Dotarizine versus flunarizine as calcium antagonists in chromaffin cells

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1 Dotarizine is a novel piperazine derivative structurally related to flunarizine that is currently being evaluated in clinical trials for its antimigraine and antivertigo effects. This clinical profile may be related to its Ca^{2+} antagonist properties. Therefore, the actions of both compounds as calcium antagonists were compared in bovine chromaffin cells.

2 Dotarizine and flunarizine blocked $^{45}\text{Ca}^{2+}$ uptake into K^{+} depolarized chromaffin cells (70 mM K^{+} /0.5 mM Ca^{2+} for 60 s) in a concentration-dependent manner, with IC_{50} s of 4.8 and 6.7 μM , respectively.

3 Dotarizine and flunarizine also inhibited the whole-cell Ca^{2+} and Ba^{2+} currents (I_{Ca} , I_{Ba}) in voltage-clamped chromaffin cells, induced by depolarizing test pulses to 0 mV, during 50 ms, from a holding potential of -80 mV. Blockade exhibited IC_{50} s of 4 μM for dotarizine and 2.2 μM for flunarizine. Dotarizine increased the rate of inactivation of I_{Ca} and I_{Ba} ; inhibition of whole-cell currents was use-dependent.

4 Transient increases of the cytosolic Ca^{2+} concentration, $[\text{Ca}^{2+}]_{\text{i}}$, produced by K^{+} stimulation (70 mM K^{+} for 5 s) of single fura-2-loaded chromaffin cells, were also inhibited by dotarizine and flunarizine with IC_{50} s of 1.2 and 0.6 μM , respectively. Upon washout of dotarizine, the $[\text{Ca}^{2+}]_{\text{i}}$ increases recovered fully after 5–10 min. In contrast, the responses remained largely inhibited 10 min after washing out flunarizine.

5 Catecholamine release induced by K^{+} stimulation (10-s pulses of 70 mM) was inhibited by dotarizine with an IC_{50} of 2.6 μM and by flunarizine with an IC_{50} of 1.2 μM . The blocking effects of both compounds developed slowly, and was fully established after 20–30 min of superfusion. While blockade by dotarizine quickly reversed upon its washout, that of flunarizine lasted even 25 min after washing out.

6 Catecholamine release from electroporated chromaffin cells triggered by 10 μM Ca^{2+} was not affected by 10 μM dotarizine or flunarizine.

7 Overall, the results suggest that dotarizine behaves as a Ca^{2+} antagonist in bovine chromaffin cells. It exhibits a potency similar to flunarizine in blocking Ca^{2+} entry, Ca^{2+} channels, Ca^{2+} signals and secretion. The dotarizine effects are readily reversible suggesting that in contrast to flunarizine, it does not accumulate in cells. Dotarizine is devoid of intracellular effects on the secretory machinery. All its blocking effects on Ca^{2+} entry, $[\text{Ca}^{2+}]_{\text{i}}$ rise and secretion are probably due to blockade of various Ca^{2+} channel subtypes in chromaffin cells. This blockade is use-dependent and seems to be due to the increase by dotarizine of the rate of Ca^{2+} channel inactivation.

Keywords: Dotarizine; flunarizine; chromaffin cell; calcium antagonists, calcium channels

Introduction

Classifications of calcium antagonists (Fleckenstein, 1983; Godfraind, 1987) vary according to their chemical structure (1,4-dihydropyridines, arylalkylamines, benzothiazepines, piperazines), pharmacological and clinical profiles (cardioselective, vasculoselective) or type of voltage-dependent Ca^{2+} channels they block (L-type selective, 'wide-spectrum' Ca^{2+} channel blockers). Though selective L-type Ca^{2+} channel blockers have useful indications in cardiovascular diseases (i.e. hypertension, cardiac ischaemic disease), 'wide-spectrum' Ca^{2+} antagonists might provide additional tissue protective effects in ischaemic conditions. These may be useful in treating clinical conditions such as migraine, in which multiple oetopathogenic factors coincide. In this condition, various vasoactive neurotransmitters including 5-hydroxytryptamine (5-HT) and catecholamines have been implicated.

Though several calcium antagonists have been useful in the treatment of migraine, the 'wide-spectrum' Ca^{2+} channel

blockers, cinnarizine and flunarizine, are being widely used for this clinical condition (Todd & Benfield, 1989). Dotarizine, 1-(diphenylmethyl)-4-[3-(2-phenyl-1,3-dioxalan-2-yl)-propyl]-piperazine, is a novel piperazine derivative structurally related to flunarizine (Figure 1). Like flunarizine, dotarizine exhibits slight histamine receptor and α -adrenoceptor blocking properties, but shows a higher anti-5-HT receptor activity, both *in vitro* and *in vivo* (Brasó *et al.*, 1989; 1994; Cartheuser *et al.*, 1994). Dotarizine is currently being investigated clinically as an antimigraine and antivertigo drug. Its therapeutic effects might be related to its anti-5-HT receptor blocking properties and/or Ca^{2+} -entry blocking properties. In fact, dotarizine inhibited $^{45}\text{Ca}^{2+}$ uptake and vessel contractility in rabbit basilar and aortic smooth muscle. The authors of this observation (Tejerina *et al.*, 1993) concluded that 'in rabbit, dotarizine inhibits Ca^{2+} entry through Ca^{2+} channels, being more selective for the basilar artery, probably by acting on multiple sites to decrease the availability of intracellular free Ca^{2+} required for activation'. In relation to this Ca^{2+} antagonist effect, more direct methodological approaches are required to ans-

