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Distinct effects of ω-toxins and various groups of Ca^{2+}-entry inhibitors on nicotinic acetylcholine receptor and Ca^{2+} channels of chromaffin cells

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Abstract

The effects of ω-toxins and various Ca^{2+} antagonist subtypes on the $^{45}Ca^{2+}$ entry into bovine adrenal medullary chromaffin cells stimulated via nicotinic acetylcholine receptors or via direct depolarization with K^+, have been compared. The conditions selected to stimulate the $^{45}Ca^{2+}$ entry consisted of a 60-s period of exposure of cells to 100 μM of the nicotinic acetylcholine receptor agonist dimethylphenylpiperazinium or to 70 mM K^+. The N-type voltage-dependent Ca^{2+} channel blockers ω-conotoxin GVIA and MVIIA (1 μM) inhibited $^{45}Ca^{2+}$ entry stimulated by dimethylphenylpiperazinium or K^+ by around 25–30%. The P-type Ca^{2+} channel blocker ω-agatoxin IVA (10 nM) did not affect the dimethylphenylpiperazinium nor the K^+ responses; 1 μM (Q-channel blockade) inhibited both responses by around 50%. The N/P/Q-type Ca^{2+} channel blocker ω-conotoxin MVIIC (1 μM) inhibited the K^+ evoked $^{45}Ca^{2+}$ entry by 70%, while dimethylphenylpiperazinium was blocked by 50% ($P < 0.001$). The L-type Ca^{2+} channel blockers nifedipine, furnidipine, diltiazem or verapamil (3 μM each) inhibited much more the dimethylphenylpiperazinium than the K^+ response. The dimethylphenylpiperazinium signal was blocked 71, 88, 89, and 53%, respectively, by nifedipine, furnidipine, diltiazem and verapamil, and the K^+ response by 38, 29, 22, and 10%. Combined ω-conotoxin MVIIC (1 μM) and furnidipine (3 μM) blocked 100% of the K^+ evoked $^{45}Ca^{2+}$ entry. However, combined ω-conotoxin GVIA (1 μM), and furnidipine left unblocked 50% of the K^+ response. The 'wide spectrum' Ca^{2+} channel antagonists flunarizine or dotarizine (3 μM each) blocked the dimethylphenylpiperazinium and the K^+ responses to a similar extent (50%); cinnarizine (3 μM) inhibited more the dimethylphenylpiperazinium (82%) than the K^+ response (21%). At 3 μM, the highly lipophilic β-adrenoceptor antagonist (±)-propranolol, reduced by 68% the dimethylphenylpiperazinium signal and by 23% the K^+ signal. Other high lipophilic β-adrenoceptor antagonists such as metoprolol and labetalol, reduced little the dimethylphenylpiperazinium and the K^+ responses. The highly lipophilic agent penfluridol blocked the dimethylphenylpiperazinium response by 30% and the K^+ response by 50%. One of the least lipophilic compounds tested, (+)-lubeluzole, blocked by 40% the dimethylphenylpiperazinium and the K^+ responses. These data are compatible with the idea that the various ω-toxin peptides used to separate pharmacologically the different voltage-dependent Ca^{2+} channels expressed by neurones, do not block the neuronal nicotinic acetylcholine receptor ion channel. In contrast the L-type Ca^{2+} channel blockers do block the nicotinic acetylcholine receptor ionophore. Lipophilicity of the compounds is not a requirement for Ca^{2+} channel or nicotinic acetylcholine receptor blockade.

Keywords: ω-Toxin; Ca^{2+} channel; Neuronal nicotinic receptor; Ca^{2+} channel antagonist; Chromaffin cell

1. Introduction

The present study poses the question of whether some of the peptide ω-toxin blockers of various neuronal voltage-dependent Ca^{2+} channel subtypes (Olivera et al., 1994), and of the non-peptidic Ca^{2+} antagonists (Fleckenstein, 1983), also recognize the ion channel of the neuronal

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nicotinic receptor for acetylcholine. This work has its foundations in the following eight previous studies, some of them showing conflicting views and results: (1) $^{45}Ca^{2+}$ entry into bovine chromaffin cells stimulated by the nicotinic acetylcholine receptor agonist dimethylphenylpiperazinium is fully blocked by the 1,4-dihydropyridine derivatives nisoldipine ($IC_{50} = 2.63$ μM) and nimodipine ($IC_{50} = 1.1$ μM); K^+-evoked $^{45}Ca^{2+}$ entry is inhibited only by 30–50% (Gandía et al., 1991). (2) Synaptosomal membranes from the electric organ of *Discopyge ommata* contain abundant low affinity ω-conotoxin GVIA receptor sites ($K_d = 0.6$ μM), which co-purify with nicotinic acetylcholine receptor (Horne et al., 1991). (3) Nimodipine and Bay K 8644 also inhibit the whole-cell current through the nicotinic acetylcholine receptor channel of bovine adrenal chromaffin cells (López et al., 1993). (4) The benzylalkylamine Ca^{2+} antagonist methoxyverapamil (D600) blocks the nicotinic acetylcholine receptor current of chromaffin cells and sympathetic neurones (Boehm and Huck, 1993), as well as $^{45}Ca^{2+}$ entry induced by nicotinic acetylcholine receptor and K^+ stimulation (Corcoran and Kirshner, 1983). (5) The benzothiazepine Ca^{2+} antagonist diltiazem also inhibits this current in bovine chromaffin cells (Gandía et al., 1996). (6) ω-Conotoxin GVIA blocks nicotine-induced catecholamine secretion by inhibiting the nicotinic acetylcholine receptor-activated inward currents in bovine chromaffin cells (Fernández et al., 1995). (7) ω-Agatoxin IVA blocks nicotinic acetylcholine receptor currents in bovine chromaffin cells (Granja et al., 1995). (8) Verapamil, nimodipine, diltiazem, isradipine, nifedipine and *R*-(+)-Bay K 8644 block the nicotinic acetylcholine receptor-mediated cation efflux in a human neuroblastoma IMR 32 cell line with IC_{50} values of 0.78–3 μM; in contrast, several micromolar concentrations of ω-conotoxin GVIA or ω-agatoxin IVA do not affect this response (Donnelly-Roberts et al., 1995).

