Br. J. Pharmacol. (1991), **103**, 1073–1078

Separation of two pathways for calcium entry into chromaffin cells

[1]Luis Gandía, Luis-Felipe Casado, Manuela G. López & [2]Antonio G. García

Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo, 4, 28029-Madrid, Spain

1 The effects of various drugs on $^{45}Ca + {}^{40}Ca$ uptake into cultured bovine adrenal chromaffin cells evoked by 1,1-dimethyl-4-phenylpiperazinium (DMPP) or high K, were studied. In the presence of 1 mM external ^{40}Ca, with ^{45}Ca as a radiotracer, unstimulated cells took up an average of 0.13 fmol/cell ^{40}Ca and 772 c.p.m./10^6 cells of ^{45}Ca ($n = 76$). Upon stimulation with DMPP (100 μM for 60 s) or K (59 mM for 60 s), Ca uptake increased to 0.92 and 1 fmol/cell, respectively.

2 Flunarizine behaved as a potent blocker of both DMPP- and K-evoked Ca uptake (IC_{50} of 1.76 and 1.49 μM, respectively for DMPP and K). A similar picture emerged with Cd ions, though Cd exhibited an IC_{50} against K (1.86 μM) slightly lower than the IC_{50} against DMPP (8.14 μM).

3 Clear cut differences were observed with amiloride, guanethidine, nimodipine and nisoldipine which behaved as selective blockers of DMPP-mediated Ca uptake responses: IC_{50} values to block DMPP effects were 290, 27, 1.1 and 1.63 μM respectively for amiloride, guanethidine, nimodipine and nisoldipine. Amiloride blocked K-evoked Ca uptake by only 35% and guanethidine did not affect it. Nisoldipine inhibited K-evoked Ca uptake only partially at low concentrations (about 30%); a second blocking component was observed at the highest concentration used (10 μM). At 10 μM, nimodipine blocked K-evoked Ca uptake by 50%.

4 Thus, it seems that the nicotinic receptor mediated Ca uptake pathway can be pharmacologically separated from the K-activated pathway. The results are compatible with the hypothesis that in cultured bovine adrenal chromaffin cells, stimulation of nicotinic receptors recruits a single type of Ca channel which is sensitive to flunarizine, Cd, amiloride, guanethidine, nimodipine and nisoldipine. The results also suggest that K depolarization might be recruiting in addition to this channel, another Ca channel which is highly sensitive to Cd and flunarizine, resistant to nisoldipine, nimodipine and amiloride, and insensitive to guanethidine.

Keywords: Chromaffin cells; calcium channels; flunarizine; guanethidine; amiloride; nisoldipine; nimodipine; cadmium

Introduction

Douglas & Rubin (1961) first demonstrated that acetylcholine or high K concentrations triggered adrenal medulla catecholamine release by activating a Ca channel located on the chromaffin cell plasmalemma. Since both nicotinic stimulation and direct depolarization activate Ca uptake and secretion in a parallel manner (Douglas & Poisner, 1962; Kilpatrick *et al.*, 1982; Holz *et al.*, 1982; Artalejo *et al.*, 1986), it seemed likely that with both types of stimuli, voltage-dependent Ca channels were used for Ca entry. These channels were demonstrated to be present in chromaffin cells by means of whole-cell recordings of Ca currents by use of patch-clamp techniques (Fenwick *et al.*, 1982).

On pharmacological grounds (i.e. inhibition of secretion by specific Ca channel antagonists) it seemed to us that both nicotinic and high-K-mediated catecholamine release were triggered by the same type of Ca channels (Ceña *et al.*, 1983). This is supported by the observation that (+)-isradipine (a 1,4-dihydropyridine derivative) blocks the nicotinic response only in the presence of Na (Cárdenas *et al.*, 1988; Abajo *et al.*, 1989). Therefore, it is likely that Na entering through the acetylcholine receptor ionophore (Amy & Kirshner, 1982; Wada *et al.*, 1985) causes cell depolarization and opening of Ca channels. Up to now, this picture has implied that external Ca entering chromaffin cells stimulated via nicotinic receptors or high K uses the same end pathway, i.e. a voltage-sensitive Ca channel of the L-subtype.

We present pharmacological data in this paper that call for a reconsideration of this widely accepted picture. By using flunarizine (a piperazine derivative), Cd (an inorganic Ca channel blocker), amiloride (a K-sparing diuretic), guanethidine (a sympathetic neuronal blocker), nimodipine and nisoldipine (1, 4-dihydropyridine derivatives) we demonstrate here that Ca channels recruited by nicotinic- or high-K stimulation can be pharmacologically separated.

