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Inhibition of nicotinic receptor-mediated responses in bovine chromaffin cells by diltiazem

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1 The effects of diltiazem on various functional parameters were studied in bovine cultured adrenal chromaffin cells stimulated with the nicotinic receptor agonist dimethylphenylpiperazinium (DMPP) or with depolarizing Krebs-HEPES solutions containing high K^+ concentrations.

2 The release of [^{3}H]-noradrenaline induced by DMPP (100 μM for 5 min) was gradually and fully inhibited by increasing concentrations of diltiazem ($IC_{50}=1.3$ μM). In contrast, the highest concentration of diltiazem used (10 μM) inhibited the response to high K^+ (59 mM for 5 min) by only 25%.

3 $^{45}Ca^{2+}$ uptake into cells stimulated with DMPP (100 μM for 1 min) was also blocked by diltiazem in a concentration-dependent manner ($IC_{50}=0.4$ μM). Again, diltiazem blocked the K^+-evoked $^{45}Ca^{2+}$ uptake (70 mM K^+ for 1 min) only by 20%. In contrast, the N-P-Q-type Ca^{2+} channel blocker ω-conotoxin MVIIC depressed the K^+ signal by 70%. In the presence of this toxin, diltiazem exhibited an additional small inhibitory effect, indicating that the compound was acting on L-type Ca^{2+} channels.

4 Whole-cell Ba^{2+} currents through Ca^{2+} channels in voltage-clamped chromaffin cells were inhibited by 3-10 μM diltiazem by 20-25%. The inhibition was readily reversed upon washout of the drug.

5 The whole-cell currents elicited by 100 μM DMPP (I_{DMPP}) were inhibited in a concentration-dependent and reversible manner by diltiazem. Maximal effects were found at 10 μM, which reduced the peak I_{DMPP} by 70%. The area of each curve represented by total current (Q_{DMPP}) was reduced more than the peak current. At 10 μM, the inhibition amounted to 80%; the IC_{50} for Q_{DMPP} inhibition was 0.73 μM, a figure close to the IC_{50} for $^{45}Ca^{2+}$ uptake (0.4 μM) and [^{3}H]-noradrenaline release (1.3 μM). The blocking effects of diltiazem developed very quickly and did not exhibit use-dependence; thus the drug blocked the channel in its closed state. The blocking effects of 1 μM diltiazem on I_{DMPP} were similar at different holding potentials (inhibition by around 30% at -100, -80 or -50 mV). Diltiazem did not affect the current flow through voltage-dependent Na^+ channels.

6 These data are compatible with the idea that diltiazem has little effect on Ca^{2+} entry through voltage-dependent Ca^{2+} channels in bovine chromaffin cells. Neither, does diltiazem affect I_{Na}. Rather, diltiazem acts directly on the neuronal nicotinic receptor ion channel and blocks ion fluxes, cell depolarization and the subsequent Ca^{2+} entry and catecholamine release. This novel effect of diltiazem might have clinical relevance since it might reduce the sympathoadrenal drive to the heart and blood vessels, thus contributing to the well established antihypertensive and cardioprotective effects of the drug.

Keywords: Diltiazem; nicotinic receptors; calcium channels; chromaffin cells

Introduction

Calcium antagonists emerged from the pioneering work of Fleckenstein *et al.* (1967) on the relaxation by various compounds of coronary artery smooth muscle. Since this study, different structurally dissimilar classes of calcium antagonists have emerged. Representative members of the main classes of calcium antagonists include the phenylalkylamine derivative verapamil, the 1,4-dihydropyridine nifedipine and the benzothiazepine diltiazem. Their widely accepted clinical use to treat various cardiovascular diseases is thought to be based on their ability to inhibit selectively Ca^{2+} entry through voltage-dependent Ca^{2+} channels of the L-subtype in cardiac and smooth muscle cells (Triggle *et al.*, 1991). However, an increasing number of other cell targets are being described for the various subtypes of calcium antagonists (Zernig, 1990). One of these targets is the nicotinic acetylcholine receptor (AChR) present in bovine chromaffin cells. The recent cloning and expression of this receptor has shown its similarity to neuronal nicotinic acetylcholine receptors (Criado *et al.*, 1992).