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Figure 1 Chemical structures of dotarizine and flunarizine.

were the following questions: (i) whether dotarizine acts only on vascular L-type Ca^{2+} channels, or also on other neuronal Ca^{2+} channels of the N-, P- or Q-types (Olivera *et al.*, 1994); (ii) if dotarizine can affect the changes in cytosolic Ca^{2+} concentrations (Ca_i^{2+}) evoked by the activation of those Ca^{2+} channel subtypes; (iii) whether dotarizine inhibits the release of neurotransmitters by blocking cytosolic Ca^{2+} signals; and (iv) whether dotarizine acts selectively on the cell plasmalemma or also has an intracellular site of action.

The chromaffin cell, maintained in primary cultures prepared from bovine adrenal glands, constitutes an excellent model to answer these questions. These cells express various Ca^{2+} channels of the neuronal subtype besides cardiovascular L-type channels (Gandia *et al.*, 1993; Albillos *et al.*, 1993). In addition, the chromaffin cell stores and secretes the catecholamines dopamine, noradrenaline and adrenaline when extracellular Ca^{2+} gains the cell interior through these diverse Ca^{2+} channels (Jiménez *et al.*, 1993; Michelena *et al.*, 1993). We present here the results of a study aimed at defining the effects of dotarizine on the following parameters in bovine chromaffin cells: (1) $^{45}\text{Ca}^{2+}$ uptake stimulated with K^+ depolarization; (2) whole-cell currents through Ca^{2+} channels using Ca^{2+} or Ba^{2+} as charge carriers; (3) increases of the $[\text{Ca}^{2+}]_i$ upon K^+ -stimulation of single fura-2-loaded cells; (4) catecholamine release from intact chromaffin cells briefly depolarized with high- K^+ solutions; and (5) catecholamine release triggered by application of Ca^{2+} /EGTA [ethylenebis(oxonitrilo)tetracetate] buffers to electroporated chromaffin cells. Because of their similar chemical structure and clinical indications (migraine, vertigo), most studies presented here with dotarizine were run in parallel to flunarizine.

Methods

Preparation and culture of bovine chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated following standard methods (Livett, 1984) with some modifications (Moro *et al.*, 1990). Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 iu ml^{-1} penicillin and 50 $\mu\text{g} \text{ml}^{-1}$ streptomycin. Cells were plated at a density of 5×10^5 cells/well in 24-multiwell Costar plates for $^{45}\text{Ca}^{2+}$ uptake studies. For measurements of $[\text{Ca}^{2+}]_i$ and whole-cell currents, cells were

plated on 1 cm diameter glass coverslips at a density of 5×10^4 cells per coverslip. For secretion experiments, 5×10^6 cells were plated in 5 cm diameter plastic Petri dishes. Cells were used 1–4 days after plating. Medium was replaced after 24 h and then after 2–3 days.

Measurements of $^{45}\text{Ca}^{2+}$ uptake

$^{45}\text{Ca}^{2+}$ uptake studies were carried out in cells after 2–3 days in culture. Before the experiment, cells were washed twice with 0.5 ml Krebs-HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) solution of the following composition (mM): NaCl 140, KCl 5.9, MgCl_2 1.2, CaCl_2 0.5, glucose 11, HEPES 10, at pH 7.2 and 37°C. $^{45}\text{Ca}^{2+}$ uptake into chromaffin cells was studied by incubating the cells at 37°C with $^{45}\text{CaCl}_2$ at a final concentration of $4 \mu\text{Ci ml}^{-1}$ in the presence of Krebs-HEPES (basal uptake) or high K^+ solution (Krebs-HEPES containing 70 mM KCl with isosmotic reduction of NaCl). This incubation was carried out for 1 min; at the end of this period, the test medium was rapidly aspirated and the uptake reaction was ended by adding 0.5 ml of a cold Ca^{2+} -free Krebs-HEPES containing 10 mM LaCl_3 . Finally, cells were washed 5 times more with 0.5 ml of Ca^{2+} -free Krebs-HEPES containing 10 mM LaCl_3 and 2 mM EGTA, at 15 s intervals.

