ELSEVIER

European Journal of Pharmacology 325 (1997) 109–119

ejp

'Wide-spectrum Ca^{2+} channel antagonists': lipophilicity, inhibition, and recovery of secretion in chromaffin cells

Baldomero Lara [c], Luis Gandía [a], Andrés Torres [c], Román Olivares [a], Rafael Martínez-Sierra [c], Antonio G. García [a,b], Manuela G. López [a,*]

[a] *Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, c / Arzobispo Morcillo 4, 28029 Madrid, Spain*
[b] *Servicio de Farmacología Clínica, Instituto de Gerontología, Hospital de la Princesa, Diego de León 62, 28006 Madrid, Spain*
[c] *Departamento de Farmacología, Facultad de Medicina, Universidad de Córdoba, 14071 Córdoba, Spain*

Received 14 November 1996; revised 31 January 1997; accepted 7 February 1997

Abstract

Repetitive application of short depolarizing K^+ pulses (70 mM K^+, 2 mM Ca^{2+} Krebs-HEPES solution, for 10 s every 5 min) produced reproducible catecholamine secretory responses from superfused bovine chromaffin cells. At 10 μM for 15 min, the piperazine derivatives dotarizine, flunarizine and lidoflazine inhibited secretion by around 90%; cinnarizine halved the secretory response. Recovery of secretion after 30-min washout with Krebs-HEPES solution amounted to 75% in the case of dotarizine, 8% for flunarizine, 46% for lidoflazine and 21% for cinnarizine. The benzothiazol derivatives (10 μM) (+)-*S*-lubeluzole and R91154 (the (−)-*R*-enantiomer of lubeluzole) blocked the response by 75%; sabeluzole inhibited secretion by only 34% and R56865 (*N*-[1-(4-(4-fluorophenoxy)butyl]-4-piperidinyl-*N*-methyl-2-benzo-thiazolamine) by 61%. Recoveries were around 70% in the case of these four benzothiazol derivatives. The diphenylbutyl-piperazine derivatives fluspirilene and penfluridol inhibited secretion by over 80%; no recovery was produced after 30-min washout. The inhibition of secretion was time dependent, as the recovery of the response was. Blockade of secretion by dotarizine and flunarizine occurred even in the absence of intermittent K^+ stimulations of the cells. No obvious correlation was seen between the octanol/water partition coefficients of the ten compounds tested (that ranged between 6 and 4.61), the rate and extent of blockade of secretion, and the recovery of the secretory response upon washout. Rather than non-specific actions on ion channels (and secretion) due to their high lipophilicity, we believe that blockade of various Ca^{2+} channels relates to their binding properties to specific channel micro and macrodomains, as the case might be for 'narrow' (ω-conotoxin GVIA) and 'wide-spectrum' (ω-conotoxin MVIIC) peptide toxins.

Keywords: Dotarizine; Flunarizine; Lubeluzole; Catecholamine release; Chromaffin cell; Ca^{2+} channel antagonist, 'wide-spectrum'

1. Introduction

The term 'Ca^{2+} channel antagonist' applies to a heterogeneous group of chemical entities having a common feature, the blockade of Ca^{2+} entry into excitable cells through the voltage-dependent L-subtype of Ca^{2+} channel. Earlier classifications separate them in two groups (Fleckenstein, 1983; Godfraind, 1987). Group I comprises those that selectively block L channels (i.e., nifedipine, verapamil, diltiazem, cinnarizine, flunarizine), and group II, those also affecting Na^+ channels (i.e., bepridil, lidoflazine). This classification did not take into consideration the existence of various Ca^{2+} channel subtypes in neurones, other than the L-type, and the fact that some toxins block specifically a channel subtype (i.e., ω-conotoxin GVIA blocks the N-type Ca^{2+} channel), while others inhibit various Ca^{2+} channel subtypes (i.e., ω-conotoxin MVIIC blocks N-, P- and Q-subtypes) (Olivera et al., 1994).

As ω-toxins, some organic non-peptidic compounds act on more than one Ca^{2+} channel subtype. For instance, flunarizine blocks various Ca^{2+} channel subtypes in neurones (Kaneda and Akaike, 1989; Takahashi and Akaike, 1991) and in bovine chromaffin cells (Villarroya et al., 1995). In fact, flunarizine and other molecules such as R56865, cinnarizine, sabeluzole, lubeluzole, R91154, fluspirilene, lidoflazine, penfluridol, and dotarizine have been

* Corresponding author. Tel.: (34-1) 397-5386; Fax: (34-1) 397-5397.

0014-2999/97/$17.00
PII S0014-2999(97)00108-8

shown to block completely the K^+-evoked $^{45}Ca^{2+}$ entry and/or the whole-cell current through Ca^{2+} channels in bovine chromaffin cells (Gárcez-Do-Carmo et al., 1993; Villarroya et al., 1995, 1997). This suggests that all these compounds, as ω-conotoxin MVIIC does, block various Ca^{2+} channels expressed by bovine chromaffin cells. These facts moved us to coin the expression 'wide-spectrum' Ca^{2+} channel blockers for compounds such as R56865, dotarizine, or flunarizine; this term better represents the pharmacological profile of these agents, as opposed to the term 'non-specific' or 'non-selective'.

The compounds selected to perform this study (see structural formulae in Fig. 1) were four piperazine derivatives (flunarizine, cinnarizine, lidoflazine and dotarizine), four benzisothiazol derivatives (sabeluzole, R56865 (*N*-[1-(4-(4-fluorophenoxy)butyl]-4-piperidinyl-*N*-methyl-2-benzo-thiazolamine), (+)-*S*-lubeluzole and R91154 (the (−)-*R*-enantiomer of lubeluzole) and two diphenylbutyl-piperidine neuroleptics (fluspirilene and penfluridol). This selection was made on the basis of previous data on their ability to block Ca^{2+} entry through various Ca^{2+} channel subtypes, as well as on their different octanol/water partition coefficients. The relevance of their lipophilicity for the rate, extent and reversibility of blockade by these compounds on Ca^{2+} channels might best be explored on a functional parameter such as the K^+-evoked catecholamine secretory response in fast-superfused bovine chromaffin cells. This is so because the on-line continuous monitoring of K^+-evoked catecholamine release from chromaffin cells is the immediate consequence of the activation of L- and non-L-type Ca^{2+} channels (López et al., 1994). Thus, the blockade of those channels by the compounds as well as the subsequent recovery of the response upon washout should be represented by the rate of blockade and recovery of secretion. In addition, this secretory signal remains stable for 1–2 h, thus facilitating the equilibrium of the