In the light of these conflicting views, it seemed appropriate to re-evaluate the ability of several peptide and non-peptide blockers of neuronal Ca^{2+} channel subtypes, to block the nicotinic acetylcholine receptor ion channel. $^{45}Ca^{2+}$ entry into cultured bovine adrenal medulla chromaffin cells is an adequate model to perform this study for the following reasons: (i) they express nicotinic acetylcholine receptor of the neuronal type (Criado et al., 1992); (ii) they express as well various neuronal Ca^{2+} channel subtypes which are sensitive to dihydropyridines (L-type), to ω-conotoxin GVIA (N-type), to nanomolar concentrations of ω-agatoxin IVA (P-type) and to micromolar concentrations of ω-agatoxin IVA or ω-conotoxin MVIIC (Q-type) (Albillos et al., 1996); (iii) the actions of ω-toxins and non-peptide molecules on nicotinic acetylcholine receptor channels and Ca^{2+} channels can be studied simultaneously and in parallel in the same batch of cells under similar experimental conditions; and (iv) a pre-incubation period to allow the equilibrium of drugs and ω-toxins with their binding sites, can be programmed at will.

2. Materials and methods

2.1. Isolation and culture of bovine chromaffin cells

Bovine adrenal medullary 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 supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were plated at a density of 5×10^5 cells $well^{-1}$ in 24-multiwell Costar plates, and were used 2–3 days after plating. In some experiments, cells were plated in 96 microwell plates at a density of 5×10^4 or 10^5 cells per microwell.

2.2. Measurements of $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ 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 solution, of the following composition (mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1, glucose 11, Hepes 10, pH 7.2, at 37°C.

$^{45}Ca^{2+}$ uptake into chromaffin cells was studied by incubating the cells at 37°C with $^{45}CaCl_2$ at a final concentration of 3 μCi ml^{-1} in the presence of Krebs-Hepes (basal uptake), high K^+ solution (Krebs-Hepes containing increasing concentrations of KCl with isosmotic reduction of NaCl), or dimethylphenylpiperazinium in Krebs-Hepes. This incubation was carried out for 1 min and at the end of this period the test medium was rapidly aspirated and the evoked $^{45}Ca^{2+}$ uptake period 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 more times 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, 0.5 ml of 10% trichloroacetic acid was added to each well and cells were scraped with a plastic pipette tip and transferred to scintillation minivials. Then, 3.5 ml scintillation fluid (Ready Micro, Beckman) were added and the samples counted in a Packard beta counter. Results are expressed as cpm 5×10^5 $cells^{-1}$ or as % of Ca^{2+} taken up by control cells. All the experiments were counted in the same beta counter with an efficiency of 60%, therefore the quenching was always the same. For this reason all the results are expressed in cpm instead of dpm.

2.3. Statistics

Data are expressed as means ± S.E.M. Statistical differences between means were estimated using the Student's *t*-test for non-paired group data. *P* values smaller than 0.05 were taken as the limit of significance.

2.4. Materials

The following materials were used: collagenase from *Clostridium histolyticum* (Boehringer-Mannheim, Madrid, Spain); Dulbecco's modified Eagle's medium, bovine serum albumin fraction V, fetal calf serum and antibiotics were purchased from Gibco (Madrid, Spain). ^{45}Ca, with a specific activity 10–40 mCi mg^{-1} Ca^{2+}, from Amersham (Amersham, UK). ω-Conotoxin GVIA and MVIIA were from Bachem (Essex, UK) ω-Conotoxin MVIIC and ω-agatoxin IVA were purchased from Peptide Institute (Osaka, Japan). 1,1-Dimethyl-4-phenylpiperazinium iodide was from Sigma (Madrid, Spain). Furnidipine was supplied by Laboratorios Alter (Madrid, Spain). Nifedipine, diltiazem, verapamil, propranolol, pindolol and atenolol were purchased from Research Biochemicals International (Natick, MA, USA). Metoprolol, labetalol, acebutolol and nadolol were from Sigma. Flunarizine, cinnarizine, R56865, penfluridol, lidoflazine, sabeluzole, (+)-Lubeluzole and (−)-lubeluzole (R91154) were gifts from the Janssen Research Foundation (Beerse, Belgium). Dotarizine was a gift from Grupo Ferrer (Barcelona, Spain).

3. Results

3.1. $^{45}Ca^{2+}$ uptake into chromaffin cells induced via stimulation of nicotinic acetylcholine receptor or through direct depolarization with high K^+ concentrations

