ELSEVIER

European Journal of Pharmacology 329 (1997) 49–53

ejp

Flunarizine, an anti-migraine agent, impairs nitroxidergic nerve function in cerebral arteries

Kazuhide Ayajiki, Tomio Okamura, Noboru Toda *

Department of Pharmacology, Shiga University of Medical Sciences, Seta, Ohtsu 520-21, Japan

Received 2 January 1997; revised 7 April 1997; accepted 11 April 1997

Abstract

Flunarizine is an anti-migraine agent that blocks the Ca^{2+} entry across cell membrane. In order to obtain a clue of mechanisms underlying the migraine headache, modifications by flunarizine of the response to nitric oxide (NO), a cerebral vasodilator and algogenic agent, derived from perivascular nerves were evaluated. Relaxations due to nerve stimulation by electrical pulses (5 Hz) and nicotine (10^{-4} M) in canine cerebral arterial strips were attenuated by treatment with flunarizine dose-dependently, whereas the responses to exogenous NO (10^{-7}–10^{-6} M) and nitroprusside (10^{-8}–10^{-6} M) were unaffected. The inhibition by the Ca^{2+} entry blocker of the response to electrical nerve stimulation and nicotine was obtained in a concentration (10^{-6} M) that did not significantly relax the arterial strips. NO derived from perivascular nerve may be one of the factors involved in the genesis of migraine attack, which is expected to be relieved by a reduction of neural NO synthase activity associated with a decreased Ca^{2+} influx by flunarizine during nerve activation.

Keywords: Migraine; Flunarizine; Nitric oxide (NO); Vasodilator nerve

1. Introduction

Flunarizine, a non-selective, piperadine-type Ca^{2+} channel inhibitor (Holmes et al., 1984), is effective in preventing attacks in migraine patients. The effectiveness has been revealed by double-blind trials performed by several research groups (Louis, 1981; Frenken and Nuijten, 1984; Mendenopoulos et al., 1985; Sorge and Marano, 1985; Sorensen et al., 1986; Sorge et al., 1988). On the other hand, dihydropyridine Ca^{2+} antagonists, such as nimodipine, have minor or no prophylactic action (Toda and Tfelt-Hansen, 1993), despite their potent cerebral vasodilator actions (Tanaka et al., 1980; Harper et al., 1981). Therefore, mechanisms of anti-migraine action of flunarizine other than a relief of cerebral vasospasm are postulated. Flunarizine passes the blood–brain barrier and reaches the brain to modulate nerve functions (Leone et al., 1991; Shibuya and Watanabe, 1992).

From the time of our discovery of nitroxidergic innervation in intra- and extracranial arteries (Toda and Okamura, 1992a), we have hypothesized that nitric oxide (NO) derived from the perivascular nerve and endothelium participates in the migraine headache, due to its potent cranial vasodilator and algogenic actions (Toda and Okamura, 1992a; Toda, 1993). Recent findings on a provocation by NO donors of the migraine attack in patients (Iversen et al., 1989) and an effectiveness of N^G-monomethyl-L-arginine in relieving the spontaneous migraine attack without aura (personal communication with Drs. Lassen and Olesen) support our hypothesis.

Therefore, the present study was undertaken to determine whether flunarizine impairs the nitroxidergic nerve function in isolated canine cerebral arteries. The neurogenic response mediated by NO was obtained by electrical pulses transmurally applied and by nicotine which stimulates nerve terminals for the release of neurotransmitters (Toda, 1982; Nedergaard, 1988).

2. Materials and methods

2.1. Preparation

The studies review board at our university approved the use of canine blood vessels in this study.

* Corresponding author. Tel.: (81-775) 48-2181; Fax: (81-775) 48-2183.

0014-2999/97/$17.00
PII S0014-2999(97)00126-X

Mongrel dogs of either sex, weighing 7–14 kg, were killed by bleeding from the carotid arteries under pentobarbital anaesthesia (50 mg/kg, i.p.). The brain was rapidly removed, and basilar and middle cerebral arteries were isolated. The arteries were helically cut into strips of approx. 20 mm long. The endothelium was removed by gently rubbing the intimal surface with a cotton ball. The specimen was vertically fixed between hooks in a muscle bath containing modified Ringer-Locke solution, which was maintained at 37 ± 0.3°C and aerated with a mixture of 95% O_2 and 5% CO_2. The hook anchoring the upper end of the strips was connected to the lever of a force-displacement transducer. The resting tension was adjusted to 1.5 g which is optimal for inducing the maximal contraction. Constituents of the solution were as follows (mM): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25.0, and dextrose 5.6. The pH of the solution was 7.38–7.43. Before the start of experiments, all of the strips were allowed to equilibrate for 60–90 min in the bathing media, during which time the fluid was replaced every 10–15 min.