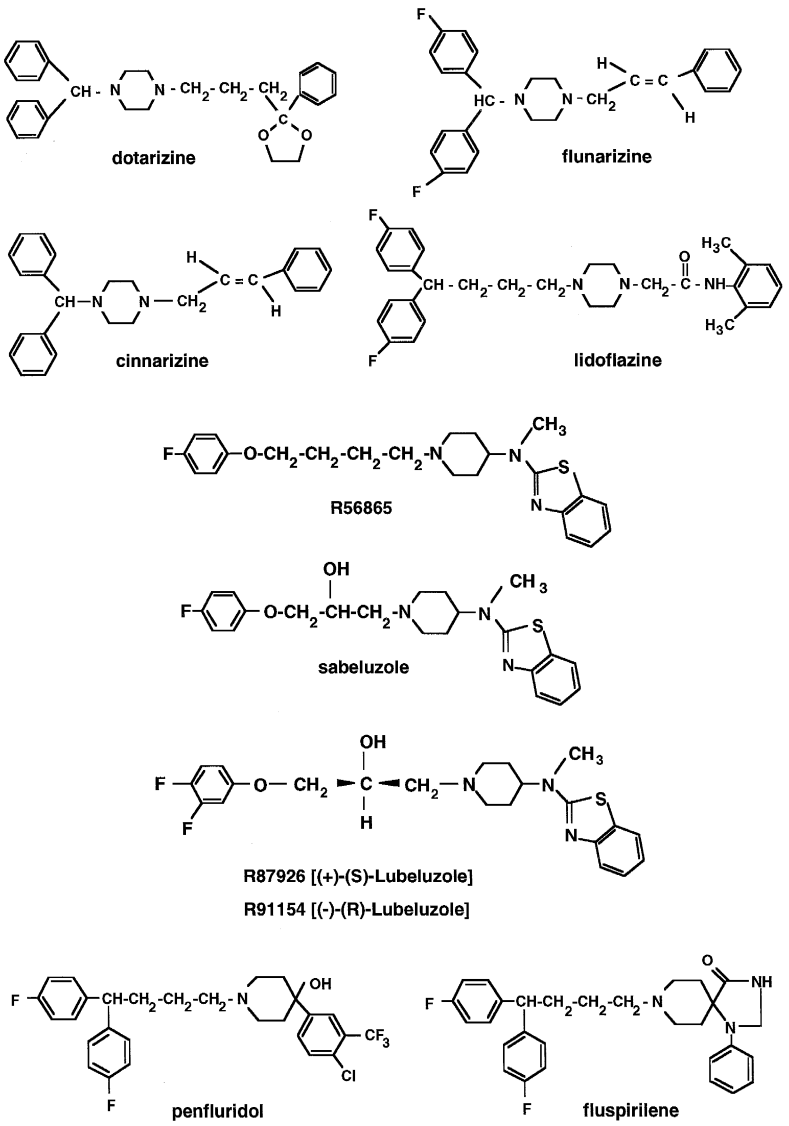

Fig. 1. Chemical structures of dotarizine, flunarizine, cinnarizine, (+)-*S*-lubeluzole, R91154, R56865, lidoflazine, sabeluzole, penfluridol, and fluspirilene.

compounds with their binding sites, as well as to follow the exit from blockade after washout. This information is difficult to be obtained through the whole-cell recordings of currents through Ca^{2+} channels, since they undergo run-down with time (Fenwick et al., 1982). Particular emphasis was made on dotarizine and flunarizine, previously shown to behave as Ca^{2+} entry blockers (Tejerina et al., 1993; Villarroya et al., 1995). Although having similar octanol/water partition coefficients and the same potency to block secretion, one was quickly reversible (dotarizine) and the other was very slowly reversible (flunarizine).

2. Materials and methods

2.1. Preparation and culture of bovine chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated following standard methods (Livet, 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, 50 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were plated at a density of 10^6 cells ml^{-1} in 5 cm diameter plastic Petri dishes (5 ml medium per dish). Medium was replaced every 2–3 days. Cells were used 1–4 days after plating.

2.2. On-line measurement of catecholamine release

Bovine chromaffin cells (5×10^6) were introduced in a microchamber for cell superfusion at room temperature (22 ± 2°C) with a Krebs-HEPES solution of the following composition (in mM): NaCl 144, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, glucose 11, HEPES 10, at pH 7.4.

The superfusion rate was 2 ml min^{-1}. The liquid flowing from the superfusion chamber reached an electrochemical detector model Metrohm AG CH9100 Hersau, that monitored 'on line' under the amperometric mode, the amount of catecholamines secreted (Borges et al., 1986).

Usually, the cells were superfused continuously with a normal Krebs-HEPES solution containing 2 mM Ca^{2+}. At 5-min intervals they were stimulated to secrete catecholamines with 10-s pulses of a K^+-enriched solution (70 mM K^+, 2 mM Ca^{2+}); the concentration of NaCl of this solution was reduced on a molar basis to maintain isotonicity. The experimental protocols were focused to study the rate and extent of inhibition of the secretory response, as well as the rate and extent of recovery after washout of the drugs.

2.3. Statistical analysis

Data are expressed throughout as means ± S.E.M. Comparison of the differences between means of data sets were made by the Student's *t*-test. Differences were accepted as significant at P values equal or smaller than 0.05. The statistical program Statworks was used to perform this analysis.

2.4. Materials and solutions

The following materials were used: collagenase from *Clostridium histolyticum* (Boehringer-Mannheim); bovine serum albumin fraction V, cytosine arabinoside, fluorodeoxyuridine and HEPES (Sigma); foetal calf serum, penicillin and streptomycin (Gibco). Dotarizine (1-(diphenylmethyl)-4-[3-(2-phenyl-1,3-dioxalan-2-1)-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), cinnarizine (1-(diphenylmethyl)-4-(3-phenyl- 2-propenyl)piperazine), lubeluzole ((+)-(*S*)-4-(2-benzothiazolylmethylamino)-α- [3,4-difluorophenoxy)-methyl]-1-piperidineethanol), sabeluzole ((±)-4-(2-benzothiazolylmethylamino)-α-[(4-fluorophenoxy)methyl]-1-piperidineethanol)), R56865 (*N*-[1-(4-(4-fluorophenoxy)butyl)]-4-piperidinyl-*N*-methyl-2-benzo-thiazolamine), R91154 ((−)-(*R*)-4-(2-benzothiazolyl-methylamino)-α-[(3,4- difluorophenoxy) methyl]1 piperidine ethanol), penfluridol (1-[4,4-bis(4-fluorophenyl)butyl]-4-[4-chloro-3-trifluoromethyl)phenyl]-4-piperidinol) and lidoflazine (4-[4,4-bis(4-fluorophenyl)butyl]-*N*-(2,6-dimethylphenyl)piperazine-acetamide) were kind gifts from Janssen (Madrid, Spain). Fluspirilene (8-[4,4-bis(4-fluorophenyl)butyl]-1-phenyl-1,3,8-triazino[4,5]decan-4-one) was a gift from Lilly-Dista (Madrid, Spain). All other chemicals were of reagent grade from Merck (Madrid, Spain).

The ten compounds were dissolved in dimethyl sulphoxide (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 the K^+-evoked secretory responses.

