ELSEVIER

European Journal of Pharmacology 366 (1999) 309–317

ejp

Effects of dotarizine and flunarizine on chromaffin cell viability and cytosolic Ca^{2+}

Jesús Novalbos [a,b], Francisco Abad-Santos [a,b], Pedro Zapater [a,b], María F. Cano-Abad [b], Javier Moradiellos [a], Pedro Sánchez-García [b], Antonio G. García [a,b,*]

[a] *Servicio de Farmacología Clínica e Instituto de Gerontología, Hospital de la Princesa, Diego de León 62; 28006, Madrid, Spain*
[b] *Instituto de Farmacología Teófilo Hernando, Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4; 28029, Madrid, Spain*

Received 10 August 1998; revised 2 December 1998; accepted 4 December 1998

Abstract

Dotarizine (a novel piperazine derivative with antimigraine properties) and flunarizine (a Ca^{2+} channel antagonist) were compared concerning: first, their ability to cause chromaffin cell damage in vitro; second, the possible correlation of their octanol/water partition coefficients and those of another 28 compounds (i.e., Ca^{2+} channel antagonists, blockers of histamine H_1 receptors, antimycotics, β-adrenoceptor antagonists, neuroleptics), with their ability to cause cell damage; third, their capacity to protect the cells against the damaging effects of veratridine; and fourth, their capabilities to enhance the basal cytosolic Ca^{2+} concentration in fura-2-loaded single chromaffin cells, or to modify the pattern of $[Ca^{2+}]_i$ oscillations elicited by veratridine. After 24-h exposure to 1–30 μM dotarizine, the viability of bovine adrenal chromaffin cells (measured under phase contrast or as lactate dehydrogenase, released into the medium) was similar to that of control, untreated cells; at 100 μM, 80% lactate dehydrogenase release was produced. At 1–3 μM flunarizine caused no cell damage; however 10 μM caused 20% lactate dehydrogenase release and 30 and 100 μM over 90% lactate dehydrogenase release. The time course of cell damage was considerably faster for flunarizine, in comparison to dotarizine. Out of 30 molecules tested (at 10 μM), having different octanol/water partition coefficients (log *P*), dotarizine was among the molecules causing no cell damage; flunarizine caused 20% cell loss, lidoflazine and verapamil over 50% cell loss, and penfluridol, draflazine, astemizole or nifedipine over 80% cell loss. No correlation was found between log *P* and cytotoxicity. Both dotarizine (10–30 μM) and flunarizine (3–10 μM) provided protection against veratridine-induced cell death; however, at 30 μM dotarizine afforded a pronounced protection while flunarizine enhanced the cytotoxic effects of veratridine. Dotarizine (30 μM) (but not flunarizine) caused a prompt transient elevation of the basal $[Ca^{2+}]_i$. Both compounds abolished the K^+-induced increases of $[Ca^{2+}]_i$ as well as the oscillations of $[Ca^{2+}]_i$ induced by veratridine. The blocking effects of dotarizine were readily reversed after washout, while those of flunarizine were long-lasting. These differences might be relevant to the clinical use of dotarizine as an antimigraine drug.

Keywords: Dotarizine; Flunarizine; Chromaffin cell viability; Ca^{2+}, cytosolic

1. Introduction

Dotarizine, a novel piperazine derivative structurally related to flunarizine (Gubert et al., 1987; Villarroya et al., 1995), is currently being evaluated in clinical trials for its antimigraine and antivertigo effects (Galiano et al., 1993). Its parent piperazine compound, flunarizine, has been used for the prophylaxis of migraine crisis; its clinical efficacy has been related with its Ca^{2+} channel antagonist properties, since Ca^{2+} as well as Na^+ channels have been implicated in the pathogenesis of migraine (Todd and Benfield, 1989; Ophoff et al., 1996). Like flunarizine, dotarizine exhibits Ca^{2+} channel blocking properties. Thus in rabbit aortic smooth muscle, dotarizine inhibits $^{45}Ca^{2+}$ uptake and vessel contractility (Tejerina et al., 1993). Furthermore, dotarizine blocks whole cell Ca^{2+} and Ba^{2+} currents through high-voltage activated Ca^{2+} channels in voltage-clamped bovine chromaffin cells. The compound also inhibits three other signals related to Ca^{2+} channel activation i.e., $^{45}Ca^{2+}$ uptake, the transient rise of cytosolic Ca^{2+} concentrations, $[Ca^{2+}]_i$, as well as the release of

* Corresponding author. Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid C/ Arzobispo Morcillo, 4 28029 Madrid Spain. Tel.: +34-91-3975388; Fax: +34-91-3975397; E-mail: agg@mvax.fmed.uam.es

0014-2999/99/$ - see front matter
PII: S0014-2999(98)00916-9

catecholamines triggered by K^+ depolarisation (Villarroya et al., 1995).

In contrast to flunarizine, dotarizine exhibits potent antiserotonergic activity, both in vitro and in vivo (Brasó et al., 1989, 1994; Cartheuser et al., 1994; Montiel et al., 1997). Various other drugs with 5-HT_2 receptor antagonist properties have been used in migraine prophylaxis, for example pizotifen, methysergide, or propranolol (Welch, 1993; Goadsby and Olesen, 1996). The 5-HT receptor blocking effect of dotarizine, in addition to its being a Ca^{2+} channel antagonist, might thus increase its therapeutic potential.

The long-term clinical use of flunarizine for the treatment of migraine, epilepsy and other diseases (Binnie et al., 1985; Todd and Benfield, 1989), revealed some undesirable side-effects, i.e., reversible extrapyramidal symptoms (Micheli et al., 1987). These side-effects might be explained in the following context. Flunarizine exhibits an extraordinary capacity to accumulate in cell membranes, where it can reach millimolar concentrations from concentrations in the low micromolar range in the extracellular solution (Scheufler and Peters, 1990; Thomas and Seelig, 1993). This could lead to the accumulation of flunarizine in brain tissue following its repeated administration to patients, thus blocking dopamine release from striatal neurones and/or striatal dopamine D_2 receptors (Brucke et al., 1995; Maroto et al., 1995; Brasó et al., 1996).