In an earlier study we showed that the 1,4-dihydropyridine derivatives nimodipine and nisoldipine completely inhibit the uptake of $^{45}Ca^{2+}$ into bovine adrenal chromaffin cells stimulated with the nicotinic receptor agonist dimethylphenylpiperazinium (DMPP). In contrast, nimodipine or nisoldipine (10 μM) reduced $^{45}Ca^{2+}$ entry into K^+-depolarized cells by only 30% (Gandía *et al.*, 1991). These results were suggested to be due either to selective recruitment of L-type Ca^{2+} channels or to blockade by dihydropyridines of the nicotinic receptor ionophore. This latter possibility was reinforced by the subsequent demonstration that nifedipine, nimodipine, nitrendipine, furnidipine and Bay K 8644 largely decreased or abolished the DMPP-mediated effects, including $^{45}Ca^{2+}$ uptake, Mn^{2+} uptake, increments of the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, cell depolarization and catecholamine release. However, they affected to a minor extent the K^+-mediated responses (López *et al.*, 1993). A similar mechanism was initially suggested by Kilpatrick *et al.* (1981) and Corcoran and Kirshner (1983) for methoxyverapamil (D600), a finding that was later confirmed by Boehm and Huck (1993) in bovine chromaffin cells and chick sympathetic neurones.

In this study we show that diltiazem powerfully inhibited

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the nicotinic receptor mediated catecholamine secretory response in bovine chromaffin cells, while the response generated by direct depolarization with high K^+ concentrations was scarcely affected. These observations gave rise to the hypothesis that diltiazem could exert its effects at a step earlier than Ca^{2+} channel activation, such as the nicotinic receptor. This hypothesis was tested by studying the effect of diltiazem on the pathways used by external Ca^{2+} to enter bovine chromaffin cells, using patch-clamp techniques to measure nicotinic receptor and Ca^{2+} channel currents, and through studies of $^{45}Ca^{2+}$ entry into those cells. This novel component in the mechanism of action of diltiazem might be clinically relevant for its antihypertensive and cardioprotecting effects (Gibson *et al.*, 1986; see Discussion).

Methods

Chromaffin cell isolation and culture

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 (DMEM) supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin and 50 $\mu g\ ml^{-1}$ streptomycin. Cells were plated at a density of 5×10^5 cells/well in 24-multiwell Costar plates for [^{3}H]-noradrenaline release and $^{45}Ca^{2+}$ uptake studies. For measurements of whole-cell currents, cells were plated on 1 cm diameter glass coverslips at a density of 5×10^4 cells per coverslip. Cells were maintained at 37°C in a humidified incubator under an atmosphere of 95% air and 5% CO_2 and were used 1–5 days after plating. Medium was replaced after 24 h and then after 2–3 days.

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

$^{45}Ca^{2+}$ uptake studies were carried out as follows. 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, at pH 7.2 and 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 5 $\mu Ci\ ml^{-1}$ in the presence of Krebs-HEPES (basal uptake), high K^+ solution (Krebs-HEPES containing 70 mM KCl with isosmotic reduction of NaCl) or 100 μM DMPP 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 uptake reaction ended by adding 0.5 ml of a cold Ca^{2+}-free Krebs-HEPES containing 10 mM $LaCl_3$. Finally cells were washed 5 times more with 0.5 ml of Ca^{2+}-free Krebs-HEPES containing 10 mM $LaCl_3$ and 2 mM EGTA, at 15 s intervals.

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

[^{3}H]-noradrenaline release

Chromaffin cells were incubated with [^{3}H]-noradrenaline (1 $\mu Ci\ ml^{-1}$) in DMEM for 3–4 h, then culture medium was replaced by Krebs-HEPES basal solution with the following composition (in mM): NaCl 134, KCl 4.7, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11, and HEPES 15, and pH adjusted to 7.4 using a NaOH solution. Cells were washed four times with this solution before the pre-incubation of monolayers with diltiazem in Krebs-HEPES basal solution. Catecholamine secretion was induced for 5 min with basal solution or by cell stimulation with high K^+ (50 mM K^+; replacing isosmotically NaCl by KCl), or with 100 μM DMPP in basal solution. During stimulation, the concentration of diltiazem was maintained. Media were then collected and the remaining cells in the well were lysed with 2% sodium dodecyl-sulphate (SDS). Secreted and total catecholamines were quantified in a liquid scintillation counter. Net secretion represents stimulated minus basal release. Data are expressed as means ± s.e.mean, for triplicates performed in two or more cell cultures.

Electrophysiological recordings

Two types of experiments were performed. In one type, currents through nicotinic receptor channels, Na^+ channels and Ca^{2+} channels, were sequentially recorded by use of the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). Briefly, nicotinic currents were elicited by fast application of 100 μM DMPP through a puffer pipette controlled by miniature solenoid valves and placed within about 100 μm of the cell. The flow rate (0.5–1 $ml\ min^{-1}$) was regulated by gravity to achieve a complete replacement of cell surroundings within 50–100 ms. External superfusion medium was (in mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 11, HEPES-NaOH 10, pH = 7.4. Soft glass patch-clamp electrodes were filled with (in mM): NaCl 10, CsCl 100, TEA.Cl 20, Mg.ATP 5, EGTA 14, GTP 0.3 and 20 HEPES-KOH, pH 7.2. The holding potential was −80 mV and electrode resistances ranged from 2 to 5 MΩ.

