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Localized L-type calcium channels control exocytosis in cat chromaffin cells

Manuela G. López[1], Almudena Albillos[1], María Teresa de la Fuente[1], Ricardo Borges[2], Luis Gandía[1], Emilio Carbone[3], Antonio G. García[1], Antonio R. Artalejo[1]

[1] Departmento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, c/. Arzobispo Morcillo, 4, E-28029 Madrid, Spain
[2] Unidad de Farmacología, Facultad de Medicina, Universidad de La Laguna, E-Tenerife, Spain
[3] Dipartimento di Anatomia e Fisiología Umana, Corso Raffaello 30, I-10125 Turin, Italy

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Abstract. Depolarizing 1-s pulses to 0 mV from a holding potential of −70 mV, induced whole-cell currents through Ca^{2+} channels (I_{Ca}) in patch-clamped cat adrenal medulla chromaffin cells. The dihydropyridine (DHP) furnidipine (3 μM) reduced the peak current by 47% and the late current by 80%. ω-Conotoxin GVIA (CgTx, 1 μM) reduced the peak I_{Ca} by 42% and the late I_{Ca} by 55%. Pulses (10 s duration) with 70 mM K^+/2.5 mM Ca^{2+} solution (70 K^+/2.5 Ca^{2+}), applied to single fura-2-loaded cat chromaffin cells increased the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) from 0.1 to 2.21 μM; this increase was reduced by 43.7% by furnidipine and by 42.5% by CgTx. In the perfused cat adrenal gland, secretion evoked by 10-s pulses of 70 K^+/2.5 Ca^{2+} was reduced by 25% by CgTx and by 96% by furnidipine. Similar results were obtained when secretion from superfused isolated cat adrenal chromaffin cells was studied and when using a tenfold lower $[Ca^{2+}]_o$. The results are compatible with the existence of DHP-sensitive (L-type) as well as CgTx-sensitive (N-type) voltage-dependent Ca^{2+} channels in cat chromaffin cells. It seems, howevever, that though extracellular Ca^{2+} entry through both channel types leads to similar increments of averaged $[Ca^{2+}]_i$, the control of catecholamine release is dominated only by Ca^{2+} entering through L-type Ca^{2+} channels. This supports the idea of a preferential segregation of L-type Ca^{2+} channels to localized "hot spots" in the plasmalemma of chromaffin cells where exocytosis occurs.

Key words: Chromaffin cells − Calcium channels − Secretion − $[Ca^{2+}]_i$ − ω-Conotoxin GVIA − Furnidipine

Introduction

Using flame-etched carbon-fibre microelectrodes of 1 μm radius for the electrochemical detection of catecholamine release from single bovine chromaffin cells, Schroeder et al. [34] have recently demonstrated the existence of highly localized "hot spots" where exocytosis occurs, alternating with other silent spots on the plasmalemma where secretion does not take place. On the other hand, results from the dialysis of Ca^{2+}/EGTA [ethylenebis(oxonitrilo)tetraacetate] buffers into single bovine chromaffin cells made Augustine and Neher [7] come to the conclusion that depolarization locally elevates the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) at the release sites to well above 10 μM. To quickly generate this highly localized $[Ca^{2+}]_i$ signal, Augustine and Neher [7] argued that the voltage-dependent Ca^{2+} channels allowing external Ca^{2+} entry must be located near exocytotic sites where chromaffin vesicles accumulate. Evidence in favour of this hypothesis has been provided by Llinás et al. [29]; by using a low-sensitivity Ca^{2+}-dependent photoprotein (n-aequorin-J) they showed $[Ca^{2+}]_i$ transient microdomains at specific points after stimulation of the pre-synaptic terminal of the giant squid synapse. However, it is unlikely that this approach can be applied to small mammalian neurosecretory cells. An alternative approach to test this hypothesis could be the use of a spherical secretory cell such as a chromaffin cell, where multiple subtypes of Ca^{2+} channels are present, but only one dominates the control of the secretory process, with the hope of separating $[Ca^{2+}]_i$ signals associated with secretion from others not triggering exocytosis.

Like neurons [28, 35, 36], bovine adrenal chromaffin cells contain L-, N- and P-type Ca^{2+} channels [4–6, 11, 12, 18, 22]. On the other hand, kinetic and pharmacological analysis of whole-cell Ca^{2+} currents (I_{Ca}) reveal the presence of only L- and N-type Ca^{2+} channels in the cat adrenal chromaffin cell [2]. To test the hypothesis that L-type, but not N-type, Ca^{2+} channels are selectively segregated to plasmalemmal hot spots where exocytosis occurs, we used the cat chromaffin cell for its greater simplicity of Ca^{2+} channel subtypes contents, and because its secretory response is highly sensitive to 1,4-dihydropyridine (DHP) Ca^{2+} channel activators and

Correspondence to: M. G. López

blockers [20]. To achieve this, we measured how L- and N-type Ca^{2+} channel blockers affected I_{Ca}, as well as the changes of $[Ca^{2+}]_i$ and the secretory activity of cat adrenal medulla chromaffin cells under experimental conditions as similar as possible.