An alternative explanation for the extrapyramidal-like symptoms could rest in the possible direct cytotoxic effect of flunarizine on striatal neurones. Flunarizine has been shown to have cytoprotectant actions in neuronal cultures (Pauwels et al., 1989, 1990, 1991; Rich and Hollowell, 1990), chromaffin cell cultures (Maroto et al., 1994), hippocampal slices (Ashton et al., 1990) and in animal models of stroke (De Ryck et al., 1989). However, at high concentrations, flunarizine also exhibits cytotoxic effects (Maroto et al., 1994). In this study, we compared the ability of dotarizine and flunarizine to cause cell death and cell protection in primary cultures of bovine adrenal medullary chromaffin cells. In addition, their effects on the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, was also studied. This comparison is particularly interesting if we consider that, in spite of having similar lipophilicity, dotarizine has readily reversible effects to block catecholamine release in chromaffin cells, opposite to the long-lasting actions of flunarizine (Lara et al., 1997).

2. Materials and methods

2.1. Materials and solutions

Flunarizine, cinnarizine, lidoflazine, R56865, sabeluzole, lubeluzole, R91154, (the R(−) enantiomer of lubeluzole), penfluridol and fluspirilene were obtained from the Janssen Research Foundation, Belgium; Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum from GIBCO, Madrid, Spain. Collagenase from *Clostridium histolyticum* (Boehringer-Mannheim) and dotarizine was obtained from Grupo Ferrer, Barcelona, Spain. Veratridine and other compounds were obtained from Sigma. Concentrated solutions (10^{-2} M) of dotarizine and flunarizine were made in dimethylsulfoxide (DMSO). Concentrated veratridine solutions (10^{-1} M) were also made in DMSO. Appropriate dilutions were then made with Krebs–HEPES solution (pH 7.4) containing (in mM): NaCl, 145; KCl, 5.9; $MgCl_2$, 1.2; $CaCl_2$, 2.5; sodium HEPES, 10; glucose, 10. At the final concentration used (up to 1%), DMSO had no effect on cell viability.

2.2. Preparation of cells

Bovine adrenal medullary chromaffin cells were isolated as previously described (Livett, 1984) with some modifications (Moro et al., 1990). To reduce the number of endothelial cells in the culture that would confuse the source of lactate dehydrogenase released into the medium, the cells were pre-plated for 30 min and subsequently plated in DMEM; proliferation inhibitors (cytosine arabinoside and fluorodeoxyuridine) were used during maintenance of the culture in the DMEM medium. Cells were plated on plastic culture wells (24-well Costar plates) with 0.01 mg ml^{-1} of poly-D-lysine at a density of 5×10^5, containing 1 ml DMEM supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cultures were maintained for 1–2 days at 37°C in a water-saturated atmosphere with 5% CO_2. Trypan blue exclusion yielded cell viability values greater than 95%.

2.3. Exposure of cells to drugs

After 24 h, the cells were incubated for different time periods with 1 ml of serum-free Krebs–HEPES medium containing flunarizine or dotarizine at various concentrations (3, 10, 30 and 100 μM). Incubations were for periods (30 min, 1, 4, 10 and 24 h), after which the medium was removed and the cells were incubated with 1 ml serum-free Krebs–HEPES medium without the drug. LDH assay was performed 24 h later. Control cells were treated with solvent, using DMSO 0.3–1%. At the end of the incubation period, the medium was removed and saved. Cells attached to the bottom of the dish were lysed by adding 1 ml of 1% Triton X100 in H_2O, for LDH determination.

2.4. Lactate dehydrogenase assay

Extracellular and intracellular lactate dehydrogenase activity was measured by following the reduction of tetrazolium at an absorbance wavelength of 492 nm (Boehringer Mannheim kit). Total lactate dehydrogenase activity

was defined as the sum of intracellular and extracellular lactate dehydrogenase activity. Released lactate dehydrogenase was defined as the percentage of extracellular compared to total lactate dehydrogenase activity.

2.5. Measurement of changes in $[CA^{2+}]_i$ in fura-2-loaded bovine chromaffin cells

Chromaffin cells were loaded with fura-2 by incubating them with fura-2/AM (4 μM) for 30 min at room temperature in Krebs–HEPES solution (pH 7.4) containing (in mM): NaCl, 145; KCl, 5.9; $MgCl_2$, 1.2; $CaCl_2$, 2.5; sodium HEPES, 10; glucose, 10. The loading incubation was terminated by washing several times the coverslip containing the attached cells, using Krebs–HEPES. The cells were then kept at 37°C in the incubator for 15 to 30 min. The fluorescence of fura-2 in single cells was measured with the photomultiplier-based system described by Neher (1989), which produces a spatially averaged measure of the $[Ca^{2+}]_i$. Fura-2 was excited with light alternating between 360 and 390 nm, using a Nikon 40× fluorite objective. Emitted light was transmitted through a 425-nm dichroic mirror and 500 to 545-nm barrier filter before being detected by the photomultiplier. $[Ca^{2+}]_i$ was calculated from the ratios of the light emitted when the dye was excited by the two alternating excitation wavelengths (Grynkiewicz et al., 1985).

2.6. Statistical analysis

Each experiment was performed in three wells and experimental data were calculated from the mean of the three wells. Data are means ± standard error of mean (S.E.M.). Effects were analysed with an analysis of variance. If significant differences were found, an appropriate multiple comparison procedure (Student–Newman–Keuls test) was done. The level of statistical significance was taken at $P < 0.05$. Analysis was performed using a SPSS software for Windows.

3. Results

The results are grouped in two categories: (i) those related to the cytotoxic effects of dotarizine, flunarizine and 28 other compounds with different octanol/water partition coefficients; (ii) those related to cytoprotection exerted by dotarizine and flunarizine against the cytotoxic effects of veratridine. In both cases, attempts were made to correlate the cytotoxic and cytoprotecting actions of dotarizine and flunarizine with the modifications of $[Ca^{2+}]_i$.

3.1. The cytotoxic effects of dotarizine and flunarizine

Cells incubated for 24 h in Krebs–HEPES containing 0.3% DMSO (the solvent for the drugs) appeared spherical or oval with a clear plasma membrane and strong birefringence. Though many cells appeared isolated, others tended to cluster in islets of 4–10 cells (Fig. 1A). Cells incubated with 30 μM dotarizine had a well delimited plasma membrane, strong birefringence and a tendency to form clusters (Fig. 1B). In contrast, cells incubated with 30 μM flunarizine lost their membrane integrity, their birefringence and appeared as cell fragments, indicating that extensive cell death had occurred (Fig. 1C).