For measuring Ba^{2+} currents through Ca^{2+} channels the external bathing solution contained 10 mM Ba^{2+} instead of Ca^{2+} and 5 μM tetrodotoxin (TTX) was added. The holding potential was as described before and the currents were digitized at 8 kHz by using pCLAMP software with a Labmaster interface (Axon Instruments) and data were stored in a PC computer. Linear leak and capacitive components were substracted by using a P/4 protocol and series resistances were compensated by 80%. Data manipulation and analysis were done by using pCLAMP and QBASIC (microsoft) written programs. Diltiazem was delivered near the cell, under continuous superfusion, by using the same puffer pipette described above. All experiments were performed at room temperature (22–24°C).

Materials and solutions

The following materials were used: collagenase from *Clostridium histolyticum* (Boehringer-Manheim); bovine serum albumin fraction V, cytosine arabinoside, fluorodeoxyuridine, 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) and EGTA (Sigma); foetal calf serum, penicillin and streptomycin (GIBCO); scintillation fluid Ready micro (Beckman); ^{45}Ca (specific activity 10–40 $mCi\ mg^{-1}$ calcium, Amersham). [^{3}H]-noradrenaline (14 $Ci\ mmol^{-1}$) was from NEN, Du Pont. Diltiazem was from Laboratorios Dr. Esteve, Barcelona, Spain. All other chemicals were reagent grade from Sigma, Merck or Bio-Rad.

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

Statistical analysis

IC_{50} values were estimated through non-linear regression analysis of ISI software, for a PC computer. Differences between groups were compared by Student's t test with the statistical program Statworks TM; a value of P equal to, or smaller than 0.05 was taken as the limit of statistical significance.

Results

Effects of diltiazem on catecholamine release induced by nicotinic receptor stimulation or by direct depolarization

Catecholamine release was studied by measuring the release of tritium from chromaffin cells whose endogenous catecholamine stores had previously been labelled with [^{3}H]-noradrenaline. Cells were treated for 5 min in basal conditions or stimulated for 5 min with concentrations of DMPP (100 μM) or K^+ (59 mM) which produce near maximal and equivalent secretory responses. Basal release was not modified by the presence of diltiazem.

Figure 1 shows the effects of increasing concentrations of diltiazem on catecholamine secretion induced by DMPP or K^+. The nicotinic receptor-mediated secretory response was inhibited in a concentration-dependent manner. The inhibition curve spanned two log units of diltiazem concentrations, and exhibited an IC_{50} of 1.3 μM. At about 7–10 μM, inhibition was almost complete. Thus, the concentration-response curve was quite steep.

K^+-evoked secretion was little affected by diltiazem. At 0.1 μM it depressed the response by 20%. However, increasing concentrations did not decrease further the release of catecholamines. At 10 μM, a new inhibition phase seemed to start (about 25% block). Higher concentrations of diltiazem produced solubility problems and the results at these high concentrations are probably meaningless; therefore, concentrations higher than 10 μM were not tested.

Actions of diltiazem on $^{45}Ca^{2+}$ uptake into chromaffin cells stimulated via nicotinic receptors or with K^+ depolarization

Nicotinic receptor stimulation and high K^+ stimulation trigger catecholamine secretory responses in chromaffin cells that are mediated by Ca^{2+} entry from the extracellular space into the cells (Douglas & Poisner, 1962; Corcoran & Kirshner, 1983; Artalejo *et al.*, 1986). Thus, it was interesting to investigate how diltiazem affected the Ca^{2+} pathways activated by DMPP (100 μM) or high K^+ (70 mM). Cells were stimulated for 60 s with concentrations of DMPP or K^+ known to trigger near maximal $^{45}Ca^{2+}$ entry responses.

Basal $^{45}Ca^{2+}$ uptake amounted to 1442 ± 135 c.p.m. per 5×10^5 cells ($n = 48$ wells from different cells batches). DMPP enhanced $^{45}Ca^{2+}$ uptake to values 2.3 fold higher than basal, and K^+ to 3.2 fold (means of 12 experiments in triplicate). As with catecholamine secretion, DMPP-triggered Ca^{2+} uptake was blocked by diltiazem in a concentration-dependent manner. At 1 μM, diltiazem blocked Ca^{2+} entry by 71% (Figure 2). Again, the concentration-response curve was rather steep, since at 3 μM inhibition was near 90%. The IC_{50} for inhibition of $^{45}Ca^{2+}$ uptake was 0.35 μM.