Materials and methods

Isolation and culture of cat chromaffin cells. Cat chromaffin cells were isolated and cultured as described by Uceda et al. [37]. Cells were plated on coverslips for electrophysiological recordings and $[Ca^{2+}]_i$ measurements and on microbeads (Cytodex 3 from Sigma, Madrid, Spain) for secretion studies, with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum containing 50 IU · ml^{-1} penicillin and 50 μg · ml^{-1} streptomycin. Cells were kept at 37°C in a water-saturated, 5% CO_2 atmosphere; they were used 1–5 days after plating.

Electrophysiological recordings. Membrane currents were measured using the patch-clamp technique in the whole-cell configuration using a List EPC-7 patch-clamp amplifier [23]. Solutions adequate to suppress Na^+ and K^+ channel currents were used. Cells were dialysed with a pipette solution of the following composition (mM): 110 CsCl, 30 tetraethylammonium (TEA) HCl, 20 EGTA, 20 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 5 MgATP, adjusted to pH 7.3 with NaOH. After gigaseal formation the external solution was switched from a Tyrode-type solution to one containing (mM): 10 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 155 NaCl and 2 μM tetrodotoxin (TTX), also adjusted to pH 7.3 with NaOH. Experiments were performed at room temperature (23–25°C). External solutions were exchanged by fast superfusion using a modified multi-barrelled ejection pipette, by means of electrovalves driven by computer commands [14]. Current recordings were filtered at 3 kHz (−3 dB, 8-pole Besel filter) and digitized at sampling intervals of 500 μs using a 12 bit A/D Tecmar Lab Master board (125 kHz) interfaced with an IBM-compatible computer. Stimulation and data adquisition were made with pClamp software (Axon Instruments, Foster City, Calif., USA). Off-line data analysis and curve fittings were made using pClamp and FIG PLOT software.

$[Ca]_i$ measurements. To determine the $[Ca^{2+}]_i$ changes, cat chromaffin cells were loaded with fura-2 by incubating them with fura-2/AM (4 μM) for 30 min at room temperature in a medium (pH 7.4) containing (mM): 145 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 NaHEPES, 10 glucose. The loading was terminated by washing the coverslips with the cells attached several times in fresh medium. The fluorescence of fura-2 in single cells was measured with the photomultiplier-based system described by Neher [31]. Fura-2 was excited with a light alternating between 360 nm and 390 nm, using a Nikon 40× objective. Emitted light was transmitted through a 425-nm dichroic mirror and 500–545 nm barrier filter before being detected by the photomultiplier. $[Ca^{2+}]_i$ was calculated from the ratios of the light emitted when the dye was excited by two alternating excitation wavelengths [21]. The cells were superfused at room temperature with the various saline solutions, with a device similar to that used for the patch-clamp experiments. Fura-2/AM was obtained from Molecular Probes, Eugene, OR, USA.

Electrochemical detection of catecholamine release. To monitor the release of catecholamines, cat adrenal glands were prepared for retrograde perfusion as shown earlier [19] and placed in a hermetic metacrilate perfusing chamber. The glands were perfused at a rate of 2 ml · min^{-1}, at room temperature, with a Krebs-HEPES solution of the following composition (in mM): 144 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.4. In some experiments, secretion from isolated superfused cat adrenal medullary chromaffin cells was measured. Cells ($n = 5\times10^6$) were trapped in a small (200 μl) chamber and superfused with Krebs-HEPES solution at a rate of 2 ml · min^{-1}. Catecholamine release was triggered by applying 10-s pulses of a high K^+ solution (70 mM K^+, with a concomitant iso-osmotic reduction of NaCl) using an electronically controlled valve. On-line catecholamine secretion was monitored by means of an electrochemical detector (Methrom 641-VA), as previously described [10].

HEPES, ω-conotoxin GVIA (CgTx) and MgATP were obtained from Sigma; CgTx was prepared as a 1-mM stock solution in distilled water and kept in aliquots at −20°C until use. Furnidipine, obtained from Laboratories Alter, Madrid, Spain, was prepared in ethanol and diluted to the required final concentration in the extracellular solution.