To measure radioactivity retained by chromaffin cells, the cells were scraped with a plastic pipette tip while adding 0.5 ml 10% trichloroacetic acid; 3.5 ml of scintillation fluid (Ready Micro, Beckman) was added and the samples counted in a Packard beta counter. Results are expressed as % of $^{45}\text{Ca}^{2+}$ taken up by control cells.

Measurements of whole-cell currents through Ca^{2+} channels

Membrane currents were measured with the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). Coverslips containing the cells were placed in an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused with a control Krebs solution containing (in mM): NaCl 145, MgCl_2 1, CaCl_2 2, HEPES/NaOH 10, tetrodotoxin (TTX) 0.005, pH 7.4. For current recording, 10 mM Ba^{2+} or 10 mM Ca^{2+} (instead of 2 mM Ca^{2+}) was used as the charge carrier. Cells were dialyzed with an intracellular solution containing (in mM): NaCl 10, CsCl 100, TEA.Cl 20, Mg.ATP 5, EGTA 14, HEPES/CsOH 20, Na.GTP 0.3, pH 7.2.

Whole-cell recordings were made with fire-polished electrodes (resistance 2–5 M Ω) mounted on the headstage of a Dagan 8900 patch-clamp amplifier, allowing cancellation of capacitance transient and compensation of series resistance. A Labmaster data acquisition and analysis board and a 386-based microcomputer with pCLAMP software (Axon Instruments, Inc., CA, U.S.A.) were used to acquire and analyse the data. Solutions were exchanged by use of multi-barrelled concentration-clamp device, the common outlet of which was placed with 100 μm of the cell to be patched.

Intracellular Ca^{2+} measurements

Cells attached to glass coverslips were loaded with fura-2/AM (2.5 μM for 60 min at 37°C, in the incubator). Then the cells were washed with Krebs-HEPES of the following composition (in mM): NaCl 140, KCl 5.9, MgCl_2 1.2, CaCl_2 2, glucose 11, HEPES 10 at pH 7.3. They were kept for 10 min at 37°C in an incubator before being placed on the stage of an inverted microscope, in a chamber allowing their continuous superfusion with Krebs-HEPES. High K^+ (70 mM in 2 mM Ca^{2+})- and dotarizine- or flunarizine (1, 3 and 10 μM)-containing solutions were applied to the cell under investigation using the fast superfusion device employed in the electrophysiological studies. Only one experimental protocol was run for each single coverslip. Single cell fluorescence

measurements were performed by exciting the fura-2-loaded cells with alternating 360 and 390 nm filtered light. The apparent $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescent signals (short over long wavelengths) according to Grynkiewicz *et al.* (1985). Calibration constants were experimentally determined as described by Almers & Neher (1985). Experimental fluorescence data were sampled every 0.5 s by a computer which provided continuously the $[Ca^{2+}]_i$ values in μM .

On-line measurement of catecholamine release from intact chromaffin cells

Bovine chromaffin cells (5×10^6) were introduced in a micro-chamber for cell superfusion at room temperature ($22 \pm 2^\circ C$) with a Krebs-HEPES of the following composition (in mM): NaCl 144, KCl 5.9, $MgCl_2$ 1.2, glucose 11, $CaCl_2$ 2 and HEPES 10, at pH 7.4. The perfusion rate was $2 ml min^{-1}$. The liquid flowing from the perfusion chamber reached an electrochemical detector model Metrohm AG CH-9100 Hersau that monitored 'on line' under the amperometric mode, the amount of catecholamines secreted (Borges *et al.*, 1986). The cells were stimulated for secretion with a Krebs-HEPES solution containing 70 mM K^+ (appropriate reduction of NaCl was made for iso-osmolarity), for 10 s at 5 min intervals by means of electrovalves.

Catecholamine release from electroporated cells

Cells were washed in Ca^{2+} -free Krebs-HEPES solution and resuspended ($3 \times 10^6 ml^{-1}$) in an electroporation buffer of the following composition (in mM): potassium glutamate 140, $MgCl_2$ 3, EGTA 1, HEPES 20, pH 7, K-ATP 2 and 0.5% bovine serum albumin. Cells were rendered permeable by 10 discharges of 1,500 V at a capacitance of $3 \mu F$ using a Gene Pulser apparatus (Bio-Rad) with 10 s in between the discharges, in a cuvette (0.5 ml) with two electrodes 0.4 cm apart. Then, cells were placed in a microchamber and superfused at room temperature with the electroporation buffer at $1 ml min^{-1}$. Catecholamine release was triggered by application of a Ca^{2+} /EGTA buffer containing $10 \mu M$ free Ca^{2+} . Catecholamine release was monitored on-line with electrochemical detection as described for intact cells.

