Multiple Calcium Pathways Induce the Expression of SNAP-25 Protein in Chromaffin Cells

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Abstract: Incubation of bovine adrenal chromaffin cells in high K^+ (38 m*M*) during 24–48 h enhanced 2.5 to five times the expression of SNAP-25 protein and mRNA, respectively. This increase was reduced 86% by furnidipine (an L-type Ca^{2+} channel blocker) but was unaffected by either ω-conotoxin GVIA (an N-type Ca^{2+} channel blocker) or ω-agatoxin IVA (a P/Q-type Ca^{2+} channel blocker). Combined blockade of N and P/Q channels with ω-conotoxin MVIIC did, however, block by 76% the protein expression. The inhibitory effects of furnidipine were partially reversed when the external Ca^{2+} concentration was raised from 1.6 to 5 m*M*. These findings, together with the fact that nicotinic receptor activation or Ca^{2+} release from internal stores also enhanced SNAP-25 protein expression, suggest that an increment of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), rather than its source or Ca^{2+} entry pathway, is the critical signal to induce the protein expression. The greater coupling between L-type Ca^{2+} channels and protein expression might be due to two facts: (a) L channels contributed 50% to the global $[Ca^{2+}]_i$ rise induced by 38 m*M* K^+ in indo-1-loaded chromaffin cells and (b) L channels undergo less inactivation than N or P/Q channels on sustained stimulation of these cells. **Key Words:** Chromaffin cells—Calcium channels—SNAP-25.
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The presence of L, N, and P/Q subtypes of high-threshold-activated, voltage-dependent Ca^{2+} channels in neurons (Nowycky et al., 1985; Llinás et al., 1989; Olivera et al., 1994) poses the interesting question of their specialization to control different cell functions. In neurons, N- and P/Q-type channels are predominantly found along the length of apical dendrites; these channels are found also in axon terminals forming synapses on dendrites (Westenbroek et al., 1992), where they control the release of various neurotransmitters (Olivera et al., 1994; Wheeler et al., 1994; García et al., 1996). However, L-type channels, predominantly located in the cell body and proximal dendrites (Ahlijanian et al., 1990; Westenbroek et al., 1990), have been associated with nonexocytotic functions such as the regulation of gene expression and enzyme activity in cortical and hippocampal neurons (Murphy et al., 1991; Bading et al., 1993; Elliot et al., 1995; Westenbroek et al., 1995; Deisseroth et al., 1998).

Because of this asymmetric distribution of Ca^{2+} channel subtypes in neurons (soma, dendrites, or axon terminals), it is easy to understand such type of specialization. However, in spherical bovine adrenal medullary chromaffin cells that, like neurons, also express L, N, and P/Q subtypes of channels (Gandía et al., 1993; Albillos et al., 1996; Lomax et al., 1997), it is harder to assign specific functions to each channel type only on the basis of a different regional distribution. In spite of this limitation, attempts have been made to assign special functions to each channel subtype, as far as its role in mediating the K^+-evoked release of catecholamine is concerned. Thus, Ca^{2+} entering through L- and P/Q-type channels triggers exocytosis, whereas Ca^{2+} entering through N-type channels does not (López et al., 1994*b*). Furthermore, using various Ca^{2+} gradients we provided evidence favoring the idea that in bovine chromaffin cells, P/Q channels are closer to secretory vesicles than L channels (Lara et al., 1998).

Considering these data, it seems that bovine chromaffin cells are an archetypical model to study whether the elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by Ca^{2+} entering through one or the other Ca^{2+}

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Abbreviations used: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_o$, external Ca^{2+} concentration; DMPP, dimethylphenylpiperazinium; SNAP-25, synaptosomal-associated protein of 25 kDa.