The morphological data are consistent with the results of experiments taking lactate dehydrogenase release as a more quantitative marker of cell lesion. In Fig. 2 the white

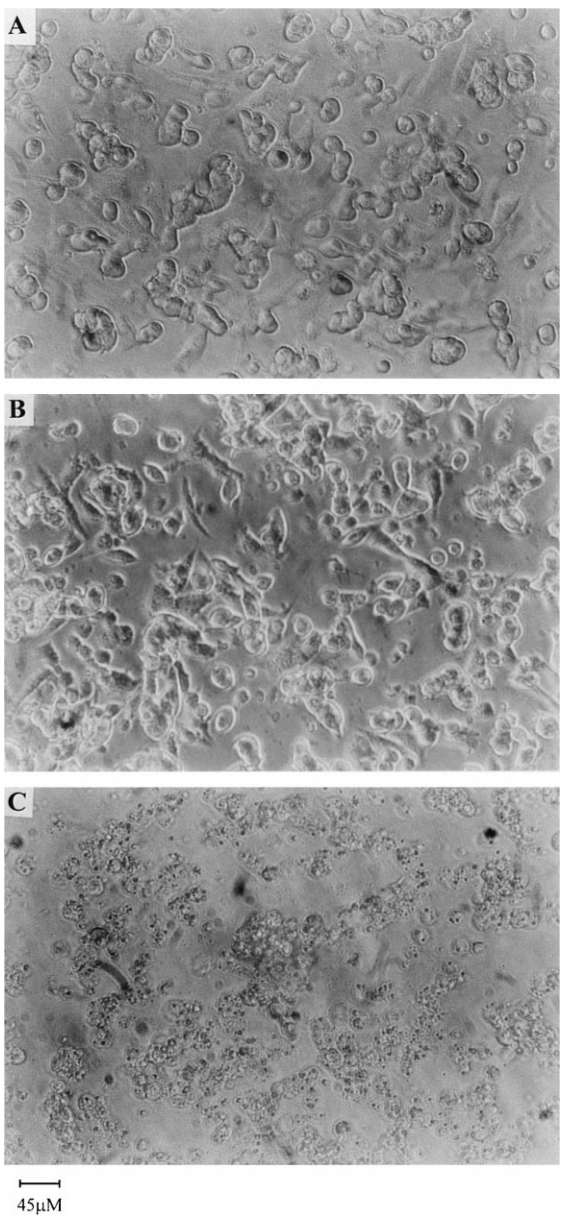

Fig. 1. Phase contrast micrographs of 2-day-old chromaffin cells incubated for 24 h in a Krebs–HEPES solution containing 0.3% DMSO (panel A), or solutions containing 30 μM dotarizine (panel B) or 30 μM flunarizine.

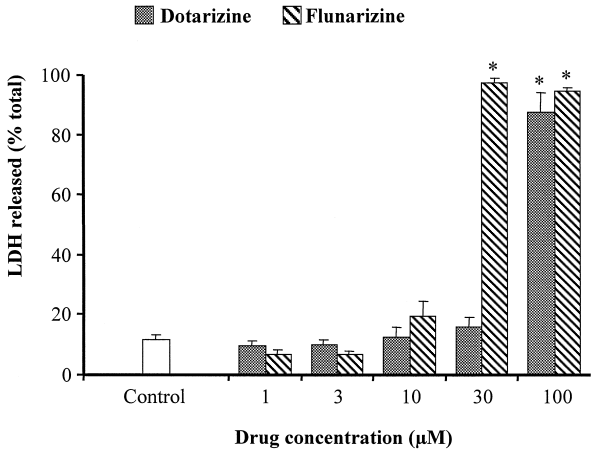

Fig. 2. Cytotoxic effects of increasing concentrations (abscissa) of flunarizine and dotarizine determined as lactate dehydrogenase (LDH) released (ordinate) after 24 h of exposure of chromaffin cells to each drug concentration. Control (white bar) corresponds to cells treated with DMSO 1%. Hatched bars represent cells treated with flunarizine at different concentrations over 24 h. Black bars represent cells treated with different concentrations of dotarizine over 24 h. Data are means ± S.E.M. of 6–7 experiments (3 wells each). * $P < 0.05$ compared with control.

bar labelled 'Control' reflects the lactate dehydrogenase spontaneously released (12 ± 1% of total enzyme) by chromaffin cells incubated for 24 h in a Krebs–HEPES solution containing 1% DMSO, the vehicle used to dissolve dotarizine and flunarizine. At the concentration of 10 μM, flunarizine showed a tendency to increase cell death; this was exaggerated at 30 μM, a concentration of flunarizine that caused near 100% cell loss. Dotarizine did not display cytotoxic effects at 10 or 30 μM, but at the very high concentration of 100 μM it was as cytotoxic as flunarizine.

The two compounds also differed as to the time course of their damaging effects. Fig. 3A shows that at 30 μM flunarizine increased lactate dehydrogenase release as soon as after 1-h exposure; at 4 h, the damaging effects of this compound were maximum. At 30 μM dotarizine caused a lactate dehydrogenase release slightly and significantly higher than that of vehicle-treated cells, only after 24-h incubation. With 100 μM flunarizine near maximal cell death was reached after only 30-min incubation. The cytotoxic effects of the 100 μM concentration of dotarizine were considerably delayed with respect to those of flunarizine; thus, after 30–60 min lactate dehydrogenase release amounted to 50%; at 4 h the release was similar to that with flunarizine.

3.2. Lipophilicity and cytotoxic effects of drugs

Flunarizine is highly lipophilic and accumulates in cell membranes to reach millimolar concentrations (Scheufler and Peters, 1990; Thomas and Seelig, 1993). Like flunarizine, dotarizine is highly lipophilic (Lara et al., 1997). Thus it could well be that lipophilicity per se might explain the cytotoxic actions of these compounds. To test this hypothesis we selected 28 compounds with octanol/water partition coefficients ranging from −1.20 to 7.6, and tested them for their ability to cause cell damage after 24 h incubation of chromaffin cells with 10 μM of each compound. LDH released in control cells amounted to about 10% (Fig. 4).

Having one of the highest log P (6), dotarizine was nevertheless the least toxic agent, together with oxatomide (log P 4.76). In contrast, with a log P similar to that of oxatomide (around 5) several compounds caused drastic cell death; this was true for draflazine, astemizole, or fluspirilene. Although, in general, the more hydrophilic compounds scarcely had cytotoxic effects, it is curious that mebendazol (log P 3.01), verapamil (log P 2), nifedipine (log P 2.86) and particularly carmidazole (log P of only 1.12) caused the release of above 50% LDH. Thus a correlation between log P and LDH release does not exist (inset to Fig. 4).