2.2. Experimental protocols

Isometric mechanical responses were displayed on an ink-writing oscillograph. The contractile response to 30 mM K^+ was first obtained, and the preparations were repeatedly washed and equilibrated.

2.2.1. Transmural electrical stimulation

The arterial strips were placed between stimulating electrodes. Intramural nerve terminals were stimulated by 0.2-ms electrical square pulses at frequencies of 2, 5 and 20 Hz for periods of 100, 40 and 10 s, respectively. In order to test the effect of flunarizine, transmural electrical stimulation at 5 Hz for 40 s was applied every 10 min, until the response was determined to be reproducible. Then, flunarizine (10^{-6} or 3×10^{-6} M) was applied, and electrical stimulation was repeated until stabilized responses were obtained. Stabilization of the responses was obtained 30–40 min after the treatment. Then, the effects of additional calcium (2.2 mM) on the response to electrical stimulation were also studied. At the end of each series of experiments, papaverine (10^{-4} M) was applied to attain the maximal relaxation.

2.2.2. Nicotine, nitric oxide and sodium nitroprusside

In the endothelium-denuded arterial strips, nicotine (10^{-4} M), NO (10^{-7} and 10^{-6} M) and sodium nitroprusside (10^{-8}–10^{-6} M) were successively applied to the bathing media. The concentration of nicotine used is submaximal in producing the relaxation (Toda and Okamura, 1991). Papaverine (10^{-4} M) was applied at the end to attain the maximal relaxation. When responses to these drugs were stabilized, the strips were treated for 30–40 min with flunarizine, before the addition of the agonists. Relaxant responses to transmural electrical stimulation and the agonists relative to those caused by papaverine were presented. Removal of the endothelium was verified by abolition of the relaxation caused by 10^{-8} M substance P (Toda and Okamura, 1991).

2.3. Statistics and drugs used

Results shown in the text and figures are expressed as mean values ± S.E. All reported n values refer to the number of strips from different dogs. Statistical analyses were made using the Student's paired and unpaired t-test or the Tukey's method after one-way analysis of variance. Drugs used were flunarizine hydrochloride (Kyowa-Hakko, Tokyo, Japan), nicotine (base), hexamethonium bromide (Nacalai Tesque, Kyoto, Japan), sodium nitroprusside (Merck, Darmstadt, Germany), substance P (Peptide Research Institute, Minoh, Japan), tetrodotoxin (Sankyo, Tokyo, Japan), prostaglandin $F_{2\alpha}$ (Pharmacia-Upjohn, Tokyo, Japan) and papaverine hydrochloride (Dainippon, Osaka, Japan). Flunarizine (10^{-3} M) was dissolved with ethanol, and 0.1 and 0.3% of the bath volume was added. In the preliminary experiments, these concentrations of the solvent alone did not affect the responses to electrical stimulation and agonists ($n = 3$). Responses to NO were obtained by adding the $NaNO_2$ solution adjusted at pH 2 (Furchgott, 1988), and the concentrations of $NaNO_2$ solution in the bathing media were expressed as those of NO.

3. Results

3.1. Response to transmural electrical stimulation

In cerebral arterial strips partially contracted with prostaglandin $F_{2\alpha}$, transmural electrical stimulation at 2, 5 and 20 Hz produced a frequency-related relaxation, which was abolished by treatment with tetrodotoxin (3×10^{-7} M). The neurogenic response induced at 5 Hz was consistent and reproducible; therefore, this frequency was used for the evaluation of flunarizine actions.

Treatment with flunarizine (10^{-6} and 3×10^{-6} M) attenuated the response to electrical nerve stimulation in a dose-dependent manner, and the inhibition was reversed by the addition of 2.2 mM Ca^{2+} (Fig. 1). The stimulation-induced relaxation was significantly greater in the strips treated with high Ca^{2+} after flunarizine, as compared with that under control conditions. Typical tracings of the response before and after flunarizine and then Ca^{2+} are illustrated in Fig. 2.