Methods

Preparation of chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated following standard methods (Livett, 1984) with the following modifications: (a) once in the cell culture unit, adrenal glands were washed three times with Ca- and Mg-free Locke buffer (mM: NaCl 154, KCl 5.6, $NaHCO_3$ 3.5, glucose 11 and HEPES buffer 10, at pH 7.2) at room temperature; (b) digestion of adrenal medulla was carried out by injecting 5 ml of a solution containing 0.25% collagenase, 0.5% bovine serum albumin and 0.01% soybean trypsin inhibitor in Ca-Mg-free Locke buffer, and incubating the glands at 37°C for 15 min; this procedure was repeated thrice; (c) collagenase was washed out with a large volume of Ca-Mg-free Locke buffer and then cells were filtered first with a 217 μm and thereafter with a 80 μm nylon mesh; (d) cells resuspended in Ca-Mg-free Locke buffer were placed on self-generated Percoll gradients containing 19 ml Percoll (17.1 ml Percoll plus 1.9 ml 10 fold concentrated Ca-Mg-free Locke buffer at pH 5), plus 21 ml of cell suspension (about 50–100 × 10^6 cells); the final pH of the mixture was 7.2. The mixture was centrifuged at 13,000 r.p.m. for 20 min (rotor SS-34, Sorvall centrifuge Model RC-5) at 22°C. Then, the lower band of the gradient (enriched in adrenaline-containing cells, Moro *et al.*, 1990) was taken, washed once with Ca-Mg-free Locke buffer and a second time with Dul-

[1] Present address: University of Pennsylvania, School of Medicine, Department of Physiology, Philadelphia, PA19104-6085, U.S.A.
[2] Author for correspondence.

becco's modified Eagle's medium (DMEM). Finally, cells were resuspended in DMEM supplemented with 10% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxiuridine, 50 u ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were plated at a density of 1 × 10^6 cells/well in 24 multiwell Costar plates and incubated at 37°C in a water-saturated, 5% CO_2/95% air atmosphere. Medium was changed 24 h later with fresh DMEM. Viability of the cells (usually greater than 90%) was estimated by Trypan blue exclusion.

Calcium uptake into chromaffin cells

Calcium 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 (composition (mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.0, glucose 11, HEPES 10, at pH 7.2) at 37°C. ^{45}Ca uptake into chromaffin cells was studied by incubating the cells at 37°C with $^{45}CaCl_2$ at a final concentration of 4 μCi ml^{-1} in the presence of Krebs-HEPES (basal uptake), high K solution (Krebs-HEPES containing 59 mM KCl with isosmotic reduction of NaCl) or DMPP solution (Krebs-HEPES with 100 μM final concentration of the nicotinic receptor agonist, 1,1-dimethyl-4-phenyl-piperazinium, DMPP). This incubation was carried out during 1 min and at the end of this period the test medium was rapidly aspirated and the uptake reaction was ended by adding 0.5 ml of a cold Ca-free Krebs-HEPES containing 10 mM $LaCl_3$. Finally, cells were washed 5 times more with 0.5 ml of cold Ca-free Krebs-HEPES containing 10 mM $LaCl_3$ and 2 mM EGTA, at 15 s intervals.

To measure radioactivity retained by chromaffin cells, the cells were scraped with a plastic pipette tip while adding 0.5 ml 10% trichloroacetic acid, 2 ml of scintillation fluid (Ready Micro, Beckman) was added and the samples counted in a Packard beta counter. Results are expressed as counts min^{-1} per 10^6 cells, fmol of total Ca taken up by a single cell or normalized as % of Ca taken up by control cells.

When possible, IC_{50}s for each drug to block DMPP or K-evoked ^{45}Ca uptake were estimated through non-linear regression analysis of inhibition curves, using a GraphPAD programme from ISI software, for a PC computer.

Materials and solutions

The following materials were used: collagenase from *Clostridium histolyticum* (Boehringer-Mannheim); bovine serum albumin fraction V, soybean trypsin inhibitor, cytosine arabinoside, fluorodeoxiuridine, guanethidine, amiloride, DMPP, EGTA (Sigma); Percoll (Pharmacia); DMEM, foetal calf serum, penicillin and streptomycin (GIBCO); scintillation fluid Ready micro (Beckman); ^{45}Ca (Specific activity 10–40 mCi mg^{-1} calcium, Amersham). All other chemicals were reagent grade. Amiloride, nimodipine, nisoldipine and flunarizine were dissolved in ethanol, and diluted in saline solutions to the desired concentrations. Experiments were performed under sodium lighting.

Results

Cell calcium uptake: signal to noise ratio

Figure 1 shows the increase in ^{45}Ca uptake by cells stimulated with increasing concentrations of DMPP or K. The threshold concentration for DMPP was 3 μM and for K, 17,7 mM; the maximum effect was obtained with 100 μM DMPP and 59 mM K. Therefore, these concentrations were selected to perform the following experiments.