3. Results

3.1. Inhibition by wide-spectrum calcium antagonists of K^+-evoked secretion and recovery from blockade

To study the inhibition by the ten compounds selected, of K^+-evoked catecholamine secretory responses from superfused bovine adrenal chromaffin cells, and the reversal of such inhibitory effects after washout of the drugs, it was necessary first to define an experimental protocol that produced reproducible responses for extended periods of time. After modifying several parameters (K^+ and Ca^{2+} concentrations of depolarizing solutions, duration of the K^+ pulses, interval of application of such pulses) we selected experimental conditions that gave reproducible secretory responses: pulses of 10-s duration with a solution containing 70 mM K^+ and 2 mM Ca^{2+} ($70K^+/2Ca^{2+}$) given at 5-min intervals. The experiment in Fig. 2A shows

an example of such a protocol. Cells were continuously superfused with a normal Krebs-HEPES solution (2 mM Ca^{2+}). After an initial 10–15 min period, the baseline of secretion stabilized. After this, K^+ pulses were applied regularly. Note that during the first three pulses the secretory response increased in a 'stair-case' manner; then the response gradually declined (around 20%) along a 60–90 min period. In 24 experiments the initial secretory response (third K^+ pulse) amounted to 448 ± 46 nA, equivalent to 1555 ± 159 ng of total catecholamine. The response to this third K^+ pulse was taken as 100% (control secretion response). The reproducibility of the secretory response to $70K^+/2Ca^{2+}$ pulses was suitable to intercalate a drug for a given time period, and study its effects on the response as well as the degree of reversibility of its effects (protocol shown by the horizontal bars at the bottom of Fig. 2A).

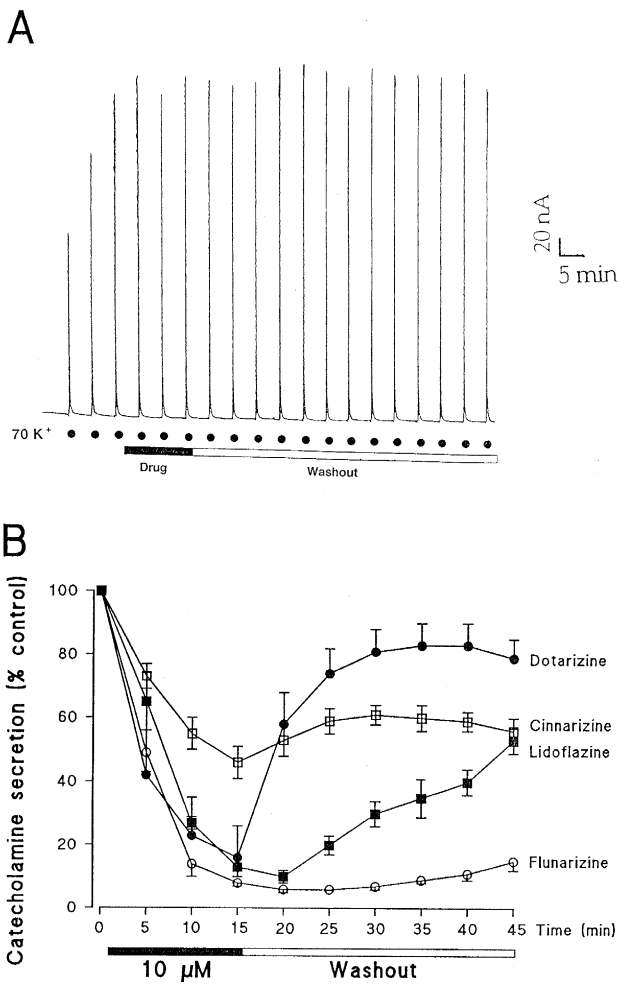

Fig. 2. Blockade and recovery of K^+-evokedcatecholamine release in chromaffin cells exposed to four piperazine derivatives. Panel A shows an example of the protocol used. Cells were superfused with Krebs-HEPES solution during an initial 10-min period to allow stabilization of baseline secretion. Then, they were stimulated to secrete catecholamines (measured as nA of current) with 10-s pulses of a K^+-enriched solution (70 mM K^+, 2 mM Ca^{2+}), given at 5-min intervals (dots at the bottom). Note the reproducibility of the secretion peaks after the third pulse. The horizontal bar at the bottom represented the time period during which the cells were exposed to the drugs. Panel B shows the effects of the four piperazine derivatives (flunarizine, dotarizine, cinnarizine and lidoflazine), on K^+-evoked secretion. Each batch of cells was superfused with a single drug at the concentration of 10 μM, during the time period (15 min) shown by the black horizontal bar. After this, the cells were superfused for a 30-min period with drug-free Krebs-HEPES solution (open horizontal bar). Secretion was triggered by application of 10-s depolarizing pulses of a solution containing 70 mM K^+. In each individual experiment, data were normalised as percentage of the third initial secretory response (448 ± 46 nA). Data are means ± S.E.M. of 17 (cinnarizine) or 3 experiments (lidoflazine, dotarizine and flunarizine).

Fig. 2B shows the effects on K^+-evoked secretion of 10 μM each of the four piperazine derivatives used. In all cases there was a time-dependent blockade and recovery of the secretory response. However, the extent of blockade and recoveries varied substantially amongst the four compounds (Table 1). The blockade ranged between 84–92% in the case of dotarizine, lidoflazine and flunarizine. Surprisingly, the blockade was considerably smaller for cinnarizine (54%; $P < 0.01$, compared with flunarizine). Again, the time constant for development of blockade was similar for flunarizine, dotarizine and lidoflazine (τ around 5 min), but was considerably higher for cinnarizine ($\tau = 13$ min). More drastic differences were seen in the recovery of secretion. So, after washout of dotarizine for 30 min, the response recovered by 75%, while in the case of flunarizine only 8% of recovery was produced. In the case of lidoflazine the recovery was 46% and for cinnarizine 21%.

The effects of the four benzothiazol derivatives (10 μM) on blockade and recovery of secretion are shown in Fig. 3A and Table 1. (+)-*S*-Lubeluzole and R91154 ((−)-*R*-lubeluzole) inhibited secretion in a time-dependent manner, by 71–75%. After a 30-min washout period, the recovery of the response amounted to 61–64%. Sabeluzole was a poorer blocker (34% inhibition of secretion), and secretion recovered faster after its washout (76%). R56865 produced 61% blockade of secretion and 64% recovery.

The effects on secretion of fluspirilene and penfluridol (twodiphenylbutyl-piperidine derivatives) are shown in Fig. 3B. Note the gradual and slow development of blockade, that amounted to 75% in the case of fluspirilene and to 85% in the case of penfluridol. Note also that the blockade of secretion continued to develop after washing out these drugs; so fluspirelene blocked almost completely the response. This 'post-drug' effect was also seen in the case of flunarizine and lidoflazine (Fig. 2B). No recovery of secretion was seen after 30-min washout of penfluridol; only a slight recovery was produced after washout of fluspirilene.

