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# Serotonergic effects of dotarizine in coronary artery and in oocytes expressing 5-$HT_2$ receptors

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**Abstract**

In strips of pig coronary arteries incubated in oxygenated Krebs-bicarbonate solution at 37°C, dotarizine blocked the phasic contractions evoked by 5-HT (0.5 μM) or $K^+$ depolarization (35 mM $K^+$) with an $IC_{50}$ of 0.22 and 3.7 μM, respectively. Flunarizine inhibited both types of contractions with $IC_{50}$ values of 1.7 μM for 5-HT and 2.4 μM for $K^+$ responses. In *Xenopus* oocytes injected with in vitro transcribed RNA encoding for 5-$HT_{2A}$ or 5-$HT_{2C}$ receptors, 5-HT (100 nM for 20 s) applied every 10 min caused, in both cases, a reproducible inward current through $Ca^{2+}$-activated $Cl^-$ channels ($I_{Cl}$). Dotarizine inhibited the 5-$HT_{2A}$ response in a concentration-dependent manner, with an $IC_{50}$ of 2.2 nM. In contrast, the 5-$HT_{2C}$ response was unaffected by 1 μM dotarizine and blocked around 62% by 10 μM of this drug. The $I_{Cl}$ activated either by intracellular injection of inositol 1,4,5-trisphosphate ($IP_3$) in oocytes or by direct photorelease of $Ca^{2+}$ in DM-nitrophen-injected oocytes was unaffected by 10 μM dotarizine. It is concluded that dotarizine blocks 5-$HT_{2A}$ receptors with a high affinity; the compound is devoid of intracellular effects on any further steps of the transduction pathway (i.e., $IP_3$ receptor). Contrary to flunarizine that blocks equally well the serotonergic and the $K^+$ vascular responses, dotarizine exhibits 17-fold higher affinity for vascular 5-HT receptors. These findings might be relevant to an understanding of the mechanism involved in the use of dotarizine and flunarizine as prophylactic agents in migraine. © 1997 Elsevier Science B.V.

*Keywords:* Dotarizine; 5-$HT_2$ receptor; $Ca^{2+}$ channels; *Xenopus* oocytes; Coronary artery

## 1. Introduction

This study was aimed at characterizing the differential effects of dotarizine on 5-$HT_2$ receptor subtypes and on vascular voltage-dependent $Ca^{2+}$ channels. Dotarizine, a novel piperazine derivative structurally related to flunarizine (Fig. 1), is currently being evaluated in clinical trials for its antimigraine and antivertigo effects. Its parent piperazine compound, flunarizine, has been used for the prophylactic treatment of migraine crisis (Todd and Benfield, 1989) and its clinical efficacy has been related with its $Ca^{2+}$ channel antagonist properties, since disorders of voltage-dependent $Ca^{2+}$ and $Na^+$ channels have been implicated in the pathogenesis of migraine. Like flunarizine, dotarizine exhibits $Ca^{2+}$ channel antagonist properties. Thus in rabbit aortic smooth muscle, dotarizine inhibits $^{45}Ca^{2+}$ uptake and vessel contractility (Tejerina et al., 1993). Furthermore, in bovine adrenal chromaffin cells dotarizine blocks whole-cell $Ca^{2+}$ and $Ba^{2+}$ currents generated by depolarizing test pulses in voltage-clamped cells, and also inhibits the $^{45}Ca^{2+}$ uptake, the transient rise of cytosolic $Ca^{2+}$ concentrations as well as the release of catecholamines triggered by $K^+$ depolarization (Villarroya et al., 1995).

Additionally, dotarizine exhibits potent antiserotonergic activity, both in vitro and in vivo (Brasó et al., 1989, 1994; Cartheuser et al., 1994). Since various drugs with 5-$HT_2$ receptor antagonist properties have been used in migraine prophylaxis, for example, pizotifen, methysergide or the β-adrenoceptor antagonist, propranolol (Welch, 1993; Goadsby and Olesen, 1996), the 5-HT receptor blocking effect of dotarizine, in addition its being a $Ca^{2+}$ channel

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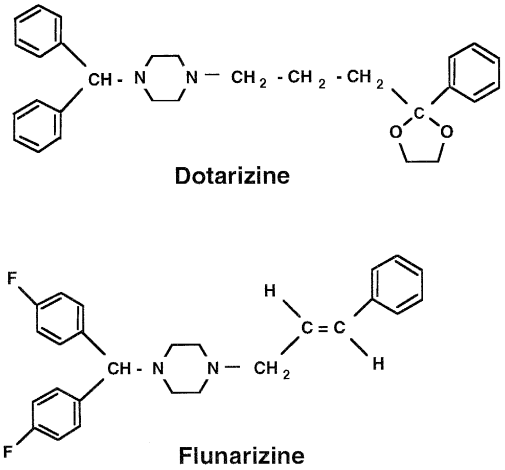


Fig. 1. Chemical structures of dotarizine and flunarizine.

antagonist, should increase its possible therapeutic potential. In this study we compared the vascular $Ca^{2+}$ channel antagonist properties of dotarizine with its antiserotoninergic effects. In addition, by using *Xenopus* oocytes as a system to express specific receptors, we analyzed the possible selectivity of dotarizine for a given 5-$HT_2$ receptor subtype. The presence of an intracellular site of action for dotarizine at inositol 1,4,5-trisphosphate ($IP_3$) receptor level was also explored.

## 2. Materials and methods

### *2.1. Preparation of the coronary artery and experimental protocols*

Hearts from Large White pigs of both sexes were obtained from a local slaughterhouse within 10 min of slaughter, and were transferred to the laboratory in ice-cold Krebs-bicarbonate solution with the following composition (in mM): NaCl 119; KCl 4.7; $MgSO_4$ 1.2; $CaCl_2$ 1.5; $KH_2PO_4$ 1.2; $NaHCO_3$ 25 and glucose 11. The first 3 cm of the left circumflex coronary artery was dissected out and the surrounding adipose and connective tissue was removed. Arteries were cut into helicoidal strips 15 mm long and 2–3 mm wide and suspended on a pair of hooks, one of which was fixed to an L-shaped rod inside the water-jacketed organ chamber (40 ml) and the other to an isometric transducer connected to an amplifier and recorder. The strips, under a tension of 1 g, were equilibrated with a Krebs-bicarbonate solution bubbled with 95% $O_2$/5% $CO_2$ at a temperature of 37°C.

After 2 h of initial equilibration, during which periodic changes of the Krebs-bicarbonate solution and readjustment of the tension were required, the strips were contracted by the addition to the bath of 5-HT or KCl from a concentrated stock solution in order to obtain the required final concentration. When the contraction peak was reached, the artery was washed twice with fresh Krebs-bicarbonate to allow its relaxation to the basal tone before application of a new stimulus, usually 30 min after the first. A few stimuli were required to obtain a reproducible contractile response before starting the experimental protocol. Subsequently it was possible to obtain the concentration–response curves for the vasorelaxant drugs assayed (dotarizine, flunarizine and elgodipine); these drugs were added to the strips 10 min before and during the corresponding addition of the vasoconstrictor agent to the bath.

Contractions were measured in mm and converted into mN force. Sometimes, relaxation induced by the drug was expressed or plotted as percentage of the initial contraction elicited by 5-HT or high $K^+$ (100%).