Results

Effects of furnidipine and CgTx on cat chromaffin cell I_{Ca}

Whole-cell I_{Ca} were measured using the patch-clamp technique in single isolated cat adrenal medulla chromaffin cells. Using 10 mM Ca^{2+} as the charge carrier, long depolarizing pulses (1 s) to 0 mV, from a holding potential of −70 mV, induced whole-cell currents through Ca^{2+} channels which underwent variable time-dependent inactivation (Fig. 1 A). The early peak I_{Ca} averaged 415 pA and the late I_{Ca} (end of the pulse) 156 pA ($n = 17$ cells). Furnidipine (3 μM), a novel DHP derivative [1], reduced the peak I_{Ca} by 47 ± 3.4% of control and the late I_{Ca} by 80 ± 3.4% ($n = 18$ cells). The blocking effects of furnidipine developed quickly (after 60 s superfusion) and were readily reversible upon wash out (Fig. 1 A, B). The substantial greater blockade of the late current suggests that, as other DHPs [17, 33], furnidipine favours the block of L-type Ca^{2+} channels in a time- and voltage-dependent manner.

CgTx (1 μM) reduced the peak I_{Ca} by 42 ± 4% and the late I_{Ca} by 55 ± 5% ($n = 15$ cells; Fig. 1 C, D). In some cells, the blockade was partially reversible, while in others it was irreversible. In three cells, the combination of furnidipine plus CgTx led to an 80% blockade of the early I_{Ca} and to full inhibition of the late I_{Ca}. Cells pre-incubated with CgTx (0.1–2 μM for 20 min to 4 h) gave currents of small amplitude (around 100 pA) that were always highly sensitive to DHP antagonists or agonists (not shown). These data suggest that cat chromaffin cells contain DHP- as well as CgTx-sensitive Ca^{2+} channels. Ca^{2+} channel subtypes with this pharmacological profile have been recently described also in bovine chromaffin cells [4–6, 11, 12, 18, 22].

Effects of furnidipine and CgTx on $[Ca^{2+}]_i$

The partial blockade of the whole-cell I_{Ca} by furnidipine or CgTx should be accompanied by parallel changes of the $[Ca^{2+}]_i$. Brief depolarizing pulses (10 s) given with a solution containing 70 mM K^+ and 2.5 mM Ca^{2+} (70 K^+/2.5 Ca^{2+}), produced peak increases of $[Ca^{2+}]_i$ averaging 2.21 ± 0.22 μM ($n = 25$ cells). Furnidipine reduced the $[Ca^{2+}]_i$ increase by 43.7 ± 4.2% ($n = 9$ cells); this effect was readily reversible upon wash out

