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Q- and L-type Ca^{2+} channels dominate the control of secretion in bovine chromaffin cells

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Abstract

Potassium-stimulated catecholamine release from superfused bovine adrenal chromaffin cells (70 mM K^+ in the presence of 2 mM Ca^{2+} for 10 s, applied at 5-min intervals) was inhibited by the dihydropyridine furnidipine (3 μM) by 50%. ω-Conotoxin MVIIC (CTx-MVIIC, 3 μM) also reduced the secretory response by about half. Combined CTx-MVIIC plus furnidipine blocked 100% catecholamine release. $^{45}Ca^{2+}$ uptake and cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in K^+-depolarized cells were partially blocked by furnidipine or CTx-MVIIC, and completely inhibited by both agents. The whole cell current through Ca^{2+} channels carried by Ba^{2+} (I_{Ba}) was partially blocked by CTx-MVIIC. Although ω-conotoxin GVIA (CTx-GVIA, 1 μM) and ω-agatoxin IVA (Aga-IVA, 0.2 μM) partially inhibited $^{45}Ca^{2+}$ entry, I_{Ba} and the increase in $[Ca^{2+}]_i$, the combination of both toxins did not affect the K^+-evoked secretory response. The results are compatible with the presence in bovine chromaffin cells of a Q-like Ca^{2+} channel which has a prominent role in controlling exocytosis. They also suggest that Q- and L-type Ca^{2+} channels, but not N- or P-types are localized near exocytotic active sites in the plasmalemma.

Key words: Ca^{2+} channels; Chromaffin cell; Control of secretion

1. Introduction

On pharmacological grounds, multiple subtypes of voltage-dependent Ca^{2+} channels have been described in neurones [1]. In the light of this growing diversity, the question arises as to which Ca^{2+} channel subtype controls the release of a given neurotransmitter in central and peripheral synapses. This question is particularly relevant in secretory cells endowed with various subtypes of Ca^{2+} channels, such as bovine chromaffin cells [2]. Catecholamine release from the intact bovine adrenal gland is insensitive to ω-conotoxin GVIA (CTx-GVIA), and only partially sensitive to dihydropyridine (DHP) L-type Ca^{2+} channel blockers [3,4]. It seems, therefore, that secretion might be partly controlled by additional Ca^{2+} channels other than the N- and L-types present in bovine chromaffin cells. One possibility is that P-like Ca^{2+} channels contribute to the control of the secretory process as well. In fact, in two recent reports, we demonstrated the presence of P-like Ca^{2+} channels in bovine chromaffin cells and suggested such a possibility [5,6].

A new toxin, from the marine snail *Conus magus*, ω-conotoxin MVIIC (CTx-MVIIC), has recently been characterized [7]. Although P- as well as N-type Ca^{2+} channels seem to recognize this toxin, a novel additional component of the whole-cell Ca^{2+} current is also blocked by CTx-MVIIC. This has led to the belief that this toxin can recognize a new subtype of neuronal Ca^{2+} channels, named Q [8] or O [9]. We were surprised to observe that the combination of CTx-MVIIC plus furnidipine, a novel DHP blocking L-type Ca^{2+} channels in chromaffin cells [5,6], but not of furnidipine plus Aga-IVA or CTx-GVIA, caused a full blockade of K^+-evoked catecholamine release from bovine chromaffin cells. In our hands, such drastic blockade could not be achieved until now using a combination of DHPs and the toxins available before CTx-MVIIC. In this article, we present those secretion experiments as well as the effects of CTx-MVIIC, furnidipine, CTx-GVIA and Aga-IVA on $^{45}Ca^{2+}$ uptake, on whole-cell Ba^{2+} currents, and on the changes in cytosolic Ca^{2+} concentrations, $[Ca^{2+}]_i$, induced by depolarizing stimuli in bovine chromaffin cells. To our knowledge, this is the first report demonstrating directly the participation of a Q subtype of Ca^{2+} channels in the regulation of the release of a chemically identified transmitter. In addition, this study emphasizes the specialization of multiple Ca^{2+} channels, as well as their highly precise location to serve different functions in the same cell.