### *2.2. Preparation of in vitro transcribed RNA*

The plasmids pSR2 and pSR1c containing the entire rat 5-$HT_{2A}$ and 5-$HT_{2C}$ receptor coding regions were linearized with the restriction enzymes *Hin*dIII and *Bam*HI, respectively. Linearized plasmids were transcribed with T7 RNA polymerase using a mCAP RNA capping Kit (Stratagene, La Jolla, CA, USA). The capped RNAs encoding 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors were extracted with phenol/chloroform, precipitated with sodium acetate and ethanol, rinsed with ethanol and suspended in RNase-free water. The concentration of RNA in each sample was determined by measuring the absorbance at 260 nm and was adjusted to a final concentration of 1 μg/μl. The samples were divided into aliquots and stored at −80°C.

### *2.3. Microinjection of the RNAs into Xenopus oocytes*

Techniques for the expression of foreign mRNA in oocytes have been described previously (Miledi et al., 1989; Sumikawa et al., 1989). Briefly, mature female *Xenopus laevis* obtained from a commercial supplier (CRBM du CNRS, Montpellier, France) were anesthetized with tricaine solution (0.125%) and ovarian lobes were dissected out. Then, follicle-enclosed oocytes were manually stripped from the ovary membranes and incubated overnight at 16°C in a modified Barth's solution containing (in mM): NaCl 88, KCl 1, $NaHCO_3$ 2.4, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, HEPES 10, buffered to pH 7.4 and supplemented with gentamicin (0.1 mg/ml) and sodium pyruvate (5 mM). The next day, healthy follicle-enclosed oocytes at stages V and VI (Dumont, 1972) were injected with 50 nl of 5-$HT_{2C}$ and 5-$HT_{2A}$ in vitro transcribed RNAs using a nanoject automatic injector (Drummond Scientific, Broomall, PA, USA). Electrophysiological recordings were made 1–3 days later. Several hours after injection, follicle-enclosed oocytes were defolliculated with collagenase (Type I, 200 units/mg at a final concentration of 0.5 mg/ml).

### 2.4. Voltage-clamping of single oocytes

Electrophysiology experiments were carried out at room temperature (22–25°C) in Ringer's solution containing (in mM): NaCl 115, KCl 2, $CaCl_2$ 1.8, HEPES 5 buffered to pH 7.4 with NaOH. Dotarizine and flunarizine were dissolved in dimethylsulphoxide (DMSO, Merck, Darmstadt, Germany) at $10^{-2}$ M, and diluted in Ringer's solution at the desired concentrations. All solutions contained DMSO at a final concentration of 0.1%. Membrane currents were recorded with a two-electrode voltage clamp (OC-725-B Warner Instrument, Hamden, CT, USA) using microelectrodes with resistances of 0.5–5 MΩ made from borosilicate glass (GC100TF-15, Clark Electromedical, Pangbourne, UK) and filled with KCl (3 M). The holding potential in all experiments was −60 mV. Single oocytes were held in a chamber with volume 0.6 ml and constantly superfused with Ringer's solution (10 ml $min^{-1}$). Voltage protocols were run and currents were recorded using a Digidata 1200 Interface and CLAMPEX software (Axon Instruments, Foster City, CA, USA).

### 2.5. Intra-oocyte injection of $IP_3$

Control non-injected oocytes previously defolliculated with collagenase were voltage-clamped as described above. Microinjections (volume 14 nl) of a solution containing D-myo-inositol 1,4,5-trisphosphate (10 μM), HEPES (5 mM), EGTA (50 μM) at a pH 7.0 were made into the animal pole of the oocyte.

### 2.6. Flash photolysis of caged $Ca^{2+}$ in oocytes

$Ca^{2+}$-activated $Cl^-$ channels in the oocyte plasma membrane were directly stimulated by the photolytic release of $Ca^{2+}$ from a caged $Ca^{2+}$ compound in control oocytes. Individual follicle-enclosed and defolliculated oocytes were injected with 41 nl of a solution containing 50 mM DM-nitrophen, 45 mM $CaCl_2$, 5 mM HEPES pH 7.0. After equilibration in the dark for at least 30 min, individual oocytes were voltage-clamped at −60 mV. Reproducible increases in the $Ca^{2+}$-induced chloride current were obtained on application of repetitive flashes of light using a flash lamp system (Cairn Research, Faversham, UK) with capacitors of 100–2000 μF charged to 200 V. Oocytes were illuminated using a light guide positioned over the vegetal pole.

### 2.7. Expression of 5-HT receptors in oocytes

In order to study the pharmacological profile of dotarizine on the different subtypes of $5\text{-}HT_2$ receptors expressed in RNA-injected oocytes, short pulses of 5-HT (100 nM, 20 s) were applied every 10 min. Activation of the receptor by the agonist was measured by recording of the $Ca^{2+}$-activated $Cl^-$ current ($I_{Cl}$) in oocytes whose membrane potential was held at −60 mV, as described previously (Gundersen et al., 1983; Nomura et al., 1987; Miledi et al., 1989; Woodward et al., 1992). Under these conditions the responses induced by successive pulses (up to 8–10) applied to the oocyte were reproducible and no desensitization was seen.

### 2.8. Statistical analysis

Data are presented as means ± S.E.M and were compared by means of 2-tailed Student's *t*-tests. Differences were accepted as significant at $P$ values equal to or smaller than 0.05. $IC_{50}$ values were calculated from a non-linear regression analysis using ISI software, with a PC computer.

### 2.9. Materials

All chemicals were obtained from Sigma (Madrid, Spain), unless otherwise stated. Dotarizine, [1-(diphenylmethyl)-4-[3-(2-phenyl-1,3-dioxalan-2-yl)-propyl]-piperazine], was a kind gift of Laboratorios Ferrer (Barcelona, spain). Flunarizine, [(*E*)-1-[bis(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)-piperazine dihydrochloride] was from Janssen Research Foundation (Beerse, Belgium). Mesulergine hydrochloride from Research Biochemicals International (Natick, MA, USA). DM-nitrophen from Calbiochem (San Diego, CA, USA). Elgodipine, isopropyl 2-[*N*-methyl-*N*-(4-fluorobenzyl)amine]-ethyl-2,6-dimethyl-4-(2′,3′-methylenedioxyphenyl)-1,4-dihydropyridine-3,5-dicarboxylate monohydrochloride, was a gift from Prodesfarma (Barcelona, Spain).

## 3. Results

### 3.1. Effects of dotarizine on the contractile response of pig coronary strips stimulated with 5-HT or high $K^+$

Initial testing of increasing concentrations of 5-HT applied cumulatively to pig coronary strips showed that the $EC_{50}$ value for the contractile response was $2.6 \times 10^{-7}$ M. Therefore, repeated applications of 5-HT, separated by 30 min washout, at a concentration double the $EC_{50}$ ($5 \times 10^{-7}$ M) were selected to induce substantial contractions in these vessels. Usually, after five or six initial challenges, the responses to 5-HT were stable and reproducible for at least ten further challenges (not shown). The amplitude of the stabilized contraction averaged 14.8 ± 3.2 mN ($n = 7$). This stabilized value was considered as the control initial contraction for each individual strip. Fig. 2A shows one original trace of the contractions induced by 5-HT and the progressive blockade by increasing concentrations of dotarizine, applied stepwise. Fig. 3A shows the averaged concentration–response curve for the blockade by dotarizine of contractions induced by 5-HT. Data are plotted as

percentage of initial 5-HT contraction. The calculated $IC_{50}$ for dotarizine was 0.22 μM. It is worth noting that mesulergine, a potent antagonist for 5-$HT_2$ receptors, had an $IC_{50}$ of 0.1 μM ($n = 7$), close to that found for dotarizine.