Flunarizine produced a slight or no relaxation at 10^{-6} M ($7.2 \pm 3.6\%$, $n = 4$, $P > 0.05$; paired t-test) but signifi-

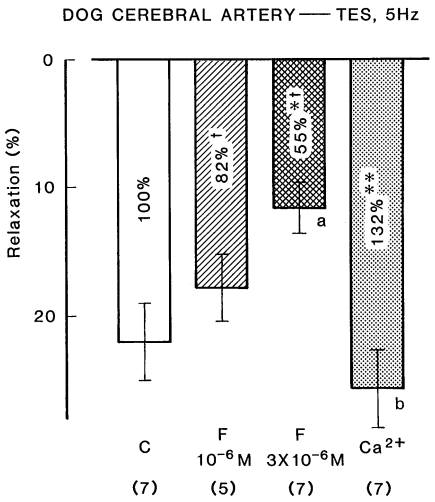

Fig. 1. Inhibition by flunarizine (F) of the relaxant response to transmural electrical stimulation (TES, 5 Hz) of cerebral arterial strips and restoration by Ca^{2+} (2.2 mM) of the response. The ordinate represents relaxations induced by nerve stimulation relative to those by 10^{-4} M papaverine. Numbers in columns denote relative values to control response (C), and numbers in parentheses indicate the number of strips from different dogs. Significantly different from control, [a] $P < 0.05$; significantly different from the value with 3×10^{-6} M flunarizine, [b] $P < 0.01$ (Tukey's method). Significantly different from control (100% in the column), [*] $P < 0.01$; [**] $P < 0.05$; significantly different from the value with Ca^{2+}, [+] $P < 0.01$ (Tukey's method). Vertical bars represent S.E.

cant relaxations at 3×10^{-6} M ($23.0 \pm 6.1\%$, $n = 5$, $P < 0.02$). On the other hand, significant inhibitions in the response to electrical stimulation were obtained at 10^{-6} and 3×10^{-6} M flunarizine; mean values were $18.5 \pm 3.6\%$ ($n = 5$, $P < 0.01$; paired t-test) and $44.7 \pm 6.5\%$ ($n = 7$; $P < 0.001$), respectively.

3.2. Responses to nicotine, NO and sodium nitroprusside

The effects of flunarizine on the relaxant responses to nicotine (10^{-4} M), NO (10^{-7} and 10^{-6} M) and sodium nitroprusside (10^{-8}–10^{-6} M) were evaluated. Typical recordings are shown in Fig. 3. The nicotine-induced

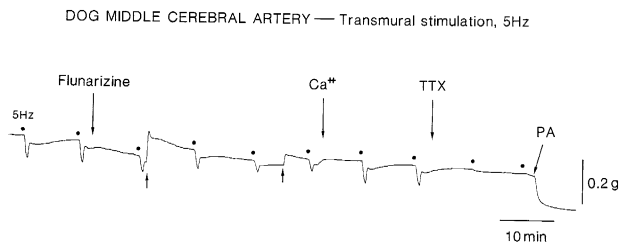

Fig. 2. Typical tracing of the response to transmural electrical stimulation (5 Hz) before and after treatment with flunarizine (3×10^{-6} M) and Ca^{2+} (2.2 mM) in a middle cerebral arterial strip contracted with prostaglandin $F_{2\alpha}$. Upward arrows indicate the application of supplemental doses of prostaglandin $F_{2\alpha}$ to raise the arterial tone. TTX, 3×10^{-7} M tetrodotoxin; PA, 10^{-4} M papaverine to attain the maximal relaxation.