Each individual plate contained 24 wells with 5×10^5 cells, and each $^{45}Ca^{2+}$ uptake data point was studied in triplicate. After a 10-min equilibration period in 0.5 ml Krebs-Hepes at 37°C, $^{45}Ca^{2+}$ uptake was determined in resting or in stimulated conditions (see Methods). In resting cells, the Krebs-Hepes solution used for the equilibration period was exchanged by fresh solution containing 1 mM $^{40}Ca^{2+}$ and 3 μCi ml^{-1} $^{45}Ca^2$ (around 8.5×10^6 cpm ml^{-1}) in a final volume of 0.3 ml. After a 60-s period, the uptake process was stopped by repeated washouts and cells were collected in trichloroacetic acid. Stimulated $^{45}Ca^{2+}$ uptake was studied by two means, activation of nicotinic acetylcholine receptor by the agonist dimethylphenylpiperazinium (indirect depolarization of cells), or by increasing K^+ concentrations (direct depolarization of cells). Increasing concentrations of dimethylphenylpiperazinium produced increasing amounts of $^{45}Ca^{2+}$ taken up by cells. The threshold concentration was 5 μM and the concentration leading to a maximum effect, 50 μM. The threshold K^+ concentration leading to augmentation of $^{45}Ca^{2+}$ entry above basal levels was 20 mM. $^{45}Ca^{2+}$ uptake was enhanced as the K^+ concentration increased, reaching near saturation at 70 mM. Concentrations of dimethylphenylpiperazinium (100 μM) and K^+ (70 mM) giving similar effects, were chosen in all subsequent experiments. Usually, experiments were performed in parallel with the two stimulating agents using two 24-well plates from the same cell culture.

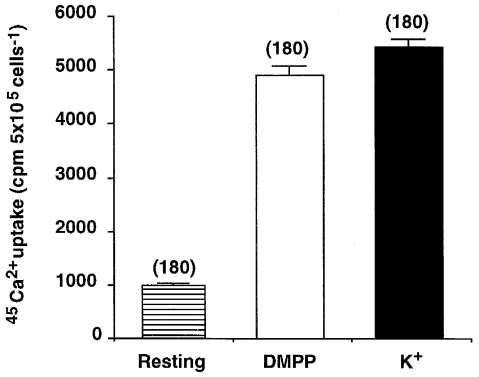

Fig. 1. $^{45}Ca^{2+}$ uptake into resting and stimulated bovine chromaffin cells. In resting conditions, cells were incubated for 60 s in a normal Krebs-Hepes solution containing 3 μCi ml^{-1} $^{45}Ca^{2+}$ and 1 mM $^{40}Ca^{2+}$. Stimulated cells were exposed for 60 s to a normal Krebs-Hepes solution containing $^{40}Ca^{2+}$ plus $^{45}Ca^{2+}$ or to dimethylphenylpiperazinium (100 μM) or high K^+ (70 mM K^+). The actual cpm of $^{45}Ca^{2+}$ taken up are shown. Data are means ± S.E.M. of the number of individual wells shown in parentheses on top of each column.

In 180 separate wells from 60 different cell cultures, the $^{45}Ca^{2+}$ taken up by resting cells amounted to 1078 ± 54 cpm 5×10^5 $cells^{-1}$. Dimethylphenylpiperazinium (100 μM for 60 s) elevated this figure to 4906 ± 166 cpm 5×10^5 $cells^{-1}$ and K^+ (70 mM for 60 s) to 5425 ± 156 cpm 5×10^5 $cells^{-1}$ (Fig. 1A). These figures correspond, respectively, to 0.25 ± 0.011, 1.25 ± 0.041 and 1.32 ± 0.039 fmol of total Ca^{2+} ($^{40}Ca^{2+} + ^{45}Ca^{2+}$) taken up by a single cell in resting or depolarizing conditions.

The ratios evoked/resting varied between 3- and 7-fold from experiment to experiment. This may be due to the use of cells from different ages (2–3-days-old) and different cultures, whose density and proportion of voltage-dependent Ca^{2+} channel subtypes might vary considerably. Therefore, normalised data are presented in all subsequent pharmacological experiments. In each individual plate, the three wells of the resting $^{45}Ca^{2+}$ were averaged. This figure was then subtracted to the individual values obtained for $^{45}Ca^{2+}$ uptake into cells stimulated with dimethylphenylpiperazinium or K^+, with or without a given toxin or drug. Thus, averaged values for a given toxin or drug refer to a number of individual wells from separate plates (3 in each plate) belonging to different cell cultures. In this manner, the statistical power of the samples analyzed improved, because the variations from plate to plate and from culture to culture decreased.

3.2. Blocking effects of various ω-toxins of $^{45}Ca^{2+}$ uptake stimulated by dimethylphenylpiperazinium or K^+

Various available ω-toxins were studied in order to identify possible differences in their effects on $^{45}Ca^{2+}$ entry when triggered by dimethylphenylpiperazinium or

K^+. 'Saturating' concentrations of each toxin to block 'selectively' a given channel subtype in bovine chromaffin cells were selected (Albillos et al., 1996). Thus, 10 nM ω-agatoxin IVA blocks P-type Ca^{2+} channels, while 1 μM will also block Q channels. At 1 μM, ω-conotoxins GVIA and MVIIA will block N-type channels, while MVIIC blocks N/P/Q-types of Ca^{2+} channels. Fig. 2 and Table 1 summarise the results obtained. ω-Conotoxin GVIA (1 μM) inhibited by 27 ± 5% the dimethylphenylpiperazinium signal and by 22 ± 2% the K^+ response. Similar blocking effects were seen with 1 μM ω-conotoxin MVIIA. ω-Agatoxin IVA (10 nM) did not affect the entry of $^{45}Ca^{2+}$, but 1 μM blocked by 50% the dimethylphenylpiperazinium as well as the K^+ response. On the other hand, 1 μM of ω-conotoxin MVIIC blocked the dimethylphenylpiperazinium response by 52 ± 1% and the K^+ response by 68 ± 2% (Fig. 2A).

