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Mechanisms of blockade by the novel migraine prophylactic agent, dotarizine, of various brain and peripheral vessel contractility

Ana Ruiz-Nuño [a], Mercedes Villarroya [a], María Cano-Abad [a], Aranzazu Rosado, Gloria Balfagón [c], Manuela G. López [a,*], Antonio G. García [a,b]

[a] *Departamento de Farmacología, Facultad de Medicina, Instituto Teófilo Hernando, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, 28029 Madrid, Spain*

[b] *Servicio de Farmacología Clínica and Instituto de Gerontología, Hospital de la Princesa, U.A.M., Diego de León 62, 28006 Madrid, Spain*

[c] *Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, 28029 Madrid, Spain*

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Abstract

The novel antimigraineur, dotarizine, inhibited 5-HT (5 hydroxytryptamine)-evoked contractions of rabbit vertebral, aorta, femoral and mesenteric arteries, with IC_{50}s of 1.35, 1.40, 0.52 and 1.09 μM, respectively. Flunarizine had little effect on these contractions, while ketanserin was more potent (IC_{50}s of 0.17 μM for vertebral, 0.22 μM for aorta, 0.05 μM for femoral and 0.03 μM for mesenteric arteries). At 10 μM, dotarizine caused 40% blockade of K^+-evoked contractions of rabbit aorta, and 70% inhibition of 5-HT-evoked responses; these values were 30% and 20% for 10 μM flunarizine. Contractions of rabbit aorta elicited by noradrenaline, angiotensin II or prostaglandin $F_{2\alpha}$ were not affected by 10 μM dotarizine or flunarizine. Ketanserin shifted to the right, in parallel, the concentration–response curves for 5-HT in rabbit aorta; however, dotarizine caused a non-competitive type of blockade, increasing the maximum 5-HT contraction at 30 nM and decreasing it at 3 and 30 μM. K^+-evoked contractions of rabbit aorta were halved by 3 μM dotarizine in a voltage-independent manner; flunarizine caused a delayed-type, non-reversible post-drug blockade, and exhibited some voltage-dependence. Blockade by nifedipine was voltage-dependent and fully reversible. Ca^{2+}-evoked contractions of depolarised bovine middle cerebral arteries were blocked by 1–3 μM dotarizine in a non-surmountable manner. Contraction of these vessels evoked by electrical stimulation was blocked 50% and 70% by 1 and 3 μM dotarizine, respectively. Dotarizine (1–3 μM) also inhibited to a similar extent the K^+-evoked [^{3}H]noradrenaline release from cultured rat sympathetic neurones. These data suggest that the mechanism of blockade by dotarizine of cerebral vessels contractility has three components: (i) presynaptic inhibition of noradrenaline release; (ii) blockade of postsynaptic vascular 5-HT receptors; (iii) blockade of Ca^{2+} entry into the vascular smooth muscle cell cytosol. The compound does not affect the vascular receptors for noradrenaline, angiotensin II or prostaglandin $F_{2\alpha}$.

Keywords: Dotarizine; Flunarizine; 5-HT receptor; Vessel contractility

1. Introduction

Dotarizine (1-(diphenylmethyl)-4-[3-(2-phenyl-1,3-dioxolan-2-yl)propyl]-piperazine) is a novel piperazine derivative structurally related to flunarizine (Gubert et al., 1987). It is currently being evaluated in clinical trials to establish its prophylactic effects in migraine and vertigo (Galiano et al., 1993; Perello et al., 1998). Dotarizine blocks Ca^{2+} uptake and vessel contractility in rabbit basilar and aorta smooth muscle (Tejerina et al., 1993), as well as the inward currents through neuronal high voltage-activated Ca^{2+} channels (Villarroya et al., 1995). In addition, dotarizine has been shown to block 5-HT receptors (Brasó et al., 1996; Montiel et al., 1997). Hence, its prophylactic actions in migraine might be related to its 5-HT receptor blocking properties and/or Ca^{2+}-entry blocking properties.

The subtypes of 5-HT receptors that mediate contraction in vascular smooth muscle are heterogeneous, as the following examples illustrate. The 5-HT-induced contraction of rabbit aorta is mediated by 5-HT_{2A} receptors (Apperly et al., 1976; Feniuk et al., 1985). Also, vasocon-

* Corresponding author. Tel.: +34-91-3975386; fax: +34-91-3975386.
E-mail address: manuela.garcia@uam.es (M.G. López).

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striction induced by 5-HT in the rabbit femoral artery (Grandaw and Purdy, 1996) and vertebral artery (Griffith et al., 1982) is mediated by 5-HT_2 receptors. On the other hand, it has been reported that 5-HT_1-like receptors are mainly involved in vasoconstriction induced by 5-HT in rabbit basilar artery (Bradley et al., 1986). It has also been shown that a mixed population of 5-HT_1-like and 5-HT_{2A} receptors seems to mediate 5-HT-induced contraction of rabbit isolated mesenteric artery (Yildiz and Tuncer, 1995).

Investigations using oocytes expressing 5-HT_2 receptors subtypes showed that dotarizine preferentially blocks 5-HT_{2A} receptors compared to 5-HT_{2C} receptors (Montiel et al., 1997). So, in the light of the heterogeneous distribution of 5-HT receptors to cause contraction of different vascular beds, we thought it of interest to carry out experiments trying to clarify the possible selectivity of dotarizine to block the 5-HT-induced contractions of various vascular beds of the rabbit. Additionally, we provide some clues as to the mechanisms involved in the vascular effects of dotarizine, its effects on contractions evoked by various vasoactive agonists, including electrical field stimulation of cerebral arteries, its voltage-dependence for blocking vascular Ca^{2+} channels and the blockade of [^{3}H]noradrenaline release from cultured sympathetic neurones.

2. Methods

2.1. Source and preparation of tissues

Male New Zealand white rabbits of approximately 2.5 kg were killed in a gas chamber with CO_2, and the following arteries were dissected: thoracic aorta, femoral, vertebral and a fourth-order branch of the mesenteric artery. Middle cerebral arteries from bovine brain, obtained from a local slaughterhouse and brought to the laboratory in iced saline solution, were also used.

Thoracic aorta and femoral arteries were cleaned of surrounding tissue, helical strips of aorta and 2-mm cylindrical segments of femoral artery were cut and mounted in an organ bath containing 40 ml of Krebs–Henseleit solution of the following composition (mM): NaCl, 119; KCl, 4.7; $CaCl_2$ 1.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; glucose, 11, and $NaHCO_3$ 25 mM, at 37°C, bubbled with a gas mixture of 95% oxygen and 5% carbon dioxide. Adrenoceptors of the α_1 subtype were inhibited by the addition of 1 μM phentolamine. Thoracic aorta and femoral arteries were allowed to equilibrate for 1 h under a resting tension of 2.5 and 0.5 g, respectively, with repeated washings. The responses were recorded isometrically with a force-displacement transducer on a polygraph or MacLab/4e acquisition system.