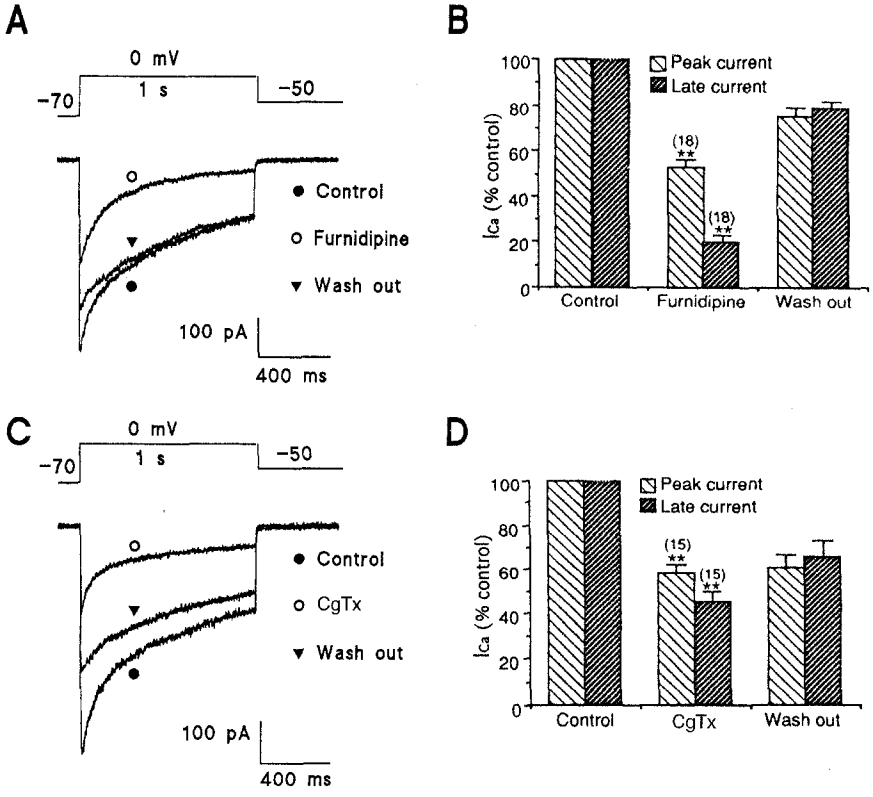

Fig. 1A–D. Partial inhibition of whole-cell Ca^{2+} currents (I_{Ca}) in cat chromaffin cells superfused with furnidipine (**A, B**) or ω-conotoxin GVIA (*CgTx*; **C, D**). Depolarizing pulses were applied to voltage-clamped cat chromaffin cells following the protocol shown at the *top* of *panels* **A** and **C**. Furnidipine (3 μM) or CgTx (1 μM) were superfused through a pipette whose tip was placed about 50 μm from the cell. They were present 60 s before and during pulse application. The wash out current were tested 60 s after removing the drugs. Quantitative average data were obtained by measuring the size of the current at its maximum peak (*peak current*) and at the end of the 1-s depolarizing pulse (*late current*). **B, D** Averaged results are shown (means ± SEM) of the number of cells given in *parentheses*, obtained using the protocols of **A** and **C**, respectively. ** $P < 0.01$ with respect to control

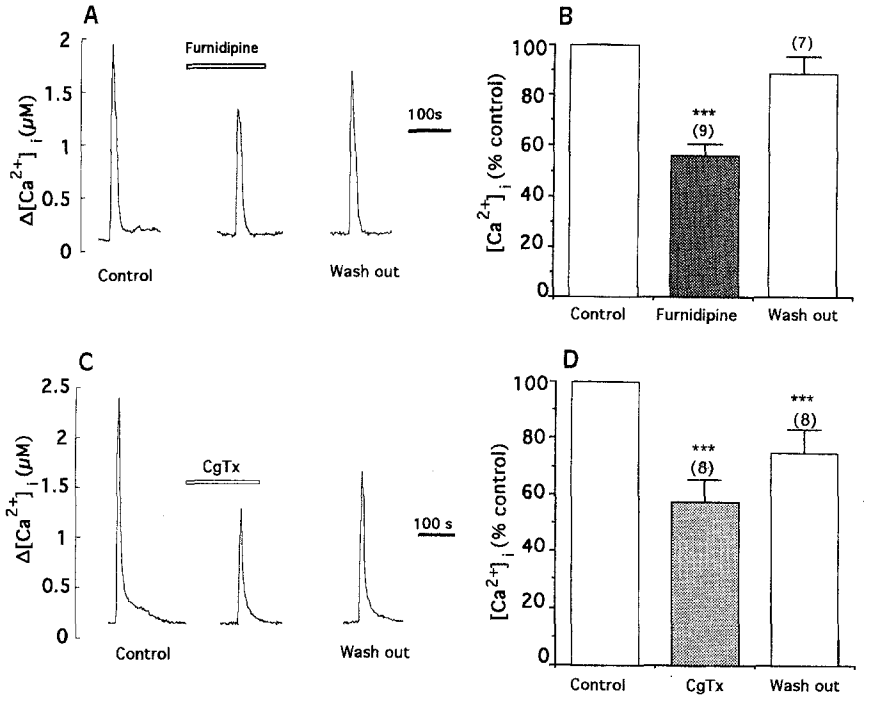

Fig. 2 A–D. Effects of furnidipine and CgTx on the transient increases of the cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in single cat chromaffin cells loaded with fura-2. A given cell was sequentially stimulated with 10-s pulses of a solution containing 70 mM K^+ (isoosmotic reduction of Na^+) and 2.5 mM Ca^{2+} (70 K^+/2.5 Ca^{2+}). Each of the three traces in **A** and **C** represent the increase of the $[Ca^{2+}]_i$ in response to three pulses of 70 K^+/2.5 Ca^{2+} given at 15-min intervals to the same cell. The second pulse was applied in the presence of furnidipine (3 μM, given 2 min before and during the pulse) or CgTx (1 μM, given 2 min before and during the pulse); the first and third pulses (control and washout) were applied in a drug-free solution. **B, D** The average results of the number of cells given in *parentheses*, are shown; they were obtained using the protocols of **A** and **C**, respectively. Data are means ± SEM. *** $P < 0.001$, compared with the control