Materials and solutions

The following materials were used: collagenase from *Clostridium histolyticum* (Boehringer-Mannheim); bovine serum albumin fraction V, cytosine arabinoside, fluorodeoxyuridine, tetrodotoxin (TTX) and EGTA (Sigma); foetal calf serum, penicillin and streptomycin (GIBCO); scintillation fluid Ready micro (Beckman); $^{45}Ca^{2+}$ (specific activity 10–40 mCi mg^{-1} calcium, Amersham). Fura-2 AM was obtained from Molecular Probes, Eugene, Oregon, U.S.A. Dotarizine was from Grupo Ferrer (Barcelona, Spain) and flunarizine from the Janssen Research Foundation, Beerse, Belgium. All other chemicals were reagent grade.

Dotarizine and flunarizine were dissolved in dimethylsulphoxide (DMSO, Merck) at $10^{-2} M$ and diluted in saline solutions to the desired concentrations. The highest concentrations of DMSO used (not more than 0.1%) had no effects on any of the parameters studied.

Statistical analysis

Data are expressed as means $\pm$ s.e.mean. IC_{50} s for each drug were estimated through non-linear regression analysis of ISI software, for a PC computer. Differences between non-paired groups were compared by Student's *t* test with the statistical programme Statworks TM; a value of *P* equal to or smaller than 0.05 was taken as the limit of statistical significance.

Results

Effects of dotarizine and flunarizine on $^{45}Ca^{2+}$ uptake

First we wished to compare the effects of dotarizine and flunarizine on $^{45}Ca^{2+}$ entry into K^+ -depolarized chromaffin cells. Thus, cells were depolarized with 70 mM K^+ (with equimolar reduction of Na^+) for 60 s and $^{45}Ca^{2+}$ uptake was evaluated in the presence of different concentrations of both drugs. Before stimulation, cells were preincubated with each concentration of the drug for 10 min.

High K^+ stimulation for 1 min induced $^{45}Ca^{2+}$ uptake (in the presence of 0.5 mM $^{40}Ca^{2+}$) of $3,840 \pm 345$ c.p.m. per 5×10^5 cells (data from 59 individual wells from 10 different batches of cells). In basal conditions (1 min incubation in Krebs-HEPES solution containing $4 \mu Ci ml^{-1}$ $^{45}Ca^{2+}$ plus 0.5 mM $^{40}Ca^{2+}$), cells retained 897 ± 151 c.p.m. (data from 32 individual wells from 10 different batches of cells). Thus, the mean ratio evoked/basal Ca^{2+} uptake was 4.3.

Both dotarizine and flunarizine inhibited K^+ -evoked Ca^{2+} uptake into chromaffin cells in a concentration-dependent manner. The IC_{50} to inhibit the K^+ response was $4.8 \mu M$ for dotarizine and $6.7 \mu M$ for flunarizine. No significant difference was found between both IC_{50} s; however, considering the individual values at given concentrations, dotarizine was slightly more potent than flunarizine in blocking $^{45}Ca^{2+}$ entry (Figure 2).

Effects of dotarizine and flunarizine on whole-cell Ba^{2+} and Ca^{2+} currents

Dotarizine and flunarizine probably interrupted Ca^{2+} entry into K^+ depolarized chromaffin cells by blocking voltage-dependent Ca^{2+} channels. It was, therefore, desirable to test this possibility by directly measuring whole-cell Ba^{2+} or Ca^{2+} currents (I_{Ba} or I_{Ca}) in voltage-clamped chromaffin cells. The blocking effects of dotarizine and flunarizine on I_{Ba} or I_{Ca} were tested by superfusing bovine chromaffin cells with increasing concentrations (1–10 μM) of dotarizine or flunarizine during 10 min. Currents were elicited by 50 ms depolarizing pulses to 0 mV applied from a holding potential of -80 mV, at 15 s intervals. Figure 3a (left panel) shows the blocking effects of dotarizine on I_{Ba} . Currents records were obtained before (control) and 10 min after superfusion of the cell with each concentration of the drug. Dotarizine blocked

Figure 2 Effects of dotarizine (●) and flunarizine (○) on $^{45}Ca^{2+}$ uptake into chromaffin cells stimulated with 70 mM K^+ for 60 s. Before stimulation, cells were preincubated with each concentration of dotarizine or flunarizine for 10 min. In each individual experiment, $^{45}Ca^{2+}$ uptake (ordinate scale) was normalized to 100% ($^{45}Ca^{2+}$ taken up by cells in the absence of drugs). Data are means $\pm$ s.e.mean of at least 4 triplicate experiments from different batches of cells. **P* < 0.05, compared to flunarizine.

Figure 3 Effects of dotarizine and flunarizine on whole-cell Ba^{2+} currents through Ca^{2+} channels. (a) Original current records in two different cells were obtained either before (control) or after treatment of the cell with increasing concentrations of dotarizine (left panel) or flunarizine (right panel). Each concentration of drug was superfused for 10 min. Cells were held at -80 mV and 50 ms depolarizing pulses to 0 mV were applied at 15 s intervals. (b) Concentration-dependency of the blocking effects of dotarizine (●) and flunarizine (○) on whole-cell Ba^{2+} currents. Data are expressed as percentage of blockade of I_{Ba} and represent the mean $\pm$ s.e.mean of 6–9 different cells.