Similar experiments were performed with repeated additions of high $K^+$ (35 mM, uncorrected for osmolarity). The amplitude of the contractions usually stabilized after two to three $K^+$ stimuli, and were reproducible for at least ten subsequent $K^+$ challenges applied at 30 min intervals. Again, the initial contractile response elicited by high $K^+$ ($24.4 \pm 5.9$ mN; $n = 4$) was blocked progressively by increasing concentrations of dotarizine. Fig. 2B shows an original trace and Fig. 3A shows averaged results from several experiments. The $IC_{50}$ for dotarizine blockade of the $K^+$ contractions was 3.7 μM, 17-fold higher than that for 5-HT.

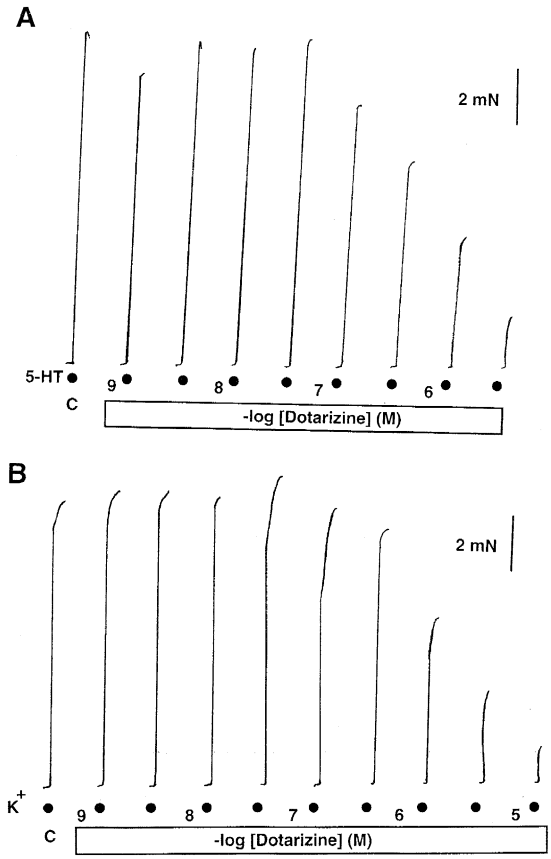


Fig. 2. Effects of increasing concentrations of dotarizine on the contractile responses of pig coronary strips to 5-HT or $K^+$. The helicoidal strips were mounted in the bath as described in Section 2. After 120 min for initial equilibration, the strips were contracted with the addition of $5 \times 10^{-7}$ M of 5-HT (panel A) or 35 mM $K^+$ (panel B). After reaching a maximal contraction, the preparation was washed out with fresh Krebs-bicarbonate solution to reach baseline contraction (usually after a 30 min washout period). Then, the vessels were contracted again. This procedure was repeated in the presence of increasing concentrations of dotarizine (bottom horizontal bar), as indicated by dots at the bottom of each trace (concentrations were increased in half-logarithmic steps). Dotarizine was added 10 min before and during the 5-HT (A) or $K^+$ (B) challenge. In the absence of drug, the contractions were reproducible for at least 10 challenges with 5-HT or $K^+$.

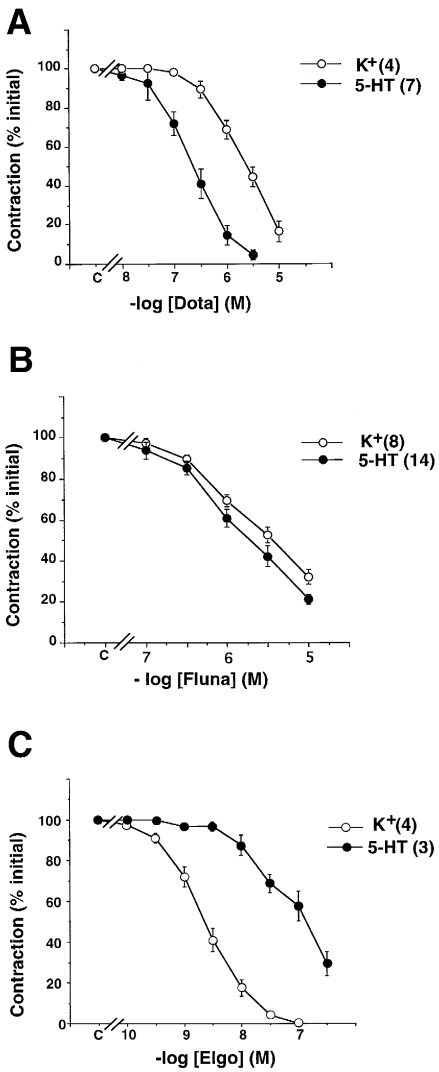


Fig. 3. Concentration–response curves for the blocking effects of dotarizine (panel A), flunarizine (panel B) or elgodipine (panel C) on the contractions induced by 5-HT ($5 \times 10^{-7}$ M) or $K^+$ (35 mM) in pig coronary strips. Results are expressed as percentages of control contraction in the absence of the drug. These curves were plotted with averaged data obtained from experiments performed with protocols similar to those described in Fig. 2. Data are means ± S.E.M. of the number of experiments shown in parentheses.

Following protocols similar to those described above, the effects of flunarizine on contractions induced by 5-HT and $K^+$ were also studied. The initial coronary contractions elicited by 5-HT and high $K^+$ were $11 \pm 1.4$ mN ($n = 14$) and $16 \pm 0.4$ mN ($n = 8$), respectively. Flunarizine blocked with a similar potency the contractions induced by the two vasoconstrictors (Fig. 3B). Thus the $IC_{50}$ to inhibit the responses was 1.7 μM for 5-HT contractions and 2.4 μM for $K^+$ contractions.