Vertebral (1.5–2 mm in length, 500–650 μm of diameter) and mesenteric (1.5–2 mm in length, 200–350 μm of diameter) artery rings were mounted in a myograph (Myo interface model 600 M) under normalised tension, as previously described (Garland and McPherson, 1992). The tissues were maintained at 37°C in Krebs–Henseleit solution bubbled with a gas mixture of 95% oxygen and 5% carbon dioxide at 37°C. The synthesis of nitric oxide (NO) was inhibited by 30 μM of L-N^G-nitro-arginine methyl ester (L-NAME). The initial equilibration period was 1 h.

Rings of the middle cerebral artery were dissected and set up in an organ bath. The experimental procedure followed to measure contractions induced by electrical stimulation is described by Marín and Balfagón (1998).

2.2. Experimental protocols to measure vessel contraction

The following experimental protocols were carried out after 1 h equilibration of the vessels.

2.2.1. Effect on 5-HT induced contraction

The vessels were contracted by the addition of 5-HT (10 μM) to the bath. When the peak contraction was reached, the arteries were washed twice with fresh Krebs–bicarbonate to allow their relaxation to the basal tone before the application of a new stimulus. Two to three initial 5-HT contractions were required until reproducible contractile responses were obtained before the start of the experimental protocol. Subsequently, the concentration–response curves for the vasorelaxant drugs were made (dotarizine, flunarizine and ketanserin); the drugs were added 15 min before and during the addition of 5-HT. The relaxation induced by the drug was expressed as a percentage of the initial contraction elicited by 5-HT (100%). To gain more insight into the mechanism of the blockade by dotarizine of 5-HT contractions, cumulative concentration–response curves for 5-HT were performed with aorta strips in the absence or presence of dotarizine or ketanserine. Only one concentration of the antagonist was tested in each preparation.

2.2.2. Voltage-dependence of the effect

Aorta helical strips were exposed 15 min before vasoconstriction was induced to a hyperpolarising Krebs–bicarbonate solution containing 1.2 mM K^+/0 Ca^{2+} or to a depolarising solution containing 100 mM K^+/0 Ca^{2+}. After this 15 min period, contraction in the hyperpolarised vessels was induced by changing the bath solution to another one containing 100 mM K^+/1.5 mM Ca^{2+} while the depolarised vessels had Ca^{2+} from a concentrated stock solution added in order to obtain a final concentration of 1.5 mM Ca^{2+} in the bath. Once maximum contraction was reached, the vessels were washed until return to the basal. After two reproducible contractions, the preparations were incubated with the drugs in the hyperpolarising (1.2 mM K^+/0 Ca^{2+}) or depolarising (100 mM K^+/0 Ca^{2+}) solution for 15 min and during the contraction.

Afterwards, the drugs were washed out to test for reversibility.

2.2.3. Effect of dotarizine and flunarizine on the contraction elicited by various vasoconstrictor agents

Aorta helical strips were contracted repeatedly with K^+ (35 mM), 5-HT (10 μM), noradrenaline (10 μM), angiotensin II (0.1 μM), or prostaglandin $F_{2\alpha}$ (3 μM), until two reproducible contractions were obtained. After an incubation period of 15 min with dotarizine or flunarizine (10 μM), a new contraction was elicited with each of the different agonists in the presence of the drugs, in order to determine their blockade.

2.2.4. Interaction between Ca^{2+} ions and dotarizine

Bovine middle cerebral artery rings were initially contracted with 70 mM K^+ and 1.5 mM Ca^{2+}, this contraction was considered as 100%. The vessels were then washed and depolarised with a Krebs–bicarbonate solution containing 70 mM K^+/0 Ca^{2+} and increasing concentrations of Ca^{2+} were added to elicit vessel contractions, both in the absence and presence of dotarizine (1 and 3 μM).

2.2.5. Electrical field stimulation

Rings from bovine middle cerebral artery were stimulated with the following parameters: 0.3 ms, 200 mA during 30 s at 4 Hz (low frequency) or 8 Hz (high frequency).

2.3. [^{3}H]noradrenaline release from cultured sympathetic neurones

Sympathetic neurones derived from the paravertebral sympathetic ganglia of 1-day-old rat pups were cultured in serum-free medium (Wakade et al., 1982) with some modifications (Wakade and Wakade, 1988). Briefly, the dissected ganglia were digested with trypsin (0.1%) in phosphate-buffered saline and the cells were dispersed by trituration (10–15 strokes). Neurones were maintained in Dulbecco's/F12 medium supplemented with insulin, transferrin and nerve growth factor on poly-lysine coated dishes. Neurones were used after 6 or 7 days in primary culture.

Detailed methods for the measurement of [^{3}H]noradrenaline release from cultured neurones have been described previously (Wood and Bunge, 1975; Wakade and Wakade, 1988). Briefly, neurones were loaded with [^{3}H]noradrenaline (4 μCi/ml Krebs–HEPES solution; incubation for 1 h at 37°C). The cells were washed repeatedly over 1 h to remove excess radioactivity and samples were collected over min periods to establish spontaneous release of [^{3}H]noradrenaline. Initially the neurones were stimulated with 70 mM K^+ and samples were again collected every 1 min, before and after stimulation; this value was considered as 100% release. Control release, complete concentration–response curves (1, 3 and 10 μM) and washout were done in a single dish of cells. The drugs were incubated for 10 min before release was stimulated with 70 mM K^+. At the end of the experiment the cells were washed with drug-free medium and samples were again collected to determine the recovery of [^{3}H]noradrenaline release.

The release of [^{3}H]noradrenaline was expressed as net c.p.m., i.e. spontaneous release (the mean c.p.m of three 1-min samples) was subtracted from the K^+-evoked release (the mean c.p.m of two 1-min samples). Then the data were plotted as percentages with respect to the initial K^+-evoked release in the absence of drug.

2.4. Statistical analysis

Averaged data are means ± S.E.M. of the mean. The statistical significance of differences between means was determined by analysis of variance (ANOVA). If significant differences were found, an appropriate multiple comparison Fisher PLSD (paired least significant differences) test was done. In some cases Student's *t*-test was used (see figure legends). Differences were considered significant at the level of $P < 0.05$. IC_{50} values were calculated from a non-linear regression analysis using ISI software, with a personal computer.

2.5. Materials

All chemicals, including nifedipine and ketanserine, were obtained from Sigma (Madrid, Spain), unless otherwise stated. Dotarizine was a kind gift of Laboratorios Ferrer (Barcelona, Spain). Flunarizine was from Janssen Research Foundation (Beerse, Belgium).

