:1 Freon tri-*n*-octylamine. The samples were vortexed and allowed to stand for 10 min before centrifugation ($12{,}000 \times g$ 10 min), and 300 μl of aqueous phase was taken for analysis of inositol phosphates. Samples were loaded on Dowex-1(X8) ion exchange columns (HCl form, 100–200 mesh, 1 ml). The columns were washed with 20 ml distilled H_2O; the inositol phosphates were eluted with 3 ml of 0.1 M HCl. Radioactivity was measured by liquid scintillation spectrometry.

Statistics

Results are presented as arithmetic means with standard error of the mean from *n* observations. Student's unpaired *t*-test was used to assess the significance of differences between mean values, significance being defined by a *P* value less than 0.05.

Drugs

Source of compounds used were as follows: 5-HT creatinin sulphate, prazosin, ketanserin, ritanserin, spiperone (Sigma, Munich, Germany), sumatriptan, GR127935 (Glaxo, Hertfordshire, U.K.). BRL15572 and SB216641 (Tocris, Bristol, U.K.). 5-HT_{1B} and 5-HT_{1D} receptor antibody (SR-1B(C-19), SR-1D (R-20), SR1D (C-18) Santa Cruz Biotechnology, CA, U.S.A.).

Results

Immunoblotting

Immunoblotting experiments using specific antisera against 5-HT_{1B} and 5-HT_{1D} receptors revealed the presence of one weak (93 and 105 kD) and one strong band (46 and 52 kD) for both 5-HT_{1B} and 5-HT_{1D} receptors in CCA, respectively (Figure 1). The strong bands were consistent with the 5-HT_{1B} and 5-HT_{1D} receptors. The molecular mass of weak bands was approximately twice as heavy as the strong bands. These bands might represent the dimerized form of these receptors (Ng *et al*., 1993; 1996; Xie *et al*., 1999). We also tested the presence of these receptors in rat cortex (using same amount of membrane protein 30 μg) as control experiments. Antibodies identified a single strong band (45 kD) for 5-HT_{1B} and a weak band (51 kD) for 5-HT_{1D} receptors in rat cortex (data not shown). We used two different antisera for 5-HT_{1D} receptor that recognized the same bands in the same preparations. The 93 and 105 kD bands were suppressed significantly by the addition of the blocking peptide of the antibody SR_{1D} (C-18) (data not shown).

Vasocontractile responses

The non-selective 5-$HT_{1B/D}$ receptor agonist sumatriptan produced a vasocontractile response in CCA with a pD_2 value of 5.1 ± 0.1. This response was antagonized by 5-HT_{1B} antagonist SB216641 with an apparent pKb value of 8.6 ± 0.11) (Figure 2). Low concentrations (0.1 μM) of the 5-HT_{1D} receptor antagonist, BRL15572 did not affect the vasocontractile response to sumatriptan whereas the higher concentrations (1 μM) produced a slight rightward shift in the concentration-response curve (apparent pKb: 6.0 ± 0.1) without affecting the maximal response (Figure 3).

The vasocontractile response induced by sumatriptan was highly sensitive to PTX-treatments of CCA. The response was also sensitive to calcium channel blockage by nicardipine (Figure 4) or nitrendipine (data not shown).

Inositol phosphate accumulation

5-HT (10 μM) and phenylephrine (10 μM), but not sumatriptan (1 mM) stimulated inositol phosphate (IP) accumula-

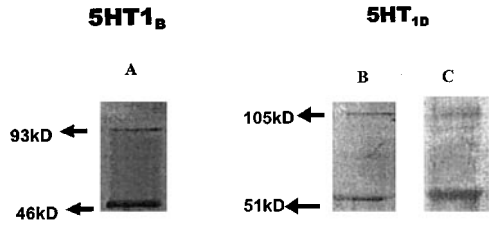

Figure 1 Membranes from carotid artery (30 μg) were subjected to SDS–PAGE and transferred to nitrocellulose membranes for immunoblotting. The (A) 5-HT_{1B} and (B,C) 5-HT_{1D} receptor subtypes were detected with specific antisera (dilution 1:5000, SR-1B(C-19), SR-$_{1D}$ (R-20), SR-$_{1D}$ (C18) Santa Cruz Biotechnology, CA, U.S.A.) by chemiluminescence. Data is representative of three independent experiments.