3.2. Inhibition of secretion upon repeated short exposures of cells to dotarizine or flunarizine

For these and the subsequent experimental protocols two compounds were selected; in spite of having similar octanol/water partition coefficients, one gave rise to a

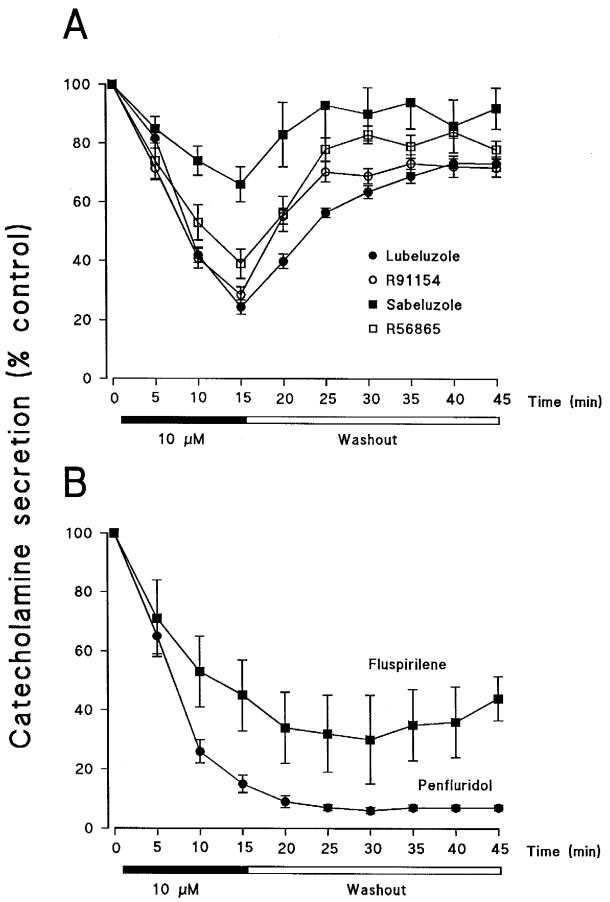

Fig. 3. Effects of the four benzisothiazol derivatives sabeluzole, R56865, (+)-*S*-lubeluzole and R91154 ((−)-*R*-lubeluzole) (panel A), and of the two diphenylbutyl-piperidine derivatives fluspirilene and penfluridol, on the K^+-evoked catecholamine secretory responses in fast-superfused bovine chromaffin cells (protocol as in Fig. 2). Data are means ± S.E.M. of 6 (R56865), 4 (sabeluzole), 8 (lubeluzole), 10 (R91154), 8 (penfluridol) and 6 experiments (fluspirilene).

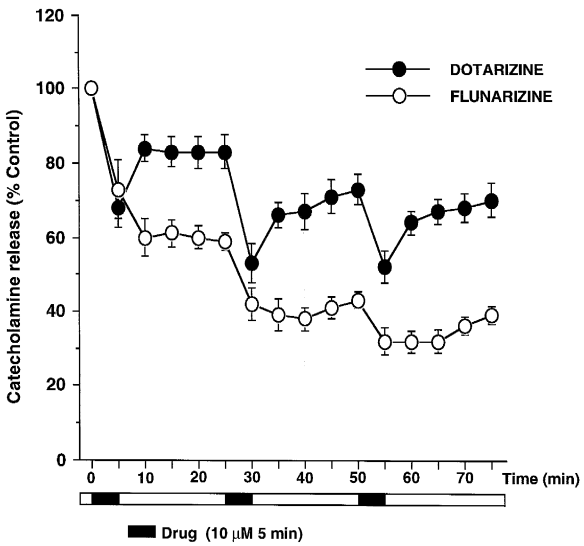

Fig. 4. Effects of intermittent application ofdotarizine and flunarizine on catecholamine secretion. Cells were stimulated with K^+ (70 mM K^+, 10 s) at 5-min intervals. After the third K^+ stimulation 10 μM dotarizine or flunarizine were applied for 5 min, as shown by the horizontal bar; then cells were superfused during 20 min with drug-free solution (open bar). This treatment was repeated twice more as indicated by the horizontal bars. Catecholamine secretion (ordinates) was normalised as percentage of the initial secretion. Data are means ± S.E.M. of 8 (dotarizine) and 5 experiments (flunarizine).

quick recovery of secretion upon its washout (dotarizine) and the other to a very mild recovery (flunarizine).

In the experiments shown above, drugs were given for a continuous 15-min superfusion period. We thought it interesting to split this period into three 5-min shorter periods of application, each followed by 20-min washout periods. In the experiment of Fig. 4 the K^+ test pulses produced initial secretory responses of around 407 ± 35 nA of cate-

Table 1
Kinetic parameters of inhibition and recovery of catecholamine release induced by 10 μM of ten 'wide-spectrum' Ca^{2+} channel antagonists with different degrees of lipophilicity

Compound	Octanol/water partition coefficient [a]	*n* [b]	Rate of inhibition [c] (τ)	Inhibition [d] (%)	Recovery [e] (%)
Dotarizine	6	3	5	84 ± 10	75
Flunarizine	5.78	3	3.9	92 ± 1	8
Cinnarizine	5.60	17	12.9	54 ± 5	21
Lidoflazine	5.20	3	5.3	87 ± 3	46
R56865	5.48	6	11	61 ± 5	64
(+)-*S*-Lubeluzole	4.89	8	7.6	75 ± 2	64
R91115 ((−)-*R*-lubeluzole)	4.89	10	8.1	71 ± 3	61
Sabeluzole	4.61	4	24.8	34 ± 6	76
Penfluridol	5.87	5	5.4	85 ± 3	0
Fluspirilene	5	6	6.7	75 ± 10	3

The parameters on the effects on secretion were calculated from the curves shown in Fig. 2Fig. 3.

[a] These data were obtained from Dr. Sigrid Stockbroeckoc (Analytical Research, Department of Physicochemistry, Janssen Research Foundation, Beerse, Belgium), except the datum of dotarizine, provided by Dr. A. Ortíz (Fundación Ferrer, Barcelona, Spain). The compounds are grouped according to their structural formula (see Fig. 1).

[b] Number of experiments.

[c] τ is the time constant in minutes for the exponential decline of the secretory response upon exposure of the cells to the drugs.

[d] Inhibition was calculated as percentage of the initial control secretory response, after 15 min superfusion of chromaffin cells with 10 μM of each compound.

[e] Recovery after 30 min washout was calculated as percentage of the fraction of the secretory response previously inhibited by each compound.