I_{Ba} by $24 \pm 4\%$ ($n = 9$ cells), $48 \pm 5\%$ ($n = 8$ cells) and $79 \pm 4\%$ ($n = 8$ cells) at 1, 3 and 10 μ M, respectively. When the effects of flunarizine were tested (right panel in Figure 3a), blockade of I_{Ba} was characterized by a slow onset of action. Averaged results on the blockade of I_{Ba} by dotarizine and flunarizine are shown in Figure 3b. A clear concentration-dependency for the blocking effects of both compounds was seen. The apparent IC_{50} s were estimated to be 2.2 μ M for flunarizine and 4 μ M for dotarizine. These values are slightly lower than those obtained measuring $^{45}Ca^{2+}$ uptake.

At the highest concentration used (10 μ M) dotarizine seemed to increase the rate of inactivation of the remaining current (see left panel in Figure 3a). It was, therefore, of interest to study further the mechanism of blockade of dotarizine. In the experiments shown in Figure 4, 5-s depolarizing test pulses were applied to cells superfused with external solutions containing 10 mM Ca^{2+} or 10 mM Ba^{2+} as charge carriers. It is interesting to note two characteristics of the dotarizine effects: (i) the blockade of peak I_{Ca} or peak I_{Ba} is time-dependent; (ii) the blockade of the late current is greater than the blockade of the initial peak current. The time-dependency of blockade is probably due to the lipophilicity of piperazine derivatives which accumulate progressively in lipid-rich membranes (see later when describing the secretion experiments). The greater blockade of the late current is better seen in Figure 4b and 4d where expanded records from panels (a) and (b) are shown. It apparently seems that dotarizine greatly accelerates the inactivation of I_{Ba} and I_{Ca} and that the drug drastically diminishes the area of the current traces. Upon wash out of dotarizine, the narrowed area quickly expanded, though the decreased peak current recovered little. The acceleration of inactivation was also seen even with much shorter pulses (15 ms instead of 5 s), as shown in the inset of Figure 5.

Another interesting point was the possibility that dotarizine inhibited I_{Ba} in a use-dependent manner. This was tested by monitoring the development of dotarizine blockade at two frequencies of stimulation (Figure 5). Short depolarizing test pulses (15 ms) were applied initially at 0.033 Hz.

Figure 4 Effects of dotarizine (10 μ M in all cases) on whole-cell currents through Ca^{2+} channels using 10 mM Ca^{2+} (a, b) or 10 mM Ba^{2+} (c, d) as charge carriers. Long depolarizing test pulses (5 s duration) to 0 mV were applied from a holding potential of -80 mV. Test pulses were sequentially applied to a voltage-clamped chromaffin cell at 30 s intervals. Dotarizine was present during the time shown by horizontal bars in (a) and (c). Panels (b) and (d) are records from (a) and (c) with an expanded time scale.

Upon addition of dotarizine the initial I_{Ba} (about 900 pA) started to slowly decline. After a 10-min stimulation period, the frequency of stimulation was increased to 0.2 Hz. The rate of blockade was accelerated, suggesting that dotarizine was blocking the Ca^{2+} channels in a use-dependent manner. Washout of dotarizine produced a partial and slowly developing recovery of I_{Ba} .

Effects of dotarizine and flunarizine on cytosolic Ca^{2+} transients

A single fura-2-loaded chromaffin cell was first perfused with Krebs-HEPES solution until a stable basal $[Ca^{2+}]_i$ was achieved (usually, around 100 nM). The compounds themselves did not increase the basal $[Ca^{2+}]_i$ at concentrations of 1–10 μ M.

Application of 5-s pulses of 70 mM K^+ in 2 mM Ca^{2+} produced a rapid increase of $[Ca^{2+}]_i$ to a peak of 1.5 μ M; the $[Ca^{2+}]_i$ quickly returned to basal levels. The peaks were very reproducible on sequential applications of K^+ at 5 min intervals. When the K^+ responses were stable, dotarizine or flunarizine were superfused for 5 min in Krebs-HEPES solution, and then the K^+ pulse was repeated in the presence of each drug (Figure 6). The percentage of blockade was measured at the peak of the third stimulus. As with I_{Ba} currents, blockade of the Ca^{2+} signals by dotarizine or flunarizine was time-dependent (see Figure 6a).