of the drug (Fig. 2 A, B). Again, CgTx action varied from cell to cell. In some cells CgTx reduced by over 80% the K^+-evoked increase of the $[Ca^{2+}]_i$; in others, it was almost devoid of blocking effects. In eight cells, CgTx reduced the $[Ca^{2+}]_i$ by 42.5 ± 7.6%. CgTx action was partially reversible in some cells, but not in others (Fig. 2 C, D). Figure 3 compares the changes of the $[Ca^{2+}]_i$ at low $[Ca^{2+}]_o$ (0.25 mM) with those obtained with high $[Ca^{2+}]_o$ (2.5 mM) in the presence of furnidipine. A given fura-2-loaded cell was first stimulated with 70 K^+/0.25 Ca^{2+} and then with 70 K^+/2.5 Ca^{2+} in the presence of furnidipine. In 0.25 mM $[Ca^{2+}]_o$ the control increase in the $[Ca^{2+}]_i$ was 63 ± 2.5% of the control increase obtained in 2.5 mM $[Ca^{2+}]_o$. In the presence of 2.5 mM $[Ca^{2+}]_o$ and furnidipine, the increase accounted for 57.5 ± 2.4% of control ($n = 6$ cells). Thus, the in-

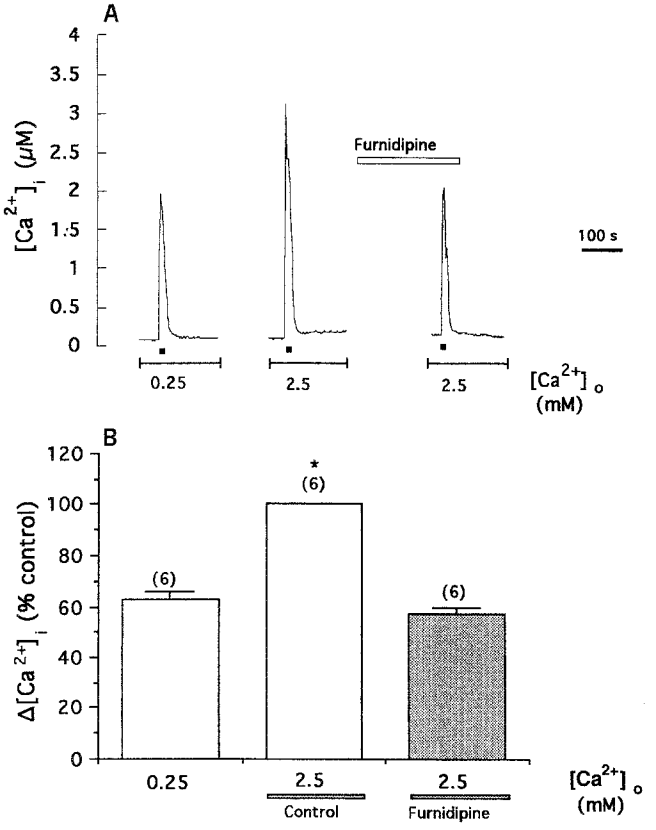

Fig. 3 A, B. The effects of furnidipine on the increase in $[Ca^{2+}]_i$ induced by 10-s pulses of solutions containing 70 mM K^+ with a low (0.25 mM) or a high (2.5 mM) external Ca^{2+} concentration ($[Ca^{2+}]_o$). **A** The original traces obtained in a single cell are shown. The cell was first stimulated with 70 K^+/0.25 Ca^{2+}, then with 70 K^+/2.5 Ca^{2+} and finally with 75K^+/2.5 Ca^{2+} in the presence of furnidipine (3 μM, present 2 min before and during the pulse). **B** The average results obtained in 6 cells are shown (means ± SEM). $*P < 0.05$ with respect to the signal obtained in 70 K^+/2.5 Ca^{2+} in the absence of furnidipine, or 70 K^+/2.5 Ca^{2+} in the presence of furnidipine

crease of the $[Ca^{2+}]_i$ induced by 70 K^+/0.25 Ca^{2+} was equivalent to that observed in 70 K^+/2.5 Ca^{2+} in the presence of furnidipine.