3. Results

3.1. Blockade by dotarizine of 5-HT-induced contractions of various arteries

Pilot experiments showed that prolonged incubation of the arteries with 5-HT did not cause sustained contractions; after a peak was reached, the artery started to relax gradually. The concentration-dependent vasorelaxant effects of dotarizine could thus not be tested using a protocol of tonic vessel contractions. Instead, reproducible phasic contractions were elicited with acute 5-HT additions at regular intervals (every 15 min). A typical experiment using this protocol is shown in Fig. 1A, where a segment of the vertebral artery was initially equilibrated for 1 h and then contracted with 10 μM 5-HT, a submaximal concentration; after peak contraction was reached 5-HT was washed out. 5-HT gave contractile responses of similar amplitudes (around 8 mN) when added repeatedly to the same artery.

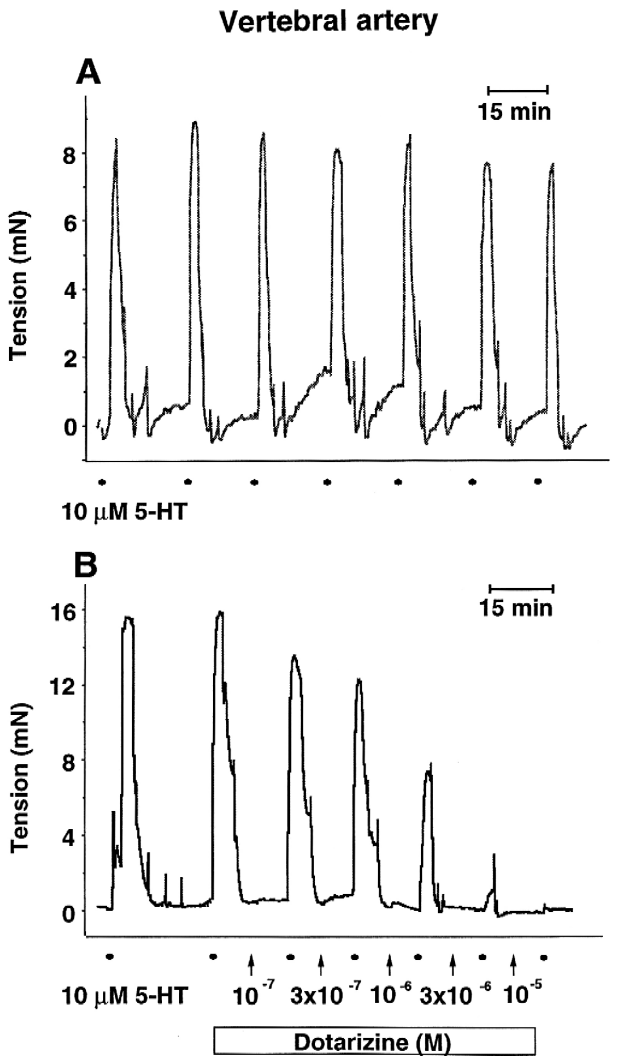

Fig. 1. Original traces of the contractions elicited by serotonin (5-HT, 10 μM) of segments of rabbit vertebral artery in the absence (panel A), or the presence of increasing concentrations of dotarizine (panel B). 5-HT was added as shown by dots at the bottom of each contraction curve, and washed out after a peak contraction was reached. Dotarizine was added 10 min before 5-HT, and was maintained, during the time of 5-HT incubation, at the increasing concentrations shown at the bottom of the figure. The force of contraction is expressed in mN (ordinates).

In the case of the vertebral artery the amplitude of the initial contractile response to 5-HT was 0.77 ± 0.13 g ($n = 8$ segments). The size of the mesenteric artery contraction was 0.31 ± 0.01 g ($n = 30$ segments). The aorta contracted with a force of 1.75 ± 0.08 g ($n = 7$ strips), and the femoral artery contraction amounted to 2.46 ± 0.23 g ($n = 10$ segments). In control tissues the repeated addition of 10 μM 5-HT elicited reproducible contractions for a period of 3 h. Dimethylsulfoxide (the vehicle of concentrated solutions of dotarizine and flunarizine) did not affect such contractions. Dotarizine, however, caused a concentration-dependent blockade of the contractions, as shown in Fig. 1B for the vertebral artery. The threshold concentration for blockade was 0.1 μM and full inhibition of 5-HT contractions was achieved with 10 μM dotarizine.

Concentration–response curves were made with dotarizine, flunarizine and ketanserin (a 5-HT_2 receptor antagonist), using the protocol followed for the vertebral artery (Fig. 1B). These curves were obtained with aorta, vertebral, femoral and mesenteric arteries; they are plotted in Fig. 2. Ketanserin was the most potent antagonist of 5-HT responses in the four vessels (IC_{50} in the submicromolar range; see Table 1); dotarizine also blocked the 5-HT contractions in all vessels, with IC_{50} around 10-fold higher (about 1 μM). Flunarizine did not affect the 5-HT contractions in aorta and femoral arteries even at 10 μM; some blockade (30%) was seen with 30 μM flunarizine in the aorta (Fig. 2A). In smaller vessels, however, flunarizine caused a clear inhibition. For instance, in vertebral arteries flunarizine (10 μM) caused 46% inhibition; in mesenteric arteries, the inhibition was similar to that caused by dotarizine (IC_{50} for flunarizine 0.48 μM, and for dotarizine 1.09 μM; see Table 1).

3.2. Not all vasoconstrictor agents were equally affected by dotarizine

To test the selectivity of dotarizine to block 5-HT-induced vessel contractions, the following experiments were performed. Rabbit aorta strips (one strip for each agonist) were repeatedly contracted with 35 mM K^+, 10 μM 5-HT or noradrenaline, 0.1 μM angiotensin II and 3 μM prostaglandin $F_{2\alpha}$. Once stabilised, these contractions had magnitudes of 1.93 ± 0.13 g ($n = 12$) for 5-HT, 2.75 ± 0.01 g ($n = 12$) for noradrenaline, 0.96 ± 0.06 g ($n = 12$) for angiotensin II, and 1.99 ± 0.12 g ($n = 12$) for prostaglandin $F_{2\alpha}$. Then, the tissues were incubated during 10 min with 10 μM dotarizine or flunarizine, and the contraction with the agonist was repeated in the presence of the antagonist (one strip for each agonist). The results of these experiments are shown in Fig. 3.

The K^+-induced contractions were blocked $37.3 \pm 5.8\%$ by dotarizine and $26.5 \pm 2.3\%$ by flunarizine. The 5-HT-induced contractions were inhibited $63 \pm 8.7\%$ by dotarizine and only $12.2 \pm 2.3\%$ by flunarizine ($P < 0.01$ when comparing dotarizine with flunarizine). The contractions elicited by noradrenaline, angiotensin II or prostaglandin $F_{2\alpha}$ were unaffected by dotarizine or flunarizine. Endothelin (3 nM)-induced contractions were also unaffected by dotarizine or flunarizine (not shown).