2. Materials and methods

Bovine adrenal medullary chromaffin cells were isolated following standard methods [10] with some modifications [11]. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxiuridine, 50 IU · ml^{-1} penicillin and 50 $\mu g \cdot ml^{-1}$ streptomycin. For $^{45}Ca^{2+}$ uptake experiments, cells were plated at a density of 5×10^5 cells/well in 24-multiwell Costar plates. For the measurements of whole-cell Ba^{2+} currents and the $[Ca^{2+}]_i$ changes, cells were plated on glass

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coverslips at a density 5×10^4 cells per 0.8 cm diameter coverslip. For secretion experiments, 5×10^6 cells were plated on plastic 3 cm diameter Petri dishes containing 10 ml DMEM. Cells were kept in a water-saturated incubator at 37°C, in a 5% CO_2/95% air atmosphere, and used 1–4 days thereafter.

$^{45}Ca^{2+}$ uptake into K^+-depolarized chromaffin cells was studied according to Gandía et al. [12]. Catecholamine release was measured by placing the cells inside a microchamber and superfusing them with a Krebs-HEPES solution; catecholamine release in response to K^+ depolarization was measured on-line with an electrochemical detector [13]. Changes of $[Ca^{2+}]_i$ after K^+ depolarization were measured in fura-2-loaded single chromaffin cells, according to Grynkiewicz et al. [14] using the microfluorometric device described by Neher [15]. Whole-cell Ba^{2+} currents were measured using the patch-clamp technique [16] with the modifications and protocols previously described [5,6]. All experiments were performed at room temperature (22 ± 3°C).

The following materials were used: collagenase from *Clostridium histolyticum* (Boehringer-Mannheim); bovine serum albumin fraction V, EGTA (Sigma); scintillation fluid Ready micro (Beckman); ^{45}Ca (specific activity 10–40 mCi·mg^{-1} calcium, Amersham). Fura-2 AM was obtained from Molecular Probes, Eugene, Oregon, USA. Furnidipine was from Laboratorios Alter (Madrid, Spain). Aga-IVA was from Peptide Institute (Osaka, Japan). CTx-GVIA and CTx-MVIIC were purchased from Bachem (UK) Ltd. (Essex, UK).

3. Results

3.1. Effects of blockers of various Ca^{2+} channel subtypes on K^+-evoked catecholamine release

Fig. 1 shows copies of original traces reflecting the continuous electrochemical detection of the rate of catecholamine release from superfused bovine chromaffin cells. The basal rate of secretion was stable for up to 3 h. Application of depolarizing pulses (70 Mm K^+, isosmotic reduction of NaCl) for 10 s in the presence of 2 mM Ca^{2+} at 5-min intervals, produced secretion peaks which were reproducible when repeated at least 10 times (not shown). These conditions allowed the sequential testing of various combinations of Ca^{2+} channel blockers in the same batch of cells.

Panel A in Fig. 1 shows an experiment where effects on secretion of the sequential addition of various Ca^{2+} channel blockers were studied. Furnidipine (3 μM) reduced the secretory responses by about 50%. When added on top of furnidipine, Aga-IVA (100 nM) plus CTx-GVIA (1 μM) did not reduce the secretion further. Panel B shows that the superfusion of combined CTx-GVIA plus Aga-IVA did not affect the initial K^+ secretory responses. Addition of furnidipine on top of those two toxins approximately halved the release of catecholamines. Panel C illustrates the initial partial blocking effect of furnidipine, and the practical abolition of any secretory signal when CTx-MVIIC (3 μM) was added on top of the DHP. Finally, panel D shows the initial partial blocking effects of CTx-MVIIC and the full inhibition of secretion upon addition of furnipine on top of the toxin. The partial recovery of the secretory response upon washout of CTx-MVIIC and furnidipine (panels C and D) may account for the slow reversibility of the blocking effects on L-type Ca^{2+} channels induced by furnidipine [5,6]. This view is strengthened by the fact that the inhibition of secretion by CTx-MVIIC alone was not reversed upon 50-min washout of the toxin, while the blockade induced by furnidipine was greatly reversed (not shown).