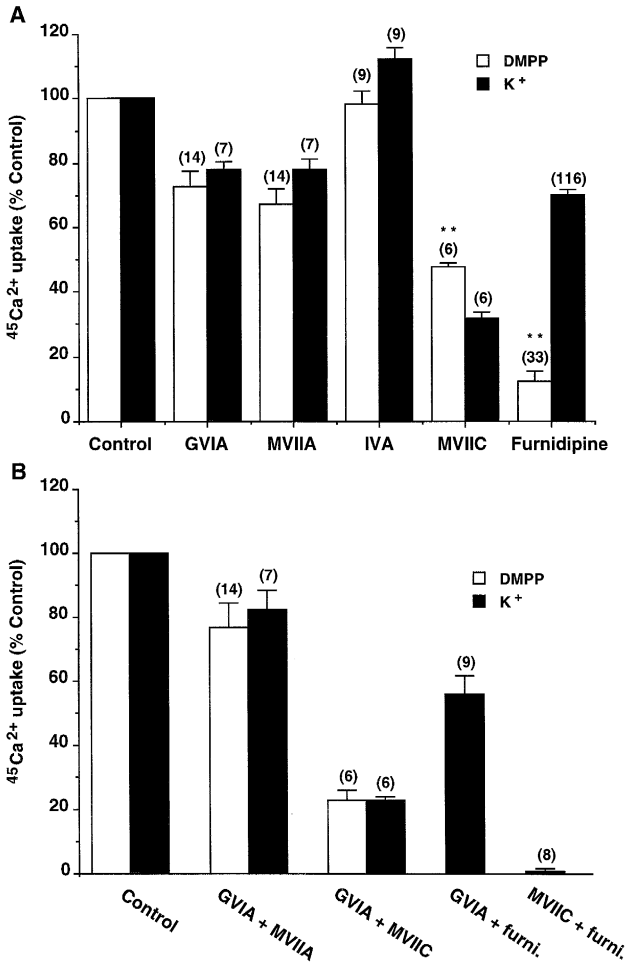

Fig. 2. Effects of single concentrations of toxins, alone (A) or in combination (B), on $^{45}Ca^{2+}$ uptake triggered by dimethylphenylpiperazinium or K^+. The toxins were added 10 min before and were maintained during stimulation with dimethylphenylpiperazinium (100 μM for 60 s) or K^+ (70 mM for 60 s). The concentrations of toxins were 1 μM for ω-conotoxin GVIA (GVIA), ω-conotoxin MVIIA (MVIIA), and ω-conotoxin MVIIC (MVIIC), 3 μM for furnidipine (Furni) and 10 nM or 1 μM for ω-agatoxin IVA (IVA). In each pair of columns, the left-hand open column represents the dimethylphenylpiperazinium signal and the right-hand black column the K^+ signal. Data are means ± S.E.M. of the number of wells shown in parentheses. All data were obtained from at least three different cell cultures, and they were normalized to percentages of $^{45}Ca^{2+}$ uptake taken up by control cells. ** $P < 0.01$, with respect to the corresponding K^+ value.

Table 1
Effects of toxins and drugs on the net $^{45}Ca^{2+}$ taken up by cells stimulated with 100 μM dimethylphenylpiperazinium (60 s) or high K^+ (70 mM K^+, 60 s) (% inhibition). Data are means ± S.E.M. of the number of individual wells shown (*n*)

Molecule	Concentration	Stimulus			
	(μM)	DMPP	(*n*)	K^+	(*n*)
ω-Conotoxin GVIA	1	27 ± 5	(23)	22 ± 2	(78)
ω-Conotoxin MVIIA	1	33 ± 5	(25)	22 ± 3	(18)
ω-Agatoxin IVA	0.01	2 ± 4	(9)	0	(9)
ω-Agatoxin IVA	1	47 ± 3	(6)	51 ± 3	(9)
ω-Conotoxin MVIIC	1	52 ± 1	(6)	68 ± 2	(6)
Nifedipine	3	71 ± 5	(9)	38 ± 4	(9)
Furnidipine	3	88 ± 3	(33)	30 ± 2	(116)
Verapamil	3	53 ± 3	(9)	10 ± 2	(9)
Diltiazem	3	89 ± 3	(12)	22 ± 3	(12)
Flunarizine	3	48 ± 3	(12)	41 ± 4	(16)
Cinnarizine	3	82 ± 4	(9)	21 ± 4	(9)
Dotarizine	3	64 ± 1	(12)	58 ± 4	(13)
R56865	3	21 ± 2	(9)	32 ± 3	(9)
Penfluridol	3	29 ± 2	(9)	47 ± 2	(9)
Lidoflazine	3	33 ± 4	(9)	33 ± 5	(9)
Sabeluzole	3	19 ± 2	(9)	29 ± 2	(9)
Lubeluzole	3	40 ± 4	(23)	33 ± 4	(23)
R91154	3	24 ± 3	(10)	33 ± 1	(8)
Propranol	3	68 ± 3	(9)	23 ± 4	(9)
Metoprolol	3	23 ± 4	(8)	16 ± 4	(8)
Labetalol	3	26 ± 3	(12)	9 ± 4	(9)
Acebutolol	3	0	(12)	6 ± 3	(12)
Pindolol	3	37 ± 3	(12)	20 ± 2	(9)
Atenolol	3	0	(9)	15 ± 3	(9)
Nadolol	3	30 ± 4	(15)	26 ± 7	(12)

Fig. 2B shows the results of the effects of combined toxins. Thus, ω-conotoxins GVIA and MVIIA (1 μM each) inhibited the dimethylphenylpiperazinium and K^+ responses by 23% and 17%, respectively. The combination of 1 μM each of ω-conotoxins GVIA and MVIIC suppressed the two signals by 77%. The association of 1 μM furnidipine (a dihydropyridine derivative) and ω-conotoxin GVIA diminished the K^+ signal by 44%; when the dihydropyridine was associated to ω-conotoxin MVIIC, the K^+-evoked $^{45}Ca^{2+}$ entry was fully suppressed.