3.3. Nature of the antagonism by dotarizine of 5-HT-induced contractions

Ketanserin produced a parallel shift to the right of the concentration–response curves for 5-HT (Fig. 4A). At 0.01 μM the shift was already visible and at 1 μM the shift

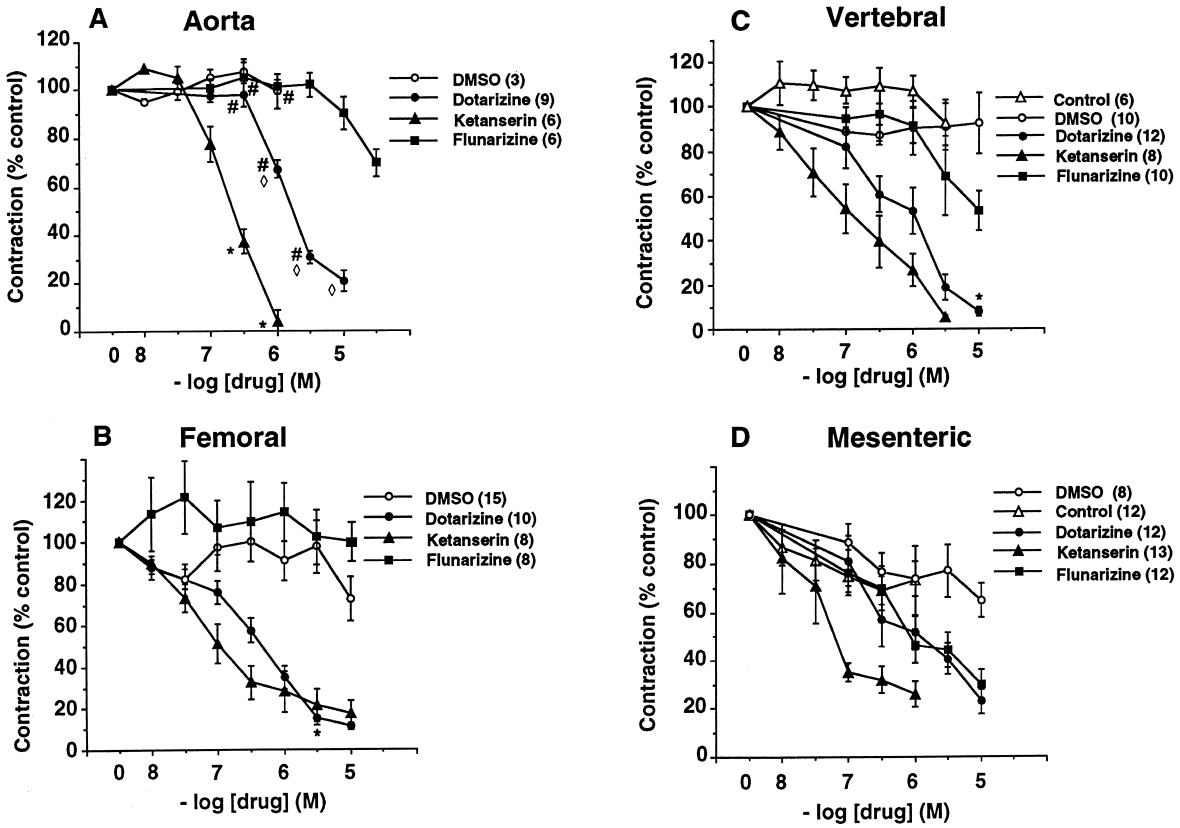

Fig. 2. Concentration–response curves for the blockade of 5-HT contractions, by dotarizine, flunarizine or ketanserin in rabbit aorta (panel A), femoral artery (panel B), vertebral artery (panel C), and mesenteric artery, fourth branch (panel D). The curves were plotted from data obtained in experiments performed with the protocol described in Fig. 1B; they were normalised as percentage of the initial contraction. Data are means ± S.E.M. of the number of experiments shown in parentheses. An ANOVA with multiple comparison (Scheffe *F*-test) was performed to determine the significance at differences. *, ◇, # indicate significant differences with respect to control (DMSO-treated preparations), flunarizine or ketanserin values, respectively.

spanned 2-log units. A Schild plot gave a pA_2 value of 7.8.

A different pattern emerged for the dotarizine blocking effects. At the lower concentration of dotarizine tested (0.03 μM), an increase of 5-HT-induced contractions was observed; this increase (about 15%) was significant at the concentrations 3–10 μM of 5-HT. Higher dotarizine concentrations produced a shift to the right of the 5-HT curves. At 3–30 μM dotarizine, the shift was not parallel (Fig. 4B); in the presence of the compound, the maximum 5-HT response could not be reached, even at agonist concentrations as high as 300 μM.

Table 1
Maximal contractions (g) induced by 5-HT (10 μM), in various rabbit vascular beds, and IC_{50}s of dotarizine, flunarizine and ketanserin to block such contractions. Data were calculated from the experiments shown in Fig. 2.

Blood vessel	Maximal contraction (g)	*n*	IC_{50}s (μM)		
			Dotarizine	Flunarizine	Ketanserin
Aorta	1.75 ± 0.08	7	1.40	NM	0.220
Femoral	2.46 ± 0.23	10	0.52	NM	0.053
Vertebral	0.77 ± 0.13	8	1.35	46.55% inhibition at 10 μM	0.175
Mesenteric (4th branch)	0.31 ± 0.06	30	1.09	0.48	0.035

NM: Not Measurable; *n* = number of experiments.