Dotarizine (1 μ M) blocked the K^+ -evoked increase of $[Ca^{2+}]_i$ by $37 \pm 2\%$ ($n = 4$ cells), while flunarizine blocked it by $68.3 \pm 2.4\%$ ($n = 6$ cells). When dotarizine 3 μ M and 10 μ M was superfused, the blockade was $84 \pm 2.2\%$ and $83 \pm 2.8\%$, respectively. At these concentrations, flunarizine blocked by $93 \pm 2\%$ and 100% the Ca^{2+} signals. The IC_{50} for dotarizine blockade was 1.2 μ M and for flunarizine 0.6 μ M (Figure 6c). These values were 4 fold and 10 fold lower than the values obtained for $^{45}Ca^{2+}$ uptake inhibition (see Table 1).

The reversibility of the blocking effects of both compounds differed considerably. For instance, only 5 min after washing out the highest concentration of dotarizine used (10 μ M), the recovery of the K^+ -evoked $[Ca^{2+}]_i$ increase was almost fully established (Figure 6a, right traces). In contrast, at 5 or 10 min after washout of flunarizine, the Ca^{2+} signal was still suppressed.

Figure 5 Use-dependent inhibitory effects of dotarizine on I_{Ba} . Short test pulses to 0 mV (15 ms duration) from a holding potential of -80 mV, were sequentially applied at the frequencies shown by the arrows. Dotarizine was present as shown by the horizontal bar. In (b) the stimulation frequency was increased from 0.033 Hz to 0.2 Hz. Inset: original traces taken at the points of the time-course curve, reflected by the letters. Observe the change in kinetics of the current (greater inactivation) of traces B and C (dotarizine present) with respect to traces A (control) and D (washout of dotarizine).

Effects of dotarizine and flunarizine on catecholamine secretion

Stimulation of bovine chromaffin cells for 10 s with 70 mM K^+ at 5 min intervals gave reproducible secretory peaks for over a 2 h perfusion period. The mean oxidation current observed with high K^+ stimulation was 300 ± 20 nA. Figure 7a shows the time-dependent blocking effects of dotarizine on K^+ -evoked catecholamine secretion. The gradual blockade of secretion suggests gradual accumulation of dotarizine in the

Table 1 Summary of IC_{50} s of dotarizine and flunarizine to block the different parameters studied in bovine chromaffin cells (values in μ M)

	Dotarizine	Flunarizine
$^{45}Ca^{2+}$ uptake	4.8	6.7
I_{Ba}	4	2.2
$[Ca^{2+}]_i$	1.2	0.6
Secretion	2.6	1.2

Figure 6 Effects of dotarizine and flunarizine on the changes in cytosolic Ca^{2+} concentrations induced by K^+ . Short depolarizing pulses (5 s) of high K^+ (70 mM) were given to a fura-2-loaded single chromaffin cell at 5 min intervals ($\bullet$). When the K^+ responses were stabilized, 10 μ M dotarizine (a) or 10 μ M flunarizine (b) were superfused for 5 min before giving the K^+ pulse. After the third stimulus in the presence of the drug, the recovery of the Ca^{2+} signal at 5 and 10 min was tested. (c) $[Ca^{2+}]_i$ values (ordinate scale) were normalized to 100% (K^+ responses in the absence of drug), and represented versus the concentration of dotarizine ($\bullet$) and flunarizine ($\circ$). $*P < 0.05$, compared to flunarizine.

Figure 7 In (a), original traces of catecholamine secretory responses are shown. Cells were stimulated at 5-min intervals with 10-s pulses of a solution containing 70 mM K^+ and 2 mM Ca^{2+} . Dotarizine (10 µM) was present as shown by the horizontal bar at the bottom of the figure. (b) Shows concentration-response curves for dotarizine (●) and flunarizine (○) on such K^+ -evoked secretory responses. Data are expressed as percentage with respect to control secretion in the absence of the drug (100%) and correspond to the mean $\pm$ s.e.mean of 4 to 8 individual experiments. The effect of dotarizine and flunarizine are represented as their effects after a 30-min superfusion period. A single concentration of each drug was assayed in individual batches of cells (protocol shown in a). * $P < 0.01$ with respect to flunarizine.

plasma membrane. The gradual recovery suggests a quick washout of the drug from such membrane. Concentration-response curves for dotarizine and flunarizine on secretion are represented in Figure 7b. Both drugs blocked secretion in a concentration-dependent manner with IC_{50} values of 1.22 µM for flunarizine and 2.6 µM for dotarizine. Statistical differences were observed only at the concentration of 3 µM ($P < 0.01$).

Figure 8a shows the time-dependent effects of dotarizine on catecholamine secretion induced by K^+ . As the cells were continuously superfused and secretion was monitored 'on line', it was possible to study the rate of instauration of the effects of the drug as well as its reversibility. Dotarizine gradually blocked secretion; a maximum effect of 87% inhibition was obtained after 25 to 30 min of perfusion with dotarizine. When washing out the drug, its blocking effects were quickly reversed. Figure 8b shows the results obtained with flunarizine. As with dotarizine, its blocking effects on secretion were established in a time- and concentration-dependent manner. After 30 min perfusion of the drug 42, 77 and 91% inhibition of secretion was obtained with 1, 3 and 10 µM flunarizine, respectively. The most outstanding difference between these two drugs was the washout of their blocking effects on secretion. Dotarizine was readily washed while flunarizine washed out only partially after a 25 min period (66% after 1 µM and 39% after 3 µM); with 10 µM, even further blockade of secretion was observed during the washout period.