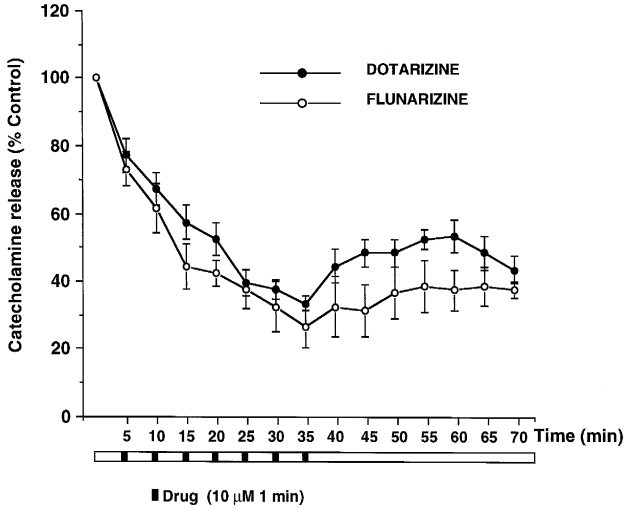

Fig. 5. Effects of repeated 1-min pulses of dotarizine and flunarizine on K^+-evoked catecholamine release. After the initial three control K^+ pulses (70 mM K^+, 10 s), dotarizine or flunarizine (10 μM) were applied for 1 min just before each K^+ stimulation, as shown by the horizontal bar. Data are means ± S.E.M. of 6 experiments for dotarizine and 4 for flunarizine.

cholamine ($n = 13$). After the first 5-min exposure to dotarizine, secretion was inhibited by 32 ± 5.3%. Washout of dotarizine prompted the recovery of secretion to about 83 ± 4.6% of the initial response. The second 5-min application of dotarizine blocked the response by 47 ± 5.2% and again, the response recovered to near 73 ± 12% of the initial response. The third exposure blocked secretion by 50% with a new fast recovery. The results obtained with 5-min applications of flunarizine considerably differed. The first application blocked the secretory response by 27 ± 8.1%, a figure similar to that obtained with dotarizine. Washout did not lead to recovery of the response; on the contrary, a post-drug effect was seen (blockade increased to 40%); no recovery was seen. The second application blocked further the response by 58 ± 4.5%; again no recovery was produced. The third application blocked secretion by 68 ± 3.8%; washout of flunarizine allowed only a tiny recovery of the response.

In a second protocol, the period of cell exposure to the drugs was further split into 1-min periods. The drugs (10 μM) were given repeatedly at 5-min intervals, only during the minute preceding each K^+ pulse. Fig. 5 shows the effects of 1-min applications of dotarizine preceding nine consecutive K^+ pulses. Secretion declined gradually with each pulse, to reach about 50% blockade of the initial response at the ninth dotarizine pulse. Recovery of secretion after stopping the dotarizine pulses reached about 75% of the initial response. The blockade induced by the intermittent 1-min pulses of flunarizine also developed gradually. Secretion was inhibited by 63% after nine pulses of flunarizine. Very little recovery of secretion was seen after interruption of the flunarizine pulses.

3.3. Effects of a high concentration of dotarizine and flunarizine on K^+-evoked secretion

In spite of the high concentration of drugs used in the previous experiments (10 μM), the blockade of secretion developed gradually. Therefore, experiments were designed to study whether shorter exposure times with even higher concentrations of drugs produced a quicker inhibition of secretion. These experiments were intended to define the relative importance of time and drug concentration, on the blockade of secretion.

Fig. 6 shows experiments in which the compounds were applied for 10 min at the concentration of 30 μM. After 5 min of superfusion, dotarizine inhibited secretion by 36%, and after 10 min by 67%. In the cells exposed to 30 μM dotarizine, the recovery of the response was markedly delayed with respect to the 10 μM concentration (compare Figs. 2 and 6). At 5 and 10 min of superfusion, flunarizine (30 μM) inhibited secretion to an extent similar to dotarizine. An interesting difference emerged after washout of the drug: flunarizine exhibited a clear post-drug effect, and secretion continued to decline during the 30-min washout period. Full blockade of secretion could not be achieved even with these high concentrations of drugs. Therefore time, more than high concentrations of drugs, seems to be required to achieve a complete inhibition of secretion.

Still more interesting was the behaviour of the K^+ secretory responses in cells exposed only once to a short pulse (1 min) of 30 μM dotarizine or flunarizine (Fig. 7). When dotarizine was given for 1 min, 5 min before the K^+

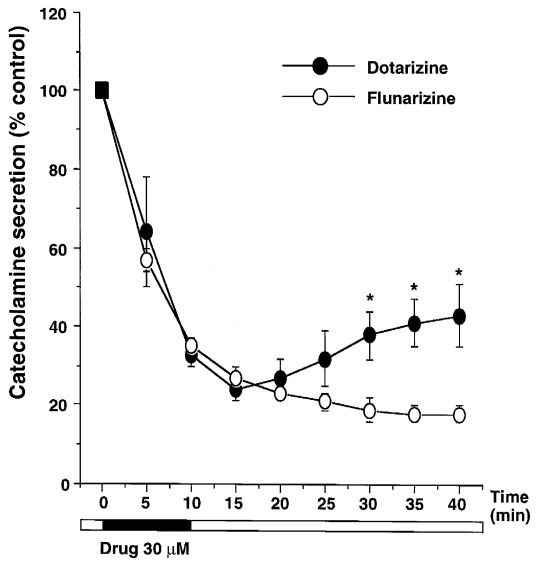

Fig. 6. Inhibition of K^+-induced secretion and recovery in cells treated with 30 μM dotarizine or flunarizine. Drugs were given during the 10-min period shown by the black horizontal bar. Data are normalised as percentage of the predrug control secretion signal (ordinate). They are means ± S.E.M. of 6 experiments for each compound, performed with different batches of cells. * $P < 0.01$ with respect to flunarizine.