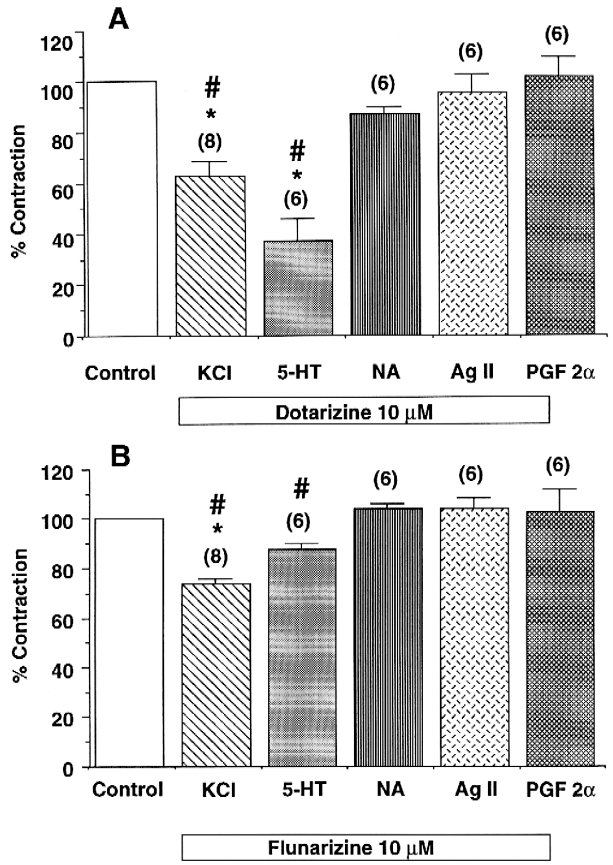

Fig. 3. Effects of dotarizine and flunarizine on the contractions of rabbit aorta strips induced by various agonists. Each agonist was tested in a separate vessel, using a protocol similar to that of Fig. 1: after two to three initial contractions with each agonist, the antagonist (10 μM) was added and 10 min later the agonist was tested in the presence of dotarizine (panel A) or flunarizine (panel B). The concentrations of the agonists were 35 mM K^+, 10 μM for 5-HT and noradrenaline (NA), 0.1 μM for angiotensin II (Ag II) and 3 μM for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Contractions obtained in the presence of the antagonist were expressed as percentage of control (ordinates). Significance of differences was determined by ANOVA with an appropriate multiple comparison (Fisher PLSD). Data are means ± S.E.M. of the number of experiments shown in parentheses. * = Significant difference with respect to control; # = significant difference with respect to other vasoconstrictor agents.

3.4. Voltage-dependence of the effects of dotarizine on isolated rabbit aorta

It is well established that dihydropyridine derivatives exhibit voltage-dependence in their vascular blocking effects (Nelson and Worley, 1989). Hence, it was of interest to compare the voltage-dependence of nifedipine (the prototype dihydropyridine) with that of dotarizine and flunarizine, for their ability to block K^+-induced vessel contraction.

Fig. 5A shows that when nifedipine was incubated in the depolarising solution (0 Ca^{2+}/100 K^+) it blocked by 70% the Ca^{2+} evoked contraction. However, when it was added to the hyperpolarising solution (0 Ca^{2+}/1.2 K^+), blockade of the contraction was significantly reduced to 40%. After washout of nifedipine, recovery of the contraction was substantially slower for the depolarised vessels, suggesting that the compound was binding to its receptor more tightly under depolarising than under hyperpolarising conditions.

As shown in Fig. 5B, the blockade exerted by dotarizine in depolarised (41 ± 3.4%) and hyperpolarised arteries (37 ± 3.6%) was very similar. Reversal of the effect after 1-h washout was about 20% under both sets of conditions. With 3 μM flunarizine (Fig. 5C) we observed a more marked blockade in depolarised (37.1 ± 9.4%) than in hyperpolarised arteries (17.7 ± 7.2%), although this difference was not statistically significant. It is interesting that after washout of flunarizine, a marked postdrug blocking effect was observed; after 60 min washout, the Ca^{2+}-induced contraction was only 5 ± 1% and 20 ± 2% of the initial contraction under depolarising and hyperpolarising conditions, respectively. This indicates that flunarizine accumulates much more markedly than does dotarizine in vessels (see Montiel et al., 1997; Lara et al., 1997).

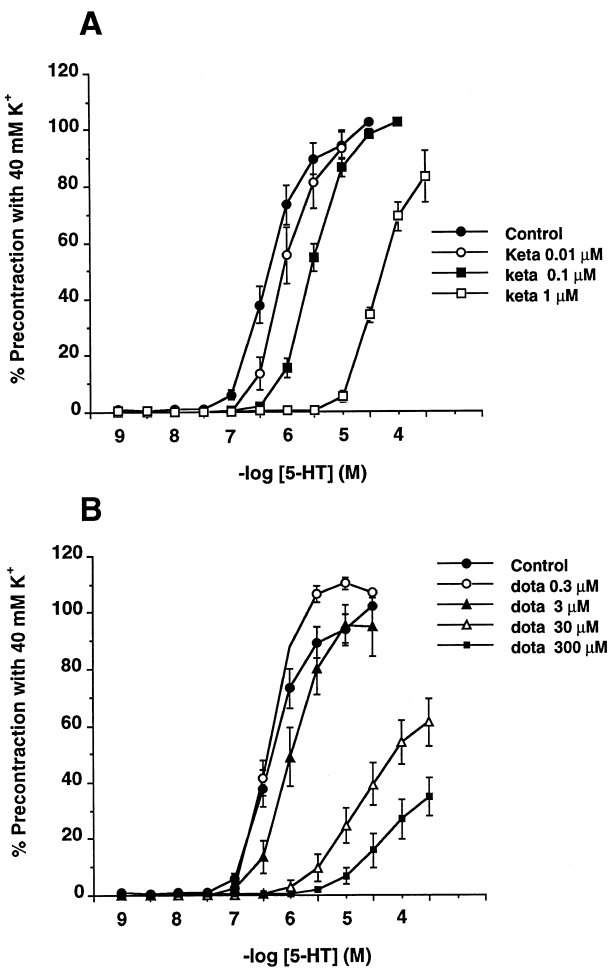

Fig. 4. Concentration–response curves for 5-HT in rabbit aorta strips: effects of ketanserin (panel A) and dotarizine (panel B). Only one concentration of the antagonist was tested in each preparation; controls were also performed with independent strips. Data were normalised in each strip, as percentage of the initial contraction induced by the addition of 40 mM K^+. They are means ± S.E.M. for six to eight strips for each antagonist concentration.