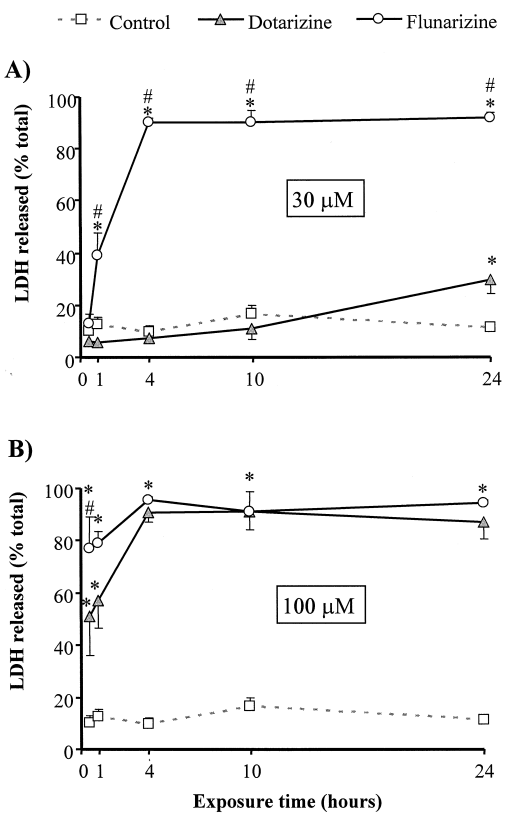

Fig. 3. Cytotoxic effects as a function of the time of cell exposure to flunarizine (circles), dotarizine (triangles) and DMSO 1% (squares), measured as lactate dehydrogenase (LDH) released (ordinate). Chromaffin cells were exposed to 30 μM (A) or 100 μM (B) of flunarizine or dotarizine for different periods of time and LDH released was measured after 24 h of exposure. Data are means ± S.E.M. of 5–7 experiments (3 wells each). * Significant differences between DMSO and flunarizine or dotarizine concentrations ($P < 0.05$). # Significant differences between flunarizine and dotarizine ($P < 0.05$).

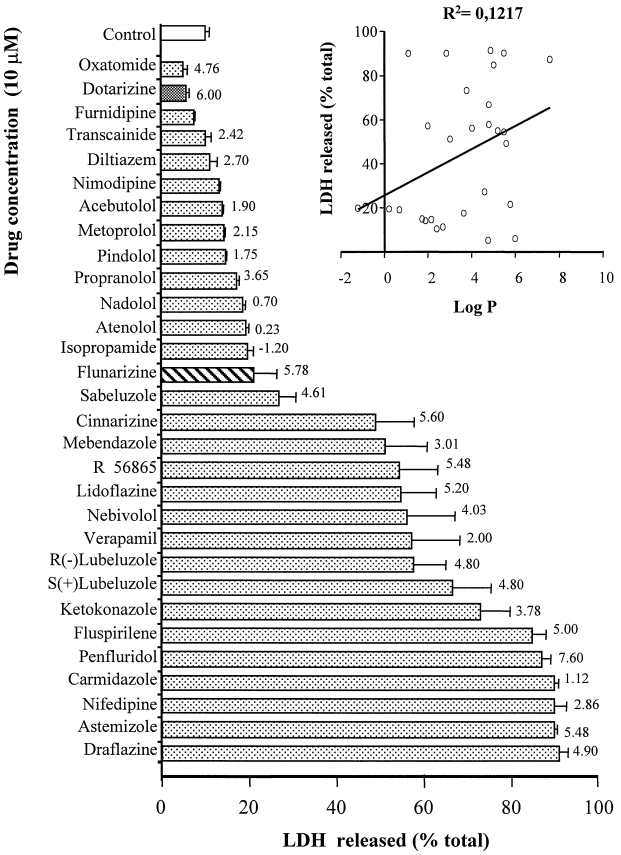

Fig. 4. Cytotoxic effects of dotarizine, flunarizine and other 28 molecules with different octanol/water partition coefficients (shown next to each bar). Cells were exposed to each drug (10 μM) for 24 h and then the lactate dehydrogenase (LDH) released (as a % of total, ordinate) was estimated as an index of cell death. Data are means ± S.E.M. of 4–6 experiments (3 wells each). Inset, correlation betwen LDH release (ordinate) and the octanol/water partition coefficient (log *P*). Furnidipine and nimodipine were excluded from the regression analysis because their log *P* was unavailable.

3.3. Cytotoxic effects of veratridine and cytoprotection afforded by dotarizine and flunarizine

In a previous study (Maroto et al., 1994) we demonstrated that flunarizine offered protection to chromaffin cells exposed to veratridine. It was therefore interesting to know whether dotarizine shares these properties with flunarizine. To achieve this goal chromaffin cells were exposed to veratridine (100 μM) for 24 h in the presence or the absence of a concentration of dotarizine (30 μM) that itself did not cause cell death. Fig. 5 shows phase contrast microphotographs of control cells (panel A), cells treated with veratridine (panel B) and cells exposed first to dotarizine and subsequently to dotarizine plus veratridine (panel C). Control cells and cells treated with dotarizine plus veratridine showed a similar pattern: well delimited plasma membranes, strong cytosolic birefringence and cells grouped in clusters. In sharp contrast, cells exposed to veratridine alone showed a broken plasma membrane, a dark punctate cytosol and signs of necrotic death.

The cytoprotecting effects of dotarizine were studied in a more quantitative way by measuring the liberation of lactate dehydrogenase into the incubation media (Fig. 6). Control cells incubated for 24 h in normal Krebs–HEPES solution released 12 ± 1.5% of enzyme. Exposure to veratridine provoked as much as 62 ± 6.7% of enzyme release. At 10–30 μM, dotarizine decreased the release of lactate