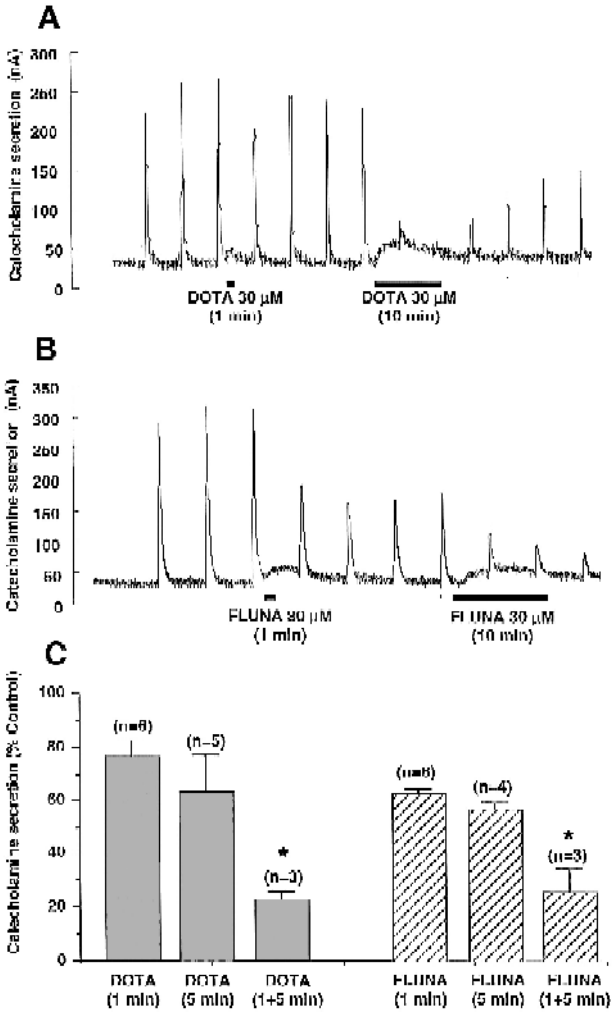

Fig. 7. Effects of single short pulses (60 s) of a high concentration (30 μM) of dotarizine or flunarizine on K^+-evoked catecholamine release. Cells were superfused with normal Krebs-HEPES solution and stimulated at 5-min intervals with a K^+-enriched solution (70 mM K^+, 2 mM Ca^{2+} for 10 s). Five minutes before the fourth K^+ pulse, cells were exposed for 60 s to a Krebs-HEPES solution containing 30 μM of dotarizine or flunarizine. In panel A, dotarizine (30 μM) was given first for 1 min and later on during 10 min. A similar design was followed with flunarizine (panel B). Panel C shows averaged experiments from this figure and from Fig. 6. The values for the blockade induced by 1-min exposure of the cells to each drug were obtained from 6 experiments with the experimental design followed in panels A and B in this figure. Those corresponding to the blockade induced by 5-min exposure were obtained from Fig. 6. The values of 1+5 min exposure were calculated again from the experiments in panels A and B of this figure, during the second application (first 5-min period) of dotarizine or flunarizine. Secretory peaks were normalised as percentage of the initial control peak (ordinates). In panel C, data are means ± S.E.M. of the number of experiments shown in parentheses, which were performed with cells from different batches. * $P < 0.05$ with respect to dotarizine or flunarizine when given for 1 or 5 min.

pulse, secretion decreased by 25–35%, but then quickly recovered. However, when the same cells were exposed to dotarizine for a longer time period (10 min), secretion was quickly and almost totally blocked (panel A). In the case of flunarizine the response was halved after 1 min of previous superfusion, and remained depressed for three additional K^+ pulses with no apparent recovery. Again, 10 min of exposure to flunarizine led to almost complete inhibition of secretion (panel B). This behaviour contrasts with the effects of 5-min exposure to 30 μM of the drugs (see Fig. 6). The 'priming' effect of 1-min pre-pulse drug treatment, on the blockade of secretion, is quantitatively illustrated in Fig. 7C. While a short pulse (1 min) of dotarizine or flunarizine caused little inhibition of secretion by itself, it however enhanced drastically the inhibitory effects of a subsequent 5-min treatment. Thus the blocking effects of 1-min and 5-min treatments were synergistic.

3.4. Effects of dotarizine and flunarizine on secretion when given to resting cells

In all experiments described up to now, dotarizine and flunarizine were applied to cells while they were stimulated with K^+ at 5-min intervals. Under these conditions, the gradual blockade of secretion could be related to such intermittent depolarization periods of the cells ('use-dependent' blockade) rather than to the time of cell exposure to the compounds. Therefore, experiments were designed in which a period of drug application elapsed without cell depolarization with K^+ (resting cells).

Cells were stimulated three times with K^+ at 5-min intervals and then they were left without stimulation for a 30-min period. During this period, dotarizine (10 μM) was

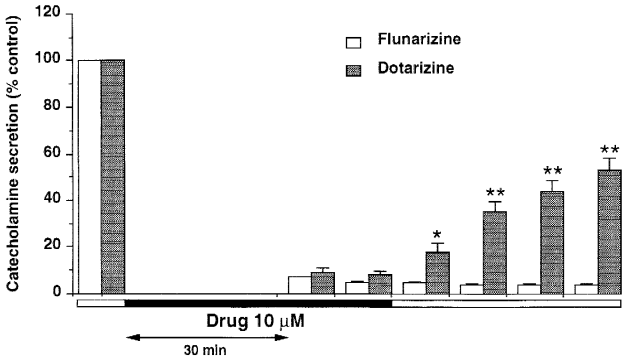

Fig. 8. Effects of dotarizine and flunarizinepretreatment on subsequent K^+-evoked catecholamine release. As usual, three K^+ pulses were initially applied to get stable responses. Then, K^+ stimulation was interrupted and 10 μM dotarizine (closed squares) or 10 μM flunarizine (open squares) were given for the 30 min 'silent' period shown by the horizontal black bar. K^+ stimulation was resumed after 30 min, still in the presence of the compounds. A washout period with Krebs-HEPES solution free of drugs followed (open horizontal bar). Data were normalised as percentage of control pre-drug secretion (third initial secretory peak), and they are means ± S.E.M. of 6 experiments. * $P < 0.05$, ** $P < 0.006$ in comparison to maximum blockade achieved.

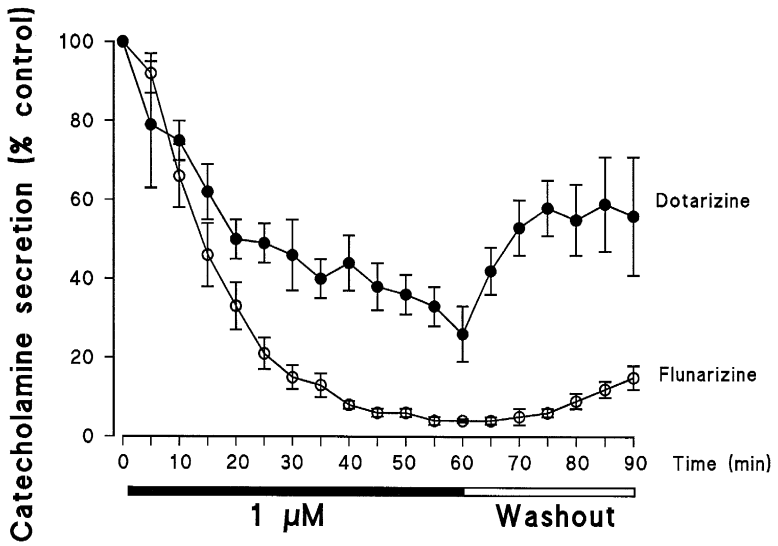

Fig. 9. Effects of a low concentration of dotarizine or flunarizine on K^+-evoked secretion. After the three initial K^+ pulses, dotarizine or flunarizine (1 μM each for 60 min) was introduced during the period shown by the black horizontal bar. Six subsequent K^+ pulses were given in the absence of the compound (30-min washout period). The values of all responses were expressed as percentage of the initial control response in each individual experiment (ordinate). Data are means ± S.E.M. of 6 experiments performed in separate batches of cells.

applied. Cells were stimulated twice more with K^+ still in the presence of the drug, and four times more in its absence (washout). Dotarizine inhibited secretion as much as 91 ± 9.2% under conditions of unstimulated cells (Fig. 8). Washout of the drug prompted the recovery of the secretory response to 53 ± 5.3% of control.

The experiment with flunarizine showed essentially the same pattern as far as blockade of secretion was concerned. Flunarizine (10 μM) given to resting cells inhibited the K^+-evoked response by 95 ± 0.3%. No recovery of secretion was produced after a 20-min washout period (Fig. 8).

3.5. Blockade of secretion during long exposure of the cells to low concentrations of dotarizine and flunarizine

In the presence of 1 μM dotarizine, the K^+-evoked secretory response declined rapidly. Blockade of secretion developed gradually and did not reach a steady state even after 60 min of continued superfusion with the drug (Fig. 9). Secretion was inhibited by 75% after 1-h superfusion. Washout of dotarizine prompted a quick recovery of secretion; the response reached a steady state after 15-min washout. Considering the decline of secretory responses in non-treated control cells (about 20% after a 90-min period), it seems that the response to K^+ recovers by over 90% after washout of dotarizine.