Figure 8 (a) Time-dependent inhibition by dotarizine of catecholamine secretion, and reversal of its blocking effects upon washout. Each column corresponds to secretion induced by a pulse of 70 mM K^+ /2 mM Ca^{2+} for 10 s, applied at 5 min intervals to intact superfused chromaffin cells. The shaded columns represent the effect of dotarizine and the lighter ones the washout. (b) Time-dependent inhibition and washout of flunarizine on secretion induced by 70 mM K^+ /2 mM Ca^{2+} for 10 s at 5 min intervals in bovine chromaffin cells. The shaded columns show the blocking effects of flunarizine at 5 min intervals and the open columns the washout of these blocking effects. Data are expressed as the mean percentage of control secretion (100%) $\pm$ s.e.mean of $n = 5$ in (a) and $n = 6$ in (b).

Catecholamine release from electroporated chromaffin cells

The results on $^{45}Ca^{2+}$ uptake, I_{Ca} , I_{Ba} and on $[Ca^{2+}]_i$ increases suggested that the blocking effects of dotarizine and flunarizine on catecholamine release were due to restriction of the access of extracellular Ca^{2+} to the secretory machinery. However, a direct intracellular effect on exocytosis could not be discarded with the above mentioned experiments. It was, therefore, interesting to test the effects of the two compounds on exocytosis triggered by micromolar concentrations of Ca^{2+} from electroporated chromaffin cells (Knight & Baker, 1982).

Figure 9a shows the secretory profiles obtained upon Ca^{2+} application (10 µM for 3 min) to electroporated cells superfused with the electroporation solution (see Methods). Ca^{2+} was applied once the base-line for secretion stabilized. Secretion rose sharply to a peak, followed by a plateau that started to decline after 2 min. When returning to the 0 Ca^{2+} solution, the secretory rate quickly went to basal levels. When present, dotarizine or flunarizine (10 µM) were added during the electroporation procedure and were kept in contact with the electroporated cells throughout the rest of the experiment. The secretory profiles obtained in the presence of these drugs were similar to the control profile. Measurements of peak secretions from various experiments produced averaged values which were similar in control cells, and in cells treated with dotarizine or flunarizine (Figure 9b).

Figure 9 Effects of dotarizine and flunarizine on catecholamine release from electroporated chromaffin cells. Secretion was triggered by the application of a Ca^{2+} /EGTA solution containing 10 μM free Ca^{2+} (see Methods). Cells were stimulated to secrete only once. In (a) copies of original traces of three secretory responses from 3 cell pools from the same batch are shown. Dotarizine or flunarizine (10 μM) were present during the electroporation procedure and during the rest of the experiment. Catecholamine release is expressed as oxidation current in nA (see calibration bar). In (b) averaged results obtained from experiments using the protocol in (a) are plotted. Results are mean $\pm$ s.e.mean of the number of experiments shown in parentheses above each column.

Discussion

The experiments presented here demonstrate that dotarizine blocks Ca^{2+} entry, whole-cell currents through voltage-dependent Ca^{2+} channels, $[Ca^{2+}]_i$ increase and catecholamine secretion in chromaffin cells subjected to depolarizing stimuli. These effects are concentration- and time-dependent, and readily reversible upon washing out the drug. In general, the effects of dotarizine were visible at 1 μM concentration and near maximal at the 10 μM concentration. The graded effects of increasing concentrations speak in favour of a selective action at a specific target. In addition, the fact that 10 μM dotarizine did not itself increase the basal $[Ca^{2+}]_i$, the spontaneous release of catecholamines nor secretion from electroporated cells also supports its specificity. Finally, the reversibility of its effects on all parameters studied strengthen this view.

As far as their potencies are concerned, dotarizine and flunarizine were equally effective (see Table 1). Thus, to block $^{45}Ca^{2+}$ uptake, dotarizine exhibited an IC_{50} of 4.8 μM and flunarizine of 6.7 μM . The increases in $[Ca^{2+}]_i$ were blocked with IC_{50} s of 1.2 and 0.6 μM and catecholamine release had IC_{50} s of 2.6 and 1.2 μM , respectively for dotarizine and