K^+-evoked Ca^{2+} uptake was inhibited by 22% at 0.3 μM diltiazem. Higher concentrations (1–10 μM) did not depress further this response. This weak effect of diltiazem might be due to the fact that K^+-evoked Ca^{2+} entry into bovine chromaffin cells takes place through L-type as well as N-, P- and Q-types of Ca^{2+} channels (López *et al.*, 1994) and diltiazem could only block the L-component. This possibility was studied in the experiment shown in Figure 3. Cells from the same culture were preincubated with Krebs-HEPES solution for 10 min, or

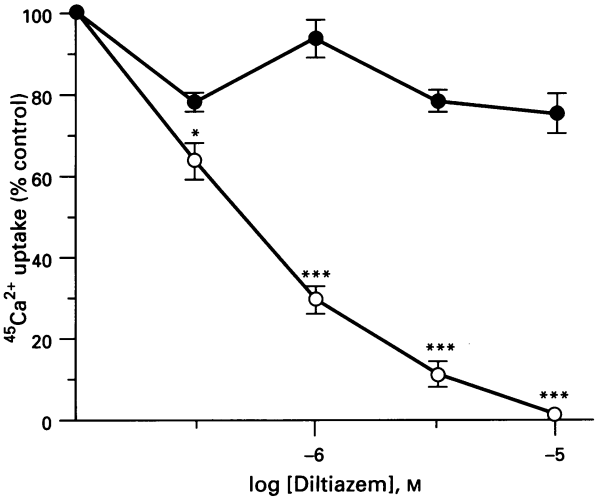

Figure 2 Effects of diltiazem on $^{45}Ca^{2+}$ uptake into bovine chromaffin cells stimulated with 70 mM K^+ (●) or 100 μM dimethylphenylpiperazinium (DMPP; ○) for 60 s. Before stimulation, cells were preincubated with each concentration of diltiazem for 10 min. In each individual experiment, $^{45}Ca^{2+}$ uptake (ordinate scale) was normalised to 100% ($^{45}Ca^{2+}$ taken up by cells in the absence of drugs). Vertical bars show s.e.mean. $*P<0.05$, $***P<0.001$ with respect to K^+-induced $^{45}Ca^{2+}$ uptake.

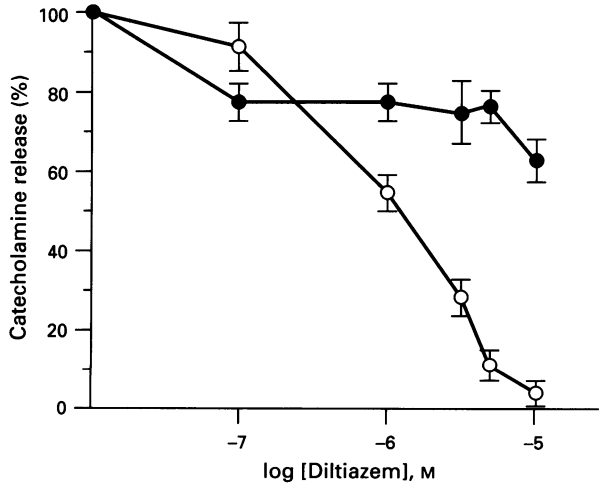

Figure 1 Effects of diltiazem on secretory responses induced by high K^+ depolarization (●) or dimethylphenylpiperazinium (DMPP) stimulation (○) of chromaffin cells. Net noradrenaline release was expressed as % of total [^{3}H]-noradrenaline cell content. Data represent means ± s.e.mean of 2 experiments performed in triplicate.

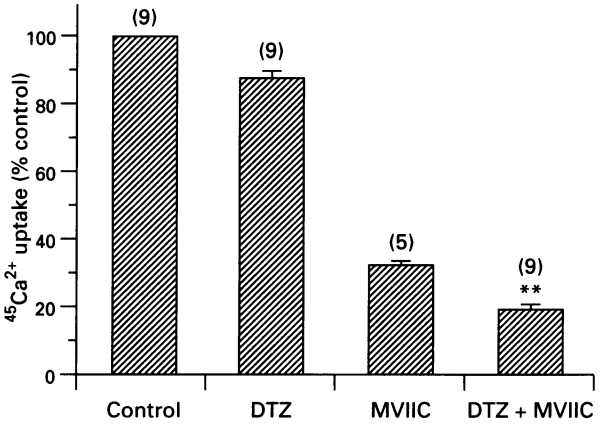

Figure 3 Effects of ω-conotoxin MVIIC (MVIIC) and diltiazem (DTZ) on $^{45}Ca^{2+}$ uptake into K^+-depolarized bovine chromaffin cells. MVIIC (1 μM), DTZ (3 μM) or DTZ + MVIIC were present 10 min before and during stimulation of the cells with a high-K^+ Krebs-HEPES solution (70 mM K^+ for 1 min). Data shown are means ± s.e.mean of the number of wells shown in parentheses at top of each column. $**P<0.01$ with respect to diltiazem alone or MVIIC alone.

with diltiazem (3 μM for 10 min), 1 μM ω-conotoxin MVIIC (a blocker of neuronal non-L-type Ca^{2+} channels; Adams *et al.*, 1993) or a combination of diltiazem and ω-conotoxin MVIIC. Then, they were stimulated for 1 min with 70 mM K^+ solution in the presence of $^{45}Ca^{2+}$. Ca^{2+} uptake was inhibited 12% by diltiazem alone and 68% by MVIIC alone. Diltiazem plus MVIIC produced additive effects (81% blockade of $^{45}Ca^{2+}$ uptake).