A recent patch-clamp study has demonstrated that both dotarizine and flunarizine block both L-type and non-L-type high-voltage-activated $Ca^{2+}$ channels (Villarroya et al., 1995). Therefore, we compared the inhibitory effects of these 'wide spectrum' $Ca^{2+}$ channel antagonists, with

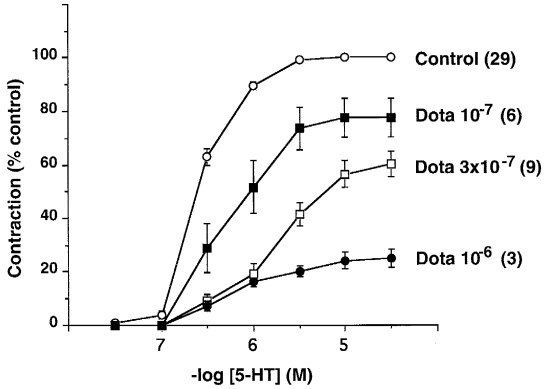


Fig. 4. Effect of different concentrations of dotarizine on the dose–response curves obtained in pig coronary strips contracted with 5-HT. Curves were obtained in arteries contracted with cumulatively increasing concentrations of 5-HT. After a 30 min washout, the contraction curve induced by 5-HT could be reproduced twice more. The second curve was considered as control ($n = 29$). In some strips dotarizine, at a fixed concentration, was added to the chamber 10 min before and was present during the third 5-HT curve. Dotarizine (Dota) concentrations used were $10^{-7}$ M; $3\times10^{-7}$ M and $10^{-6}$ M. Results are expressed as percentages of control contraction in the absence of dotarizine and represent means ± S.E.M. of the number of experiments shown in parentheses.

those of a highly selective blocker of vascular L-type $Ca^{2+}$ channels, the novel 1,4-dihydropyridine derivative, elgodipine (Tamargo et al., 1991). Elgodipine, used with a protocol similar to that described above, selectively blocked the $K^+$-induced contraction with an $IC_{50}$ of 2.3 nM. This value was two orders of magnitude lower than that required to block by 50% the initial contraction evoked by 5-HT ($IC_{50}$ = 330 nM).

### *3.2. Effects of dotarizine on the coronary contractions elicited by increasing 5-HT concentrations*

Since dotarizine exhibited potent antiserotonergic activity, subsequent experiments were designed to study the type of antagonism exerted by this drug. Coronary strips were contracted with cumulatively increasing concentrations of 5-HT, added to the bath when the previous dose had caused its maximum effect. The control 5-HT curve could be reproduced twice more in the same strip if the preparation was washed in between with fresh Krebs-bicarbonate solution for 30 min. Thus three concentration–response curves with 5-HT, 30 min apart, were obtained with each strip; the second curve was used as an internal control for the experimental curve (third one). In each strip, 10 min before the start of the third 5-HT curve, a fixed concentration of dotarizine was added to the chamber. The blocking effects of dotarizine on the contraction induced by 5-HT were expressed as percentages of the internal control values obtained during the second curve, and are shown in Fig. 4. Note that as the dotarizine concentration was increased from 0.1 to 1 μM, the blockade of the contractions evoked by 5-HT became unsurmountable.

### *3.3. Duration of the blockade by dotarizine and flunarizine of 5-HT and $K^+$-evoked contractions*

Further experiments were carried out to study the reversibility of the blocking effects of dotarizine and flunarizine on the coronary contraction elicited by 5-HT (1 μM) and high $K^+$ (35 mM). In the same strip, several alternate applications of 5-HT and $K^+$, separated by 15 min washout periods, were employed to make sure that a stable contractile response was obtained (initial control contraction). Then, three new pulses of 5-HT were applied, but in the presence of either dotarizine (1 μM) or flunarizine (1 μM), to two different strips. Each drug was added to the chamber 10 min before the relevant 5-HT pulse. Afterwards the two vasorelaxing agents were washed out and

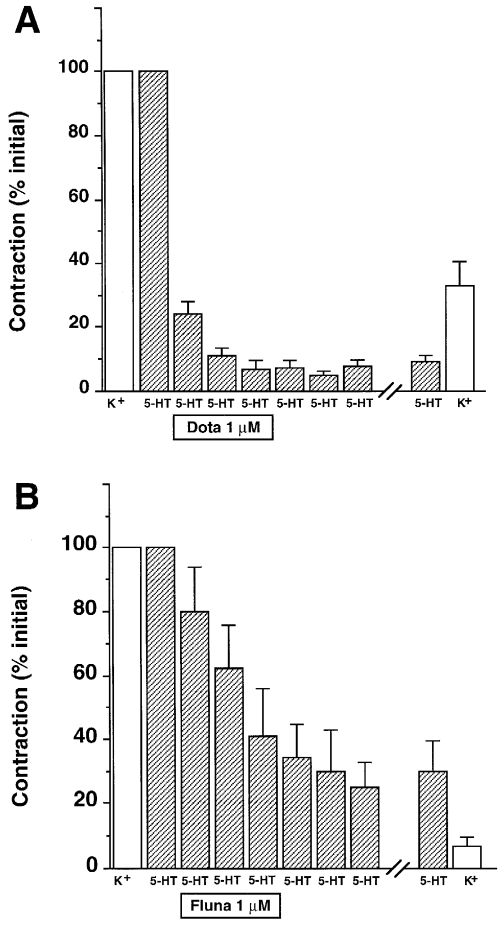


Fig. 5. Reversibility of the 5-HT receptor blocking effects of dotarizine (Dota) and flunarizine (Fluna) in pig coronary strips. Strips were alternatively contracted with $K^+$ (35 mM, white bars) or 5-HT (1 μM, hatched bars), separated by 15 min of washout. Both stimuli were repeated until a stable contraction was reached (initial control response, 100%). Then three new pulses of 5-HT were applied but in the presence of 1 μM dotarizine (panel A) or 1 μM flunarizine (panel B). After washout, the arteries were newly contracted with successive 5-HT applications. At the end of the experiment, and after 90 min of washing, the contractions elicited by two final 5-HT and $K^+$ additions were tested. Contractile responses were expressed as percentages of control initial contraction. Data are means ± S.E.M. of 5 (panel A) and 3 (panel B) experiments.

fresh 5-HT applications were carried out every 15 min. The results are plotted in Fig. 5A and B which show the effects of dotarizine and flunarizine respectively, expressed as percentages of the control contractions. The two bars at the right represent the contractions produced when 5-HT and $K^+$ were applied 90 min after the washout of dotarizine or flunarizine. Fig. 5A shows the blockade by dotarizine of the response to 5-HT, as well as the lack of recovery of this effect during the washout period studied. The same effect of dotarizine was observed with high $K^+$ upon washout of the drug, which may reflect the prolonged blockade by dotarizine of voltage-dependent $Ca^{2+}$ channels, in addition to that of 5-HT receptors. A similar long-term blocking effect on the contraction induced by 5-HT was observed with flunarizine (Fig. 5B), although the blockade developed more slowly and was considerably smaller.