Effects of furnidipine and CgTx on catecholamine secretion

The blockade of Ca^{2+} channels and the inhibition of the K^+-induced $[Ca^{2+}]_i$ increase did not run parallel to the effects of furnidipine and CgTx on catecholamine release. In the perfused cat adrenal gland, 10-s pulses of a 70 K^+/2.5 Ca^{2+} solution given at 5-min intervals, induced reproducible secretion peaks when monitored on-line by electrochemical detection (Fig. 4 A). CgTx reduced secretion evoked by 70 K^+/2.5 Ca^{2+} by only 25 ± 3% of control. Furnidipine, given after wash out of CgTx, reduced the K^+ secretory response by 96 ± 3%. Upon wash out, secretion recovered to 60% of the initial response (Fig. 4 B). A similar picture was observed when $[Ca^{2+}]_o$ was reduced tenfold (Fig. 4 C). The initial secretory response to 10-s pulses with 70 K^+/0.25 Ca^{2+} was reduced by 20% with respect to the response in 2.5 mM $[Ca^{2+}]_o$. CgTx reduced this response by 30%, but furnidipine blocked it completely. Finally, without previous treatment with CgTx, furnidipine alone still blocked by 97% the secretory response to 70 K^+/2.5 Ca^{2+}; upon wash out of furnidipine, secretion recovered to 41 ± 5% in 15–20 min (Fig. 4 D). This slow reversibility, in comparison to the rapid relief of I_{Ca} blockade, may be due to the slower wash out and to longer pre-perfusion times in the intact glands with respect to single chromaffin cells. Secretion experiments were performed in intact glands because of the scarcity of available isolated chromaffin cells. However, in three experiments, the protocols used in intact glands were repeated in superfused isolated cat chromaffin cells. In response to 10-s pulses of 70 K^+/2.5 Ca^{2+} the catecholamine released was again decreased by only 10% in the presence of CgTx, while addition of furnidipine after CgTx wash out blocked the secretion fully (Fig. 5).

Discussion

Three conclusions emerge from this study: (1) The whole-cell I_{Ca} can be partially blocked by furnidipine or CgTx, suggesting that cat chromaffin cells contain L-type, as well as N-type, voltage-dependent Ca^{2+} channels; (2) each of the two channels equally contribute to the increase of the $[Ca^{2+}]_i$ evoked by high K^+ depolarization of fura-2-loaded chromaffin cells; and (3) the external Ca^{2+} entering through the L-type channels has a more direct access to the secretory machinery than Ca^{2+} entering through N-type Ca^{2+} channels.

One possible explanation for the separation of the effects of CgTx and furnidipine on I_{Ca}, $[Ca^{2+}]_i$ and secretion could reside in poor binding of CgTx and/or access of CgTx to its receptor in the conditions used to study secretion. Because Ca^{2+} delays the CgTx binding to its receptor in chromaffin cell membranes [9], experiments were also performed with a $[Ca^{2+}]_o$ as low as 0.25 mM. Under these conditions, blockade of secretion by CgTx was still poor, yet furnidipine fully suppressed the secretory signal. Poor diffusion of CgTx to its receptors in the intact gland can also be discarded as an explanation of its scarce blocking action on secretion because: (1) in sympathetically innervated intact tissues, CgTx fully blocks noradrenaline release at submicromolar concentrations [16, 30]; (2) another small peptide, apamin, markedly potentiates the muscarinic secretory response in intact cat adrenal glands [37]; and (3) the same block of secretion was obtained in superfused cat chromaffin cells, where the diffusion barriers present in intact adrenal glands do not exist.

Supramaximal concentrations of CgTx and furnidipine were used in this study. In the case of furnidipine it is beyond doubt that this is so, because 3 μM always caused full blockade of secretion. On the other hand, in sympathetic neurons, submicromolar concentrations of CgTx block N-type currents and secretion [26]. It seems, therefore, that the experimental conditions and the results obtained in the present study strongly speak in