channel subtype, or on the release of Ca^{2+} from intracellular stores, is important to regulate functions unrelated with the immediate exocytotic event. To explore this question we studied the expression of synaptosomal-associated protein of 25 kDa (SNAP-25), one of the critical proteins of the SNAP–receptor complex (SNARE). This protein forms part of the exocytotic machinery in neurons (Blasi et al., 1993; Binz et al., 1994) and in bovine chromaffin cells (Gutiérrez et al., 1995, 1997; Foran et al., 1996; Kannan et al., 1996; Lawrence et al., 1997), and its expression is enhanced by the rise of $[Ca^{2+}]_i$ on depolarization of hippocampal explants and PC12 cells (Sepúlveda et al., 1998). We have therefore measured the expression of this protein after exposing the chromaffin cells to depolarizing concentrations of K^+ to activate the different subtypes of Ca^{2+} channels; also, the protein levels were determined on nicotinic receptor activation of the cells with dimethylphenylpiperazinium (DMPP) or with caffeine, which induces the release of Ca^{2+} from intracellular stores. All these maneuvers produce the rise of $[Ca^{2+}]_i$ in bovine chromaffin cells, a signal for gene expression in many cell types (Bito et al., 1997; Finkbeiner and Greenberg, 1997; Ginty, 1997; Hardingham et al., 1997). Dissection of the various Ca^{2+} entry pathways has been achieved through the use of selective blockers of the different subtypes of voltage-dependent Ca^{2+} channels.

EXPERIMENTAL PROCEDURES

Cell culture and treatments

Bovine adrenal chromaffin cells were isolated following standard methods (Livett, 1984) with some modifications (Moro et al., 1990). Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Cells (1.8×10^7) were plated on plastic 10-cm-diameter culture plates at a density of 1×10^6/ml or on 25-mm-diameter glass coverslips at a density of 2.5×10^4 cells/ml, for the immunoblot and cytosolic Ca^{2+} experiments, respectively. Cells were kept in a water-saturated incubator at 37°C, in a 5% CO_2/95% air atmosphere. Treatments with high K^+ for SNAP-25 expression experiments were carried out in cells after 1 day in culture. To obtain a final concentration of 38 m*M* KCl, 2 ml of an osmotically balanced solution with a high concentration of K^+ (170 m*M* KCl, 0.9 m*M* $CaCl_2$, 1.3 m*M* $MgCl_2$, and 10 m*M* HEPES, pH 7.4) was added to 8 ml of Dulbecco's modified Eagle's medium. In all experiments, except when indicated, the final concentration of external Ca^{2+} used was 1.6 m*M*. Control cells were manipulated as experimental cells, but they were incubated in a medium in which high KCl has been replaced by NaCl. For the cytosolic Ca^{2+} experiments, cells after 2–3 days in culture were used.

Isolation of RNA and northern blot analysis

Total RNA was extracted from chromaffin cells using a guanidinium-thiocyanate method (Chomczynski and Sacchi, 1987). To assess that equal amounts of the two RNA samples were loaded onto the gel, the fluorescence intensities of 28S and 18S ribosomal RNAs were monitored. Northern blot analysis was performed as described previously (Lara et al., 1990) by hybridization with an 881-bp complementary probe synthesized from a mouse SNAP-25 cDNA cloned in the *Eco*RI site of pBluescript (Oyler et al., 1989). The RNA probe was obtained from 500 ng of *Sal*I-linearized plasmid using a Promega transcription kit with T_7 polymerase and 100 μCi of [α-^{32}P]CTP (New England Nuclear–DuPont, Claremont, CA, U.S.A.). Hybridization was done with 2×10^6 cpm/10 ml of the antisense probe (10^9 cpm/μg). Membranes were washed, air-dried, and exposed to Kodak Biomax-MS film for 3 days. Autoradiograms were analyzed by densitometry using an Action Scanner II (Epson) to digitize the image. Results are expressed as the ratio of SNAP-25 signals in 38 m*M* K^+-treated cells compared with those in control 38 m*M* Na^+-treated cells.

Immunoblot analysis

Equal amounts of cell lysate proteins (typically 70 μg of total proteins) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in Mini-Protean electrophoresis cells (Bio-Rad) using the Laemmli buffer system (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose membranes by standard procedures. Membranes were incubated with a monoclonal anti-SNAP-25 antibody (Sternberger Monoclonals) diluted in phosphate-buffered saline (1:1,000) for 1 h at room temperature. After washing, the blots were incubated for 1 h with a secondary antibody labeled with horseradish peroxidase, and the immunoblot was developed with 4-chloro-1-naphthol as substrate. To compare changes in the expression of SNAP-25, the blots were scanned, and the density and area of each band were determined with the NIH Image Version 1.6 program. Results are expressed as mean $\pm$ SEM values of the relative increase of SNAP-25 expression in depolarized cells in relation to control cells (considered as 1). When the effects of the different Ca^{2+} channel blockers on SNAP-25 expression induced by high K^+ were considered, the data are represented as percentages of expression induced by the depolarizing stimulus in the presence of the blocker, compared with the K^+-induced expression (defined as 100%).