Effects of diltiazem on whole-cell barium currents

Figure 4a shows representative traces of whole-cell Ba^{2+} currents (I_{Ba}) obtained in a voltage-clamped chromaffin cell. The holding potential was maintained at −80 mV and test depolarizing pulses to 0 mV were applied at 15 s intervals. Under these conditions, the application of 3 μM diltiazem inhibited I_{Ba} by 12%. A higher concentration (10 μM) reduced the current by 18%. Washout of diltiazem prompted a quick though partial recovery of I_{Ba}. Figure 4b shows averaged results of 5 cells. At 3 μM, diltiazem reduced I_{Ba} by 12±4% and at 10 μM, the inhibition amounted to 18±5%.

Effects of diltiazem on whole-cell nicotinic receptor currents (I_{DMPP})

Whole-cell currents through the nicotinic AChR were evoked by applications of 250 ms pulses of an extracellular solution containing 100 μM DMPP at 30 s intervals. Figure 5a shows the time course of I_{DMPP} inhibition by increasing concentrations of diltiazem (1−10 μM). The initial current in this cell amounted to 2.5 nA. Inhibition was concentration-dependent and took place in discrete single steps after addition of each drug concentration. It is interesting that the inhibition of each diltiazem concentration was fully established at the first DMPP pulse application after addition of the drug. With subsequent DMPP pulses (5 pulses applied at 30 s intervals) further blockade of current was not seen. Upon washout of diltiazem, the current recovered very rapidly to almost its initial values. Figure 5b shows original traces of I_{DMPP} obtained from a typical cell in control conditions (first two traces) and in the presence of 0.1, 1 or 10 μM diltiazem. It is interesting that diltiazem did not seem to delay the rate of activation of the current. The blocking effects of diltiazem were completely reversed 1 min after washout of the drug.

The selectivity of diltiazem to block the nicotinic receptor currents, but not Na^+ or Ca^{2+} currents was studied by use of a protocol which measured these three currents sequentially (Figure 6). A voltage-clamped cell (−80 mV of holding potential) was stimulated first with a 250 ms pulse of DMPP (100 μM) and 2 s later with a depolarizing pulse to 0 mV of 50 ms duration, to measure I_{Na} and I_{Ca} (see the ionic composition of the extracellular solution in Methods). The original

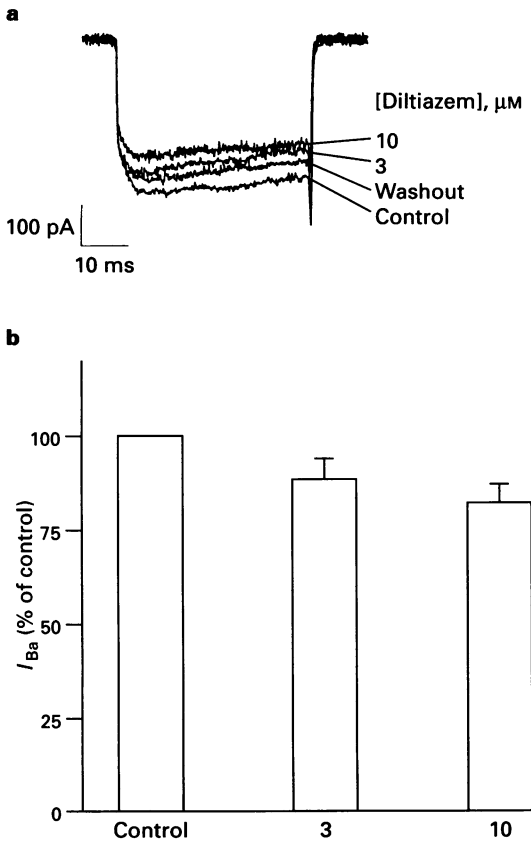

Figure 4 The effects of diltiazem on Ba^{2+} currents in bovine chromaffin cells. (a) Representative traces obtained in the absence (control) or the presence of 3 and 10 μM diltiazem. Also shown is the Ba^{2+} current obtained after the washout of the drug. Depolarizing pulses were applied from a resting potential of −80 mV to 0 mV for 40 ms at 15 s intervals. Linear capacitative and leak currents were subtracted. (b) Normalised data (means±s.e.mean) from experiments performed in 5 cells in conditions described above. Control currents were calculated by interpolation between the initial control and washout currents.