At 1 μM, flunarizine relaxed the secretory responses in a faster and more efficient manner than dotarizine (Fig. 9). The response was halved by about 10–15 min of superfusion with flunarizine; after 40–50 min, full blockade of secretion developed. Recovery from blockade started at 10-min washout, and accounted for only 10% 30 min later.

4. Discussion

Attempts were made at correlating the octanol/water partitioncoefficients of the ten compounds with the blockade and recovery of secretion, through a linear regression analysis (Fig. 10). The dispersion of the points was apparent for the rate of blockade of secretion (panel A), the degree of inhibition of secretion (panel B) and the recovery of secretion after washout (panel C). This suggests that the pharmacological effects of these classes of compounds cannot simply be explained on the basis of their high lipophilicity and their ability to impregnate tissue and cells, and to dissolve and accumulate in their membranes.

This lipophilic hypothesis might be valid when considering the case of flunarizine in isolation. In fact, flunarizine shows an extraordinary capacity to concentrate in cell membranes, where it can reach millimolar concentrations from medium concentrations in the low micromolar range (Scheufler and Peters, 1990; Thomas and Seelig, 1993). In line with this is the time-dependent effect of flunarizine in blocking secretion, and the fact that at 1 μM it blocks secretion as much as 10–30 μM concentrations, provided that cells were exposed for a sufficient length of time to the drug (Fig. 9). However, the hypothesis is untenable if the effects of flunarizine are compared with those of the other compounds here studied. This is particularly true for

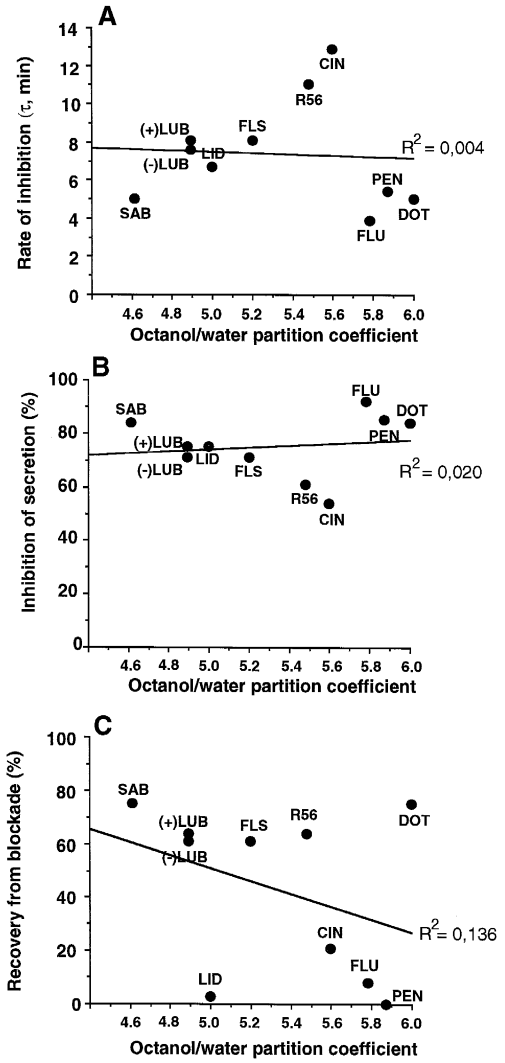

Fig. 10. Linear regression analysis of thecorrelation between the octanol/water partition coefficients of the ten compounds studied, and the rate of blockade of K^+-evoked catecholamine secretion (panel A), the degree of inhibition (panel B), or the recovery upon washout (panel C). Data are taken from Table 1. DOT, dotarizine; FLU, flunarizine; CIN, cinnarizine, LID, lidoflazine; R56, R56865; (+)LUB, (+)-*S*-lubeluzole; (−)LUB, (−)-*R*-lubeluzole (R91115); SAB, sabeluzole, PEN, penfluridol; FLS, fluspirilene.

the case of dotarizine, a compound structurally related to flunarizine (both are piperazine derivatives) and with an even higher degree of lipophilicity. As flunarizine, dotarizine inhibited markedly the K^+-evoked secretory responses in a time-dependent manner. However, unlike flunarizine, dotarizine exhibited no post-drug effects; its blockade was readily reversed upon washout, and it showed concentration dependence (compare Figs. 8 and 9 where the blockade of secretion by flunarizine was similar at 10 and 1 μM, respectively, while that of dotarizine differed).

A common feature to all compounds tested was the time dependence of secretion blockade. Difficult access to their binding sites, slow equilibrium binding or use-dependent block could explain this time dependence. This last possibility can be discarded on the basis of the results of the experiment shown in Fig. 8. Dotarizine and flunarizine inhibited the K^+-evoked secretory responses even in the absence of prior depolarizations. As suggested by their blocking effects of $^{45}Ca^{2+}$ entry and whole-cell currents through Ca^{2+} channels (Gárcez-Do-Carmo et al., 1993; Villarroya et al., 1995), dotarizine and flunarizine inhibit secretion by blocking Ca^{2+} channels; it seems therefore that previous opening of these channels is not required to block Ca^{2+} channels, Ca^{2+} entry and secretion.

The slow time-course of secretion blockade keeps pace with also a slow development of blockade by R56865 (Gárcez-Do-Carmo et al., 1993), flunarizine and dotarizine (De La Fuente et al., 1992; Villarroya et al., 1995) of whole-cell currents through Ca^{2+} channels in voltage-clamped bovine chromaffin cells. Even at 30 μM the blockade of secretion by flunarizine and dotarizine needed a time period of 10–15 min to develop. Because a 'use-dependent' blockade of Ca^{2+} channels and secretion can be discarded, it seems that the access and/or equilibrium binding of the molecules to their binding sites on Ca^{2+} channels must be responsible for the slow development of blockade.

Bovine chromaffin cells express several of the neuronal types of the high-threshold voltage-dependent Ca^{2+} channels (Olivera et al., 1994). Thus, the whole-cell current through Ca^{2+} channels in these cells consists of 15–20% L-type Ca^{2+} channels, 30–40% N-type Ca^{2+} channels and 35–45% P/Q-type Ca^{2+} channels (Albillos et al., 1996). By measuring $^{45}Ca^{2+}$ entry into K^+-depolarized chromaffin cells, we could recently show that all these Ca^{2+} entry pathways can be blocked by the compounds studied here (Villarroya et al., 1997). Because the K^+-evoked catecholamine release response is controlled by L- as well as non-L-type Ca^{2+} channels (López et al., 1994), it seems that the blocking effects of secretion by these compounds seen in this study relate to their ability to inhibit L- as well as non-L-subtypes of Ca^{2+} channels. This is the reason why we would like to name these compounds as 'wide-spectrum Ca^{2+} channel antagonists'. This is not only a concept of academic interest, since it also has clinical implications. For instance, R56865, flunarizine, and lubeluzole protected against the Ca^{2+}-dependent cytotoxic effects of veratridine, which are due to Ca^{2+} overloading by excess Ca^{2+} entry through different Ca^{2+} channel subtypes in bovine chromaffin cells (Maroto et al., 1994). While the more selective Ca^{2+} channel blockers furnidipine (L-type) or ω-conotoxin GVIA (N-type) did not protect against the cytotoxic effects of veratridine, ω-agatoxin IVA (P/Q-type) affords some protection and ω-conotoxin MVIIC (N/P/Q-type) markedly prevents cell death (Maroto et al., 1996). Thus organic compounds mimicking ω-conotoxin MVIIC ('wide-spectrum' Ca^{2+} channel blocker) are potentially more useful than 'narrow-spec-

trum' channel blockers in exerting protection against neuronal damage.