3.2. Correlation between blockade of Ca^{2+} entry and secretion

Ca^{2+} entry was tested by stimulation of chromaffin cells with a solution containing 70 mM K^+, 1 mM Ca^{2+} and 6 μCi·ml^{-1} of $^{45}Ca^{2+}$ during 60 s. Averaged results of $^{45}Ca^{2+}$ taken up by cells were compared with averaged catecholamine release results. Fig. 2 shows that furnidipine (3 μM) blocked Ca^{2+} uptake by 30% and catecholamine release by 50%. CTx-MVIIC inhibited Ca^{2+} uptake by as much as 76% but blockade of secretion was similar to that of furnidipine (45%). The combination of furnidipine plus CTx-MVIIC led to 99% inhibition of Ca^{2+} entry and 98% inhibition of secretion.

Combined Aga-IVA (100 nM) and CTx-GVIA (1 μM) produced as much as 65% blockade of Ca^{2+} entry yet secretion was not significantly affected (9% reduction). The combination of these two toxins with furnidipine produced an additive effect on Ca^{2+} uptake (87% blockade) but not on secretion (50% blockade). Thus, it seems clear that a dissociation exists between the effects of the blockers on Ca^{2+} entry and secretion. All of them reduced Ca^{2+} entry to some extent and their effects were additive. However, only CTx-MVIIC and furnidipine inhibited secretion by themselves and, when combined, their blocking effects were additive, causing a full blockade of the secretory response.

3.3. Blockade of whole-cell Ba^{2+} currents

Although the sequential addition of CTx-GVIA, Aga-IVA and furnidipine blocked different components of whole-cell Ba^{2+} currents in bovine chromaffin cells, a small residual current (10–20%) was left unblocked [6]. Thus, the possibility was raised that CTx-MVIIC could block this residual component, and that in addition to L-, N- and P-type Ca^{2+} channels, bovine chromaffin cells could also express Q-type Ca^{2+} channels.

Whole cell Ba^{2+} currents through Ca^{2+} channels (I_{Ba}) were recorded in the presence of 5 mM Ba^{2+} as the charge carrier. Effects of the different Ca^{2+} channel blockers were studied by fast superfusion of chromaffin cells with toxin-containing solutions. When first given, CTx-MVIIC (3 μM) blocked a large fraction of I_{Ba} (63 ± 2%; $n = 6$). Upon washing out the toxin from the perfusion solution, a slow recovery was observed (Fig. 3A), probably reflecting the unblocking of N-type Ca^{2+} channels. If CTx-GVIA (1 μM) and/or Aga-IVA (200 nM) were then superfused, the remaining current was hardly inhibited. Finally, furnidipine (3 μM) caused a small additional blockade of the residual current.

More critical was the experiment in which CTx-GVIA and Aga-IVA were applied before CTx-MVIIC. Three