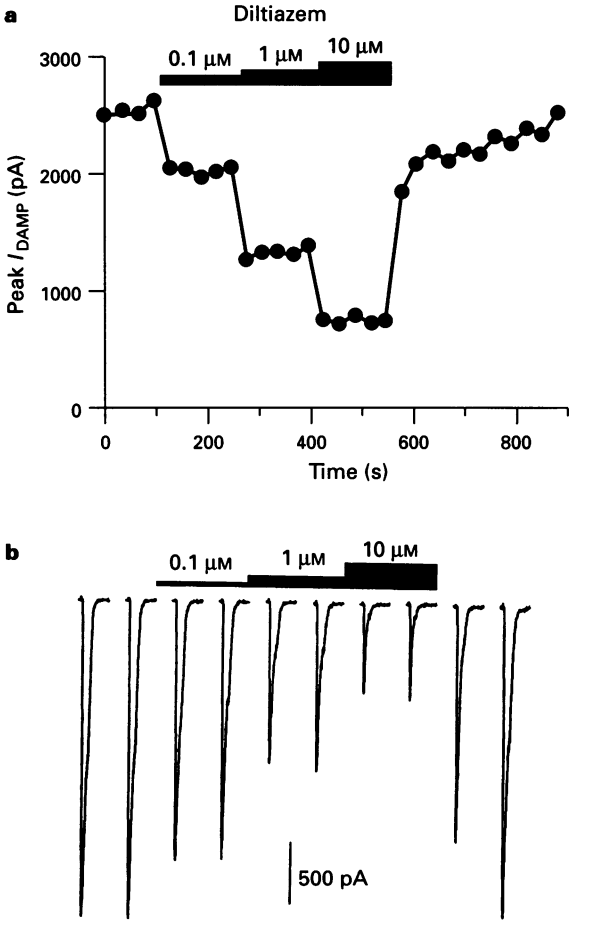

Figure 5 Effect of diltiazem on dimethylphenylpiperazinium (DMPP) evoked currents in cultured chromaffin cells. (a) Time course of the effects of increasing concentrations of diltiazem on I_{DMPP}. Currents were elicited by 250 ms pulses of a 100 μM solution of DMPP given at 30 s intervals. Diltiazem was applied at the concentrations and time periods shown by the top horizontal bars. This cell was voltage-clamped at −80 mV. (b) Representative experiment showing current traces induced by 250 ms pulses with 100 μM DMPP. Conditions were in the absence (first two traces) or in the presence of different concentrations of diltiazem (from 0.1 to 10 μM) incubated for 30 s before the first application of DMPP. The cell was voltage-clamped at −80 mV.