3.3. Effects of L-type Ca^{2+} channel blockers on $^{45}Ca^{2+}$ entry induced by dimethylphenylpiperazinium or K^+

Three main chemical entities are accepted as selective blockers of L-type Ca^{2+} channels: 1,4-dihydropyridines (nifedipine, furnidipine), benzothiazepines (diltiazem) and dibenzylalkylamines (verapamil). They were tested at the single concentration of 3 μM, which is supramaximal for a

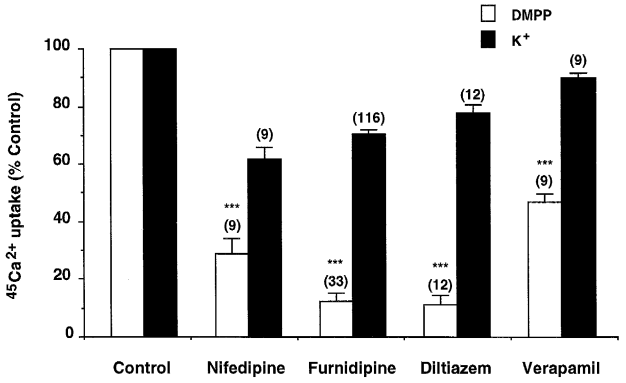

Fig. 3. Effects of L-type Ca^{2+} channel blockers on $^{45}Ca^{2+}$ entry induced by dimethylphenylpiperazinium (open columns) or K^+ (closed columns). Before challenging with dimethylphenylpiperazinium (100 μM for 60 s) or K^+ (70 mM for 60 s), cells were preincubated for 10 min with 3 μM each of nifedipine, furnidipine, diltiazem or verapamil. These compounds were also present during the dimethylphenylpiperazinium or K^+ challenge. Data are normalized to % of controls and are means ± S.E.M. of the number of wells shown in parentheses on top of each column. Cells from at least three different cultures were used in all the studies. *** $P < 0.001$, with respect to K^+.

series of cardiovascular effects mediated by these compounds (Fleckenstein, 1983). Nifedipine inhibited the dimethylphenylpiperazinium signal by 71%, furnidipine by 88%, diltiazem by 89%, and verapamil by 53.2% (Fig. 3). Inhibition of the K^+ response was substantially and significantly lower, 38% by nifedipine, 30% by furnidipine, 22% by diltiazem and 10% by verapamil. Thus, it seems clear that blockade of $^{45}Ca^{2+}$ entry induced by nicotinic acetylcholine receptor activation cannot solely be accounted for by inhibition of L-type Ca^{2+} channels (see Discussion).

3.4. Effects of 'wide-spectrum' Ca^{2+} channel blockers on dimethylphenylpiperazinium- and K^+-evoked $^{45}Ca^{2+}$ uptake

We have coined the expression 'wide-spectrum Ca^{2+} channel blockers' for those molecules generally possessing a piperazine ring, which block the various components of the whole-cell current through the various Ca^{2+} channel subtypes of bovine chromaffin cells (Gárcez-do-Carmo et al., 1993; Maroto et al., 1994; Villarroya et al., 1995). Various of these agents were tested to define a possible selective action on the dimethylphenylpiperazinium or K^+ responses. Fig. 4 and Table 1 show the results of such experiments. At 3 μM, flunarizine reduced the dimethylphenylpiperazinium signal by 48% and the K^+ signal by 41%. Cinnarizine (3 μM) blocked the nicotinic response by 82% and the K^+ response by 21%. Finally, the third piperazine derivative assayed, dotarizine (3 μM) inhibited 64% of the dimethylphenylpiperazinium signal and 58% of the K^+ signal.

3.5. Lipophilicity and blockade of Ca^{2+} entry

Most Ca^{2+} antagonists are highly lipophilic drugs. This is true for the selective L-type Ca^{2+} channel blockers such as nifedipine, verapamil and diltiazem, as well as for the less selective so called 'wide-spectrum' Ca^{2+} channel blockers flunarizine, R56865 or dotarizine (Gárcez-do-Carmo et al., 1993; Maroto et al., 1994; Villarroya et al., 1995). In a recent report we correlated the octanol/water partition coefficients of some of these drugs with their ability to deplete the accumulated mitochondrial Ca^{2+} (Uceda et al., 1995). The greater blockade of dimethylphenylpiperazinium-evoked responses by nifedipine, verapamil, diltiazem or furnidipine could be due to their lipophilicity. Furthermore, the higher lipophilicity could imply greater blockade of Ca^{2+} entry triggered either by dimethylphenylpiperazinium or K^+. To test these possibilities two approaches were followed: (1st) testing of various β-adrenoceptor antagonists with different degrees of lipophilicity (Opie et al., 1995) on dimethylphenylpiperazinium- and K-evoked $^{45}Ca^{2+}$ entry; (2nd) studying the effects of 'wide-spectrum' Ca^{2+} channel blockers and related drugs with different known octanol/water partition coefficients. All drugs were tested at the concentration of 3 μM, in order to compare their effects with those of selective L-type Ca^{2+} channel blockers, which were tested also at 3 μM (see above).