Cytosolic calcium measurements

Chromaffin cells plated on coverslips were incubated with 10 μg/ml indo-1 acetoxymethyl ester (Molecular Probes) for 30 min at 37°C. Changes in $[Ca^{2+}]_i$ evoked by different stimuli were measured as described previously (Grynkiewicz et al., 1985; Sepúlveda et al., 1998), using a fluorescence inverted microscope (Diaphot-200; Nikon Corp.).

RESULTS

High K^+ enhances mRNA and protein levels of SNAP-25

To examine whether a depolarizing stimulus, which raises $[Ca^{2+}]_i$ by opening voltage-dependent Ca^{2+} channels, also elevates SNAP-25 mRNA levels, bovine chromaffin cells were incubated with 38 m*M* K^+ for 24 h. In all experiments referred to here, control cells were manipulated like experimental cells, but elevating the NaCl instead of KCl level. Northern blot analysis revealed the presence of mRNA species of 2.2 kb (Fig. 1A), corresponding to SNAP-25 mRNA (Oyler et al., 1989). There was a fivefold increase of SNAP-25 mRNA levels in K^+-treated cells, compared with control cells of the same culture. This mRNA level rise was correlated with an increase of SNAP-25 protein expression. Thus, when chromaffin cells were incubated with 38 m*M* KCl for 48 h, protein expression increased 2.5 ± 0.2-fold ($n = 28$

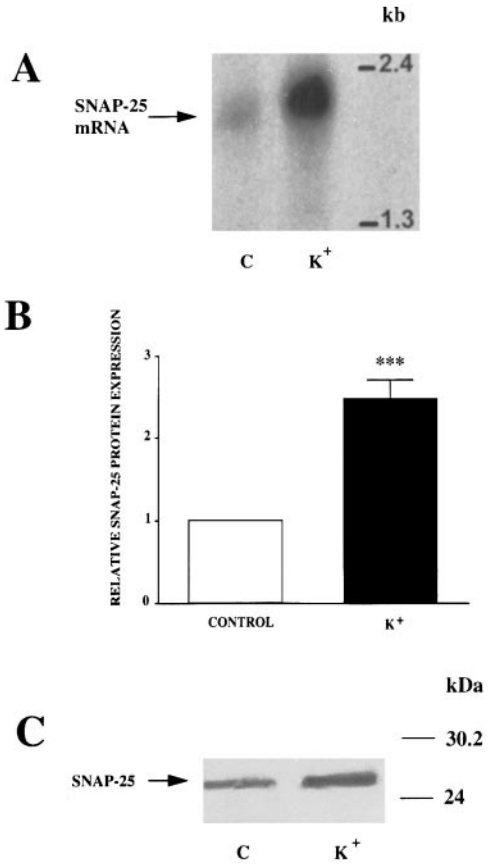

FIG. 1. Effect of a high K^+ concentration on SNAP-25 mRNA and protein levels in bovine chromaffin cells. Cells were incubated with 38 m*M* K^+ for 24 or 48 h, respectively, for mRNA and protein determinations. **A:** A typical autoradiogram (of three) of an original northern blot. Total RNAs (25 μg) obtained from control (C) or depolarized (K^+) cells were loaded in the first and second lane, respectively. The position of the synthetic poly(A)$^+$ RNA markers (2.4 and 1.3 kb) is indicated on the right side of the autoradiogram. **B:** Relative increment of SNAP-25 protein expression obtained in cells treated with K^+ for 48 h compared with control cells. ***$p < 0.001$. Data are mean ± SEM (bars) values obtained in 28 different batches of cells. **C:** An original immunoblot of SNAP-25 expression obtained from control cells (C) and cells treated with high K^+ for 48 h (K^+).

batches of cells) compared with parallel control cells (Fig. 1B). Figure 1C shows an original immunoblot of SNAP-25 expression in control and K^+-treated cells.