We wished to compare the effects of various drugs on DMPP- and K-evoked Ca uptake. Thus, concentrations of the stimulants provoking maximal signals were selected (Figure 2). DMPP (100 μM for 60 s) induced ^{45}Ca uptake (in the presence of 1 mM ^{40}Ca) of 5663 ± 407 c.p.m./10^6 cells (data from 42 individual wells from 14 different batches of cells). This figure represents 0.92 ± 0.07 fmol of total Ca taken up by a single chromaffin cell upon stimulation of its nicotinic receptors during 60 s.

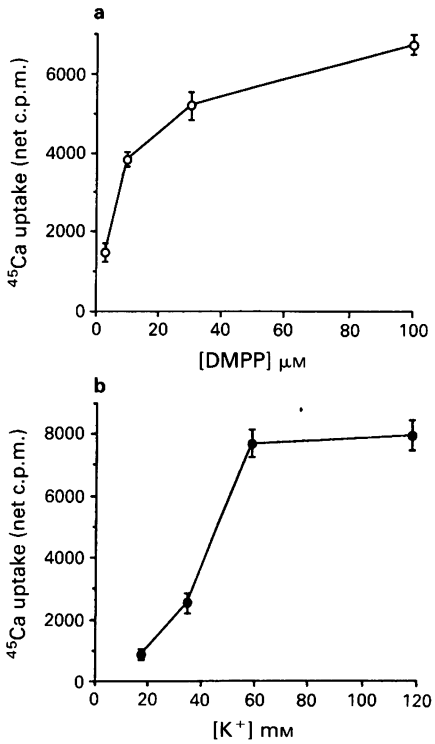

Figure 1 ^{45}Ca uptake into chromaffin cells stimulated with increasing concentrations of 1,1-dimethyl-4-phenylpiperazinium (DMPP) (a) or K (b). Cells in different wells were exposed to different concentrations of DMPP or K (abscissa scale) for 60 s. ^{45}Ca uptake is expressed in net c.p.m./well (ordinates); basal ^{45}Ca taken up by resting cells was subtracted from evoked ^{45}Ca uptake. Data are means of 8 wells from two separate batches of cells; s.e.mean shown by vertical bars.

High K concentrations (59 mM for 60 s) produced comparable signals. The average ^{45}Ca taken up amounted to 6145 ± 483 c.p.m./10^6 cells (data from 42 individual wells from 14 different batches of cells). This figure represents

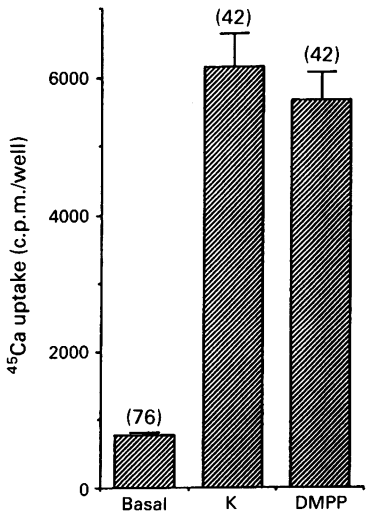

Figure 2 ^{45}Ca uptake into resting or stimulated chromaffin cells. ^{45}Ca retained by unstimulated cells (Basal) or cells stimulated with 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or K (59 mM for 60 s) is expressed in c.p.m./10^6 cells (ordinate scale). The ratio DMPP/Basal was 7.3 and K/Basal, 7.9. Data are means of the number of experiments shown in parentheses; vertical bars show s.e.mean.

1.00 ± 0.08 fmol of total Ca taken up by a single chromaffin cell depolarized for 60 s with 59 mM K.

In basal conditions (1 min incubation in Krebs-HEPES solution containing 4 μCi ml^{-1} ^{45}Ca plus 1 mM ^{40}Ca) cells retained 771 ± 35 c.p.m. (data from 76 individual wells from 25 different batches of cells). In individual experiments, stimulated cells took up about 5–10 fold the amount of ^{45}Ca retained by unstimulated cells. Thus, signal to noise ratio was adequate to perform studies with increasing concentrations of drugs to block Ca uptake.

Flunarizine blocks equally well DMPP- and K-evoked Ca uptake into chromaffin cells

Flunarizine (Figure 3) inhibited DMPP- and K-evoked Ca uptake into chromaffin cells in a concentration-dependent manner. Full blockade was achieved at a concentration of 10 μM. The IC_{50} to inhibit the DMPP response was 1.76 μM; the K response exhibited an IC_{50} of 1.49 μM.

Effects of cadmium on calcium uptake by chromaffin cells

Cd ions blocked both DMPP- and K-evoked Ca uptake into chromaffin cells. The IC_{50} for the K response was 1.86 μM and that for DMPP 8.14 μM (Figure 4). At 30 μM Cd, blockade of ^{45}Ca uptake was complete with both stimuli.