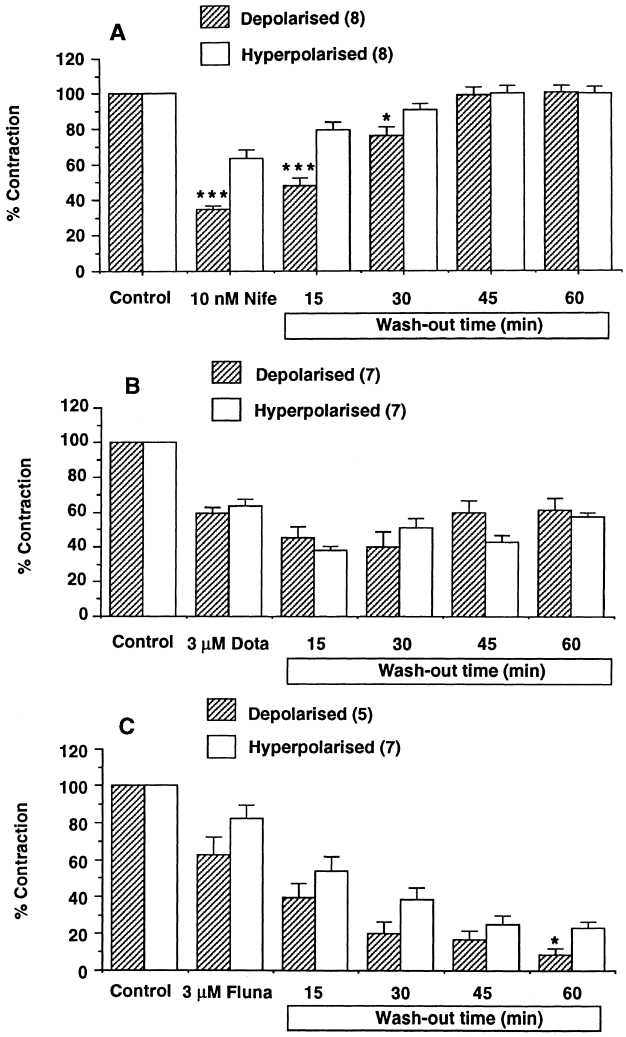

Fig. 5. Blockade and recovery of Ca^{2+}-induced contractions in depolarised (100 $K^+/0$ Ca^{2+}; shaded columns) and Ca^{2+}/K^+-induced contractions in hyperpolarised (1.2 $K^+/0$ Ca^{2+}; white columns) rabbit aorta strips, elicited by 10 nM nifedipine (panel A), 3 μM dotarizine (panel B) or 3 μM flunarizine (panel C). After two consecutive contractions of the same magnitude, the drugs were incubated in the depolarising (100 $K^+/0$ Ca^{2+}) or hyperpolarising (1.2 $K^+/0$ Ca^{2+}) Krebs solution for 15 min before contraction was induced by adding 1.5 mM Ca^{2+} in the depolarised vessels, or changing to a solution containing 100 $K^+/1.5$ Ca^{2+} in the hyperpolarised vessels. Data are means ± S.E.M. of the number of experiments shown in parentheses $^{*}P < 0.05$, $^{***}P < 0.001$ compared with hyperpolarised tissues.

3.5. Blockade by dotarizine of Ca^{2+} induced contractions of bovine middle cerebral arteries is insurmountable by increasing concentrations of Ca^{2+}

These experiments were designed to find more about the nature of the interaction of dotarizine and Ca^{2+} in cerebral vessels. In order to normalise the results, all the vessels were initially contracted with 70 mM K^+ and 1.5 mM Ca^{2+}; this contraction was considered as 100%. Thereafter the preparations were washed until basal a tone was reached and were preincubated in a 0 $Ca^{2+}/70$ K^+ depolarising solution; then, cumulative additions of Ca^{2+} (0.1–2 mM) were performed, to elicit gradual vessel contractions. The threshold Ca^{2+} concentration capable of eliciting a measurable contraction of middle cerebral arteries was 0.1 mM (0.29 ± 0.03 g, $n = 4$, which corresponds to 25.4 ± 6.63% of the initial control 70 $K^+/1.5$ Ca^{2+} contraction); the maximum contraction was achieved at the Ca^{2+} concentration of 2 mM (2 ± 0.4 g; 164 ± 23%, $n = 4$).

In the presence of 1 μM dotarizine, the amplitudes of the contractions was reduced at all Ca^{2+} concentrations tested (Fig. 6). Increasing concentrations of Ca^{2+} tended to evoke greater contractions, but could not reach the amplitude of the control contractions in the absence of dotarizine. At most Ca^{2+} concentrations tested, the contractions in the presence of dotarizine were about half of the control contractions. However, at 2 mM Ca^{2+} the contraction approached control values (124 ± 6% in the presence of 1 μM dotarizine, vs. 164 ± 23% in its absence). At 3 μM, dotarizine reduced by over 90% the Ca^{2+} contractions, even at 2 mM Ca^{2+}; in the presence of dotarizine, the contraction was only 0.30 ± 0.09 g, compared with 2 ± 0.3 g for the control contraction (Fig. 6).

3.6. Blockade by dotarizine of contractions elicited by electrical stimulation of bovine middle cerebral arteries

In addition to vascular Ca^{2+} channels, dotarizine blocks the neuronal type of Ca^{2+} channels that control neuro-

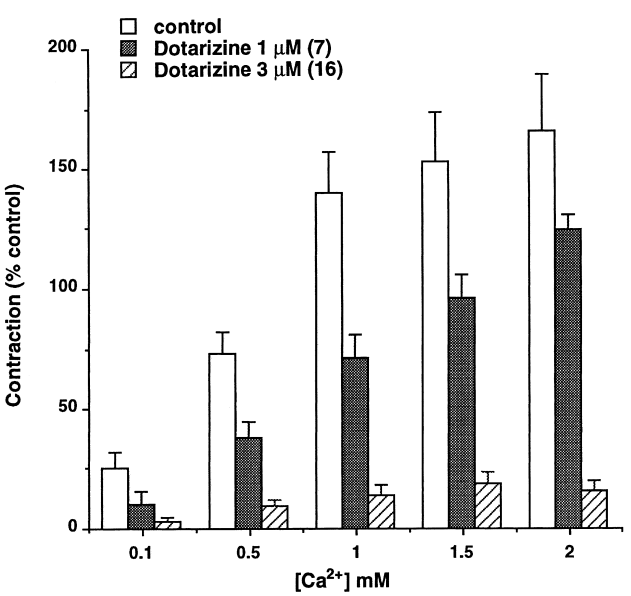

Fig. 6. Blockade by dotarizine of Ca^{2+}-induced contractions of bovine middle cerebral arteries was not surmountable by excess Ca^{2+}. To normalise the results, the vessels were contracted initially with 70 mM K^+ and 1.5 mM Ca^{2+}; this contraction was considered as 100%. Thereafter, the preparations were washed and incubated in 0 Ca^{2+} solution containing 70 mM K^+. They were then contracted by cumulative additions of Ca^{2+} (abscissa), in the absence (control) or presence of 1 or 3 μM dotarizine. Data are the mean values ± S.E.M. for the number of strips shown in parentheses.

transmitter release (Villarroya et al., 1995). It was therefore of interest to know whether the drug modifies the electrically induced brain artery contractions, which are due to the Ca^{2+}-dependent release of noradrenaline from sympathetic nerve endings (Hardebo, 1992).

Fig. 7 shows the effect of dotarizine (1 and 3 μM) and flunarizine (3 μM) on contractions elicited by electrical stimulation (0.3 ms, 4 or 8 Hz, 200 mA during 30 s). The results are shown as percentage of an initial contraction induced by 75 mM K^+; this contraction amounted to 3.5 ± 0.7 g ($n = 29$). The mean amplitude of the contractions induced by the low (4 Hz) and high (8 Hz) frequency stimulation was around 7.5% and 14% of the initial K^+-evoked contraction.

At 1 μM, dotarizine blocked the contractions by 53% ($n = 5$) and 21% ($n = 5$) at 4 and 8 Hz, respectively. At 3 μM dotarizine the blockade of the contraction induced by low frequency stimulation was rather similar to that obtained with 1 μM, i.e. 44% ($n = 15$). However, at high frequency stimulation, blockade of the contraction increased to 53% ($n = 15$). Flunarizine also caused blockade of electrically evoked contractions, amounting to 29% and 42% ($n = 9$), respectively, for 4 and 8 Hz.