flunarizine. Slight differences between the various parameters studied might be due to unavoidable differences in protocols. For instance, $^{45}Ca^{2+}$ uptake was studied by exposing the cells for 60 s to a solution containing 70 mM K^+ and 0.5 mM Ca^{2+} . Each group of cells was stimulated only once. Increments in $[Ca^{2+}]_i$, however, were tested by applying repetitive 10 s pulses of a solution containing 70 mM K^+ and 2 mM Ca^{2+} . To study secretion, a similar protocol was used (repetitive 10-s pulses with a solution containing 70 mM K^+ and 2 mM Ca^{2+}). Finally, the effects on I_{Ba} were checked by applying short, 50 ms depolarizing pulses. Such differences can also be explained by the fact that the changes in $[Ca^{2+}]_i$ are not proportional to Ca^{2+} entry through Ca^{2+} channels. In this way, Ca^{2+} mobilization from intracellular stores present in bovine chromaffin cells (Burgoyne *et al.*, 1989) also contribute to the final Ca^{2+}_i signal. This Ca^{2+}_i mobilization could be particularly sensitive to dotarizine and flunarizine. Experiments now under way will clarify this issue.

An interesting and drastic difference between dotarizine and flunarizine relied on the different reversibility of their effects. Thus, after blocking the $[Ca^{2+}]_i$ increments and secretion, washout of flunarizine did not lead to a reversal of its blocking effects, at least for a 30-min period. This might be due to the high lipophilicity of flunarizine which favours its accumulation in lipid-rich membranes. For instance, the partitioning/transfer of flunarizine from an aqueous phase into phospholipid bilayers shows a binding/partitioning coefficient of 28,700 M^{-1} , supporting the notion that this drug is particularly membrane active (Thomas & Seelig, 1993). On the other hand, at 1 μM concentration, flunarizine can reach tissue/medium ratios as high as 682 in rat aorta and 784 in left atrium (Scheufler & Peters, 1990).

In contrast to flunarizine, the blocking effects of dotarizine were readily reversible upon washout of the drug. In fact, the Ca^{2+}_i signals and the secretory responses recovered after 5–10 min washout of dotarizine, suggesting that dotarizine does not accumulate in cells. It is likely that the extra-pyramidal, Parkinson-like effects described for flunarizine are due to the accumulation of the drug in basal ganglia, leading to a strong blockade of dopamine release (Todd & Benfield, 1989). Because of its ready reversibility, it is unlikely that dotarizine might suffer a similar accumulation process in the brain. Therefore, dotarizine might be devoid of the adverse effects associated to flunarizine.

All the effects of dotarizine and flunarizine on $^{45}Ca^{2+}$ uptake, $[Ca^{2+}]_i$ and secretion can be explained through their ability to block voltage-dependent Ca^{2+} channels. As in neurones (Olivera *et al.*, 1994), multiple types of voltage-dependent Ca^{2+} channels have been discovered in bovine chromaffin cells. Thus, they contain ω -conotoxin GVIA-sensitive N-type Ca^{2+} channels (Hans *et al.*, 1990), ω -agatoxin IVA-sensitive P-type Ca^{2+} channels (Albillos *et al.*, 1993), and dihydropyridine-sensitive Ca^{2+} channels (Garcia *et al.*, 1984). Dihydropyridine Ca^{2+} antagonists block only partially the whole-cell current through Ca^{2+} channels (Gandia *et al.*, 1993; Albillos *et al.*, 1993) and catecholamine release from the intact bovine adrenal gland (Gandia *et al.*, 1990; Jiménez *et al.*, 1993) and from isolated chromaffin cells (Boarder *et al.*, 1987; but see Ceña *et al.*, 1983). In contrast, dotarizine and flunarizine inhibited almost completely whole-cell I_{Ba} and catecholamine release in bovine chromaffin cells (this study) and intact glands (Fuente *et al.*, 1992), suggesting that they have a wider-spectrum than most Ca^{2+} channel blockers known to date. They seem to act on a site different from the sites for dihydropyridines, ω -conotoxin GVIA and ω -agatoxin IVA which might be common to the various Ca^{2+} channel subtypes identified in neurones. Recently, Olivera *et al.* (1994) have suggested that Ca^{2+} channels might have a macrosite common to all of them, but with various microsites having different affinities for various toxins blocking specific channel subtypes. In this context, dotarizine and flunarizine might eventually become interesting probes to investigate molecular features related to those macrosites and microsites

on various Ca^{2+} channel subtypes. In fact, flunarizine has been shown to block low-threshold as well as high-threshold Ca^{2+} channels in neurones (Kaneda & Akaike, 1989; Takahashi & Akaike, 1991).

In conclusion, like flunarizine, dotarizine behaves as a wide-spectrum calcium antagonist in bovine chromaffin cells. However, dotarizine presents the advantage over flunarizine of the reversibility of its blocking effects. This may have clinical implications, since the accumulation of drugs in tissue cells is associated with a greater incidence of adverse reac-

tions. In this context, dotarizine might prove clinically safer than flunarizine in treating patients suffering migraine or vertigo. Its Ca^{2+} antagonist effects (this paper) as well as those on 5-HT₁ and 5-HT₂ receptors (Brasó *et al.*, 1994) might contribute to this clinical profile.

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