Selective block by amiloride of the nicotinic receptor-evoked calcium uptake into chromaffin cells

K-evoked Ca uptake into chromaffin cells was affected little by increasing concentrations of amiloride; only at 1 mM, could a 35% blockade be seen. In contrast, DMPP-evoked Ca uptake was gradually inhibited by increasing concentrations of this drug (Figure 5). The IC_{50} was 290 μM, and full blockade of Ca uptake was achieved at 1 mM.

Selective inhibition by guanethidine of nicotinic receptor-evoked calcium uptake into chromaffin cells

Guanethidine did not affect K-evoked Ca uptake into chromaffin cells; the Ca uptake mechanism was depressed by only 20% at a concentration of 10 μM. This concentration fully blocked DMPP-evoked Ca uptake. In contrast to amiloride, the inhibition curve was very steep (Figure 6). Guanethidine had little effect on the Ca uptake mechanism in concentrations up to 10 μM; at 30 μM, 70% blockade was achieved. The IC_{50} was 27 μM.

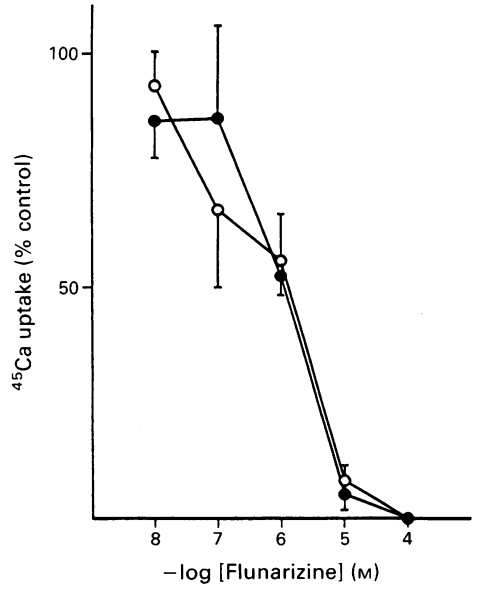

Figure 3 Flunarizine blocks equally well the uptake of Ca into chromaffin cells stimulated with (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60s) or (●) K (59 mM for 60 s). Before stimulation, cells were preincubated with each concentration of flunarizine for 10 min. ^{45}Ca uptake (ordinate scale) was normalized to 100% (^{45}Ca taken up by cells in the absence of flunarizine). Data are means of 4 triplicate experiments from different batches of cells; s.e.mean shown by vertical bars.

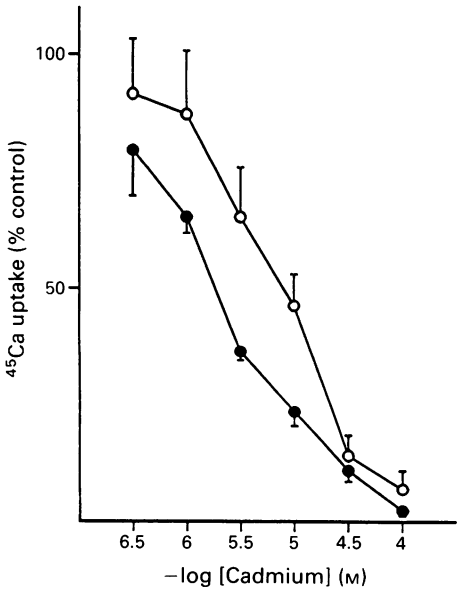

Figure 4 Effects of Cd ions on ^{45}Ca uptake into chromaffin cells evoked by (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or (●) K (59 mM for 60 s). Experimental protocol as in Figure 3 but here, Cd instead of flunarizine was used as blocking agent. Data (normalized to 100% of control ^{45}Ca uptake) are means of 4 triplicate experiments done with cells from different batches; s.e.mean shown by vertical bars.

Effects of nimodipine and nisoldipine on nicotinic receptor and K-evoked calcium uptake into chromaffin cells

Blockade of K-evoked ^{45}Ca uptake by nimodipine and nisoldipine exhibited a biphasic pattern. At submicromolar concentrations, a 10–30% blockade was observed. Then, a second