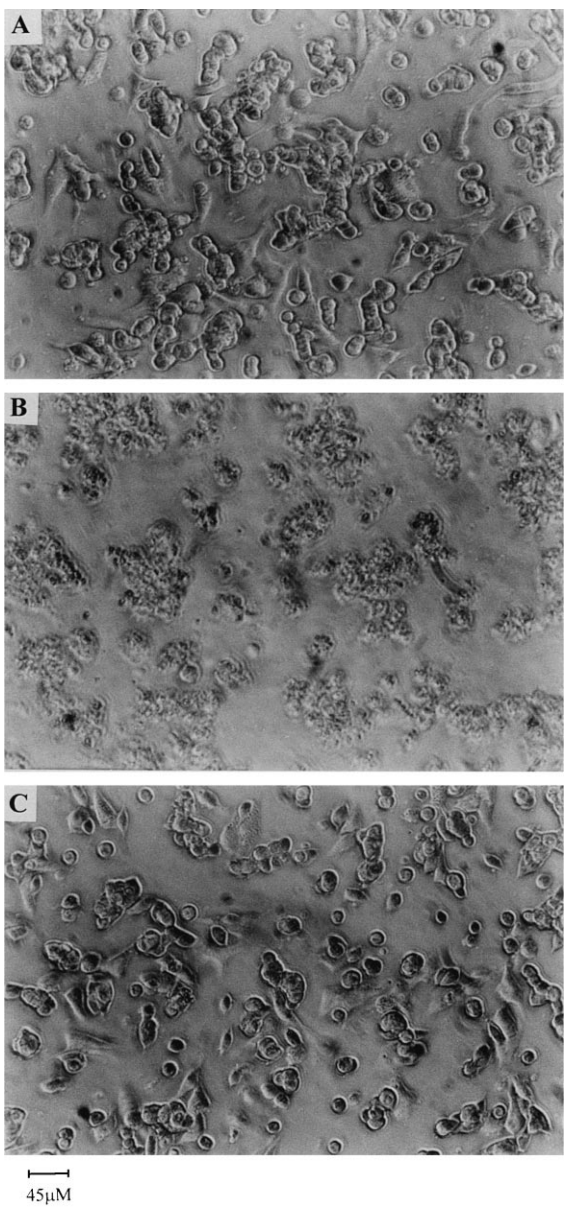

Fig. 5. Cytoprotection by dotarizine against the cytotoxic effects of veratridine. Control chromaffin cells were incubated for 24 h in a Krebs–HEPES solution containing 0.4% DMSO (panel A). Veratridine-treated cells were incubated for 24 h with a Krebs–HEPES solution containing 100 μM veratridine (panel B). The cytoprotection by dotarizine was studied by adding first 30 μM of this compound and after 30 min, 100 μM veratridine; cells were subsequently incubated with the two compounds for an additional 24-h period (panel C). Phase contrast microphotographs were taken at the end of the 24-h incubation period.

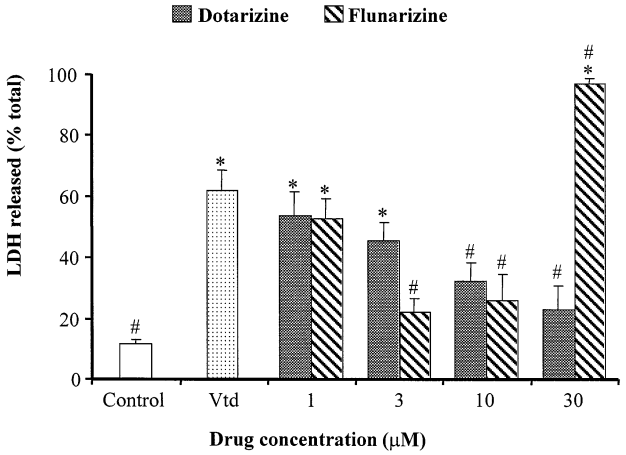

Fig. 6. Cytoprotection afforded by dotarizine (black bars) and flunarizine (hatched bars) against the cytotoxicity induced by veratridine (100 μM for 24 h; Vtd). Control cells (white left bar) were incubated in a solution with 0.4% DMSO. The protocols for incubation of the cells with veratridine in the presence of increasing concentrations of dotarizine and flunarizine were similar to those described in legend to Fig. 5. Data are means ± S.E.M. of 4–5 experiments in triplicate. # Significant differences compared with Vtd ($P < 0.05$). * Significant differences compared with control ($P < 0.05$).

dehydrogenase, thus suggesting that the drug caused a concentration-dependent, significant, cytoprotection against the cytotoxic effects of veratridine. This pattern substantially differed from that observed with flunarizine. At concentrations of 3–10 μM flunarizine caused clear cytoprotection, thus confirming previous results from this laboratory (Maroto et al., 1994). However, at 30 μM flunarizine showed the opposite effect, an augmentation of the cytotoxic actions of veratridine. This was not unexpected considering that 30 μM flunarizine itself caused near 100% enzyme release (Fig. 2).

3.4. Effects of dotarizine and flunarizine on basal $[CA^{2+}]_i$ and K^+-induced increase of $[CA^{2+}]_i$

Again we observed clear differences in the actions of dotarizine and flunarizine on the basal and K^+-induced changes of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ traces shown in Fig. 7A were obtained from a fura-2-loaded single chromaffin cell that was stimulated first with K^+ (70 mM K^+, 2.5 mM Ca^{2+}, 5 s pulses), then exposed for 20 min to 30 μM dotarizine and stimulated intermittently with K^+, at 5-min intervals (small circles at the bottom). The first K^+ pulse caused a rapid $[Ca^{2+}]_i$ spike that quickly declined to reach the basal $[Ca^{2+}]_i$ of about 0.1 μM. Subsequent superfusion of the cell with dotarizine caused a prompt increase of basal $[Ca^{2+}]_i$ to a small peak of about 0.5 μM, followed by a slow decline to near basal levels, in spite of sustained superfusion with the compound. K^+ pulsing failed to produce a $[Ca^{2+}]_i$ increase while dotarizine was present; however, after washout of dotarizine, K^+ elicited increasing $[Ca^{2+}]_i$ spikes at the beginning of its application (see traces in Fig. 7B). $[Ca^{2+}]_i$ remained low during the first 20 min of flunarizine superfusion; later on, the $[Ca^{2+}]_i$ began to increase first slowly, and then rapidly, to reach saturation of the fura-2. Afterwards, the cell detached, probably because it was flooded with cytotoxic levels of $[Ca^{2+}]_i$. During flunarizine treatment, the K^+ pulses did not give rise to any modification of the $[Ca^{2+}]_i$ levels measured at that moment. After washout of flunarizine the cell continued to be insensitive to the K^+ challenge.

3.5. Effects of dotarizine and flunarizine on the oscillations of $[CA^{2+}]_i$ elicited by veratridine

When fura-2-loaded bovine chromaffin cells are exposed to veratridine their $[Ca^{2+}]_i$ changes and follows a sustained non-desensitizing oscillatory pattern (López et al., 1995). This pattern was reproduced in the chromaffin cell shown in Fig. 8A. After an initial K^+ pulse to test cell viability, veratridine (30 μM) was continuously superfused. Soon, the $[Ca^{2+}]_i$ started to rise and to descend to basal levels following an irregular oscillatory pattern. Dotarizine (10 μM) rapidly abolished the oscillations.