Effects of voltage-dependent Ca^{2+} channel blockers on $[Ca^{2+}]_i$ transients induced by brief depolarizations

It was important to be sure that a slight depolarization, such as that produced by 38 m*M* K^+, was able to open the three Ca^{2+} channel subtypes present in chromaffin cells; therefore, the $[Ca^{2+}]_i$ rise induced by such stimulus in the absence or presence of different Ca^{2+} channel blockers was studied in single indo-1-loaded chromaffin cells. To obtain reproducible $[Ca^{2+}]_i$ signals induced by repetitive 38 m*M* K^+ pulses, brief stimulation periods (15 s) were applied every 15 min. After two or three initial K^+ pulses, successive depolarizations gave reproducible $[Ca^{2+}]_i$ signals (>1 μM). Addition of ω-conotoxin MVIIC (1 μM), a blocker of non–L-type Ca^{2+} channels (N and P/Q subtypes), 15 min before and during the next K^+ pulse, halved the $[Ca^{2+}]_i$ signal. Subsequent addition of the dihydropyridine furnidipine (3 μM), a selective blocker of L-type Ca^{2+} channels, combined with ω-conotoxin MVIIC practically abolished the K^+-induced response. A typical record of the $[Ca^{2+}]_i$ changes obtained with this experimental protocol in a single cell is shown in Fig. 2A. Figure 2B shows averaged results from different cells obtained assaying each Ca^{2+} channel blocker in separate cells. ω-Conotoxin MVIIC blocked the $[Ca^{2+}]_i$ signal by 43 ± 3%, furni-

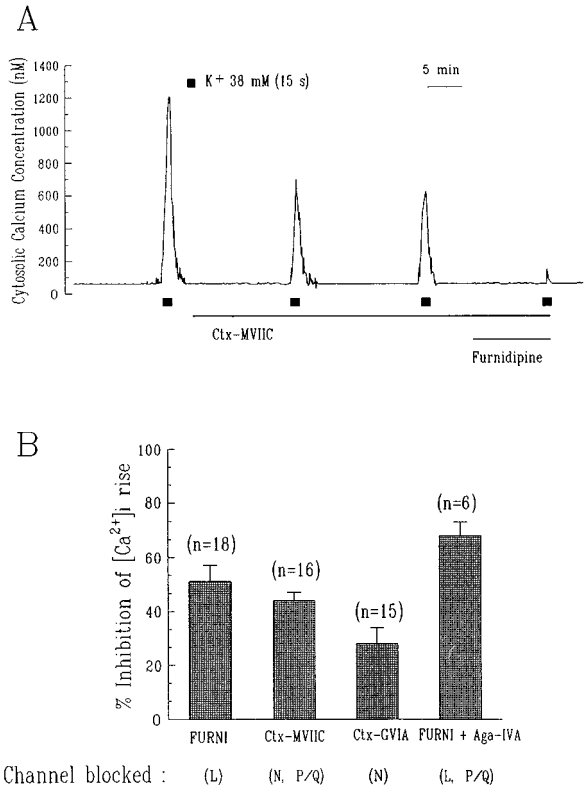

FIG. 2. Effect of different voltage-dependent Ca^{2+} channel blockers on the $[Ca^{2+}]_i$ rise induced by brief high K^+ pulses in single indo-1-loaded chromaffin cells. To generate $[Ca^{2+}]_i$ signals, a depolarizing solution containing 38 m*M* KCl was applied for 15 s at 15-min intervals. **A:** Original record of the $[Ca^{2+}]_i$ increments obtained on successive depolarization pulses (black squares at the bottom of the panel), in the absence or presence of ω-conotoxin MVIIC (Ctx-MVIIC; 1 μM). The first $[Ca^{2+}]_i$ rise represents the control response, when a stable $[Ca^{2+}]_i$ peak evoked by the K^+ pulse was obtained. At this moment, Ctx-MVIIC alone or combined with furnidipine (3 μM) was added for the intervals indicated by the horizontal lines at the bottom of the panel. **B:** Inhibitory effect of furnidipine (FURNI; 3 μM), Ctx-MVIIC (1 μM), ω-conotoxin GVIA (Ctx-GVIA; 1 μM), and FURNI (3 μM) plus ω-agatoxin IVA (Aga-IVA; 1 μM) on the $[Ca^{2+}]_i$ rise evoked by K^+ pulses with protocols similar to those described in A. Data, expressed as percent inhibition of the control response, are mean ± SEM (bars) values obtained in the number of cells shown in parentheses.