Fig. 5 and Table 1 show the results obtained with β-adrenoceptor antagonists. The highly lipophilic (±)-propranolol reduced the dimethylphenylpiperazinium signal by 68% and the K^+ signal by 23%. Similar results were obtained with *R*-(+)-propranolol and *S*-(−)-propranolol. Metoprolol and labetalol, another two highly lipophilic agents had, however, very little Ca^{2+} entry blocking effect; labetalol reduced the dimethylphenylpiperazinium signal by 26%, a figure significantly different from the reduction of the K^+ signal (9%). The two β-adrenoceptor antagonists having an intermediate lipophilicity had quite a different behavior; acebutolol exhibited no blocking ef-

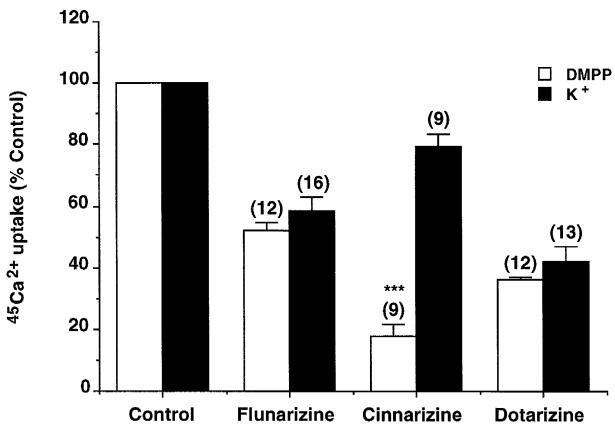

Fig. 4. Effects of piperazine derivatives on $^{45}Ca^{2+}$ entry into chromaffin cells stimulated with dimethylphenylpiperazinium (100 μM, 60 s; open columns) or high K^+ (70 mM, 60 s; closed columns). Drugs (3 μM each) were present 10 min before and throughout the stimulation period. Data are means ± S.E.M. of the number of wells shown in parentheses; cells from at least 3 different cultures were used to test each compound. *** $P < 0.001$, with respect to K^+.

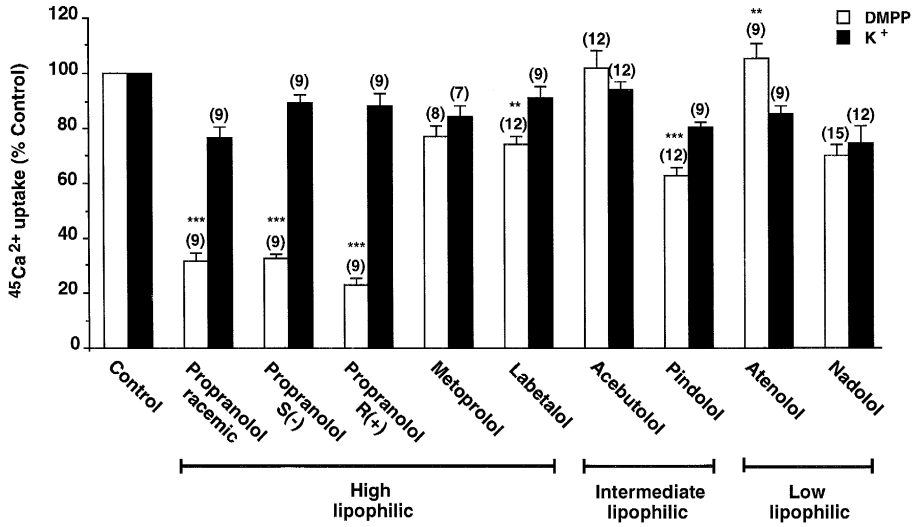

Fig. 5. The effects of β-adrenoceptor antagonists with different degree of lipophilicity, on $^{45}Ca^{2+}$ uptake into chromaffin cells stimulated with dimethylphenylpiperazinium (100 μM, 60 s; open columns) or high K^+ (70 mM K^+, 60 s, closed columns). High lipophilic β-adrenoceptor antagonists (propranolol racemate and its enantiomers, metoprolol, labetalol), intermediate lipophilic (acebutolol, pindolol) and low lipophilic compounds (atenolol, nadolol) were tested at the concentration of 3 μM. Data are means ± S.E.M. of the number of individual wells shown on top of each column in parentheses; cells from at least three different cultures were used to test each compound. ** $P < 0.01$, *** $P < 0.001$, with respect to K^+.

fects, but pindolol inhibited the dimethylphenylpiperazinium signal by 37% and the K^+ signal by 20%. Finally, of the two more hydrophilic β-adrenoceptor antagonists, atenolol did not affect Ca^{2+} uptake and nadolol blocked 30% of the dimethylphenylpiperazinium signal and 26% of the K^+-signal.

Another way of testing the existence of a possible correlation between lipophilicity and blockade of Ca^{2+} entry was the selection of substances with different octanol/water partition coefficients, some of them belonging to the 'wide-spectrum' Ca^{2+} channel antagonist group (i.e., flunarizine). Fig. 6 shows that penfluridol, the most lipophilic compound (octanol/water partition coefficient of 5.87) blocked by 30% the Ca^{2+} entry triggered by dimethylphenylpiperazinium, and by 50% the K^+ response. This slightly higher effect on K^+ was also observed with compound R56865 and with the least lipophilic sabeluzole (octanol/water partition coefficient of 4.61). A