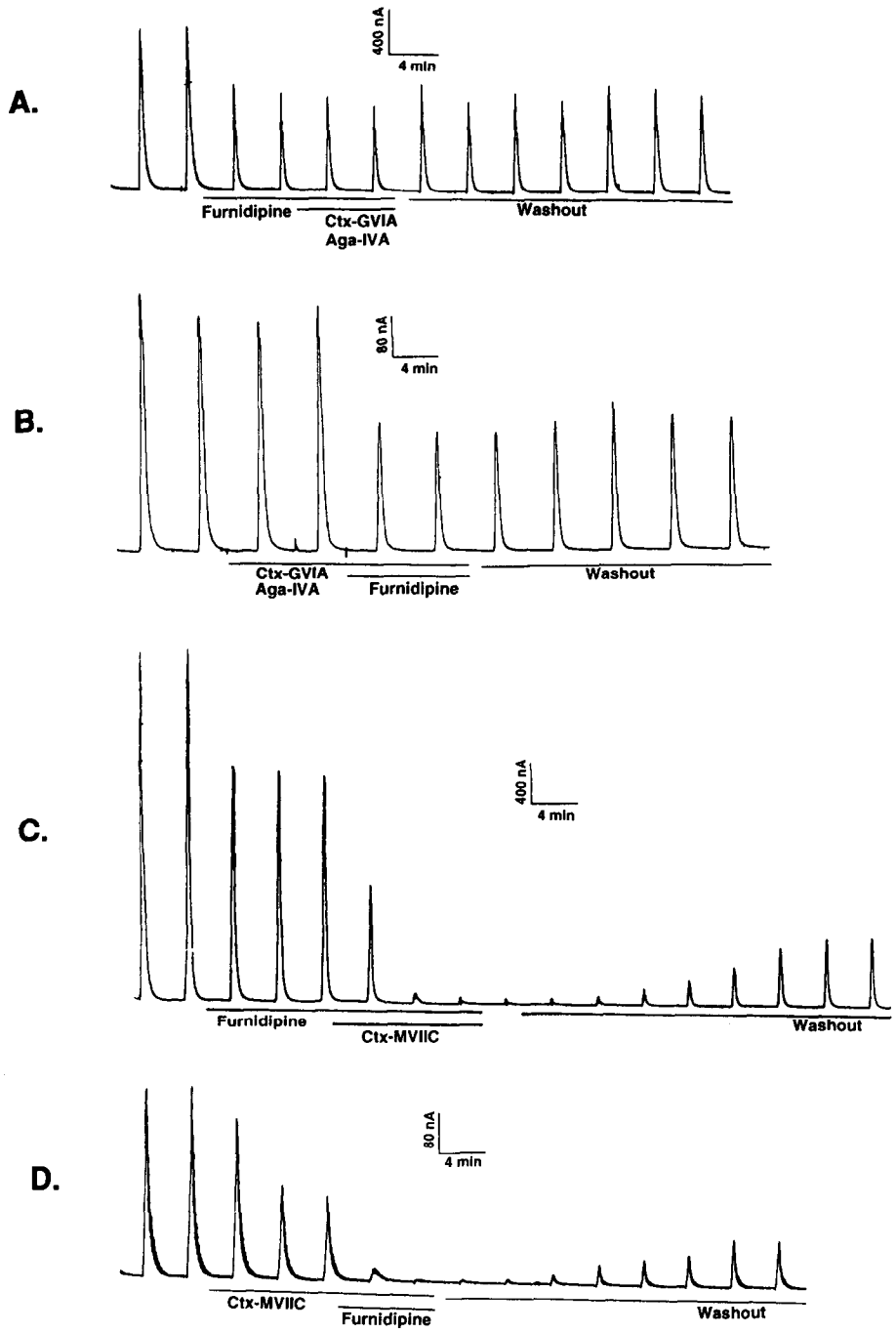

Fig. 1. Effects of the sequential addition of blockers of various Ca^{2+} channel subtypes on catecholamine release responses from chromaffin cells (5×10^6 cells in each experiment) superfused with Krebs–HEPES solution and stimulated for 10 s with a solution containing 70 mM K^+ (isosmotic reduction of NaCl) and 2 mM Ca^{2+}. Furnidipine (3 μM), CTx-GVIA (1 μM), Aga-IVA (100 nM) and CTx-MVIIC (3 μM) were present during the time periods indicated by the horizontal lines at the bottom of panels A, B, C and D, respectively. Catecholamine release is expressed as nA of oxidation current (see calibration bars).

clear-cut inhibitory steps were seen upon addition of each of the three toxins (Fig. 3B). Combined CTx-GVIA plus Aga-IVA blocked I_{Ba} by 52 ± 8% (n = 5). The fact that CTx-MVIIC alone blocked 63% of the current suggests that this last toxin might be blocking an additional component of the whole cell current. This was confirmed in experiments where CTx-MVIIC was superfused once the irreversible blockade of CTx-GVIA and Aga-IVA was reached (Fig. 3B). Under these conditions, CTx-MVIIC inhibited the remaining current by 42 ± 3% (n = 5). Thus, it seems that CTx-MVIIC blocks an additional component, not associated to the CTx-GVIA-,