dipine by 51 ± 5%, and ω-conotoxin GVIA (1 μ*M*), a selective N-type Ca^{2+} channel blocker, by 28 ± 5%. Simultaneous blockade of L- and P/Q-type Ca^{2+} channels with furnidipine plus ω-agatoxin IVA (1 μ*M*) reduced the $[Ca^{2+}]_i$ signal induced by high K^+ by 67 ± 5%. This relative contribution of each Ca^{2+} channel subtype to the $[Ca^{2+}]_i$ increase induced by 38 m*M* K^+ differs from previous data concerning the relative densities of Ca^{2+} channel subtypes in bovine chromaffin cells. Thus, using $^{45}Ca^{2+}$ uptake in cells stimulated with 70 m*M* K^+ (Villarroya et al., 1997) or depolarizing pulses from −80 mV to 0 mV in voltage-clamped cells (Albillos et al., 1996), we came to the conclusion that L channels account for 20%, N channels for 30%, and P/Q channels for 50% of the total population of Ca^{2+} channels present in bovine chromaffin cells. In neurons and chromaffin cells, L-type channels are activated at more negative potentials than non–L-type channels (Carbone et al., 1990; Albillos et al., 1994); also, in chromaffin cells N and P/Q subtypes are more prone than the L subtype to inactivation at depolarizing voltages (Villarroya et al., 1999). Thus, the milder depolarization used in this study (38 m*M* K^+) might condition different grades of activation/inactivation of the various channel subtypes; in spite of their lower density, L channels had a greater contribution to the $[Ca^{2+}]_i$ rise under the present experimental conditions.

Effects of furnidipine and ω-conotoxin MVIIC on expression of SNAP-25 protein

These experiments were designed to test how Ca^{2+} ions entering through L- or non–L-type Ca^{2+} channels affected the increase of SNAP-25 protein expression induced by high K^+. Cells were incubated with furnidipine (3 μ*M*) 10 min before and during the entire depolarization period (38 m*M* K^+, 48 h). The same was done in the presence of ω-conotoxin MVIIC (1 μ*M*) or with a combination of furnidipine plus ω-conotoxin MVIIC.

Results obtained in different batches of cells are shown in Fig. 3A. In these batches, the relative enhancement of the protein expression level induced by high K^+, compared with control nondepolarized cells, was 2.9 ± 0.4-fold; this value was defined as 100%. Furnidipine inhibited protein expression by 86 ± 5%. A similar result was obtained using ω-conotoxin MVIIC, which caused 78 ± 5% blockade, or a cocktail of 1 μ*M* ω-conotoxin GVIA plus 1 μ*M* ω-agatoxin IVA, which caused 80 ± 6% blockade (experiment not shown). The incubation of cells with a combination of furnidipine plus ω-conotoxin MVIIC completely prevented the increase of protein expression. One original immunoblot of the effects of furnidipine and/or ω-conotoxin MVIIC on SNAP-25 expression, induced by high K^+, is shown in Fig. 3B. Note the sharp increase of the band density corresponding to K^+-treated cells compared with control and its attenuation on treatment with the various Ca^{2+} channel blockers.