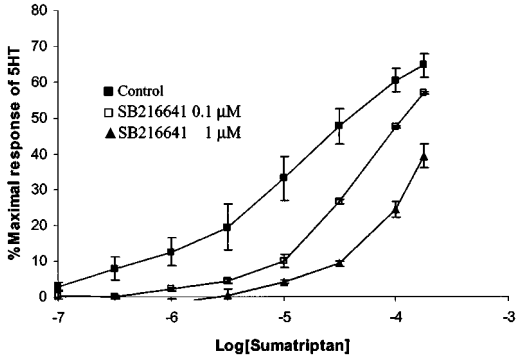

Figure 2 Effect of 5-HT_{1B} receptor antagonist SB216641 on concentration-response curves of sumatriptan in common carotid artery. Maximal contractions induced by 5-HT were (mN) 19.4 ± 1.9 in carotid artery. Data are presented as mean ± s.e.mean ($n = 5-6$).

tion in CCA (Figure 5). Higher concentration of 5-HT did not increase IP accumulation, any further. Spiperone (0.1 μM) or GR127935 (10 μM) was used to test the involvement of 5-HT2 and 5-$HT_{1B/D}$ receptors in IP accumulation induced by 5-HT (10 μM). The response was antagonized by spiperone (0.1 μM) but not by GR127935 (10 μM) (Figure 5). Neither spiperone (0.1 μM) nor GR127935 (10 μM) affected the basal IP accumulation (data not shown).

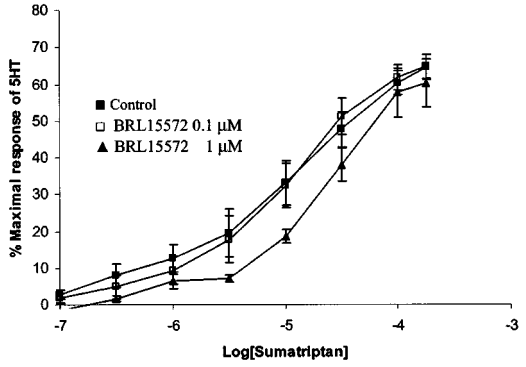

Figure 3 Effect of 5-HT_{1D} receptor antagonist BRL15572 on concentration-response curves of sumatriptan in common carotid artery. Maximal contractions induced by 5-HT were (mN) 19.4±1.9 in carotid artery. Data are presented as mean±s.e.mean (n=5–6).

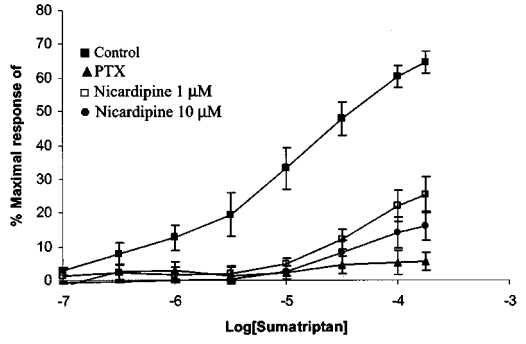

Figure 4 Effect of PTX treatment or nicardipine on concentration-response curves of sumatriptan in common carotid artery. Maximal contractions induced by 5-HT were (mN) 19.4±1.9 in carotid artery. Data are presented as mean±s.e.mean (n=5–6).

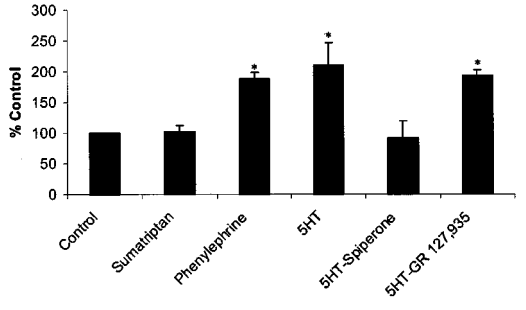

Figure 5 5-HT, phenylephrine, but not sumatriptan stimulated IP accumulation in carotid artery; spiperon (0.1 μM), GR127935 (10 μM). Responses were expressed as a percentage of the values from unstimulated rings. The basal count is 665±100 d.p.m. Data are presented as mean±s.e.mean (n=5–6). (*indicates statistical difference from control, student t-test, $P<0.05$).

Discussion

In the present study, we intended to determine the role of 5-HT_{1B} and 5-HT_{1D} receptor subtypes in vasocontractile response of CCA to sumatriptan.

Ullmer *et al.* (1995) and Sgard *et al.* (1996) have been able to show the presence of mRNA of 5-HT_{1B} but not that of 5-HT_{1D} receptors in rat, porcine, canine vascular tissues and in endothelial or smooth muscle primary cultures from several vessels of human. However there are some other studies where the presence of 5-HT_{1D} receptor mRNA has been demonstrated in bovine, human cerebral and coronary arteries (Bouchelet *et al.*, 1996; Ishida *et al.*, 1999). Nevertheless, even in these studies the authors have admitted the difficulty of such detection.

We observed two bands for 5-HT_{1B} (93 and 46 kD) or 5-HT_{1D} (105 and 51 kD) receptors by using the relevant antisera. Low molecular weight bands perfectly matched the receptor molecule. Heavier bands may represent the dimerized form of these subtypes, since the molecular weights of these bands were twice as much as the light ones. Dimerization of 7-TM receptors has been shown in several studies by using similar immunoblotting studies (Cvejic & Devi, 1997; Hebert *et al.*, 1996; Devi, 2000) including 5-HT_{1B} and 5-HT_{1D} receptors (Ng *et al.*, 1993; 1996; Xie *et al.*, 1999).