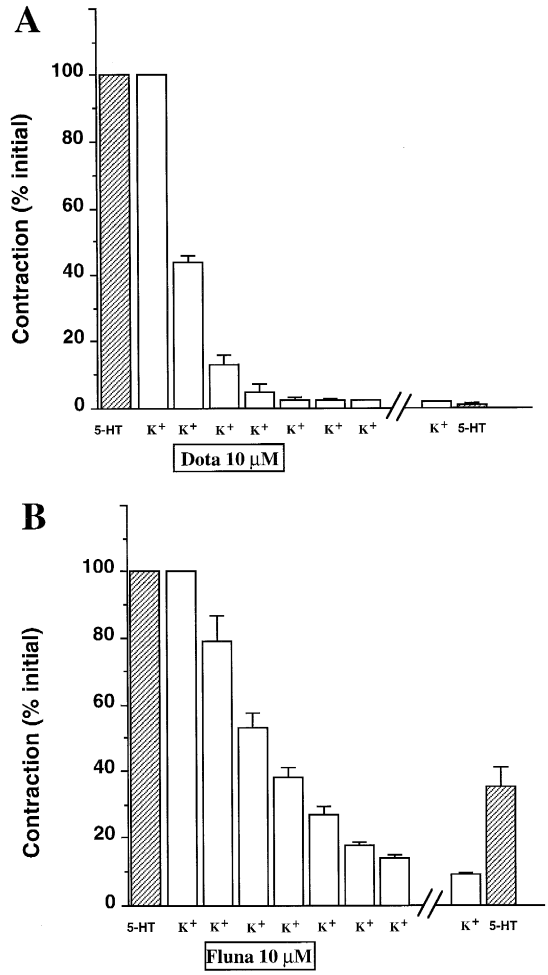


Fig. 6. Reversibility of the blocking effects of dotarizine (Dota) and flunarizine (Fluna) on the contraction induced by direct recruitment of voltage-dependent $Ca^{2+}$ channels in pig coronary strips. Strips were alternatively contracted with 5-HT (1 μM, hatched bars) or $K^+$ (35 mM, white bars) separated by 15 min of washout until a stable contraction was reached (initial control response, 100%). Next, after the control $K^+$ pulse, three new pulses of $K^+$ were applied but in the presence of 10 μM dotarizine (panel A) or 10 μM flunarizine (panel B). Upon washout, arteries were again contracted with successive $K^+$ applications. At the end of the experiment, and after 90 min of washing, the contractions elicited by two final $K^+$ and 5-HT pulses were tested. Contractile responses were expressed as percentages of the control initial contraction. The figure represents means ± S.E.M. of the data obtained in 5 (panel A) and 3 (panel B) experiments.

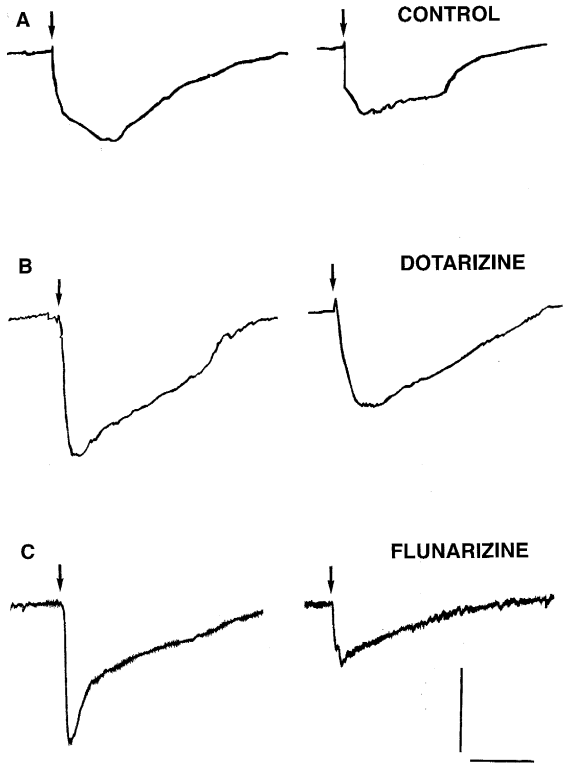


Fig. 7. Effects of dotarizine and flunarizine on $IP_3$-induced currents in *Xenopus* oocytes. Membrane currents were recorded at a holding potential of −60 mV and downward deflections represent inward currents. Oocytes A, B and C were microinjected with 0.14 pmol $IP_3$ at the points shown by the arrows. After a 20 min washout, by which time the current had returned to baseline, a second $IP_3$ injection was carried out in the control state (A), or in the presence of dotarizine (B) or flunarizine (C). Both drugs (at a concentration of 10 μM) were added to the chamber 10 min before the $IP_3$ injection. The vertical scale bar represents 100 nA (trace A), 200 nA (trace B) and 400 nA (trace C), and the horizontal scale bar represents 1 min (all traces).

Experiments similar to those described above were repeated but, this time, to study the effects of dotarizine and flunarizine on contractions induced directly by the opening of voltage-dependent $Ca^{2+}$ channels with high $K^+$ applications. After reproducible responses to alternate applications of 5-HT (1 μM) and high $K^+$ (35 mM) had been obtained, the coronary strips were repeatedly contracted with high $K^+$, applied at 15 min intervals in the presence of dotarizine or flunarizine (the three first $K^+$ pulses) or upon washing out of these drugs. In these protocols high concentrations (10 μM) of dotarizine and flunarizine were employed because, in previous experiments (see Fig. 3A and B), these concentrations had blocked the contractions evoked by the direct opening of $Ca^{2+}$ channels by 90% and 70%, respectively. Once again dotarizine strongly blocked, in a sustained manner, the

contractions induced by high $K^+$ and 5-HT (Fig. 6A, first and second bar on the right). Similar results were obtained with flunarizine although the blocking effect of this agent on the contraction evoked by high $K^+$ and 5-HT was smaller and started more slowly (Fig. 6B).

### 3.4. Effects of dotarizine and flunarizine on $IP_3$ receptors in Xenopus oocytes

As described above, foreign 5-$HT_2$ receptors expressed in oocytes are able to couple with the native G-protein-$IP_3$ production, intracellular $Ca^{2+}$ rise and $I_{Cl}$ activation. To determine whether dotarizine and flunarizine, as putative 5-$HT_2$ antagonist drugs, exert their effect at the receptor level or at any of the steps involved in the transduction and amplification of the signal, it was convenient to test if these two compounds had any intracellular effect on the $IP_3$ receptor in the oocyte. To do this we investigated the effects of flunarizine and dotarizine on $I_{Cl}$ when oocytes were microinjected with $IP_3$.

Injection of $IP_3$ (0.14 pmol) into control oocytes at a holding potential of $-60$ mV generated an inward $I_{Cl}$ with a mean rise of $225 \pm 32$ nA ($n = 31$ oocytes), whereas injection of the same volume of an $IP_3$-free solution evoked no such current ($n = 4$ oocytes). A second injection of $IP_3$ 20 min later elicited an $I_{Cl}$ that was $77 \pm 8\%$ of the first one ($n = 10$ oocytes), as shown in an original trace in Fig. 7A. The inward $I_{Cl}$ induced by $IP_3$ injection was blocked in the presence of 5 mM caffeine (data not shown), an $IP_3$ receptor antagonist in the oocyte (Parker and Ivorra, 1991). In oocytes in which the second injection of $IP_3$ was carried out in the presence of dotarizine (10 μM, added 10 min before the second injection), the magnitude of this second response to $IP_3$ was not changed with respect to the control second response in the absence of the drug ($79 \pm 5\%$, $n = 4$ oocytes). A typical trace of one such experiment is shown in Fig. 7B. This result suggests that dotarizine does not affect either the $IP_3$ receptor or the $Ca^{2+}$-activated $Cl^-$ channel. However, in the presence of flunarizine (10 μM, added 10 min before the second $IP_3$ injection) the amplitude of the second response was only $40 \pm 7\%$ of the first ($n = 7$ oocytes, $P < 0.01$ compared with control second response, unpaired *t*-test). An original trace of the flunarizine effect is presented in Fig. 7C.