3.7. Dotarizine blocks [^{3}H]noradrenaline release in sympathetic neurones

Complete concentration–response curves with dotarizine or flunarizine were obtained in a single dish of sympathetic neurones. Before incubation with the drugs, control release of [^{3}H]noradrenaline was evoked by incubating the cells for 1 min with 70 mM K^+ and 2 mM Ca^{2+}. Three basal samples of 1-min were collected before stimulation of the cells with K^+, and an extra 1-min sample was collected after the 1-min stimulation with K^+. Between stimulations, the cells were kept in Krebs–HEPES for 15 min. The drugs were present, starting from the

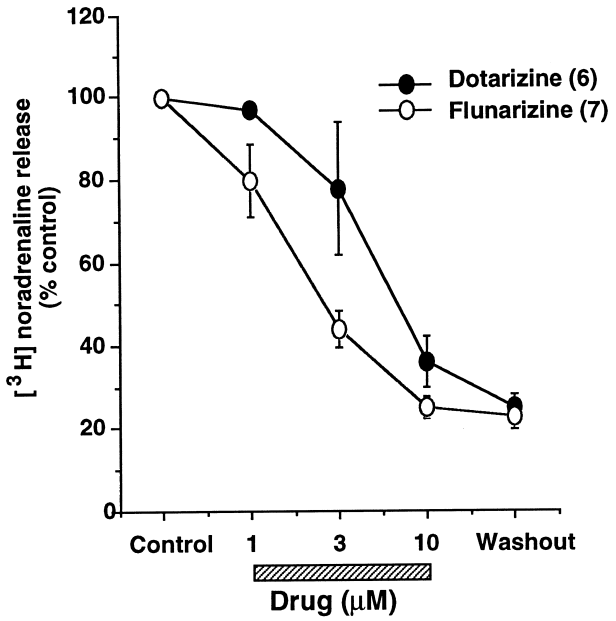

Fig. 8. Blockade by dotarizine and flunarizine of [^{3}H]noradrenaline release from cultured sympathetic neurons stimulated with 70 mM K^+ for 1 min. The drugs were preincubated for 10 min before release was induced with high K^+. Data are expressed as percentages of net release evoked by K^+ in the absence of drugs and correspond to the mean ± S.E.M. for six and seven experiments for dotarizine and flunarizine, respectively.

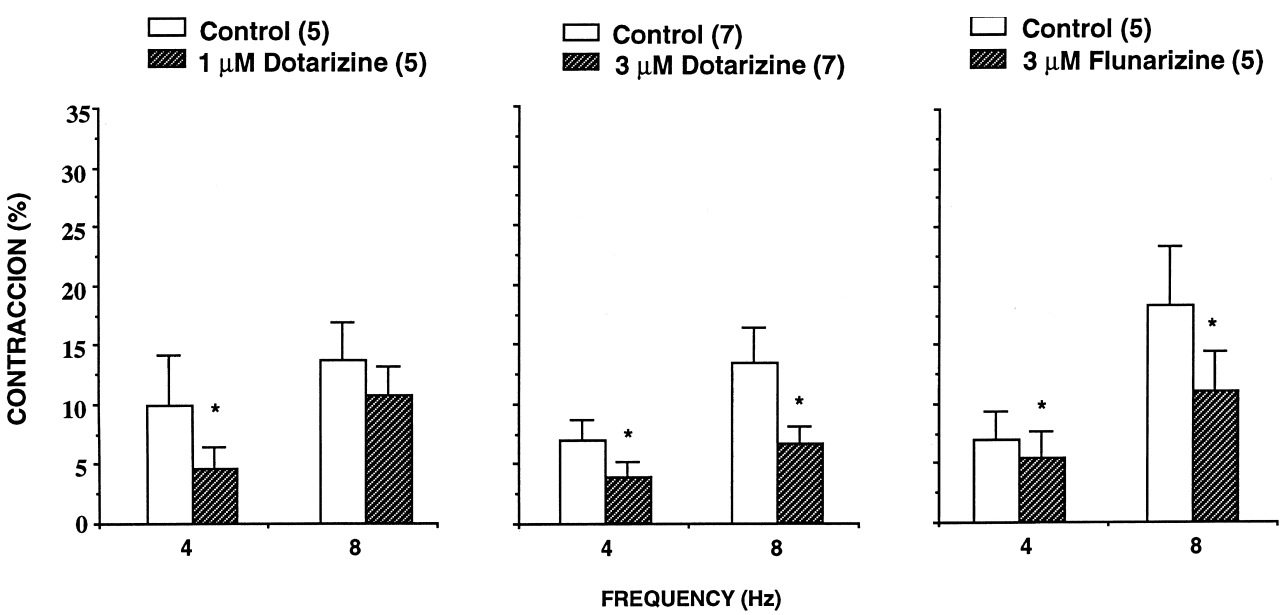

Fig. 7. Effect of dotarizine (1 and 3 μM) and flunarizine (3 μM) on the contraction induced by electrical stimulation (stimulation 0.3 ms, 4 or 8 Hz, 200 mA for 30 s) in bovine cerebral arteries without endothelium. The data are percentages of the contractile response to 75 mM K^+, which was 3.5 ± 0.7 g. Data are given as mean values ± S.E.M. of the number for experiments expressed in parentheses. $^*P < 0.05$, $^{**}P < 0.01$ with respect to control.

lowest concentration, 10 min before stimulation with K^+. Basal release amounted to 780 ± 46 c.p.m. ($n = 39$) and K^+-evoked release to 5022 ± 574 c.p.m. ($n = 13$).

As shown in Fig. 8, dotarizine and flunarizine blocked the K^+-evoked [^{3}H]-noradrenaline release from sympathetic neurones in a concentration-dependent manner, with IC_{50} values of 6.9 μM and 2.8 μM for dotarizine and flunarizine, respectively.