The long-term clinical use of flunarizine for the treatment of migraine, epilepsy and other diseases (Binnie et al., 1985; Todd and Benfield, 1989; Diener, 1994), gave rise to the discovery of some undesirable side-effects, i.e., reversible extrapyramidal symptoms (Micheli et al., 1987). These effects could be explained by the accumulation of flunarizine in brain tissue, blocking dopamine release from striatal neurones and/or striatal dopamine D_2 receptors (Brucke et al., 1995; Maroto et al., 1995). In addition to the 'wide-spectrum Ca^{2+} channel antagonist' properties of flunarizine, dotarizine is a potent blocker of 5-HT receptors, both in vitro and in vivo (Agut et al., 1990; Brasó et al., 1989, 1994; Cartheuser et al., 1994). Both properties might be responsible for its beneficial effects in the prophylaxis of migraine (personal communication of J.F. Horga). It is interesting that in spite of having a similar logP the blocking effects upon catecholamine release were readily reversed in the case of dotarizine, but not with flunarizine. If these differences are present in vivo, it is plausible that dotarizine is washed off readily from brain tissue, thus precluding the production of side-effects of the type seen with flunarizine. In fact, chronic oral administration of high doses of dotarizine to rats was devoid of neuroleptic-like actions, yet flunarizine exhibits mild neuroleptic effects (K. Füxe; personal communication).

Acknowledgements

This work was supported by Grupo Ferrer (Barcelona, Spain) and Janssen España (Madrid). Also by grants from Fundación Ramón Areces, DGICYT No. PB94-0150 to A.G.G., and from Universidad de Córdoba to B.L. We thank Mrs. M.C. Molinos for typing the manuscript and Dr. Richard Lomax for reviewing the manuscript.

References

Agut, J.A., A. Sacristán and J.A. Ortíz, 1990, Dotarizine: action on 5-HT_2 receptor binding in rat frontal cortex, Eur. J. Pharmacol. 183, 2147.

Albillos, A., A.G. García, B. Olivera and L. Gandía, 1996, Re-evaluation of the P/Q Ca^{2+} channel components of Ba^{2+} currents in bovine chromaffin cells superfused with low and high Ba^{2+} solutions, Pflügers Arch. Eur. J. Physiol. 432, 1030.

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

Borges, R., F. Sala and A.G. García, 1986, Continuous monitoring of catecholamine release from perfused cat adrenals, J. Neurosci. Methods 16, 389.

Brasó, A., C. Cartheuser, A. Sacristán, and J.A. Ortíz, 1989, Dotarizine, a promising *N*-benhydryl-piperazine for migraine prophylaxis due to an additional antiserotoninergic effect. 4th Int. Symp. Calcium Antagonists, Pharmacol. Clin. Res., Florence, May 25–27, p. 261.

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

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

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

De La Fuente, M.T., J.M. Guantes, M. Del Valle and A.G. García, 1992, Mechanism of blockade by flunarizine of bovine adrenal catecholamine release, Eur. J. Pharmacol. 229, 189.

Diener, H.C., 1994, A review of current treatments for migraine, Eur. Neurol. 34 (Suppl. 2), 18.

Fenwick, E.M., A. Marty and E. Neher, 1982, Sodium and calcium channels in bovine chromaffin cells, J. Physiol. 331, 599.

Fleckenstein, A., 1983, History of Ca^{2+} channel antagonists, Circ. Res. 52 (Suppl. 1), 3.

Gárcez-Do-Carmo, L., A. Albillos, A.R. Artalejo, M.T. De La Fuente, M.G. López, L. Gandía, P. Michelena and A.G. García, 1993. R56865 inhibits catecholamine release from bovine chromaffin cells by blocking calcium channels, Br. J. Pharmacol. 110, 1149.

Godfraind, T., 1987, Classification of Ca^{2+} channel antagonists, Am. J. Cardiol. 59, 11B.

Kaneda, M. and N. Akaike, 1989, The low-threshold Ca current in isolated amygdaloid neurons in the rat, Brain Res. 497, 187.

Livet, B.G., 1984, Adrenal medullary chromaffin cells in vitro, Physiol. Rev. 64, 1103.

López, M.G., M. Villarroya, B. Lara, R. Martínez-Sierra, A. Albillos, A.G. García and L. Gandía, 1994, Q- and L-type Ca^{2+} channels dominate the control of secretion in bovine chromaffin cells, FEBS Lett. 349, 331.

Maroto, R., M.T. De La Fuente, A.R. Artalejo, F. Abad, M.G. López, J. García-Sancho and A.G. García, 1994, Effects of Ca^{2+} channel antagonists on chromaffin cell death and cytosolic Ca^{2+} oscillations induced by veratridine, Eur. J. Pharmacol. 279, 331.

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

Maroto, R., M.T. De La Fuente, P. Zapater, F. Abad, E. Esquerro and A.G. García, 1996, Effects of ω-conotoxin MVIIC on veratridine-induced cytotoxicity and cytosolic Ca^{2+} oscillations, Brain Res. 714, 209.

Micheli, F., M.F. Pardal, M. Gatto, M. Torres, G. Paradiso, I.C. Parera and R. Giannaula, 1987, Flunarizine and cinnarizine-induced extrapyramidal reactions, Neurology 37, 881.

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

Olivera, B.M., G. Miljanich, J. Ramachandran and M.E. Adams, 1994, Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins, Annu. Rev. Biochem. 63, 823.

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

Takahashi, K. and N. Akaike, 1991, Calcium antagonist effects on low-threshold (T-type) calcium current in rat isolated hippocampal CA1 pyramidal neurons, J. Pharmacol. Exp. Ther. 256, 169.

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

Thomas, P.G. and J. Seelig, 1993, Binding of the Ca^{2+} channel antagonist flunarizine to phosphatidylcholine bilayers: charge effects and thermodynamics, Biochem. J. 291, 397.

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

Villarroya, M., L. Gandía, B. Lara, A. Albillos, M.G. López and A.G. García, 1995, Dotarizine versus flunarizine as Ca^{2+} channel antagonists in chromaffin cells, Br. J. Pharmacol. 114, 369.

Villarroya, M., M.-T. De La Fuente, M.G. López, L. Gandía and A.G. García, 1997, Distinct effects of ω-toxins and various groups of Ca^{2+}-entry inhibitors on nicotinic acetylcholine receptor and Ca^{2+} channels of chromaffin cells, Eur. J. Pharmacol. 320, 249.