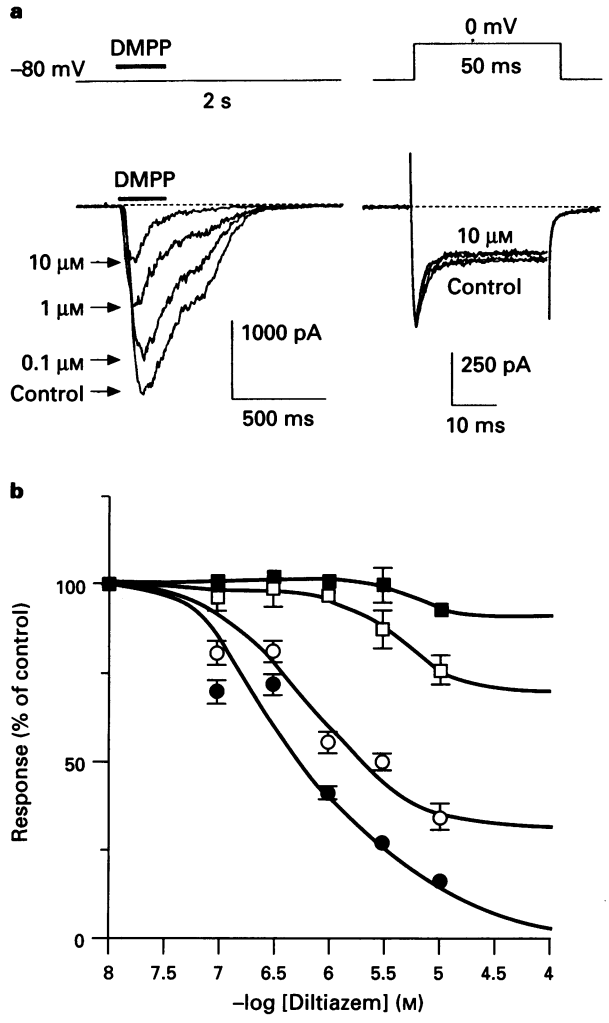

Figure 6 Experiments showing the selectivity of diltiazem at blocking nicotinic receptor currents (I_{DMPP}) but not voltage-dependent Na^+ (I_{Na}) or Ca^{2+} (I_{Ca}) currents. A voltage-clamped chromaffin cell (holding potential −80 mV) was subjected to the stimulation protocol shown on top. First, a dimethylphenylpiperazinium (DMPP) pulse (100 μM for 250 ms) was applied; 2 s later a depolarizing pulse to 0 mV, of 50 ms duration was given. This alternating pattern of chemical and electrical stimulation was repeated at 30 s intervals. In (a), original traces of I_{DMPP}, I_{Na} and I_{Ca} are shown, both in the absence or the presence of increasing concentrations of diltiazem. In (b), averaged results on the effects of diltiazem on I_{Na} (■), I_{Ca} (□), I_{DMPP} (○), and Q_{DMPP} (●) (area of I_{DMPP} traces) are plotted. Results are means ± s.e.mean of 4–18 cells.

traces shown in Figure 6a illustrate the effects of diltiazem on these three currents. Diltiazem decreased the peak I_{DMPP} in a concentration-dependent manner; however, the peak I_{Na} was unaffected. I_{Ca} was decreased by about 10–15% by 10 μM diltiazem. Figure 6b shows the concentration-response curves for each current. The IC_{50} for blockade of I_{DMPP} was around 1 μM. It is interesting that the total amount of charge carried by ions after each DMPP pulse (Q_{DMPP}) was even more depressed by increasing concentrations of diltiazem ($IC_{50} = 0.6$ μM).

The possible voltage-dependence of the blocking effects of diltiazem on I_{DMPP} were explored at three different holding potentials. Figure 7 shows original current traces elicited by DMPP pulses (100 μM for 250 ms) given at 30 s intervals to a cell whose holding potential was first fixed at −80 mV, then at −50 mV and finally at −110 mV. I_{DMPP} was obviously greater at more hyperpolarizing potentials. However, the % blockade of I_{DMPP} by diltiazem (1 μM) was similar, 39% at −80 mV, 31% at −50 mV and 31% at −110 mV.

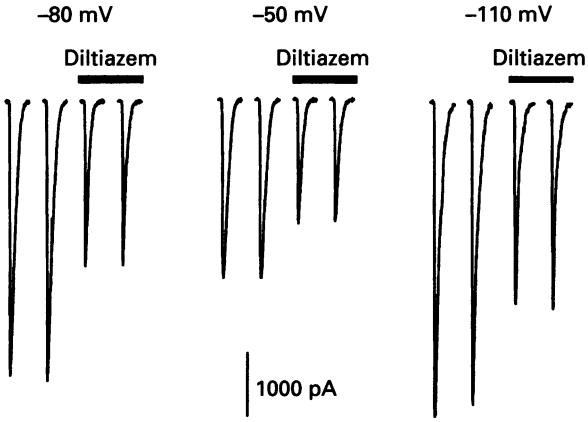

Figure 7 The effects of diltiazem (1 μM) on nicotinic receptor currents (I_{DMPP}) at different holding potentials. A chromaffin cell was voltage-clamped at −80 mV, −50 mV and −110 mV. At each holding potential I_{DMPP} was elicited by 250 ms pulses of a 100 μM dimethylphenylpiperazinium (DMPP) solution. Four DMPP pulses were given at each potential at 30 s intervals; two of them were in the absence and two in the presence of 1 μM diltiazem (top horizontal bar). Note a similar depression of I_{DMPP} caused by diltiazem at the three holding potentials.

Discussion

The central finding of this study is that diltiazem behaved as a poor blocker of K^+-evoked responses, yet it efficiently inhibited the responses triggered by activation of nicotinic AChR of bovine chromaffin cells. K^+ depolarization of bovine chromaffin cells leads to recruitment of various subtypes of voltage-dependent Ca^{2+} channels. In fact, L-type Ca^{2+} channels carry only a minor component of the whole-cell current through Ca^{2+} channels in bovine chromaffin cells (Albillos *et al.*, 1993; Gandía *et al.*, 1993). Thus, it was not unexpected that diltiazem inhibited only a minor component of the K^+-evoked $^{45}Ca^{2+}$ uptake (Figures 2 and 3) implying that only the L-type, but not N-P-Q-types of Ca^{2+} channels are blocked by this drug. In fact, in the presence of ω-conotoxin MVIIC, a blocker of N, P and Q channels in these cells (López *et al.*, 1994) diltiazem still inhibited an additional component of K^+-evoked $^{45}Ca^{2+}$ uptake (Figure 3).