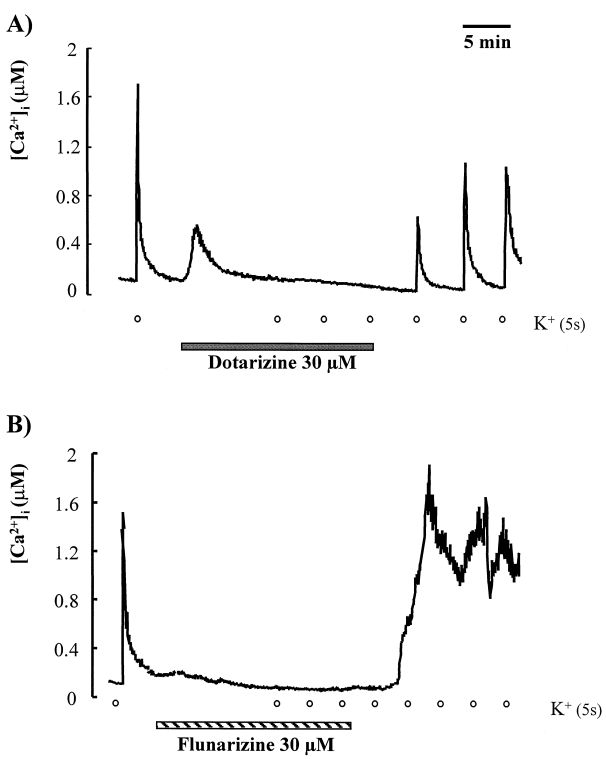

Fig. 7. Changes in the $[Ca^{2+}]_i$ in fura-2-loaded single chromaffin cells superfused for 20 min with 30 μM dotarizine (panel A) or 30 μM flunarizine (panel B). The compounds were superfused for the time period shown by the bottom horizontal bars. Before, during and after washout of the compounds, K^+ pulses (70 mM isotonic K^+, 2.5 mM Ca^{2+}, 5 s) were applied as shown by the small circles at the bottom. The $[Ca^{2+}]_i$ is expressed in μM (ordinates). DMSO (0.3%) did not modify the $[Ca^{2+}]_i$ (not shown). These experiments were repeated with 10 cells (dotarizine) and 6 cells (flunarizine) with similar results.

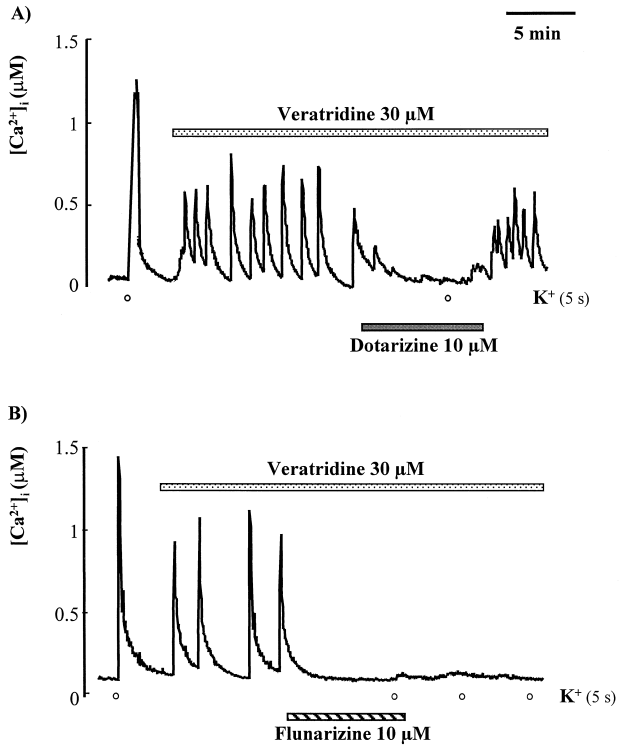

Fig. 8. Effects of dotarizine and flunarizine on the oscillations of $[Ca^{2+}]_i$ induced by veratridine (30 μM) in single fura-2-loaded chromaffin cells. The experiment was initiated by applying a 5-s pulse of K^+ (70 mM K^+, 2.5 mM Ca^{2+}) to test cell viability. Then veratridine was added, as indicated by the horizontal bars at the top. In the middle of the veratridine application, dotarizine (10 μM, panel A) or flunarizine (10 μM, panel B) was applied during the time period shown by the bottom horizontal bars. These experiments were repeated with 5 cells (dotarizine) and 4 cells (flunarizine), with similar results.

While superfusion with dotarizine proceeded, a K^+ pulse (dot) failed to elevate the $[Ca^{2+}]_i$. Upon washout of dotarizine the oscillations resumed promptly. This was not the case for flunarizine (10 μM) in that after the $[Ca^{2+}]_i$ oscillations were suppressed they did not recover so fast following its washout.

4. Discussion

Dotarizine and flunarizine are piperazine derivatives with similar chemical structures but dissimilar pharmacological profiles. The differences extend to blockade of 5-HT receptors, reversibility of their effects on Ca^{2+} entry and catecholamine release (see Section 1 for references), and to their ability to cause cell damage (this study). Thus dotarizine damaged the chromaffin cells only at the very high concentration of 100 μM, while flunarizine was cytotoxic at concentrations 10–100 μM; damage developed faster with flunarizine. The IC_{50} for dotarizine to block 5-HT-induced contractions of pig coronary artery is 0.22 μM, and the IC_{50} to inhibit 5-HT_{2A} receptors expressed in *Xenopus* oocytes is 2.2 nM (Montiel et al., 1997). On the other hand, the blockade by dotarizine of $^{45}Ca^{2+}$ entry, whole-cell Ca^{2+} channel currents and K^+-evoked $[Ca^{2+}]_i$ increases and catecholamine release in bovine chromaffin cells has IC_{50}s of 1.2–5 μM (Villarroya et al., 1995). Thus, the pharmacological actions of dotarizine plausibly responsible for its antimigraine effects (i.e., blockade of 5-HT receptors and calcium antagonism) are exerted at concentrations 50–5000-fold lower than those required to cause cell damage.