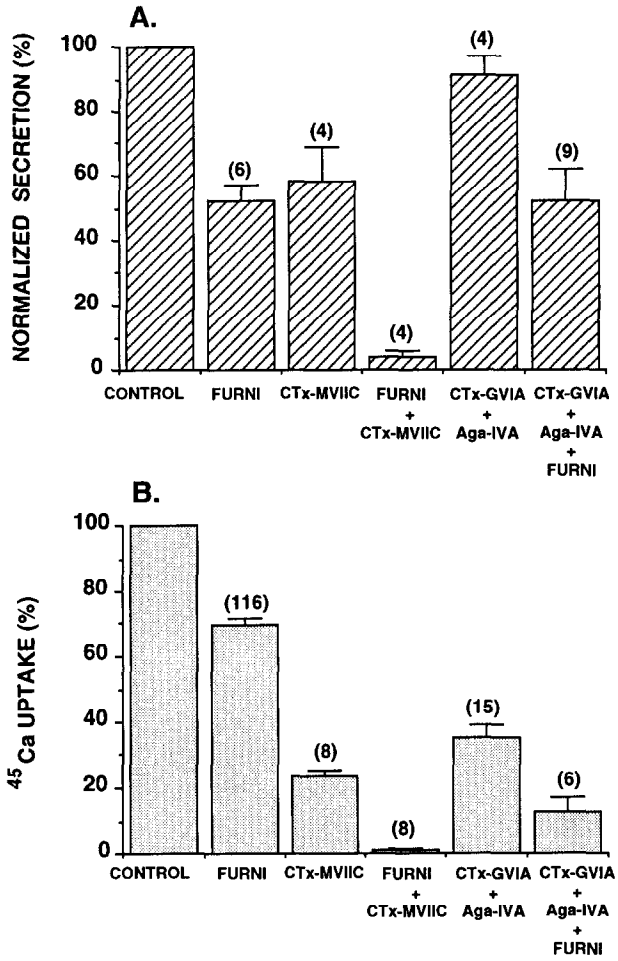

Fig. 2. Averaged data on the effects of various combinations of Ca^{2+} channel blockers on catecholamine release responses (A) and $^{45}Ca^{2+}$ uptake (B) into K^+ depolarized bovine adrenal chromaffin cells. Secretion data were obtained from experiments using protocols similar to those in Fig. 1. $^{45}Ca^{2+}$ uptake was measured in 5×10^5 cells attached to the bottom of 24-well plates. Cells were stimulated for 60 s with 70 mM K^+ (NaCl isoosmotically reduced) in the presence of 1 mM Ca^{2+} and 6 $\mu Ci \cdot ml^{-1}$ $^{45}Ca^{2+}$. Data are expressed as a % of the initial control responses. In the case of $^{45}Ca^{2+}$ uptake experiments, furnidipine (FURNI, 3 μM), CTx-GVIA (1 μM), CTx-MVIIC (3 μM) and Aga-IVA (100 nM) were present 10 min before, and during, the stimulation period. Data are means ± S.E.M. of the number of experiments shown in parentheses.

Aga-IVA- and DHP-sensitive components; this may be associated to the Q-type Ca^{2+} channel similar to that described in hippocampal neurones [8].

3.4. *Blockade of the increase in cytosolic Ca^{2+} concentrations*

The increase in $[Ca^{2+}]_i$ induced by depolarizing pulses (70 mM K^+, 2 mM Ca^{2+} applied for 10 s at 5-min intervals) was studied in single fura-2-loaded chromaffin cells. Fig. 4A shows a prototype experiment. CTx-MVIIC reduced by around 60% the peak increase of the $[Ca^{2+}]_i$ induced by K^+. Addition of furnidipine on top of CTx-MVIIC caused a full blockade of the Ca^{2+}_i signal, which partially recovered upon washing out. Fig. 4B shows averaged results from various cells. Furnidipine (3 μM) and CTx-MVIIC (3 μM) reduced the increase in $[Ca^{2+}]_i$ by around 50%. The combination of both compounds suppressed the increase in $[Ca^{2+}]_i$. Combined CTx-GVIA plus Aga-IVA reduced the $[Ca^{2+}]_i$ around 50% and CTx-GVIA + Aga-IVA + furnidipine left about 20% of the $[Ca^{2+}]_i$ signal unblocked.

4. Discussion

By using as tools selective toxins which block various Ca^{2+} channel subtypes in neurones [1] we have recently come to the conclusion that bovine adrenal medulla chromaffin cells contain L-, N- as well as P-type Ca^{2+} channels. In studies of the secretory process, however, the combination of a DHP with CTx-GVIA and Aga-IVA always left unblocked a fraction of the whole-cell Ba^{2+} current. Now, we have demonstrated that an additional component of I_{Ba} can be blocked by CTx-MVIIC, a toxin that exhibits high affinity for P-type Ca^{2+} channels, lower affinity for N-type Ca^{2+} channels, and very high affinity for a novel Ca^{2+} channel subtype in neurones named Q [7,8].