4. Discussion

Various aspects of the mechanism of dotarizine modulation of vascular contractility were now studied. The first one concerns possible differences among large capacitance vessels (rabbit aorta and femoral artery) and smaller resistance vessels (4th branch of the mesenteric artery and vertebral artery of the rabbit). It is known that dotarizine is a good 5-HT_2 receptor antagonist (Brasó et al., 1996; Montiel et al., 1997). Hence, it was not surprising that the drug blocked equally well the 5-HT induced vessel contractility in the four vessels studied (IC_{50}s around 1 μM). It was interesting, however, that flunarizine, which does not exhibit 5-HT receptor blocking properties (Montiel et al., 1997), caused some blockade of small, but not large vessels. In larger capacitance arteries, receptor agonists contract the vessels by mobilising Ca^{2+} from intracellular stores; thus, though these arteries express L-type Ca^{2+} channels (Godfraind, 1989) they are likely not recruited because agonists causing vessel contraction do not depolarise the smooth muscle fibres (Cauvin and Van Breemen, 1985). In contrast, resistance smaller vessels are contracted by receptor agonists by recruitment of L-type channels to favour external Ca^{2+} entry into smooth muscle cells (Benham and Tsien, 1984). This explains why the noradrenaline-induced contraction of rabbit aorta is resistant to blockade by the L-type Ca^{2+} channel antagonist, diltiazem, and why this compound potently inhibits the agonist-induced contraction of small vessels of the mesenteric artery tree (Cauvin and Van Breemen, 1984; Tejerina et al., 1992). This may also explain why flunarizine, which blocks vascular L-type Ca^{2+} channels but not 5-HT receptors (Montiel et al., 1997), did not block the 5-HT-induced contractions of aorta (Fig. 2A) and femoral arteries (Fig. 2B), yet it blocked the small 4th branch of mesenteric arteries (Fig. 2D) and the vertebral artery.

A second aspect of this study concerned the receptor selectivity of dotarizine. The compound did not block the vessel contractions elicited by activation of alpha-adrenergic, angiotensin or prostaglandin receptors (Fig. 3). Thus, as for ketanserin, the vascular effects of dotarizine are likely mediated through 5-HT_2 receptors. However, the IC_{50} for dotarizine was 7–8-fold higher than that of ketanserin to block 5-HT-induced contractions of aorta, femoral and vertebral arteries, and 30-fold higher for the 4th branch of the mesenteric artery (Table 1). This suggests that dotarizine and ketanserin may be blocking different subtypes of 5-HT receptors in each artery. Different 5-HT receptor subtypes have been described as mediating contractions of various vascular beds (Parsons, 1991). It is, nevertheless, interesting, that contrary to that by ketanserin, the blockade by dotarizine of 5-HT contractions is non-competitive. It is also curious that at submicromolar concentrations, dotarizine slightly enhanced the maximum vessel contraction elicited by 5-HT (Fig. 4B). This suggests a complex interaction of dotarizine with 5-HT receptors and/or an interaction with more than one receptor subtype, depending on its concentration range and the type of vessel. Because 5-HT_{2B} receptors have been recently implicated in the pathogenesis of migraine (Schmuck et al., 1996; Johnson et al., 1998) we are now trying to express those receptors in oocytes, to see whether dotarizine binds to them.

In addition to blocking 5-HT_2 receptors, dotarizine has a second component in its vascular stabilising actions, i.e. Ca^{2+} channel blockade (Figs. 3A and 5B; see also Tejerina et al., 1993; Villarroya et al., 1995; Montiel et al., 1997). The Ca^{2+} channel responsible for the depolarisation-mediated vessel contraction is of the L-subtype, whose prototype antagonist is nifedipine (Fleckenstein-Grun and Fleckenstein, 1990). We now continued that the inhibition of these channels by nifedipine was voltage-dependent, as shown by Nelson and Worley (1989) with the rabbit mesenteric artery for nisoldipine, another dihydropyridine. However, dotarizine did not show such voltage-dependence for blocking Ca^{2+}-evoked vessel contraction (Fig. 5B). These experiments uncovered an interesting difference between dotarizine and flunarizine. After washout, the vessels exposed to dotarizine tended to recover their initial contractility slowly; this did not happen with flunarizine, which tended to show a progressive vessel blockade even after its washout. This agrees with previous observations that flunarizine, but not dotarizine has a marked tendency to accumulate in cultured chromaffin cells (Lara et al., 1997); and as a consequence, flunarizine showed greater cytotoxic effects than did dotarizine (Novalbos et al., 1999).

Blockade by dotarizine of K^+-evoked contractions of cerebral vessels was dependent on the Ca^{2+} concentration of the medium and thus was consistent with the concept of calcium antagonism formulated by Fleckenstein-Grun and Fleckenstein (1990) for verapamil, diltiazem and 1,4-dihydropyridine derivatives. Hence, at the low concentration (1 μM), excess Ca^{2+} was able to reverse the blockade of the middle cerebral artery contraction induced by dotarizine. However, Ca^{2+} could not reverse the blockade elicited by the higher concentration of dotarizine (Fig. 6). These experiments support the view that dotarizine blocks Ca^{2+} entry through L-type Ca^{2+} channels, thus preventing the K^+-induced cerebral vessel contraction.

The last effect of dotarizine that was studied was the blockade of cerebral vessel contractions evoked by electri-

cal field stimulation, an action shared by flunarizine but to a lesser extent (Fig. 7). This contractile response was likely due to the release of noradrenaline from sympathetic nerve terminals innervating cerebral vessels. It is known that noradrenaline release is controlled by Ca^{2+} entry through N-type Ca^{2+} channels (Lipscombe et al., 1989; Vega et al., 1995; Hirata et al., 1997), and that dotarizine blocks the whole cell currents through this and other subtypes of neuronal Ca^{2+} channels (Villarroya et al., 1995). An additional, more direct, proof for the blockade by dotarizine of noradrenaline release arises from the experiments with cultures of sympathetic neurons. Dotarizine blocked the K^+-evoked [^{3}H]noradrenaline release with an IC_{50} of 6.9 μM (Fig. 8), similar to the concentration that inhibited by 50% the cerebral vessel contractions elicited by electrical field stimulation (Fig. 7). It seems that the sympathetic inervation of cerebral vessels comes from fibres from the superior cervical ganglion. If so, the sympathetic tone will modulate cerebral vessel contractility and dotarizine will reduce such control to cause vasodilatation. In fact, in experiments in vivo using transcranial Doppler sonography, dotarizine abolished the cerebral vasoconstrictor effect of hyperventilation in cats (Czernicki et al., 1996) and rabbits (Kuridze et al., 2000), causing vasodilatation and an increase in cerebral blood flow.

It is widely accepted that in the pathogenesis of migraine, there occurs a failure of the regulation of the cerebrovascular blood flow; this has been substantiated by using transcraneal Doppler ultrasounds in patients suffering migraine attacks (Silvestrini et al., 1995). Also, it has been shown that during headache-free intervals, migrainers exhibit a reduced vasodilatory response to hypercapnia of cerebral arterioles (Totaro et al., 1997). During these intervals, dotarizine could have a stabilising effect on these vessels by acting presynaptically to limit the sympathetic drive to brain vessels, and postsynaptically by modulating the contractile effects mediated by 5-HT receptors in large and smaller vessels as well as Ca^{2+} entry through L-type Ca^{2+} channels in smaller vessels. This triple mechanism could explain the efficacy of dotarizine in the prophylaxis of migraine attacks, which was shown in various clinical trials (data on file, Laboratorios Ferrer, Barcelona, Spain).

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