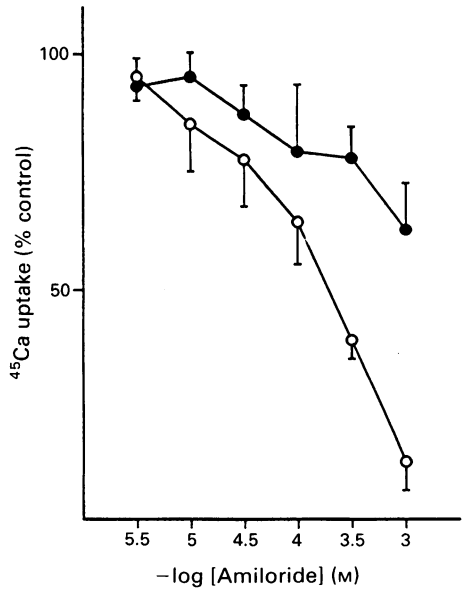

Figure 5 Effects of amiloride on ^{45}Ca uptake into chromaffin cells stimulated with (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or (●) K (59 mM for 60 s). Experimental protocol as in Figure 3. Data (normalized to 100% of control ^{45}Ca uptake) are means of 4 triplicate experiments made with cells from different batches; s.e.mean shown by vertical bars.

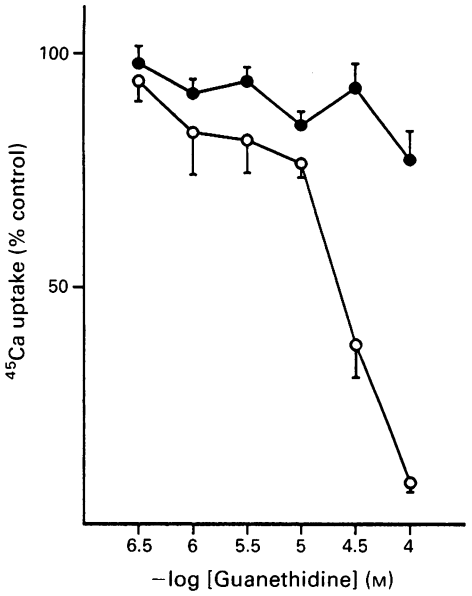

Figure 6 Effects of guanethidine on ^{45}Ca uptake into chromaffin cells stimulated with (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or (●) K (59 mM for 60 s). Experimental protocol as in Figure 3. Data (normalized to 100% of control Ca uptake) are means of 4 triplicate experiments made with cells from different batches; s.e.mean shown by vertical bars.

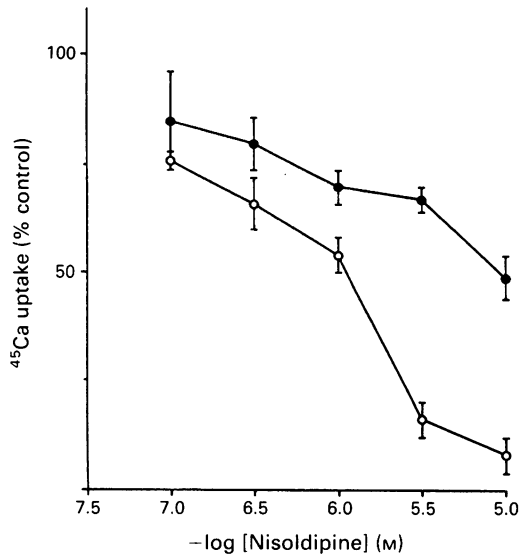

Figure 8 Effects of nisoldipine on ^{45}Ca uptake into chromaffin cells evoked by (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 10 μM for 60 s) or (●) K (59 mM for 60 s). The experimental protocol was as in Figure 7, but here each concentration of nisoldipine was present for 90 min before and during stimulation of the cells with DMPP or K. Data (normalized to 100% of control ^{45}Ca uptake) are means of 2 triplicate experiments made with cells from different batches; s.e.mean shown by vertical bars.

component seemed to arise at concentrations between 3 and 10 μM (Figure 7). Because nisoldipine is a slowly acting dihydropyridine derivative and these experiments were performed with preincubation times of 10 min, we tested its effects on Ca uptake after exposure of the cells to this drug for 90 min. Figure 8 shows that the blocking effects of nisoldipine on K- and DMPP-evoked Ca uptake were similar to those seen when cells were pre-exposed to the drug for only 10 min. The IC_{50} to block DMPP-evoked Ca uptake in these conditions was 1.63 nM, about 3 fold lower than with a 10 min preincubation. However, K-evoked Ca uptake was affected similarly with a 10- or 90-min pre-incubation period with nisoldipine.

In the case of nimodipine, another dihydropyridine derivative, a partial blockade of K-evoked Ca uptake (about 50% at 10 μM) was obtained. DMPP-evoked Ca uptake was completely blocked by nimodipine following a biphasic pattern (Figure 9). At 30 nM 30–40% blockade was obtained; then, at μmolar concentrations, nimodipine blocked DMPP-evoked Ca uptake completely (IC_{50} = 1.1 μM).