The presence of such diversity of Ca^{2+} channels in chromaffin cells has given rise to the hypothesis concerning their role in controlling the exocytotic release of catecholamines, as well as to general questions related to the specialization of such Ca^{2+} channels to serve other functions in addition to secretion. We first reported a full blockade of secretion by submicromolar concentrations of DHPs in the cat adrenal gland [17,18]. For 10 years, we believed that these cells expressed only L-type Ca^{2+} channels. However, we have recently demonstrated that cat chromaffin cells also express CTx-GVIA-sensitive Ca^{2+} channels in about equal proportion to L-type channels [19]. We have demonstrated that these two types of Ca^{2+} channels contribute equally to the increase in $[Ca^{2+}]_i$ in K^+-depolarized single cat chromaffin cells, yet secretion is mostly controlled by L-type Ca^{2+} channels. On this basis we have proposed the hypothesis that L channels are closely located to secretory sites at the plasmalemma, but N channels are segregated from the secretory machinery, most likely serve other non-secretory functions [20].

The picture is more complex in bovine chromaffin cells, where L-, N-, P-, as well as Q-type (this study) Ca^{2+} channels seem to be present. The finding that CTx-MVIIC blocks a new component in the whole-cell I_{Ba} and that these channels make an important contribution to secretion, might resolve a previous puzzling observation: that the catecholamine release responses in intact bovine adrenal glands are insensitive to CTx-GVIA and only partially sensitive to DHPs [3,4]. After demonstrating an