### 3.5. Effects of flunarizine and dotarizine on the $I_{Cl}$ current in oocytes

In order to ascertain whether the flunarizine effect observed was due to a direct action of this drug on the $IP_3$ receptor and/or to a blockade of the $Cl^-$ channel in the oocyte, $I_{Cl}$ was activated directly by increasing the cytosolic free $[Ca^{2+}]$. This was done by flash-photolysis of the 'caged' $Ca^{2+}$ compound, DM-nitrophen, which had previously been microinjected into the oocyte (see Section 2). As shown in Fig. 8, at a holding potential of $-60$ mV, brief flashes of ultraviolet light generated reproducible and transient inward currents due to the opening of $Ca^{2+}$-activated $Cl^-$ channels. Dotarizine (10 μM) had no effect on this transient current in 4 oocytes tested, whereas flunarizine (10 μM) caused a small ($10.3 \pm 4.8\%$) reduction of the current, suggesting only a minor inhibition on

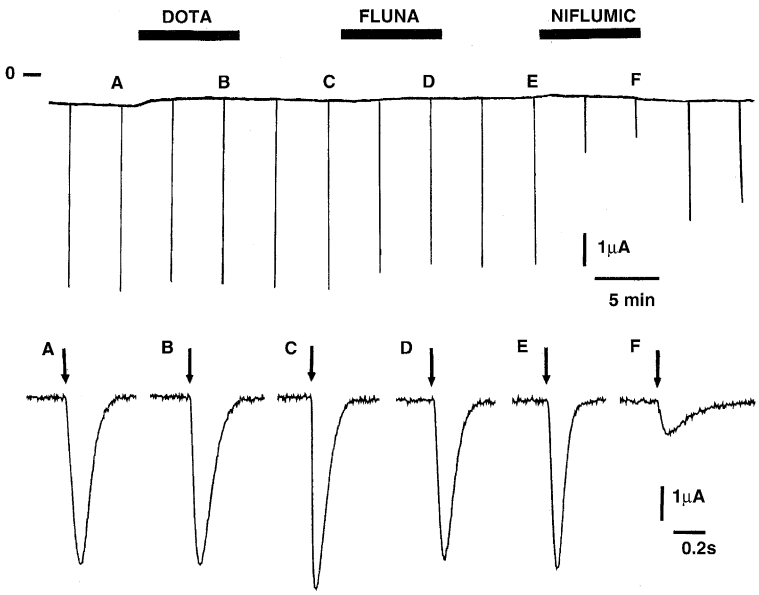


Fig. 8. Effects of dotarizine (DOTA, 10 μM), flunarizine (FLUNA, 10 μM) and niflumic acid (NIFLUMIC, 100 μM) on $Ca^{2+}$-activated $Cl^-$ currents in a voltage-clamped oocyte. Photorelease of intracellular $Ca^{2+}$ from the caged compound DM-nitrophen previously injected in the oocyte was performed by repetitive application, at 4 min intervals, of a flash of light using a flash-lamp system. The lower panel shows expanded time records of currents in the control state (A, C, E) and in the presence of dotarizine (B), flunarizine (D) and niflumic acid (F). The light flashes are indicated by arrows.

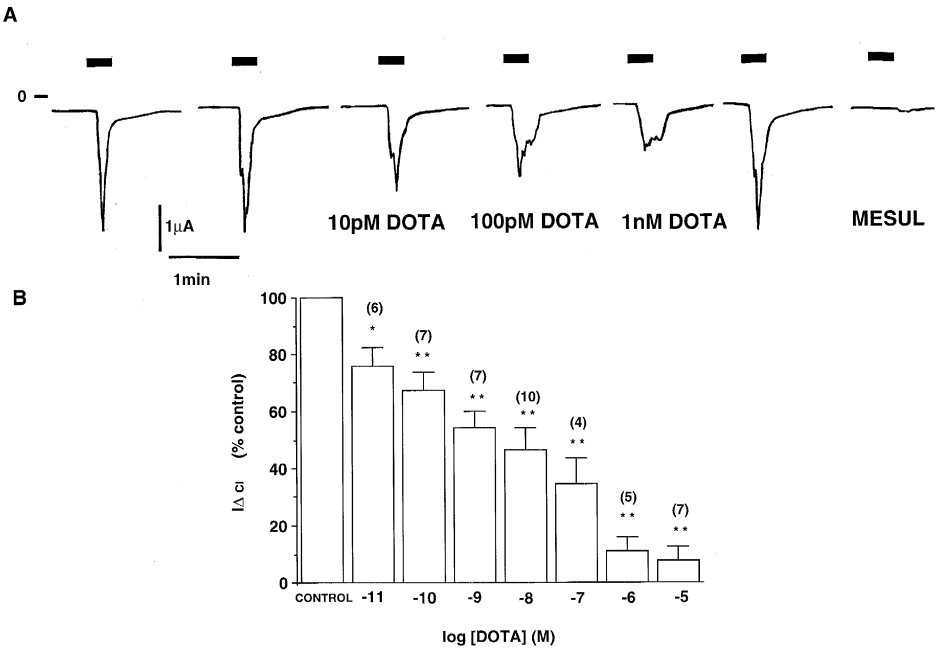


Fig. 9. Effects of dotarizine (DOTA) on $Ca^{2+}$-activated chloride currents ($I_{Cl}$) evoked by 5-HT in oocytes expressing the $5HT_{2A}$ receptor. In panel (A), a typical trace of the current obtained in one oocyte injected with the RNA encoding for the 5-$HT_{2A}$ receptor is shown (see Section 2). The agonist pulse (100 nM, 20 s) was applied repeatedly at 10 min intervals, as shown by the solid bars. Dotarizine (DOTA), at different concentrations, or the 5-$HT_2$ receptor antagonist, mesulergine (MESUL, 1 μM), was added 6 min before 5-HT application. In panel (B), the mean currents obtained in the presence of increasing concentrations of dotarizine are plotted. Currents are expressed as percentages of the control, with the number of oocytes tested in parentheses ($^{*}P < 0.05$ and $^{**}P < 0.005$ compared with control, paired *t*-tests).