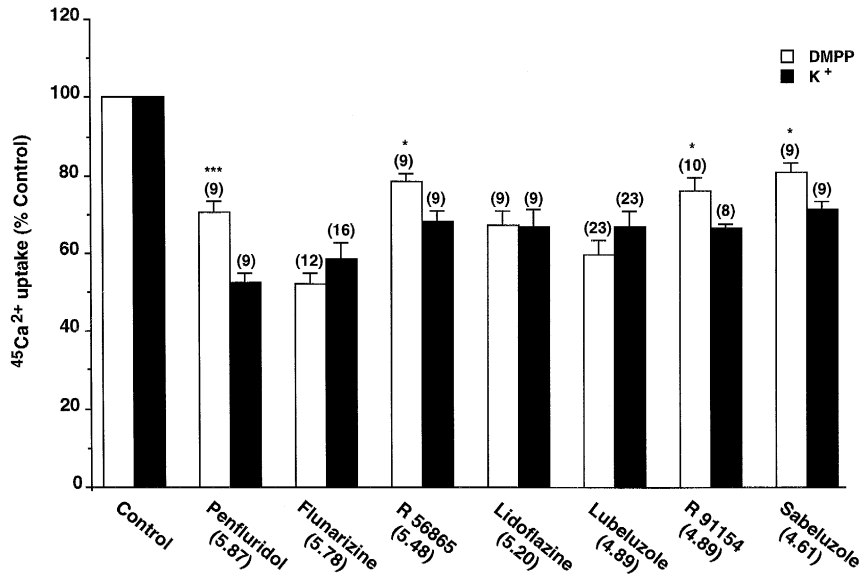

Fig. 6. Blocking effects of compounds with different degrees of lipophilicity, on $^{45}Ca^{2+}$ entry into chromaffin cells stimulated with dimethylphenylpiperazinium (100 μM, 60 s; open columns) or high K^+ (70 mM, 60 s; closed columns). Compounds (3 μM each) were added 10 min before and present during the stimulation period. The octanol/water partition coefficients are written in parentheses below the name of each compound. Data are means ± S.E.M. of the number of individual wells shown in parentheses on top of each column; cells from at least three different cultures were used to test each compound. * $P < 0.05$ and *** $P < 0.001$, with respect to K^+.

correlation between blockade of Ca^{2+} entry and lipophilicity was not apparent. So, penfluridol, flunarizine, R56865, lidoflazine, lubeluzole and sabeluzole inhibited the dimethylphenylpiperazinium signal by 30, 48, 21, 33, 40 and 19%, respectively, and the K^+ signal by 47, 41, 32, 33, 33 and 29%.

4. Discussion

The permeability to Ca^{2+} ions of the nicotinic acetylcholine receptor channel of bovine chromaffin cells is only 2.5% of the total permeability to cations (Zhou and Neher, 1993). Therefore, the net $^{45}Ca^{2+}$ entry after stimulation of nicotinic acetylcholine receptor with dimethylphenylpiperazinium, in our experimental conditons, must take place mostly through voltage-dependent Ca^{2+} channels recruited by cell depolarization. The same occurs when cells are directly depolarized with high K^+. Thus, it was expected that a ω-toxin or drug acting preferentially on nicotinic acetylcholine receptor will block more the dimethylphenylpiperazinium-evoked $^{45}Ca^{2+}$ uptake than the K^+-induced $^{45}Ca^{2+}$ uptake. This was the rationale for using this experimental approach for testing the conflicting hypothesis that some ω-toxins blocking Ca^{2+} channels (i.e., N-channel blocker ω-conotoxin GVIA, and P-Q channel blocker ω-agatoxin IVA) would also block the nicotinic acetylcholine receptor channel, thus limiting their usefulness as selective tools to target Ca^{2+} channels.

The ω-toxins studied here are considered to be highly specific for voltage-sensitive non-L-type Ca^{2+} channels. This idea was challenged in two recent reports showing that ω-conotoxin GVIA (Fernández et al., 1995) and ω-agatoxin IVA (Granja et al., 1995) blocked the nicotinic acetylcholine receptor channel in bovine chromaffin cells. Our results do not support this concept. In our hands, 1 μM ω-conotoxin GVIA inhibited the $^{45}Ca^{2+}$ entry evoked by dimethylphenylpiperazinium or K^+ only by 20%; however, these concentrations reduced by 50 and 80% the total net charge influx through nicotinic acetylcholine receptor channels (Fernández et al., 1995). On the other hand, 10 nM ω-agatoxin IVA had no effect on dimethylphenylpiperazinium-evoked Ca^{2+} entry. In contrast, Granja et al. (1995) observed that at 1 nM ω-agatoxin IVA reduced the nicotinic current by 30–40%, and 10 nM by 60–70%. The sharp discrepancy between our data and those of Fernández et al. (1995) and Granja et al. (1995) could be explained on the basis of differences in experimental conditions, protocols used and parameters measured (current versus $^{45}Ca^{2+}$ fluxes). Our data agree with those of Donnelly-Roberts et al. (1995) which could not show any blocking effects on human neuronal nicotinic acetylcholine receptor of ω-conotoxin GVIA or ω-agatoxin IVA, even at concentrations as high as 100 μM. Concerning the binding site for ω-conotoxin GVIA seen by Horne et al. (1991) in the *Discopyge* electric organ, the co-precipitation with nicotinic acetylcholine receptor does not necessarily imply the presence of a selective binding site for the toxin. In any case, these authors did not study any functional consequences of this 'receptor site', and we therefore do not know if the nicotinic acetylcholine receptor ion channel in this preparation would have been blocked by ω-conotoxin GVIA.

Though the presence in bovine chromaffin cells of N and Q channels seems to be clearly established, the data with low concentrations of ω-agatoxin IVA raise doubts on the presence of a P-type Ca^{2+} channel. In previous electrophysiological patch-clamp studies, concentrations of 100 nM or higher of ω-agatoxin IVA were used (Albillos et al., 1993). These concentrations also inhibit Q-type Ca^{2+} channels (Randall and Tsien, 1995). Since 10 nM of ω-agatoxin IVA did not affect Ca^{2+} entry induced by dimethylphenylpiperazinium or K^+, we are tempted to conclude that P-type Ca^{2+} channels are poorly represented in bovine chromaffin cells. So, in previous studies where 100 nM and higher concentrations of ω-agatoxin IVA were used, the toxin was likely targetting Q-type Ca^{2+} channels that are recognised by ω-conotoxin MVIIC (López et al., 1993; Albillos et al., 1996; Lomax et al., 1996; this study). In principle, the present data are compatible with the idea that bovine chromaffin cells maintained in primary cultures for 2–3 days express L-, N- as well as Q-type Ca^{2+} channels. Recent patch-clamp experiments show that high concentrations of ω-agatoxin IVA combined with furnidipine and ω-conotoxin GVIA fully block the whole cell current through Ca^{2+} channels; in addition, the fact that ω-conotoxin MVIIC mimics the high concentrations of ω-agatoxin IVA, in blocking Ba^{2+} currents support this conclusion (Albillos et al., 1996).