Although we observed only two bands by using anti-human 5-HT_{1D}-receptor antiserum in our Western blots, the interpretation of these immunoreactivities could not be obvious for the following reasons. First, Geerts *et al.* (2000) have been unable to show the presence of mRNA of 5-HT_{1D} receptor, and they only observed nonspecific bands in their Western blot analysis in this preparation. Secondly, our functional studies did not support the functional presence of 5-HT_{1D} receptors in CCA (see also Akin *et al.* this issue). These observations, when taken together, may indeed suggest the absence of 5-HT_{1D} receptors in this artery and our Western blots may be interpreted as a casual artefact. However, other possibilities can still be considered in this case since we observed 5-HT_{1D} immunoreactivity by using two different antisera: (1) 5-HT_{1D} receptor may be there and may be coupled to functions other than contraction and adenylate cyclase inhibition, but its mRNA levels in the cells may be below the detection limits; or (2) we are facing a different subtype of 5-HT_{1D} receptor (still uncoupled from vasocontraction and cyclase inhibition) which cannot be detected in RT–PCR due to the configuration of the probes currently used to detect mRNA of this receptor, but can be recognized by the relevant antibodies.

Sumatriptan is a non-selective agonist and can bind and stimulate both 5-HT_{1B} and 5-HT_{1D} receptors. Considering the fact that sumatriptan-induced vasocontraction in the present preparation was antagonized: (1) by SB216641 with an apparent pKb value of 8.6 (which was consistent with its affinity for 5-HT_{1B} receptor (Price *et al.*, 1997; Saxena *et al.*, 1998)); and (2) weakly by BRL15572 with an apparent pKb value of 6.0 (which was consistent with its affinity for 5-HT_{1B} but not for 5-HT_{1D} (Price *et al.*, 1997; Saxena *et al.*, 1998)), we concluded that the observed response was mainly mediated by 5-HT_{1B} and that the involvement of 5-HT_{1D} was insignificant or none.

The second question concerns the coupling mechanism of 5-HT_{1B} receptors to vasocontraction in CCA. In general,

pharmacomechanical coupling of Gi/o-coupled receptors in vasocontraction is poorly understood. Zgombick *et al*. (1993) have shown that sumatriptan increases inositol phosphate accumulation by stimulating human 5-HT_{1B} and 5-HT_{1D} receptors in heterologue expression systems through activating a pertussis toxin (PTX)-sensitive G protein, which might be a potential explanation for sumatriptan-induced vasocontraction. However, we were unable to show sumatriptan-induced IP accumulation in the present preparations. The lack of IP response is unlikely to be a technical problem in the present preparation, since the α_1-adrenoceptor agonist phenylephrine, which is well known for its PLC coupling, induced a perfectly measurable IP response in this preparation. Likewise, 5-HT itself stimulated IP accumulation in this artery, which was blocked by the 5-HT_2 receptor antagonist spiperone and not by 5-$HT_{1B/D}$ receptor antagonist GR127935. Therefore, we concluded that sumatriptan-induced vasocontration is mediated by 5-HT_{1B} subtype in a manner that is seemingly independent of IP accumulation.

Sensitivity of sumatriptan mediated vasocontraction to nicardipine and PTX treatment suggests the involvement of L-type voltage dependent calcium channels and Gi/o proteins in the 5-HT_{1B}-receptor stimulation. In agreement with our results, Sumner *et al*. (1992) have also shown that sumatriptan-induced contraction depends on the extracellular calcium in dog saphenous vein. However, the question as to how 5-HT_{1B} receptors are coupled to L-type voltage dependent calcium channels still remains. The only conclusion we can draw from the present data is that 5-HT_{1B}-L type voltage dependent calcium channel coupling is not directly mediated by the inhibition of adenylate cyclase (see also Akin *et al*. this issue). If this is indeed the case, to investigate the functional importance of 5-HT_{1B}-mediated inhibition of adenylate cyclase in vascular tissues may be an interesting experimental task. Such an attempt may also contribute to the clarification of the therapeutic potency of sumatriptan in migraine.

In conclusion (1) CCA expresses 5-HT_{1B} receptor subtype and sumatriptan induces vasocontraction by stimulating these receptors; (2) this response is mainly mediated by the activation of L-type voltage dependent calcium channels, but not by the PLC pathway; (3) the vasocontraction is mediated by PTX sensitive Gi/o proteins; and (4) the presence of 5-HT_{1D} receptor or an unknown receptor protein which carries the same antigenic epitopes as 5-HT_{1D} in CCA needs further clarification.

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