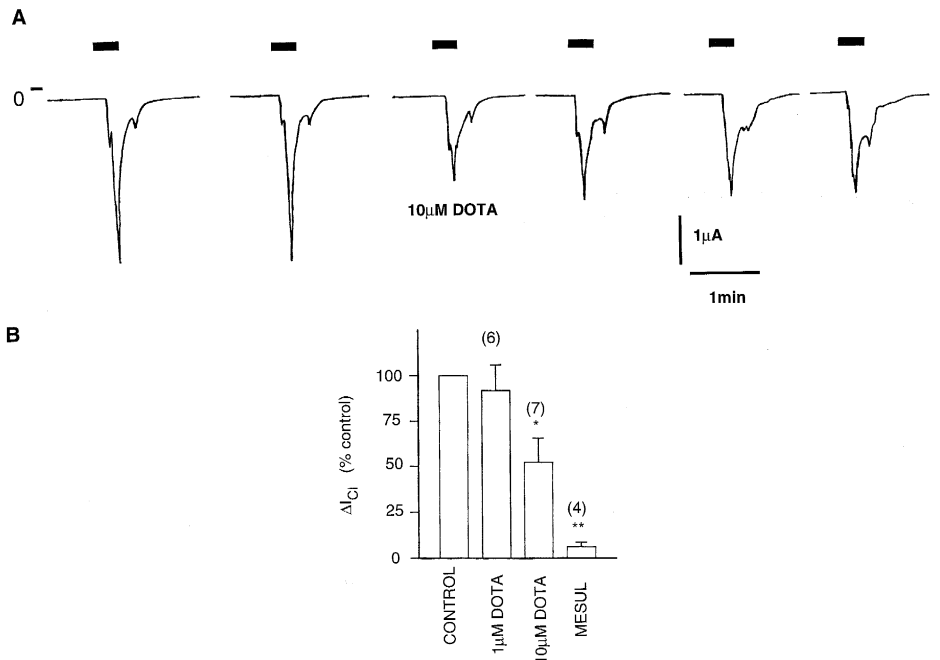


Fig. 10. Effects of dotarizine (DOTA) on $Ca^{2+}$-activated chloride currents ($I_{Cl}$) evoked by 5-HT in oocytes expressing the $5HT_{2C}$ receptor. Panel (A) shows a typical trace of the current induced by the repeated application of 5-HT pulses (100 nM every 10 min) to one oocyte injected with the RNA encoding for the 5-$HT_{2C}$ receptor. Solid bars represent the time of 5-HT application. Dotarizine (DOTA, 10 μM) was added 6 min before the 5-HT pulse. In panel (B), the mean currents obtained in the presence of dotarizine (DOTA, 1 μM or 10 μM) and mesulergine (MESUL, 1 μM) are shown; they are expressed as percentages of the control current ($^{*}P < 0.05$ and $^{**}P < 0.001$ compared with control, paired *t*-tests). The number of oocytes is shown in parentheses.

the chloride channel. In contrast, in the presence of niflumic acid (100 μM), a specific blocker of the oocyte $Ca^{2+}$-activated $Cl^-$ channel (White and Aylwin, 1990), the $I_{Cl}$ was blocked by 74 ± 2%. The upper part of Fig. 8 shows an original trace of this experiment; in the lower part of the same figure, the same trace is shown expanded.

### *3.6. Effects of dotarizine on two pure populations of 5-HT$_2$ receptors expressed in oocytes*

Control non-injected oocytes did not respond to the application of 5-HT. However, in oocytes injected with RNA encoded for 5-$HT_{2A}$ receptors the application of 5-HT (100 nM for 20 s) resulted in a mean increase of 1.48 ± 0.29 μA in the inward current ($n = 23$ oocytes from 4 different donors). A typical record is shown in Fig. 9A. The inward current response was reproducible with a wash period of 10 min between stimuli, and was inhibited by 95 ± 2% by the 5-$HT_2$ receptor antagonist, mesulergine, at 1 μM ($n = 4$, $P < 0.001$ compared with control, paired *t*-test). Application of increasing concentrations of dotarizine during the 6 min previous to the 5-HT pulse caused a dose-dependent inhibition of the inward current (Fig. 9A and B) with an $IC_{50}$ of 2.2 ± 0.9 nM. When the drug was applied at low concentrations (up to 1 nM) the blocking effect of dotarizine was completely reversed on washing for 10 min, whereas when used at a concentration of 10 μM the dotarizine blockade was not reversed upon washing.

In 8 oocytes from 3 different donors expressing the 5-$HT_{2C}$ receptor subtype, the application of 5-HT (100 nM for 20 s) caused a mean increase of 1.93 ± 0.47 μA in the inward current. At 1 μM dotarizine did not significantly decrease the $I_{Cl}$ induced by 5-HT (Fig. 10B). At a concentration of 10 μM, the drug caused a 48 ± 13% inhibition of the $I_{Cl}$ evoked by 5-HT ($P < 0.05$ compared with control current, paired *t*-test, $n = 7$ oocytes). The 5-$HT_2$ receptor antagonist, mesulergine (1 μM), caused a 94 ± 3% inhibition of the inward current induced by 5-HT ($P < 0.001$ compared with control, paired *t*-test, $n = 4$ oocytes). A typical trace of this experiment is shown in Fig. 10A. Fig. 10B shows the means ± S.E.M. of the $I_{Cl}$ values (expressed as percentages of control) obtained in the presence of 10 μM of dotarizine and 1 μM of mesulergine.

## 4. Discussion

Though of similar chemical structure, the two piperazine derivatives, dotarizine and flunarizine, have important pharmacological differences (Table 1). Thus dotarizine blocked the phasic contractions induced by 5-HT in pig coronary arteries with a 17-fold greater potency than the $K^+$-induced responses. On the contrary, flunarizine inhibited the 5-HT and $K^+$ responses with similar $IC_{50}$ values. From previous experiments with rabbit basilar and aorta smooth muscle and in bovine chromaffin cells, it seems that dotarizine and flunarizine inhibit the contractile vessel response and catecholamine secretion by blocking $Ca^{2+}$ entry through voltage-dependent $Ca^{2+}$ channels (Tejerina et al., 1993; Villarroya et al., 1995). However, the present experiments showed that in the case of dotarizine, its vasorelaxing effects in coronary artery strips contracted with 5-HT have a high component of blockade of 5-HT receptors. That the profile of dotarizine is not typical of a selective L-type $Ca^{2+}$ channel blocker was clearly demonstrated by the experiments with the 1,4-dihydropyridine derivative, elgodipine (Tamargo et al., 1991). This compound inhibits the $K^+$-evoked contractions with an $IC_{50}$ of only 2.3 nM, 140-fold lower than its $IC_{50}$ to block the 5-HT-induced coronary contractions. Thus it seems clear that, in exerting an anti-vasoconstricting and/or vasodilator effect, elgodipine acts by preferential blockade of L-type $Ca^{2+}$ channels, while dotarizine acts by blocking 5-HT receptors in addition to $Ca^{2+}$ channels.

The effects of 5-HT in coronary artery are complex and are probably mediated by various receptor subtypes which cause vascular contraction or relaxation (Martin, 1994). In fact the use of reverse transcriptase and polymerase chain reaction technique (RT-PCR), has allowed the identification of mRNAs for 5-$HT_{1D\beta}$, 5-$HT_{2A}$, 5-$HT_{2B}$, 5-$HT_4$ and 5-$HT_7$ receptors in coronary artery of pig and other rat and human blood vessels (Ullmer et al., 1995). Some of these receptors are located in endothelial cells (5-$HT_{1D\beta}$ and

Table 1
Summary of compared effects of dotarizine and flunarizine on contractions of the pig coronary artery and on inward $Cl^-$ current ($I_{Cl}$) triggered by various manipulations in *Xenopus* oocytes

| Parameter measured | Dotarizine | Flunarizine [a] | Ratio Fluna /Dota |
|---|---|---|---|
| Contraction of coronary artery induced by 5-HT ($IC_{50}$, μM) | 0.22 | 1.7 | 7.72 |
| Contraction of coronary artery induced by $K^+$ ($IC_{50}$, μM) | 3.7 | 2.4 | 0.65 |
| $I_{Cl}$ activated by $IP_3$ injection in oocytes | no effect | 48% blockade at 10 μM | — |
| $I_{Cl}$ induced by photorelease of $Ca^{2+}$ in oocytes | no effect | 10% blockade at 10 μM | — |
| $I_{Cl}$ activated by 5-HT in oocytes injected with RNA encoding for 5-$HT_{2A}$ receptors ($IC_{50}$, nM) | 2.2 | — | — |
| $I_{Cl}$ activated by 5-HT in oocytes injected with RNA encoding for 5-$HT_{2C}$ receptors ($IC_{50}$, nM) | 62% blockade by 10 μM | — | — |

[a] Because of the blockade of $IP_3$ receptors by flunarizine, its effects on expressed 5-$HT_2$ receptors in oocytes could not be tested (see Section 4).