Discussion

The various Ca^{2+} channel blockers used in this study behave quite differently when their effects on ^{45}Ca uptake into chro-

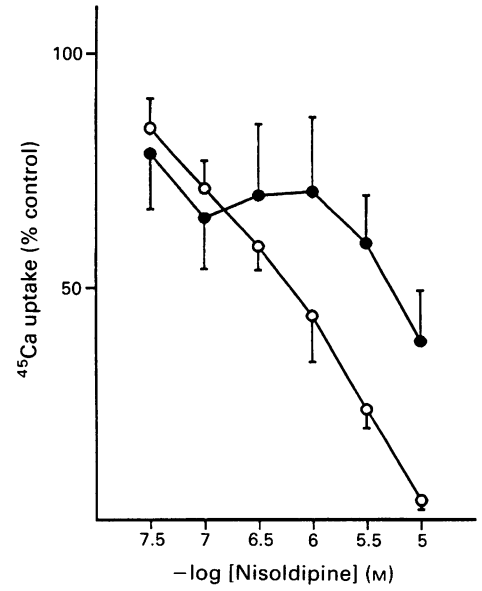

Figure 7 Effects of nisoldipine on ^{45}Ca uptake into chromaffin cells evoked by (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or (●) K (59 mM for 60 s). Nisoldipine was present for 10 min before and during stimulation of the cells with DMPP or K. Experimental protocol as in Figure 3. Data (normalized to 100% of control ^{45}Ca uptake) are means of 7 triplicate experiments made with cells from different batches; s.e.mean shown by vertical bars.

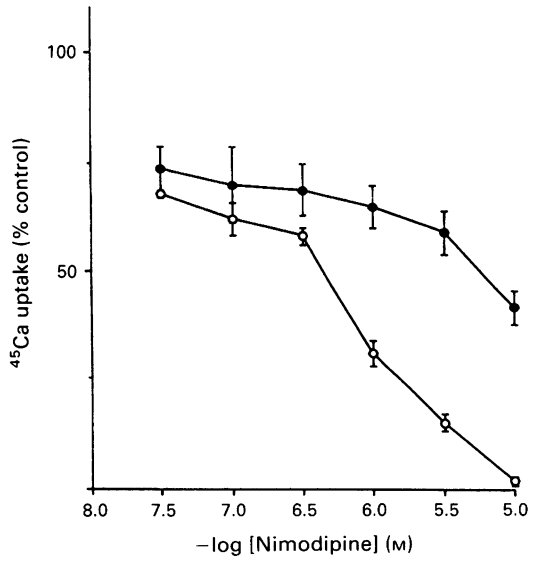

Figure 9 Effects of nimodipine on ^{45}Ca uptake into chromaffin cells stimulated by (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or (●) K (59 mM for 60 s). Nimodipine was present for 90 min before and during stimulation of the cells with DMPP or K^+. The rest of the experiment followed a protocol similar to that described in Figure 3. Data are means of 2 triplicate experiments; s.e.mean shown by vertical bars.

Table 1 IC_{50}s for various agents to block ^{45}Ca uptake into chromaffin cells stimulated with 1,1-dimethyl-4-phenylpiperazinium (DMPP) or K

	n	*DMPP*	*K*
Flunarizine	4	1.76	1.49
Cadmium	4	8.14	1.86
Amiloride	4	290	—
Guanethidine	4	27	—
Nisoldipine (10 min)	7	5	—
Nisoldipine (90 min)	2	2.63	—
Nimodipine	2	1.1	—

Values were calculated from the data in Figures 3–9, by a non-linear regression analysis computer programme (see Methods). IC_{50}s are expressed in μM; *n* shows the number of experiments in triplicate from different batches of cells.

maffin cells, stimulated with DMPP or K, are studied (see summary of IC_{50}s in Table 1). Bovine chromaffin cells may contain two different populations of Ca channels; in our experimental conditions, DMPP may recruit only one population of those channels but high K may activate all available channels. With this hypothesis in mind, our results could be explained as follows.

Flunarizine blocks L and T channels in cardiac cells (Tytgat *et al.*, 1988); thus it seems to be a rather non-specific Ca channel blocker, justifying in this manner its ability to block DMPP- and K-evoked Ca uptake. The same applies to Cd, an inorganic cation with little selectivity for Ca channel subtypes (Tsien *et al.*, 1988; Kostyuk, 1989). Though Cd seemed to block better K-evoked Ca uptake, DMPP was also highly sensitive to the cation.

A different picture can be drawn with the other drugs. Amiloride is reported to be a selective T-type Ca channel blocker in cardiac cells (Tang *et al.*, 1988). This might explain why this molecule fully blocks DMPP-evoked Ca uptake, yet K effects are affected little. A similar reasoning applies for nimodipine and nisoldipine, two 1,4-DHP derivatives selectively blocking L-type Ca channels in several excitable cells (Tsien *et al.*, 1988; Kostyuk, 1989). The drugs blocked DMPP effects much more efficiently than the effects induced by K depolarisation.