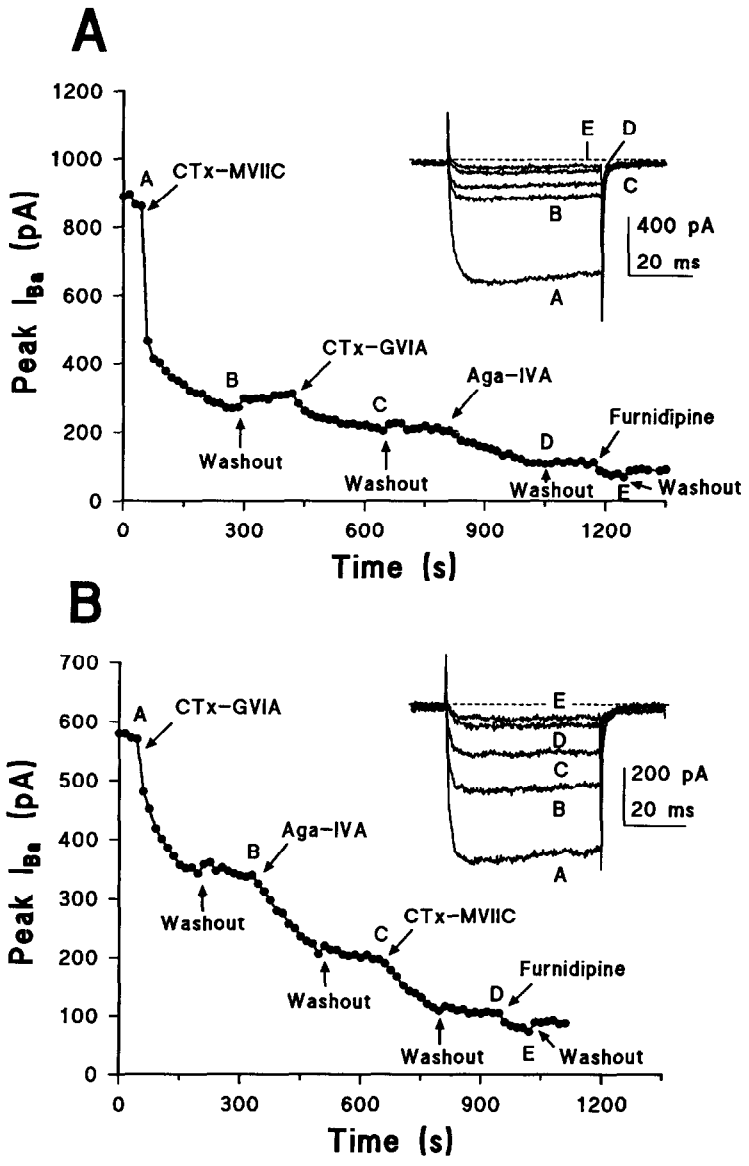

Fig. 3. Effects of CTx-MVIIC on whole-cell Ba^{2+} currents. Panel A shows the time-course for the blocking effects of CTx-MVIIC (3 μM) applied to an untreated bovine chromaffin cell. Further addition of CTx-GVIA (1 μM) and Aga-IVA (0.2 μM) induced additional blocking effects. At the end, furnidipine (3 μM) was superfused to block L-type Ca^{2+} channels. Depolarizing pulses (50 ms to 0 mV) were applied from a holding potential of −80 mV at 15 s intervals. The inset shows the original current traces obtained at the points indicated in the figure. In panel B, CTx-MVIIC was applied after the irreversible blocking effects of CTx-GVIA and Aga-IVA were established. The inset shows the original current records obtained at the points indicated in the figure.

Aga-IVA-sensitive component of I_{Ba} we suggested that the missing second channel controlling secretion in bovine chromaffin cells could be a P-like Ca^{2+} channel [6]. However, we demonstrate here that secretion was also insensitive to Aga-IVA (Figs. 1 and 2). The availability of the novel toxin CTx-MVIIC has facilitated the identification of the missing link between Ca^{2+} channel diversity and catecholamine release in bovine chromaffin cells. Thus, the present results support the idea that these cells express four pathways for extracellular Ca^{2+} entry into their cytosol, but that only two (the L- and the Q-type Ca^{2+} channels) control the secretory process through the regulation of the access of external Ca^{2+} to the secretory machinery.

Our present results and interpretation disagree with those presented in a recent study in which whole-cell Ba^{2+} currents and membrane capacitance as an index of exocytosis were measured [21]. This study concluded that the whole-cell Ca^{2+} current in bovine chromaffin cells could be resolved into only three components, L, N and P; these authors concluded that although the L-type Ca^{2+} channels dominate the control of secretion, P- as well as