5-$HT_{2B}$) where they elicit a vasorelaxant effect mediated by inhibition of cAMP formation or activation of nitric oxide release, respectively (Schoeffter and Hoyer, 1990; Weinshank et al., 1992; Glusa and Richter, 1993). Other receptors (5-$HT_{2A}$, 5-$HT_{1D\beta}$, and 5-$HT_7$) are expressed in coronary smooth muscle cells. In these cells 5-$HT_{2A}$ receptors mediate a strong constrictor activity through intracellular $Ca^{2+}$ release after phosphatidylinositol hydrolysis (Doyle et al., 1986). 5-$HT_{1D\beta}$ receptors found in porcine coronary smooth muscle mediate an additional endothelium-independent contractile response, which is probably induced by an intracellular $Ca^{2+}$ rise similar to that described for the activation of 5-$HT_1$-like receptors in smooth muscle cells from bovine basilar artery (Ebersole et al., 1993). Therefore, the marked blocking effect of dotarizine to inhibit the 5-HT-induced contractions in coronary artery seems to be related to its 5-$HT_{2A}$ receptor effect, although we cannot exclude that the drug also has antagonist properties on coronary smooth muscle 5-$HT_{1D\beta}$/5-$HT_1$-like receptors. In fact, dotarizine inhibits the 5-HT-induced contraction of rabbit basilar artery, induced mainly through 5-$HT_1$-like receptor activation (Brasó et al., 1994).

To distinguish the 5-$HT_2$ receptor antagonist effect of dotarizine from the 5-$HT_1$-like effect it was convenient to use a system capable of expressing pure subtypes of 5-$HT_2$ receptors. *Xenopus* oocytes provide an excellent system for translating different subtypes of 5-$HT_2$ receptors. Although the oocytes lack endogenous 5-HT receptors, they are able to express foreign 5-$HT_2$ receptors which are coupled efficiently to the oocyte's own G-protein/phosphoinositide/$Ca^{2+}$ pathway. The rise in intracellular [$Ca^{2+}$] activates $Cl^-$ channels in the oocyte membrane. Therefore, the activation of 5-$HT_2$ receptors expressed in oocytes can be monitored by measuring the plasma membrane $Ca^{2+}$-activated chloride current, $I_{Cl}$ (Gundersen et al., 1983; Nomura et al., 1987, Miledi et al., 1989; Woodward et al., 1992). Using this expression model, dotarizine exhibited high selectivity for the 5-$HT_{2A}$ receptor, showing an $IC_{50}$ as low as 2.2 nM. This $IC_{50}$ is 100-fold lower than that obtained with coronary strips to counteract the 5-HT contractile effects. This discrepancy could have two causes: (1) the optimal concentration of 5-HT used to contract the vessel was 500 nM, while that selected in oocytes to elicit 5-$HT_{2A}$-mediated $I_{Cl}$ was 100 nM; and (2) the 5-HT contractile effects in coronary arteries might be mediated, in addition to 5-$HT_{2A}$ receptors, by other 5-HT receptor subtypes less sensitive to dotarizine, as mentioned above.

An interesting additional feature of the blockade by dotarizine of the 5-HT coronary contractions was the long duration of this blockade. Even after a 90 min washout of dotarizine, the responses to 5-HT remained greatly depressed. A surprising finding was that dotarizine also blocked, in a sustained manner, the $K^+$-evoked coronary contractions (Fig. 6A). This contrasts with the readily reversible blockade by dotarizine of $K^+$-evoked catecholamine release from superfused bovine adrenal chromaffin cells. This might have been due to differences in the efficiency of the washout procedure in a small volume of isolated cells superfused continuously at a high rate (Villarroya et al., 1995), compared with a static incubation of a large volume of tissue (i.e. the coronary artery in this study). However, this discrepancy could also have been due to intrinsic differences in the binding properties of dotarizine to vascular 5-$HT_{2A}$ receptors, to expressed 5-$HT_{2A}$ receptors in oocytes, to vascular L-type $Ca^{2+}$ channels, and to L- and non-L-subtypes $Ca^{2+}$ channels in bovine chromaffin cells.

Endothelial 5-$HT_{2B}$ receptors have been shown to be involved in the pathogenesis of migraine; there still remains doubt about the presence of endothelial 5-$HT_{2C}$ receptors. Serotonin released from platelets, or more likely from perivascular 5-HT-containing neurons, would activate 5-$HT_{2B}$ receptors and induce nitric oxide release which is an important trigger for migraine (Fozard and Kalkman, 1994; Fozard, 1995). Our experiments expressing 5-$HT_{2C}$ receptors in oocytes show that the effect of dotarizine on this receptor subtype is weak compared with its 5-$HT_{2A}$ receptor effect or with the 5-$HT_{2C}$ receptor antagonist properties of mesulergine (a potent blocker of different 5-$HT_2$ receptor subtypes); mesulergine inhibited the 5-HT induced $I_{Cl}$ by 94%. This result suggests that the antimigraine effect of dotarizine is not related to the 5-$HT_{2C}$ receptor blockade, although we cannot discard the possibility that the drug has an effect on the other 5-$HT_{2B}$ receptors implicated in the pathogenesis of migraine. Since cloned 5-$HT_{2B}$ receptors have been expressed in oocytes and are efficiently coupled to $Ca^{2+}$-dependent chloride channels (Foguet et al., 1992), additional experiments with dotarizine in oocytes expressing this receptor subtype should be done to address this question.

Use of oocytes to study the effects of dotarizine on different steps involved in the activation of $I_{Cl}$ by 5-HT also reveal some interesting features of the mechanism of action of this drug. Our results with oocytes injected with $IP_3$ or caged-$Ca^{2+}$ show that dotarizine is devoid of an intracellular effect on $IP_3$ receptors or on membrane $Ca^{2+}$-activated $Cl^-$ channels. Blockade by flunarizine of $IP_3$ receptors prevented its study on 5-HT-generated $I_{Cl}$. Similar intracellular effects of flunarizine have been described in platelets (Seiler et al., 1987). Dotarizine thus seems to be more selective than flunarizine to block specifically 5-$HT_{2A}$ receptors.

The clinical relevance of the dotarizine profile described here relates to its possible application in the prophylactic treatment of migraine (see Section 1). Its parent compound, flunarizine, has been prescribed for years for this clinical condition. In fact, in a recent double-blind multicentre clinical trial, dotarizine showed more effectiveness than pizotifen in the prophylaxis of migraine with and without aura, with the additional advantage of less frequent and severe side-effects (Horga et al., 1996). It seems that selectivity of dotarizine for blocking 5-$HT_{2A}$ receptors,

might not be the sole aspect of its therapeutic profile; its ability to block $Ca^{2+}$ channels might represent a major component for its antimigraine prophylactic effect, although additional experiments to study the effect of the drug on 5-$HT_{2B}$ receptors are required.

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