A particular case can be made with guanethidine, a potent blocker of noradrenaline release from sympathetic nerve terminals (Kirpekar, 1975) which, as far as we know, is not yet catalogued as a Ca channel blocker. Guanethidine dissociated the K from the DMPP response, blocking the nicotinic effects but leaving intact the Ca pathway activated by direct depolarization. Since adrenal chromaffin cells are quite similar, structurally and functionally, to sympathetic neurones, it might be that guanethidine is blocking N-type Ca channels which are probably mediating transmitter release at sympathetic nerve terminals (Lipscombe *et al.*, 1989). If so, the Ca channel recruited by nicotinic stimulation in bovine chromaffin cells may be pharmacologically, similar to the N channel in sympathetic neurones; and guanethidine might block these channels. Guanethidine blocks high-threshold Ca currents in bovine adrenal chromaffin cells (personal communication of Gandía, Lacinova & Morad). Of course, a direct blockade of nicotinic receptors by guanethidine cannot be discarded at present.

Heterogeneity of Ca channels in bovine chromaffin cells is a polemic issue. Radioligand binding studies with [^{3}H]-isradipine (Castillo *et al.*, 1989), [^{3}H]-nitrendipine and [^{3}H]-ω-conotoxin (Ballesta *et al.*, 1989; 1990) suggest the presence of various binding sites associated with different Ca channels. Measurements of ^{45}Ca uptake (Ballesta *et al.*, 1989) and Ca_i transients (Rosario *et al.*, 1989) also indicate the presence of heterogeneous populations of Ca channels. However, direct measurement of Ca currents with whole-cell or single-channel recording patch-clamp techniques favour the presence of homogeneous (Fenwick *et al.*, 1982; Hoshi *et al.*, 1984; Ceña *et al.*, 1989) or heterogeneous (Artalejo *et al.*, 1989) populations of Ca channels. The results of our present experiments are in line with the view that different Ca channel subtypes might be present in cultured bovine adrenal chromaffin cells. This is not the case for the cat adrenal gland which seems to be equipped mostly with Ca channels highly sensitive to DHPs and controlling very efficiently the secretory process (see review by Artalejo *et al.*, 1988).

In conclusion, by using a pharmacological approach to dissect Ca entry pathways in cultured bovine adrenal chromaffin cells, we have demonstrated that nicotinic- and high-K stimulation recruit different subtypes of voltage-dependent Ca channels.

This work has been supported in part by grants from Fundación Ramón Areces, CICYT and FISS. We thank Mrs N. Tera for typing the manuscript. L. Gandia is a Fellow of F.I.S.S., Spain.

References

ABAJO, F.J., CASTRO, M.A.S., LOPO, C.R., GARIJO, B., SÁNCHEZ-GARCÍA, P. & GARCÍA, A.G. (1989). Sodium-dependent and sodium-independent nicotine-evoked catecholamine release from cat adrenals. *Neurosci. Lett.*, **101**, 101–106.

AMY, C. & KIRSCHNER, N. (1982). Na^{+1} uptake and catecholamine secretion by primary cultures of adrenal medulla cells. *J. Neurochem.*, **39**, 132–142.

ARTALEJO, C.R., BADER, M.F., AUNIS, D. & GARCÍA, A.G. (1986). Inactivation of the early calcium uptake and noradrenaline release evoked by potassium in cultured chromaffin cells. *Biochem. Biophys. Res. Commun.*, **134**, 1–7.

ARTALEJO, C.R., DAHMER, M., PERLMAN, R.L. & FOX, A.P. (1989). Facilitation of Ca current in bovine chromaffin cells: recruitment of a different class of Ca channels? *Biophys. J.*, **55**, 593a.

ARTALEJO, C.R., LÓPEZ, M.G., CASTILLO, C.F., MORO, M.A. & GARCÍA, A.G. (1988). L-type calcium channels and adrenomedullary secretion. In *The Calcium Channel: Structure, Function and Implications*. ed. Morad, M., Nayler, W., Kazda, S. & Schramm, M. pp. 347–362. Berlin: Springer-Verlag.

BALLESTA, J.J., GARCÍA, A.G., GUTIERREZ, L.M., HIDALGO, M.J., PALMERO, M., REIG, J.A. & VINIEGRA, S. (1990). Separate [^{3}H]-nitrendipine binding sites in mitochondria and plasma membranes of bovine adrenal medulla. *Br. J. Pharmacol.*, **101**, 21–26.

BALLESTA, J.J., PALMERO, M., HIDALGO, M.J., GUTIÉRREZ, L.M., REIG, J.A., VINIEGRA, S. & GARCÍA, A.G. (1989). Separate binding and functional sites for ω-conotoxin and nitrendipine suggest two types of calcium channels in bovine chromaffin cells. *J. Neurochem.*, **53**, 1050–1056.