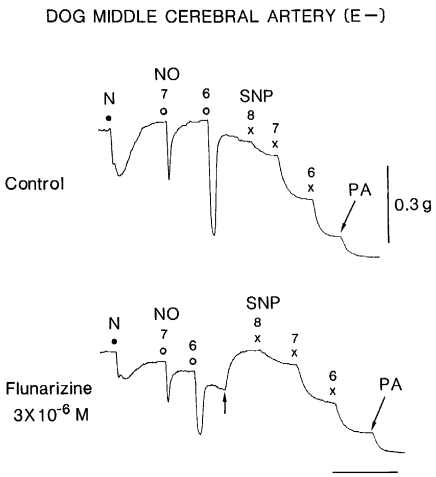

Fig. 3. Typical tracings of the responses to nicotine (10^{-4} M), NO (10^{-7} and 10^{-6} M) and sodium nitroprusside (SNP, 10^{-8}–10^{-6} M) before (control) and after treatment with flunarizine in a middle cerebral arterial strip contracted with prostaglandin $F_{2\alpha}$. The upward arrow denotes the application of a supplemental dose of prostaglandin $F_{2\alpha}$. PA represents 10^{-4} M papaverine that produced the maximal relaxation.

relaxation was significantly attenuated by the Ca^{2+} channel inhibitor (10^{-6} and 3×10^{-6} M) in a dose-dependent manner, whereas the responses to NO and nitroprusside were not influenced. Quantitative data are summarized in Fig. 4. Hexamethonium (10^{-5} M) abolished the response. The relaxations elicited by NO and nitroprusside were not affected by flunarizine in a concentration (3×10^{-6} M) sufficient to moderately depress the response to nerve stimulation.

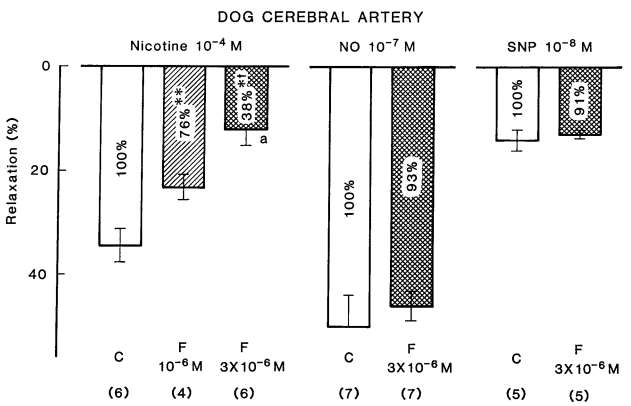

Fig. 4. Modifications by flunarizine (F) of the responses to nicotine, NO and sodium nitroprusside (SNP) of cerebral arterial strips contracted with prostaglandin $F_{2\alpha}$. The ordinate represents relaxations relative to those by 10^{-4} M papaverine. Numbers in the columns indicate the value relative to control (C); numbers in parentheses represent the number of strips from different dogs. Significantly different from control, [a] $P < 0.01$ (Tukey's method). Significantly different from control (100% in the column), [*] $P < 0.01$, [**] $P < 0.05$; significantly different from the value with 10^{-6} M flunarizine, [†] $P < 0.05$ (paired t-test). Vertical bars represent S.E.

4. Discussion

It is hypothesized that vasodilatation induced by perivascular nerve stimulation is mediated by NO liberated as a neurotransmitter in canine intracranial extracerebral (middle cerebral, basilar, etc.) and extracranial (retinal central and superficial temporal) arteries (Toda and Okamura, 1990, 1991, 1992a; Toda et al., 1994) that are expected to participate in vascular headache (Toda, 1993). The reasons are: (1) neurogenic vasodilatation is abolished by NO synthase inhibitors, and is restored by the addition of L-arginine; (2) release of NO_X from superfused arteries is increased during nerve stimulation, and the effect is abolished by NO synthase inhibitors; (3) oxyhemoglobin and methylene blue abolish the relaxation elicited by nerve stimulation and exogenous NO; and (4) the presence of perivascular nerve fibers containing NO synthase immunoreactivity is histologically demonstrated.