In general the calcium antagonists are highly lipophilic molecules with high log *P*. Lipophilicity favours the accumulation of these molecules in cells. The case of flunarizine is an illustration, since it can reach millimolar concentrations in cell membranes from concentrations in the low micromolar range in the extracellular solution (Scheufler and Peters, 1990; Thomas and Seelig, 1993). A priori, it is tempting to link this cell accumulation of highly lipophilic molecules to their potential to cause cell damage; however, this does not seem to be the case for several reasons: first, dotarizine and flunarizine have similar high log *P* (near 6), yet they differ in the rate of washout of some of their pharmacological effects (Villarroya et al., 1995; Lara et al., 1997); second, in spite of having similar log *P*, the cytotoxic actions of dotarizine were substantially less and developed much more slowly than those of flunarizine (Figs. 2 and 3); third, the cytotoxic actions of 30 molecules with a wide range of log *P* (−1.2 to 7.6) did not correlate with their degree of lipophilicity (Fig. 4). In fact, dotarizine was the least cytotoxic molecule of the 30 assayed, in spite of being highly lipophilic (log *P* 6).

Both dotarizine and flunarizine afforded cell protection against the cytotoxic actions of veratridine. In the case of flunarizine, these actions are well documented in neurones (Pauwels et al., 1989, 1990, 1991; Rich and Hollowell, 1990) and chromaffin cells (Maroto et al., 1994) but they were unknown for dotarizine. Again, differences were observed in the cytoprotecting actions of the two compounds. Dotarizine exhibited a concentration-dependent cytoprotecting action at 10–30 μM. In contrast, flunarizine exhibited a biphasic pattern; at low concentrations (3–10 μM) the drug was cytoprotectant and at the higher concentrations (particularly at 30 μM) flunarizine had the opposite effect, augmentation of the cytotoxic effects of veratridine. Considering the intrinsic cytotoxic actions of flunarizine (Fig. 2), this finding was expected.

The mechanism of cell protection offered by dotarizine and flunarizine may be linked to their ability to block the L-, N-, P- and Q-subtypes of Ca^{2+} channels expressed by bovine chromaffin cells (Villarroya et al., 1995). This 'wide-spectrum' Ca^{2+} channel blockade stops the oscillations of Ca^{2+} induced by veratridine (Fig. 8). Such oscillations are known to be due to cyclic membrane depolarisation and cyclic opening of Ca^{2+} channels and Ca^{2+} entry into the cytosol (López et al., 1995), and they might be responsible for the Ca^{2+} overloading of the cells that

might constitute the primary signal that initiate the cascade of events leading to cell death (Pauwels et al., 1989, 1990, 1991; Maroto et al., 1994).

In a previous study Koh and Cotman (1992) found that the calcium antagonists, flunarizine, nifedipine, diltiazem and verapamil, caused neuronal cell death; this agrees with our present results for chromaffin cells (Fig. 4). These authors attempted to explain their results by the suggestion that both high and low levels of $[Ca^{2+}]_i$ can be detrimental to neuronal survival (Koike et al., 1989). The dual effects of dotarizine and flunarizine seen here may also be explained in this way. When excess Ca^{2+} entry is produced, i.e., in the case of glutamate neurotoxicity (or veratridine in our case), excess $[Ca^{2+}]_i$ can lead to activation of Ca^{2+}-dependent proteolytic enzymes (Lynch and Seubert, 1989) and/or the generation of free radicals (Choi, 1988) to cause cell death. By preventing the increase of $[Ca^{2+}]_i$, dotarizine and flunarizine would protect chromaffin cells against the lethal actions of Ca^{2+} overloading induced by veratridine. On the other hand, the decreased $[Ca^{2+}]_i$ in cells exposed to high concentrations of calcium antagonists may be a trigger for the synthesis of proteins leading to cell death.

Whatever the mechanism, we can conclude that dotarizine and flunarizine share cytotoxic and cytoprotecting properties in chromaffin cells; however, dotarizine causes substantially lower cytotoxicity than flunarizine, and affords cytoprotection against veratridine in a range of concentrations wider than that of flunarizine. These in vitro differences might be clinically relevant when dotarizine is used as an antimigraine drug.

Acknowledgements

Supported by Fundación Ferrer, Barcelona, Spain and by Janssen-Cilag, Spain. Also by grants from Dirección General de Investigación Científica y Técnica, DGICYT No. PB94-0150 and Comunidad Autónoma de Madrid, CAM No. 07/050/96. We thank Mr. Ricardo de Pascual for the preparation of cell cultures and Mrs. Maricarmen Molinos for typing the manuscript. JN is a fellow of Formación de Personal Investigador of Comunidad Autónoma de Madrid, CAM, Spain and MFCA is a fellow of Formación de Personal Investigador, FPI, Ministerio de Educación y Ciencia, Spain. We thank Luis Gandía for useful comments and criticism of the manuscript.

References

Ashton, D., Willems, R., Marrannes, R., Janssen, P.A., 1990. Extracellular ions during veratridine-induced neurotoxicity in hippocampal slices: neuroprotective effects of flunarizine and tetrodotoxin. Brain Res. 528, 212–222.

Binnie, C.D., De Beukelaar, F., Meijer, J.W.A., Meinardi, H., Overweg, J., Wauquier, A., Van Wieringen, A., 1985. Open dose ranging trial of flunarizine as add-on therapy in epilepsy. Epilepsia 26, 424–428.

Brasó, A., Cartheuser, C., Sacristán, A., Ortíz, J.A., 1989. Dotarizine, a promising *N*-benhydrylpiperazine for migraine prophylaxis due to an additional antiserotoninergic effect. 4th Int. Symp. Calcium Antagonists. Pharmacol. Clin. Res., Florence, 261.

Brasó, A., Agut, J. Ruíz, A. Cartheuser, C.F., Sacristán, A., Ortíz, J.A., 1994. Dotarizine effects on 5-HT_2, and 5-HT_1 receptors. XIIth Int. Congr. Pharmacol. Montreal, Canada.

Brasó, A., Martínez, L., Planas, J.M., Cartheuser, C.F., Sacristan, A., Ortíz, J.A., 1996. Pharmacological profile and actions of dotarizine as a prophylactic antimigraine drug. J. Neurol. 243 (suppl. 2,6), 531, Abst.

Brucke, T., Wober, C., Podreka, I., Wober-Bingol, C., Asembaum, S., Aull, S., Wenger, S., Ilieva, D., Harasko-Van-der-Meer, C., Wessely, P., 1995. D_2 receptor blockade by flunarizine and cinnarizine explains extrapyramidal side effects. J. Cereb. Blood Flow Metab. 15, 513–518.

Cartheuser, C.F., Brasó, A., Sacristán, A., Ortíz, J.A., 1994. Effects of dotarizine on peripheral and pulmonary circulation and cardiac dynamics in dogs. Pharmacology 48, 187–193.

Choi, D.W., 1988. Glutamate neurotoxicity and diseases of the nervous system. Neuron 1, 623–634.