In experiments measuring I_{DMPP}, I_{Na} and I_{Ca} in the same voltage-clamped cell it was clearly shown that diltiazem did not affect I_{Na}, slightly inhibited I_{Ca} and clearly blocked, in a concentration-dependent manner I_{DMPP}. The blockade was readily reversible and did not show either voltage- or use-dependence. Since the inhibition exhibited a low IC_{50} (around 1 μM), did not affect I_{Na} and only partially blocked I_{Ca} or I_{Ba}, it seems that the effects of diltiazem on neuronal nicotinic receptors are selective and specific in these cells. Concerning the mechanism of blockade of I_{DMPP}, it is interesting that diltiazem inhibits more the integrated I_{DMPP} than the peak I_{DMPP} (Figure 6). Though this difference was not pronounced, it suggests that diltiazem might speed up the desensitization of the nicotinic receptor. The fact that diltiazem reduces the peak current response suggests an interference with the channel activation. An increased rate of desensitization or induction of channel blockade has been proposed to be the mechanisms involved in the inhibition of nicotinic receptor current by substance P (Clapham & Neher, 1984) and somatostatin (Inoue & Kuriyama, 1991).

In physiological conditions, the catecholamine secretory response is triggered by stimulation of the nicotinic AChR by presynaptically released acetylcholine at the splanchnic nerve-chromaffin cell synapse (Douglas & Rubin, 1961). This depolarizes the chromaffin cell, triggers action potentials and activates the various subtypes of voltage-dependent Ca^{2+} channels, leading to Ca^{2+} entry and secretion. Because diltiazem barely inhibits Ca^{2+} entry through Ca^{2+} channels, it

seems that its powerful blocking effects on secretion must be exerted at an earlier or a later step in this chain of events. Blockade by diltiazem of the inward current through the nicotinic AChR channel strongly suggests that interruption of ionic fluxes through such channels by this drug might prevent cell depolarization and all subsequent events leading to catecholamine release. The observation that diltiazem inhibited Ca^{2+} entry and secretion triggered by DMPP, but not by high K^+ supports this hypothesis. As stated in the Introduction, it is widely accepted that the clinically relevant calcium antagonists are effective in hypertension, arrhythmias or coronary ischaemic diseases because they impair Ca^{2+} entry through L-type voltage-dependent Ca^{2+} channels of cardiac muscle and vascular smooth muscle. While this mechanism should play an unquestionable role, other mechanisms such as that described here for diltiazem might also contribute to their therapeutic profile. In fact, oversecretion of catecholamines from sympathetic nerve terminals or adrenal medullary chromaffin cells is mainly responsible for the pathogenic mechanism of cardiac ischaemia, arrhythmia and sudden cardiac death.

Thus, moderate blockade of nicotinic receptors to the adrenal chromaffin cells, as shown in this paper for diltiazem, and in previous papers for dihydropyridines (López *et al.*, 1993) and methoxyverapamil (Boehm & Huck, 1993) might limit the release of catecholamines in stressful conditions. Diltiazem completely inhibited the release of catecholamines and decreased by over 80% the acetylcholine receptor currents. This inhibition of the ionic flow through the receptor, will prevent the increase of the membrane potential required to open the Ca^{2+} channels to a significant degree. Consequently, diltiazem causes a total inhibition of the secretory response. In addition, because chromaffin cell nicotinic acetylcholine receptors are of the neuronal type (Criado *et al.*, 1992), it seems plausible that nifedipine, diltiazem and verapamil also block the nicotinic receptor at sympathetic ganglia and central neurones, contributing in this manner to slowing down the sympathetic drive to heart and vessels. In fact, D600 has been also shown to block the nicotinic receptor-mediated response in chick sympathetic ganglionic neurones (Boehm & Huck, 1993). These effects on nicotinic receptors might be less pronounced for the more vasodilator dihydropyridines (IC_{50} to cause vasorelaxation around 1 nM) than for verapamil or diltiazem under clinical conditions. However, the IC_{50} for diltiazem to cause vasorelaxation (around 1 μM) is in the range of its IC_{50} value to block nicotinic receptor-mediated responses, as shown in this study. Taken together, it is likely that a decrease of sympathetic drive to the heart, the effects on blood vessels and the decrease of circulating catecholamines during stressful situations caused by diltiazem could be important contributing factors to the antihypertensive and cardioprotective effects of this drug.

In conclusion, diltiazem blocks catecholamine release from bovine adrenal chromaffin cells by acting directly on their nicotinic acetylcholine receptor ionophore to block cell depolarization and subsequent Ca^{2+} entry. A direct effect on voltage-dependent Ca^{2+} entry contributed little to the actions of diltiazem on nicotinic AChR-mediated catecholamine secretion.

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