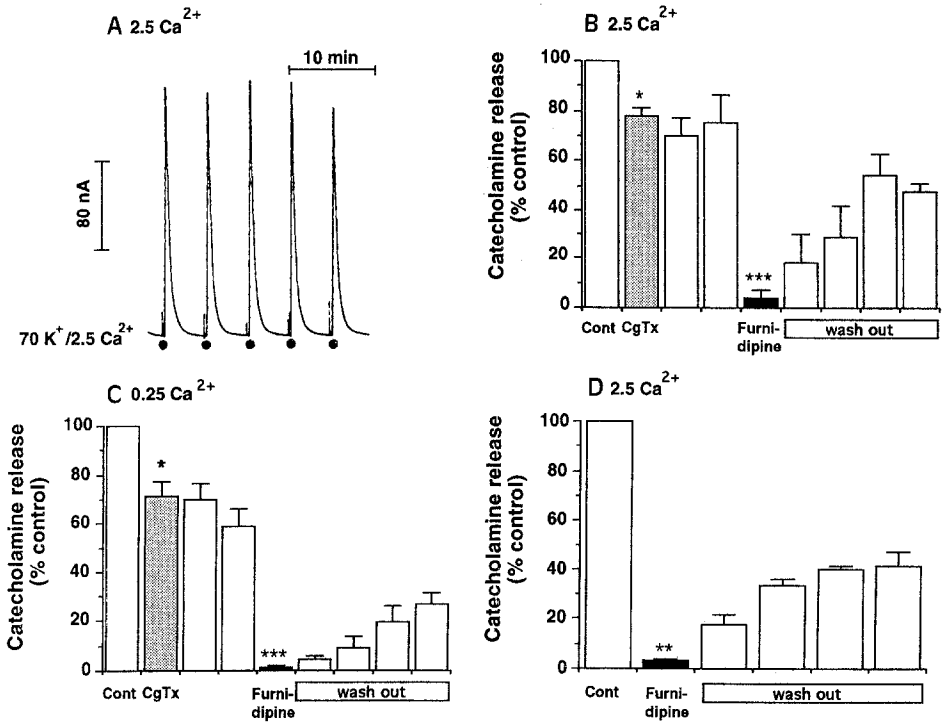

Fig. 4 A–D. Effects of CgTx and furnidipine on the release of catecholamines evoked by 10-s pulses of a solution containing 70 mM K^+ in high (2.5 mM) or low (0.25 mM) $[Ca^{2+}]_o$, from perfused cat adrenal glands. **A** Typical traces obtained upon stimulation with 70 mM K^+ in 2.5 mM $[Ca^{2+}]_o$ (70 K^+/2.5 Ca^{2+}), at 5-min intervals, are shown. **B–D** The effects of CgTx and furnidipine on the secretory responses obtained in 2.5 mM $[Ca^{2+}]_o$ (**A** and **D**) or in 0.25 mM $[Ca^{2+}]_o$ (**C**) are shown. CgTx (1 μM) or furnidipine (3 μM) were present 2 min before and during the high K^+ pulse. The results are expressed as a % of the initial secretion obtained in the absence of drug (Control, *Cont*) in the presence of CgTx or furnidipine, and after wash out of the compounds. For calibration purposes, 1 nA of oxidation signal is equivalent to 71 ng · ml^{-1} of adrenaline. Data are means ± SEM of 7 (**B**), 7 (**C**) and 3 (**D**) glands. $*P < 0.05$; $***P < 0.001$ compared with control

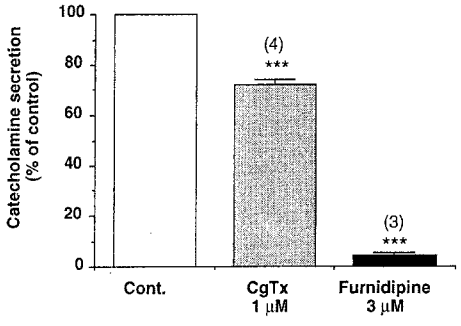

Fig. 5. Effects of CgTx and furnidipine on the release of catecholamines from superfused isolated cat adrenal medullary chromaffin cells. Secretion was evoked by 10-s pulses of a solution containing 70 mM K^+ and 2.5 mM Ca^{2+}. CgTx or furnidipine were present 2 min before and during the high K^+ pulse. The results are expressed as a % of the initial control (*Cont*) secretion. Data are means ± SEM of the number of experiments shown in *parentheses*. $***P < 0.001$ with respect to the controls

favour of the presence of two Ca^{2+} entry pathways in feline chromaffin cells. A detailed analysis of the kinetics and pharmacological properties of Ca^{2+} channel currents supports this conclusion [2].

To clearly correlate the results from the three types of parameters measured, the experimental conditions were as reproducible as possible. Currents were measured with depolarizing potentials from −70 to 0 mV; $[Ca^{2+}]_i$ and secretion were measured with 70 mM K^+ pulses, which shift the resting membrane potential of chromaffin cells from around −65 mV to values close to 0 mV [3, 13]. On the other hand, the length of the depolarizing pulses (1 s for I_{Ca}, 10 s for $[Ca^{2+}]_i$ and secretion measurements) were also as close as possible. In spite of this, the changes of secretory responses did not parallel the changes in $[Ca^{2+}]_i$. This separation was particularly relevant in experiments performed at low and high $[Ca^{2+}]_o$. In 2.5 mM $[Ca^{2+}]_o$, furnidipine or CgTx reduced the K^+-evoked increase of $[Ca^{2+}]_i$ by around 50%. Secretion was fully blocked by furnidipine in spite of the fact that the DHP blocked by only 50% the $[Ca^{2+}]_i$ increase. Conversely, the 50% reduction of the $[Ca^{2+}]_i$ signal by CgTx in 2.5 mM $[Ca^{2+}]_o$, or by a tenfold lowering of the $[Ca^{2+}]_o$, did not affect the secretory response. On the other hand, although 0.25 mM $[Ca^{2+}]_o$ and furnidipine reduced the $[Ca^{2+}]_i$ by around 40% with respect to the $[Ca^{2+}]_i$ increase induced by 2.5 mM $[Ca^{2+}]_o$, 0.25 mM $[Ca^{2+}]_o$ reduced secretion by 37%, whilst furnidipine completely blocked secretion. Thus, it seems clear that not all the procedures used to reduce the K^+-evoked increase in $[Ca^{2+}]_i$ by approximately 50%, produced a similar degree of secretion blockade. Therefore, it seems that the elevation of the averaged $[Ca^{2+}]_i$ is not sufficient to trigger an efficient secretory response. When such elevation is secondary to L-type Ca^{2+} channel activation the secretory mechanism is fully activated; on the contrary, Ca^{2+} entry through N-type channels led to a poor secretory response. The most plausible explanation for this observation is that L-type Ca^{2+} channels are located adjacent to secretory sites and that N-type Ca^{2+} channels are away from those sites. In contrast, noradrenaline release from sympathetic neurons seems to be dominated by N-type Ca^{2+} channels [26]. At present, we have no explanation for the control