De Ryck, M., Van Reempts, J., Borgers, M., Wauquier, A., Janssen, P.A.J., 1989. Photochemical stroke model: flunarizine prevents sensorimotor deficits after neocortical infarcts in rats. Stroke 20, 1383–1390.

Galiano, L., Matias-Guiu, J., Horga, J.F., Martín, R., Falip, R., Montiel, I., 1993. Dotarizine: a double-blind trial in prophylactic treatment of migraine. Cephalalgia 13, 251, suppl. 13.

Goadsby, P.J., Olesen, J., 1996. Diagnosis and management of migraine. Br. Med. J. 312, 1279–1283.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Gubert, S., Brasó, M.A., Sacristán, A., Ortíz, J.A., 1987. Synthesis of some *N*-benzhydryl-piperazine derivatives. Arzneimitz-Forsch. Drug. Res. 37, 1103–1107.

Koh, J.Y., Cotman, C.W., 1992. Programmed cell death: its possible contribution to neurotoxicity mediated by calcium channel antagonists. Brain Res. 587, 233–240.

Koike, T., Martin, D.P., Johnson, E.M. Jr., 1989. Role of Ca^{2+} channels in the ability of membrane depolarization to prevent neuronal death induced by trophic factor deprivation: evidence that levels of internal Ca^{2+} determine nerve growth factor dependence of sympathetic ganglion cells. Proc. Natl. Acad. Sci. USA 86, 6421–6425.

Lara, B., Gandía, L., Torres, A., Olivares, R., Martínez-Sierra, R., García, A.G., López, M.G., 1997. 'Wide-spectrum Ca^{2+} channel antagonists': lipophilicity, inhibition, and recovery of secretion in chromaffin cells. Eur. J. Pharmacol. 325, 109–119.

Livett, B.G., 1984. Adrenal medullary chromaffin cells in vitro. Physiol. Rev. 64, 1103–1161.

López, M.G., Artalejo, A.R., García, A.G., Neher, E., García-Sancho, J., 1995. Veratridine induces oscillations of cytosolic calcium and membrane potential in bovine chromaffin cells. J. Physiol. 489, 15–27.

Lynch, G., Seubert, P., 1989. Links between long-term potentiation and neuropathology. An hypothesis involving calcium-activated proteases. Ann. N.Y. Acad. Sci. 568, 171–180.

Maroto, R., De la Fuente, M.-T., Artalejo, A.R., Abad, F., López, M.G., García-Sancho, J., García, A.G., 1994. Effects of Ca^{2+} channel antagonists on chromaffin cell death and cytosolic Ca^{2+} oscillations induced by veratridine. Eur. J. Pharmacol. 270, 331–339.

Maroto, R., López, M.G., Del Valle, M., Naranjo, J.R., Meström, B., García, A.G., 1995. Expression of the bovine striatal D_2 receptor, but not the D_1 receptor, in bovine adrenal medulla. Mol. Pharmacol. 47, 40–50.

Micheli, F., Pardal, M.F., Gatto, M., Torres, M., Paradiso, G., Parera,

I.C., Giannaula, R., 1987. Flunarizine and cinnarizine-induced extrapyramidal reactions. Neurology 37, 881–884.

Montiel, C., Herrero, C.J., García-Palomero, E., Renart, J., García, A.G., Lomax, R.B., 1997. Serotonergic effects of dotarizine in coronary artery and in oocytes expressing 5-HT_2 receptors. Eur. J. Pharmacol. 332, 183–193.

Moro, M.A., López, M.G., Gandía, L., Michelena, P., García, A.G., 1990. Separation and culture of living adrenaline- and noradrenaline-containing cells from bovine adrenal medullae. Anal. Biochem. 185, 243–248.

Neher, E. 1989. Combined fura-2 and patch-clamp measurements in rat peritoneal mast cells. In: Sellin, L.C., Libelius, R., Thesleff, S. (Eds.), Neuromuscular Junctions. Elsevier, Amsterdam, pp. 65–76.

Ophoff, R.A., Terwindt, G.M., Vergouwe, M.N., van Eijk, R., Oefner, P.J., Hoffman, S.M.G., Lamerdin, J.E., Mohrenweiser, H.W., Bulman, D.E., Ferrari, M., Haan, J., Lindhout, D., van Ommen, G.-J.B., Hofker, M.H., Ferrari, M.D., Frants, R.R., 1996. Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca^{2+} channel gene CACNL1A4. Cell 87, 543–552.

Pauwels, P.J., Van-Assouw, H.P., Leysen, J.E., Janssen, P.A., 1989. Ca^{2+} mediated neuronal death in rat brain neuronal cultures by veratridine: protection by flunarizine. Mol. Pharmacol. 36, 525–531.

Pauwels, P.J., Van-Assouw, H.P., Peeters, L., Leysen, J.E., 1990. Neurotoxic action of veratridine in rat brain neuronal cultures: mechanism of neuroprotection by Ca^{2+} antagonists nonselective for slow Ca^{2+} channels. J. Pharmacol. Exp. Ther. 255, 1117–1122.

Pauwels, P.J., Leysen, J.E., Janssen, P.A., 1991. Ca^{2+} and Na^{+} channels involved in neuronal cell death. Protection by flunarizine. Life Sci. 48, 1881–1893.

Rich, K.M., Hollowell, J.P., 1990. Flunarizine protects neurons from death after axotomy or NGF deprivation. Science 248, 1419–1421.

Scheufler, E., Peters, T., 1990. Phosphatidylserine monolayers as models for drug uptake into membranes and tissue. Cell Biol. Int. Reports 14, 381–388.

Tejerina, T., Chulia, T., González, P., 1993. Effects of dotarizine on $^{45}Ca^{2+}$ movements and contractile responses in vascular smooth muscle. Eur. J. Pharmacol. 239, 75–81.

Thomas, P.G., Seelig, J., 1993. Binding of the calcium antagonist flunarizine to phosphatidylcholine bilayers: charge effects and thermodynamics. Biochem. J. 291, 397–402.

Todd, P.A., Benfield, P., 1989. Flunarizine. A reappraisal of its pharmacological properties and therapeutic use in neurological disorders. Drugs 38, 481–499.

Villarroya, M., Gandía, L., Lara, B., Albillos, A., López, M.G., García, A.G., 1995. Dotarizine versus flunarizine as calcium antagonists in chromaffin cells. Br. J. Pharmacol. 114, 369–376.

Welch, K.M.A., 1993. Drug therapy of migraine. N. Engl. J. Med. 329, 1476–1483.