The relaxant response to nerve stimulation by electrical pulses or nicotine was attenuated by treatment with flunarizine, whereas the responses to NO and nitroprusside, a NO donor, was not influenced. The concentration (10^{-6} M) sufficient to depress the response to electrical stimulation and nicotine did not significantly relax the arteries contracted with prostaglandin $F_{2\alpha}$. The flunarizine-induced inhibition of the neurogenic response was reversed by raising the external Ca^{2+} concentration. These findings suggest that this Ca^{2+} channel inhibitor blocks the transmembrane influx of Ca^{2+} due to action potentials into nerve terminals, resulting in a reduced activation of NO synthase and a decreased production/release of NO. Nicardipine, an L-type Ca^{2+} channel inhibitor, does not inhibit the neurogenic relaxation (Toda and Okamura, 1992b) and the binding of ω-conotoxin, an N-type Ca^{2+} channel inhibitor (McCleskey et al., 1987), to synaptosomes of the rat brain (Hosono et al., 1995). ω-Conotoxin, an N-type Ca^{2+} channel inhibitor, suppresses the response to electrical nerve stimulation (Toda et al., 1995). Therefore, flunarizine impairs nitroxidergic nerve function possibly by interference with the influx of Ca^{2+} through non-L type channels, including the N-type. Inhibition of the N-type Ca^{2+} channel by flunarizine has been reported in hippocampal neurons (Tytgat et al., 1991). Recently developed dihydropyridine Ca^{2+} channel inhibitors reportedly decrease the autonomic efferent nerve function; AE0047 attenuates vascular responses of adrenergic as well as nitroxidergic nerve stimulation by a decreased release of neurotransmitters (Okamura et al., 1992; Nishikawa et al., 1995), and cilnidipine inhibits the sympathetic nerve function in vivo and the ω-conotoxin binding to synaptosomes in vitro (Hosono et al., 1995). It would be intriguing to know if these inhibitors effectively prevent attacks in migraine patients, as does flunarizine.

Plasma concentrations of flunarizine in healthy volunteers at an oral dose of 10 mg are in a range of 82–115 ng/ml (Araki et al., 1982) (approx. equivalent to (2.0–2.9) $\times 10^{-7}$ M), which are 1/5–1/3 of the effective concentration of the Ca^{2+} entry blocker in the present study (10^{-6} M). Tissue accumulation of flunarizine (Leone et al., 1991), its lipid solubility, in vivo effectiveness of the drug and species variation of its efficacy may bridge the gap of effective concentrations in plasma in vivo and bathing medium in vitro. Involvement of NO, a potent vasodilator and algogenic agent, in the pathogenesis of migraine has been suggested from studies on migraine patients (Olesen et al., 1994). The present study may indicate that the anti-migraine action of flunarizine is associated with a suppression of synthesis/release of NO in perivascular nerve terminals in intra- and extracranial arteries.

Acknowledgements

This work was supported in part by Grant-in Aid for Scientific Research (B) and (C) from the Ministry of Education, Science, Sports and Culture, Japan.

References

Araki, G., Saito, T., Ishii, A., 1982. Phase I study of KW-3149 (flunarizine hydrochloride). Kiso to Rinsho 16, 171–177, in Japanese.

Frenken, C.W.G.M., Nuijten, S.T.M., 1984. Flunarizine, a new preventive approach to migraine. Clin. Neurol. Neurosurg. 86, 17–20.

Furchgott, R.F., 1988. Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: Vanhoutte, P.M. (Eds.), Vasodilatation, Raven Press, New York, NY, pp. 401–414.

Harper, A.M., Craigen, L., Kazda, S., 1981. Effect of the calcium antagonist, nimodipine, on cerebral blood flow and metabolism in the primate. J. Cerebr. Blood Flow Metab. 1, 349–356.

Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M., Avery, G.S., 1984. Flunarizine. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic use. Drug 27, 6–44.

Hosono, M., Fujii, S., Hiruma, T., Watanabe, K., Hayashi, Y., Ohnishi, H., Takata, Y., Kato, H., 1995. Inhibitory effect of cilnidipine on vascular sympathetic neurotransmission and subsequent vasoconstriction in spontaneously hypertensive rats. Jpn. J. Pharmacol. 69, 127–134.

Iversen, H.K., Olesen, J., Tfelt-Hansen, P., 1989. Intravenous nitroglycerin as an experimental model of vascular headache. Basic characteristics. Pain 38, 17–24.

Leone, M., Grazzi, L., La Mantia, L., Bussone, G., 1991. Flunarizine in migraine: a minireview. Headache 31, 388–391.

Louis, P., 1981. A double-blind placebo-controlled prophylactic study of flunarizine in migraine. Headache 21, 235–239.

McCleskey, E.W., Fox, A.P., Tsien, R.W., Yoshikami, D., 1987. ω-Conotoxin, direct and persistent blockade of specific types of calcium channels in neurons but not muscle. Proc. Natl. Acad. Sci. USA 84, 4327–4331.

Mendenopoulos, G., Manafi, T., Logothesis, I., Bostantjopoulou, S., 1985. Flunarizine in the prevention of classical migraine: a placebo-controlled evaluation. Cephalalgia 5, 31–37.