Patch-clamp experiments studying the blockade of the whole-cell current through Ca^{2+} channels by some of the compounds used showed that there is a good correlation with the Ca^{2+} channel blockade. Since not all the compounds were studied, those data were not included here.

A different story emerges for the non-peptide, 'selective' L-type Ca^{2+} channel blockers that seem to block the nicotinic acetylcholine receptor channels in addition to Ca^{2+} channels. Thus, nimodipine (López et al., 1993), methoxyverapamil (Boehm and Huck, 1993), and diltiazem (Gandía et al., 1996) have been shown to inhibit directly the nicotinic acetylcholine receptor current induced by nicotinic agonists in voltage-clamped bovine chromaffin cells. This could explain the potent blockade of dimethylphenylpiperazinium-evoked Ca^{2+} uptake seen in this study with nifedipine, furnidipine, verapamil and diltiazem. Such an effect cannot be explained solely by blockade of Ca^{2+} channels, since these compounds only inhibit the L-component of the whole-cell current through Ca^{2+} channels, which in bovine chromaffin cells accounts for only around 20% of the total current (Albillos et al., 1993; Gandía et al., 1993). Thus, these Ca^{2+} antagonists seem to recognise a specific site on the nicotinic acetylcholine

receptor itself. This is in line with Zernig (1990), who viewed a widening potential for Ca^{2+} antagonists at targets unrelated to L-type Ca^{2+} channels. It is interesting, however, that none of those targets seem to be associated to non-L voltage-sensitive Ca^{2+} channels of the N-P-Q-types. In fact, these compounds, even at concentrations as high as 10 μM (data not shown), did not inhibit the K^+-induced Ca^{2+} entry more than 15–20%.

Because of their high lipophilicity, L-type Ca^{2+} channel blockers and some 'wide spectrum' Ca^{2+} antagonists might block the various Ca^{2+} channel subtypes in a non-selective manner; this inhibition could also affect the nicotinic acetylcholine receptor channel. In the chromaffin cell we have an excellent model expressing all these channels to test the hypothesis of a possible relationship between lipophilicity and channel blockade. As far as the dimethylphenylpiperazinium response was concerned, the most lipophilic compound penfluridol (octanol/water partition coefficient of 5.87) caused only a 30% inhibition. The other five compounds tested (flunarizine, R56865, lidoflazine, lubeluzole and sabeluzole) had decreasing octanol/water partition coefficients of 5.5, 5.3, 5.1, 4.9 and 4.7, respectively. The inhibition of dimethylphenylpiperazinium-evoked $^{45}Ca^{2+}$ uptake amounted, respectively, to 48, 21, 33, 40 and 19%. A similar picture emerged from the study of the K^+ response. Thus, an apparent relationship between lipophilicity and blockade of $^{45}Ca^{2+}$ entry by these 'wide-spectrum' Ca^{2+} antagonists was not apparent. This lack of correlation between lipophilicity and inhibition of $^{45}Ca^{2+}$ uptake was reinforced by the data obtained with various β-adrenoceptor antagonists having different degrees of lipophilicity. The most lipophilic compounds were not necessarily the most potent blockers. Thus, flunarizine, cinnarizine, dotarizine or lubeluzole could be considered as blockers of various Ca^{2+} channel subtypes through a mechanism more specific than their lipophilicity would predict.

Recently, doubts arose as to the cardioprotective effects of Ca^{2+} antagonists, particularly of nifedipine (SPRINT 2, 1993). A greater cardiac risk in hypertensive patients treated with this drug might be associated to reflex activation of the sympatho-adrenal axis in response to brisk oscillations of the blood pressure. We raised the hypothesis that the inhibition of sympatho-adrenal catecholamine release by Ca^{2+} antagonists could contribute to their overall cardiovascular protection effect. However, differences between them exist. For instance, nifedipine causes vasodilatation at nanomolar concentrations, and inhibits the nicotinic acetylcholine receptor-mediated Ca^{2+} entry and catecholamine release only at three orders of magnitude higher concentrations (López et al., 1993; this study). This is not the case for diltiazem and verapamil, which cause vasodilatation and inhibition of nicotinic acetylcholine receptor responses at similar micromolar concentrations (Boehm and Huck, 1993; Gandía et al., 1996; this study). It is therefore plausible that contrary to nifedipine, the clinically relevant concentrations of diltiazem and verapamil are causing both milder vasodilatation and inhibition of the sympatho-adrenal drive to the heart and blood pressures. Hence their better cardioprotective clinical effects were shown in secondary prevention clinical trials on patients who suffered a previous myocardial infarction (MDPIT, 1988; DAVIT II, 1990).

In conclusion, our results clearly support the idea that the ω-toxins used routinely as tools to block various neuronal voltage-dependent Ca^{2+} channels, are highly selective and do not block ligand activated channels such as the ionophore associated to the nicotinic acetylcholine receptor. In contrast, various of the non-peptidic blockers of L-type Ca^{2+} channels also block the nicotinic acetylcholine receptor ion channel at concentrations that might be clinically relevant. Highly lipophilic Ca^{2+} antagonist drugs do not necessarily exhibit the property of non-specifically blocking voltage- and/or receptor-operated Ca^{2+} channels.

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