CÁRDENAS, A.M., MONTIEL, C., ARTALEJO, A.R., SÁNCHEZ-GARCÍA, P. & GARCÍA, A.G. (1988). Sodium-dependent inhibition by PN200-110 enantiomers of nicotinic adrenal catecholamine release. *Br. J. Pharmacol.*, **95**, 9–14.

CASTILLO, C.J.F., FONTERIZ, R.I., LÓPEZ, M.G., ROSENHECK, K. & GARCÍA, A.G. (1989). (+)-PN200-110 and ouabain binding sites in purified bovine adrenomedullary plasma membranes and chromaffin cells. *J. Neurochem.*, **53**, 1442–1449.

CEÑA, V., NICOLÁS, G.P., SÁNCHEZ-GARCÍA, P., KIRPEKAR, S.M. & GARCÍA, A.G. (1983). Pharmacological dissection of receptor-associated and voltage-sensitive ionic channels involved in catecholamine release. *Neurosci.*, **10**, 1455–1462.

CEÑA, V., STUTZIN, A. & ROJAS, E. (1989). Effects of calcium and Bay-K-8644 on calcium currents in adrenal medullary chromaffin cells. *J. Membr. Biol.*, **112**, 255–265.

DOUGLAS, W.W. & POISNER, A.M. (1962). On the mode of action of acetylcholine in evoking adrenal medullary secretion: increased uptake of calcium during the secretory response. *J. Physiol.*, **162**, 385–392.

DOUGLAS, W.W. & RUBIN, R.P. (1961). The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J. Physiol.*, **159**, 40–57.

FENWICK, E.M., MARTY, A. & NEHER, E. (1982). Sodium and calcium channels in bovine chromaffin cells. *J. Physiol.*, **331**, 599–635.

HOLZ, R.W., SENTER, R.A. & FRYE, R.A. (1982). Relationship between Ca^{2+} uptake and catecholamine secretion in primary dissociated cultures of adrenal medulla. *J. Neurochem.*, **39**, 635–646.

HOSHI, T., ROTHLEIN, J. & SMITH, S.J. (1984). Facilitation of Ca^{2+}-

channel currents in bovine adrenal chromaffin cells. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 5871–5875.

KILPATRICK, D.L., SLEPETIS, R.J., CORCORAN, J.J. & KIRSHNER, N. (1982). Calcium uptake and catecholamine secretion by cultured bovine adrenal medulla cells. *J. Neurochem.*, **38**, 427–435.

KIRPEKAR, S.M. (1975). Factors influencing transmission at adrenergic synapses. *Prog. Neurobiol.*, **4**, 63–98.

KOSTYUK, P.G. (1989). Diversity of calcium ion channels in cellular membranes. *Neurosci.*, **28**, 253–261.

LIPSCOMBE, D., KONGSAMUT, S. & TSIEN, R.W. (1989). Alpha-adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium-channel gating. *Nature*, **340**, 639–642.

LIVETT, B.G. (1984). Adrenal medullary chromaffin cells in vitro. *Physiol. Rev.*, **64**, 1103–1161.

MORO, M.A., LÓPEZ, M.G., GANDIA, L., MICHELENA, P. & GARCÍA, A.G. (1990). Separation and culture of living adrenaline- and noradrenaline-containing cells from bovine adrenal medullae. *Analyt. Biochem.*, **185**, 243–248.

ROSARIO, L.M., SORIA, B., FEUERSTEIN, G. & POLLARD, H.B. (1989). Voltage-sensitive calcium flux into bovine chromaffin cells occurs through dihydropyridine-sensitive and dihydropyridine- and w-conotoxin-insensitive pathways, *Neurosci.*, **29**, 735–747.

TANG, C.M., PRESSER, F. & MORAD, M. (1988). Amiloride selectively blocks the low threshold (T) calcium channel. *Science*, **240**, 213–215.

TSIEN, R.W., LIPSCOMBE, D., MADISON, D.V., BLEY, K.R. & FOX, A.P. (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.*, **11**, 431–438.

TYTGAT, J., VEREECKE, J. & CARMELIET, E. (1988). Differential effects of verapamil and flunarizine on cardiac L-type and T-type Ca channels. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**, 690–692.

WADA, A., TAKARA, H., IZUMI, F., KOBAYASHI, H. & YANAGIHARA, N. (1985). Influx of ^{22}Na through acetylcholine receptor-associated Na channels: Relationship between ^{22}Na influx, ^{45}Ca influx and secretion of catecholamines in cultured bovine adrenal medulla cells. *Neurosci.*, **15**, 283–292.

(Received July 31, 1990
Revised December 12, 1990
Accepted January 14, 1991)