Nedergaard, O.A., 1988. Effect of nicotine on neuroeffector transmission in blood vessels. In: Rand, M., Thurau, K. (Eds.) The Pharmacology of Nicotine. IRL Press, Washington, DC, pp. 143–162.

Nishikawa, M., Okamura, T., Toda, N., 1995. Effect of the novel calcium antagonist AE0047 on responses of isolated dog blood vessels to vasoconstricting agents and to nerve stimulation. Folia Pharmacol. Jpn. 106, 19–29, text in Japanese, abstract in English.

Okamura, T., Nishikawa, M., Yoshida, K., Toda, N., 1992. AE0047, a new dihydropyridine Ca^{2+} entry blocker, inhibits the responses to adrenergic nerve stimulation and substance P in dog mesenteric arteries. Eur. J. Pharmacol. 220, 27–33.

Olesen, J., Thomsen, L.L., Iversen, H., 1994. Nitric oxide is a key molecule in migraine and other vascular headaches. Trends Pharmacol. Sci. 15, 149–153.

Shibuya, T., Watanabe, Y., 1992. Central effect of Ca^{2+} channel blockers: multiple sites of action. Folia Pharmacol. Jpn. 100, 239–247, text in Japanese, abstract in English.

Sorensen, P.S., Hansen, K., Olesen, J., 1986. A placebo-controlled, double-blind, cross-over trial of flunarizine in common migraine. Cephalalgia 6, 7–14.

Sorge, F., Marano, E., 1985. Flunarizine v. placebo in childhood migraine. A double-blind study. Cephalalgia 5 (Suppl. 2), 145–148.

Sorge, F., De Simone, R., Marano, E., Nolano, M., Orefice, G., Carrieri, P., 1988. Flunarizine in prophylaxis of childhood migraine. A double-blind, placebo-controlled, cross-over study. Cephalalgia 8, 1–6.

Tanaka, K., Gotoh, F., Muramatsu, F., Fukuuchi, Y., Amano, T., Okayasu, H., Suzuki, N., 1980. Effects of nimodipine (Bay e 9736) on cerebral circulation in cats. Arzneim.-Forsch. 30, 1494–1497.

Toda, N., 1982. Relaxant responses to transmural stimulation and nicotine of dog and monkey cerebral arteries. Am. J. Physiol. 243, H145–H153.

Toda, N., 1993. Endothelial and neural nitric oxide-induced cerebral artery dilatation. In: Olesen, J., Schmidt, R.F. (Eds.), Pathophysiological Mechanisms of Migraine, VCH Publishers, New York, NY, pp. 37–43.

Toda, N., Okamura, T., 1990. Possible role of nitric oxide in transmitting information from vasodilator nerve to cerebroarterial muscle. Biochem. Biophy. Res. Commun. 170, 308–313.

Toda, N., Okamura, T., 1991. Role of nitric oxide in neurally induced cerebroarterial relaxation. J. Pharmacol. Exp. Ther. 258, 1027–1032.

Toda, N., Okamura, T., 1992a. Regulation by nitroxidergic nerve of arterial tone. News Physiol. Sci. 7, 148–152.

Toda, N., Okamura, T., 1992b. Different susceptibility of vasodilator nerve, endothelium and smooth muscle functions to Ca^{++} antagonists in cerebral arteries. J. Pharmacol. Exp. Ther. 261, 234–239.

Toda, N., Tfelt-Hansen, P., 1993. Calcium antagonists, In: Olesen, J., Tfelt-Hansen, P., Welch, K.M.A. (Eds.), Raven Press, New York, NY, pp. 383–390.

Toda, N., Kitamura, Y., Okamura, T., 1994. Role of nitroxidergic nerve in dog retinal arterioles in vivo and arteries in vitro. Am. J. Physiol. 266, H1985–H1992.

Toda, N., Uchiyama, M., Okamura, T., 1995. Prejunctional modulation of nitroxidergic nerve function in canine cerebral arteries. Brain Res. 700, 213–218.

Tytgat, J., Pauwels, P.J., Vereecke, J., Carmeliet, E., 1991. Flunarizine inhibits a high-threshold inactivating calcium channel (N-type) in isolated hippocampal neurons. Brain Res. 549, 112–117.