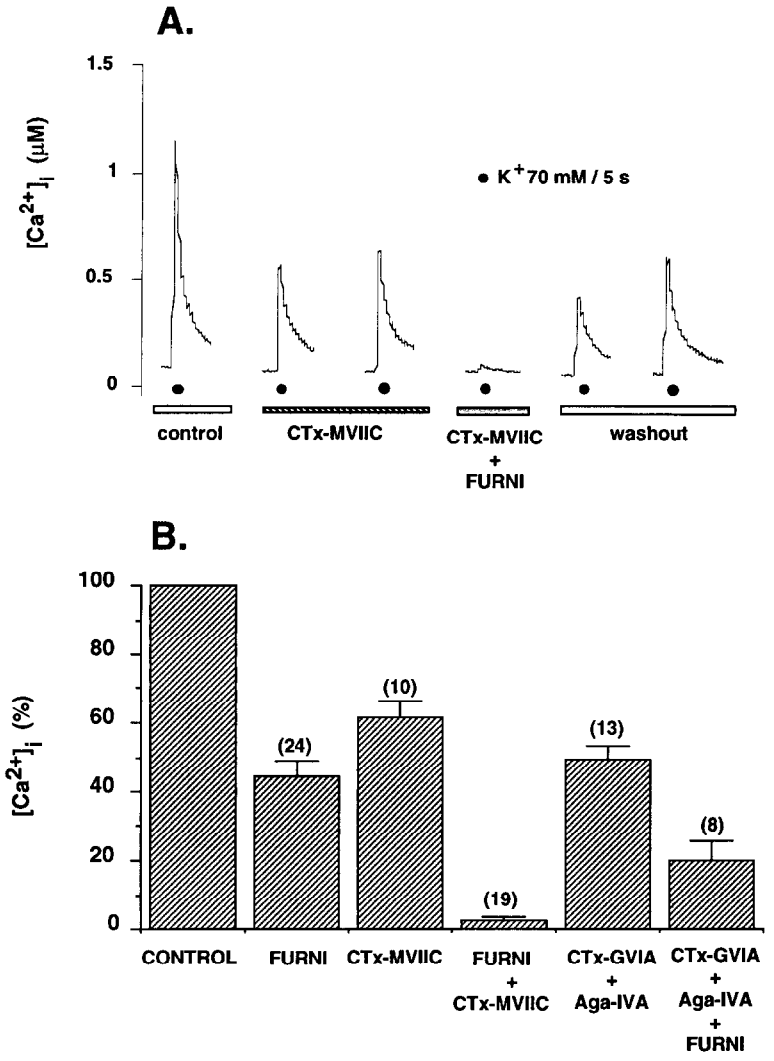

Fig. 4. Effects of different Ca^{2+} channel blockers on the changes of $[Ca^{2+}]_i$ induced by depolarizing pulses (70 mM K^+ + 1 mM Ca^{2+} for 10 s) applied to single bovine chromaffin cells loaded with fura-2, at 5-min intervals. Panel A represents a typical tracing of one of these experiments. After two reproducible control responses, 3 μM CTx-MVIIC was applied to the cell; under these conditions, the cell was stimulated twice with 70 mM K^+ at 5 min intervals and finally furnidipine (3 μM) was added together with the toxin. The last two pulses correspond to the washout of the compounds. Panel B summarizes the mean effect ± S.E.M. of the DHP, furnidipine and toxins, used alone or in combination. Data are normalized as the percentage of the $[Ca]_i$ increase obtained in the absence of the drugs. On top of each bar, the number of experiments is indicated in parentheses.

N channels also contribute to exocytosis. We do not know the basis for this discrepancy. In the present experiments, we are measuring the exocytotic activity of the secretory machinery directly through on-line electrochemical detection of catecholamines being released in response to each depolarizing pulse. In contrast, capacitance indirectly measures exocytosis as well as endocytosis. In addition, we are complementing our patch-clamp measurements of I_{Ba} with direct measurements of $^{45}Ca^{2+}$ entry into K^+-depolarized cells as well as on $[Ca^{2+}]_i$ changes in experimental conditions which allow a better equilibration of toxins with the 'receptor' sites on the bovine chromaffin cell. However, in spite of these methodological and protocol considerations, the differences between the conclusions of Artalejo et al. [21] and our conclusion cannot be reconciled with two facts emerging from the present study: first, that bovine chromaffin cells seem to contain an additional Q channel subtype; and second, that this channel contributes to the control of secretion in a manner much more important than that of N- and P-types Ca^{2+} channels.

In intact adrenal medullary tissue, chromaffin cells directly release their catecholamines into the circulation. Thus, the secretory active sites must be located at the cell surface facing the blood vessels. This geographic specialization of secretion strongly suggests that Q- and L-type Ca^{2+} channels must also be located at this secretory surface to quickly deliver the Ca^{2+} required for activation

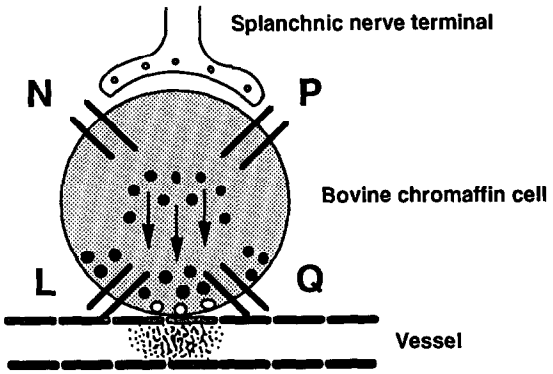

Fig. 5. Schematic diagram of the cholinergic nerve terminal–chromaffin cell synapse in the intact adrenal gland. Proposal of a model for a distinct geographic localization of Ca^{2+} channel subtypes in chromaffin cells, to explain the selective blockade of secretion by agents acting on L- or Q-type Ca^{2+} channels, but not by those acting on P- or N-type Ca^{2+} channels. Channels located away from the secretory machinery might provide Ca^{2+} for functions other than exocytosis, requiring lower concentrations of the cation, i.e. chromaffin vesicle transport from the Golgi apparatus to active secretory sites underneath the plasmalemma, activation of catecholamine-synthesizing enzymes by Ca^{2+}-dependent phosphorylation or gene expression.

of the secretory machinery during stressful activities. N- and P-like Ca^{2+} channels might be located away from the secretory sites (see Fig. 5) to serve other Ca^{2+}-dependent cell functions, i.e. the intracellular transport of organelles, including chromaffin vesicles, to active secretory sites nearby the plasmalemma [22], the synthesis of catecholamines or the expression of genes.

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