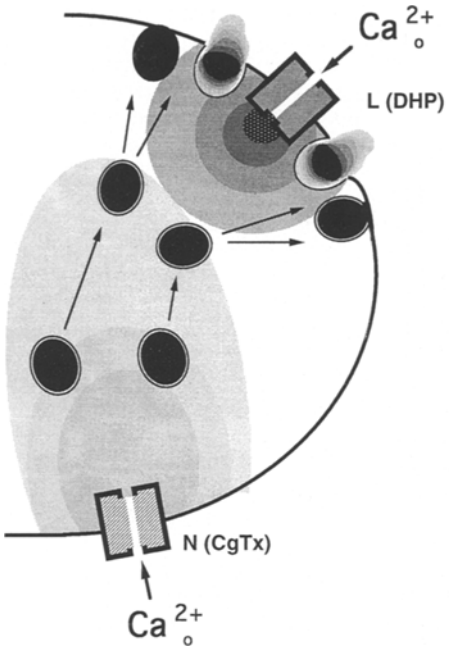

Fig. 6. Scheme showing that the access of extracellular Ca^{2+} to the cat chromaffin cell cytosol can follow two distinct pathways. The L-type of Ca^{2+} channel pathway is located adjacent to the secretory machinery to provide highly localized increases of $[Ca^{2+}]_i$ at subplasmalemmal sites, to trigger a fast release of catecholamines from docked vesicles. The N-type of Ca^{2+} channels [N(CgTx)] are located away from active secretory zones; the external Ca^{2+} entering through them is likely to serve as an earlier step of exocytosis, the transport of vesicles from a reserve pool to secretory plasmalemmal sites, requiring lower increases of $[Ca^{2+}]_i$ [24, 32]

of exocytosis by N- or L-type channels in two catecholaminergic cells as closely related.

An alternative explanation could lie in a much higher density of L-versus N-type channels. However, the opposite seems to be true. A single bovine chromaffin cell contains around 18 000 N-type channels [6, 9] and less than 2000 L-type channels [15]. In spite of this, the physiological acetylcholine-mediated response in these cells was fully insensitive to CgTx, was blocked by 70% by nisoldipine and was drastically potentiated by Bay K 8644 [27]. Therefore, these findings support once more the idea that L-type Ca^{2+} channels are highly localized to active zones were exocytosis occurs. The tenfold higher density of N-type Ca^{2+} channels present in these cells seems to control, if anything, only a very minor component of the overall secretory response. Thus, it is plausible that extracellular Ca^{2+} entering through CgTx-sensitive Ca^{2+} channels bypasses the subplasmalemmal exocytotic sites travelling to inner parts of the cell. In fact, extracellular Ca^{2+} may transmit signals to the nucleus of depolarized sympathetic neurons [25] and regulate gene transcription [8]. A similar specialization has just been suggested in hippocampal neurons, where Ca^{2+} entry through *N*-methyl-D-aspartate receptors or through L-type Ca^{2+} channels activates two distinct Ca^{2+} signalling pathways [8].

Our results could also be explained in the frame of a recent hypothesis on a two-step model of secretion in chromaffin cells [24]. In this model, not only the rate of consumption (exocytosis), but also the rate of vesicle supply to the pool of release-ready granules are Ca^{2+} dependent. However, a fast rate of exocytosis requires a higher $[Ca^{2+}]_i$ than the vesicle transport to subplasmalemmal sites [32]. It may well be that in cat chromaffin cells, the higher $[Ca^{2+}]_i$ serving exocytosis can be reached very quickly because L-type Ca^{2+} channels are strategically located near the secretory active sites in the plasmalemma. In its turn, Ca^{2+} entering through N-type Ca^{2+} channels located away from secretory sites will reach only milder $[Ca^{2+}]_i$ elevations at deeper areas of the cell cytosol where vesicle transport takes place (see scheme in Fig. 6).

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