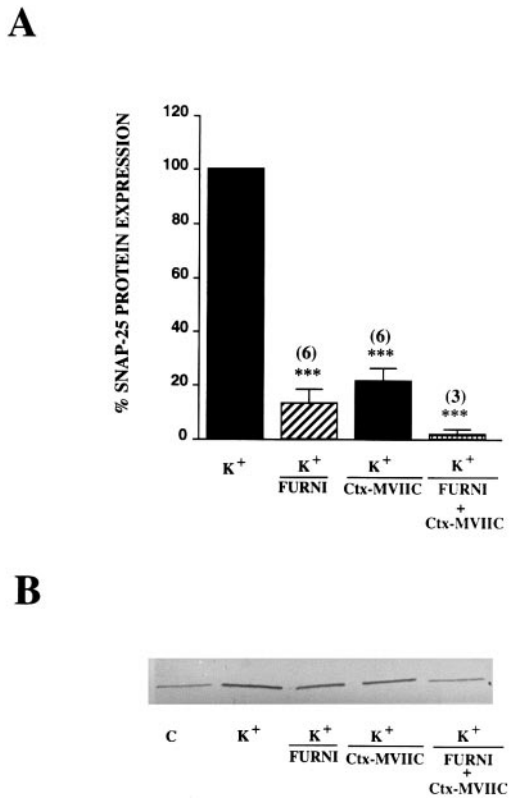

FIG. 3. Inhibitory effects of furnidipine (FURNI) and ω-conotoxin MVIIC (Ctx-MVIIC) on SNAP-25 protein expression induced by high K^+. Cells were incubated with 38 m*M* K^+ for 48 h in the absence or presence of furnidipine (3 μ*M*) or Ctx-MVIIC (1 μ*M*). In three batches of cells FURNI and Ctx-MVIIC were added together. Blockers were present since 10–20 min before and during the entire depolarization period (48 h). **A:** Mean ± SEM (bars) protein expression values obtained in cells depolarized with K^+ in the absence or presence of Ca^{2+} channel blockers. The number of experiments done is in parentheses. Values are expressed as percentages of protein expression induced by high K^+ in the absence of blockers (defined as 100%). ***$p < 0.001$ compared with K^+ values in the absence of blockers. **B:** A representative immunoblot of SNAP-25 expression in nondepolarized (control) and in depolarized chromaffin cells (K^+), incubated in the absence or the presence of FURNI or Ctx-MVIIC.

Effects of ω-conotoxin GVIA and ω-agatoxin IVA on expression of SNAP-25 protein

Simultaneous blockade of the two non–L-type Ca^{2+} channels (N and P/Q) with ω-conotoxin MVIIC drastically reduced the expression of SNAP-25 induced by depolarization. It was of interest to assess the contribution of each of the two non–L channel subtypes to the regulation of protein expression. Thus, experiments similar to those described above were performed using ω-conotoxin GVIA (1 μ*M*) and ω-agatoxin IVA (1 μ*M*) to block N- or P/Q-type Ca^{2+} channels separately. In these batches of cells, 38 m*M* K^+ increased the protein expression 1.9 ± 0.1- (n = 4) and 2.0 ± 0.2-fold (n = 3), respectively, in experiments carried out in the presence of ω-conotoxin GVIA and ω-agatoxin IVA. Results on the effects of ω-toxins are shown in Fig. 4; the selective

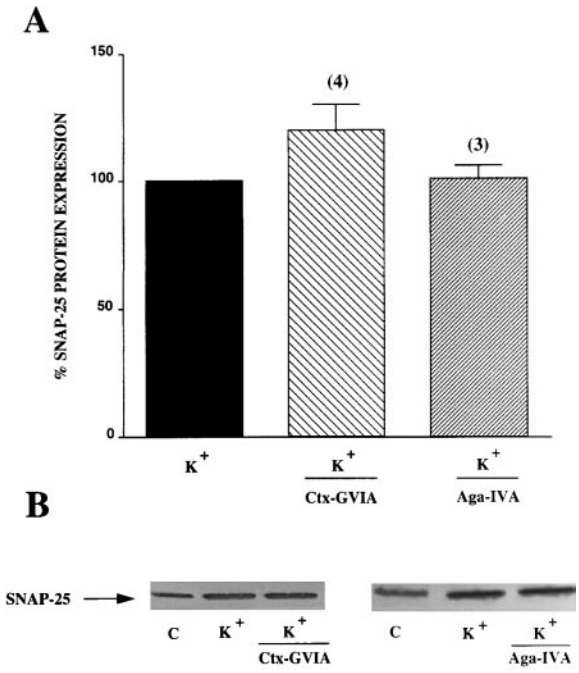

FIG. 4. Effects of ω-conotoxin GVIA (Ctx-GVIA) and ω-agatoxin IVA (Aga-IVA) on SNAP-25 protein expression induced by high K^+. Cells were depolarized with 38 m*M* K^+ for 48 h in the absence or presence of Ctx-GVIA (1 μ*M*) or Aga-IVA (1 μ*M*). Each ω-toxin was assayed in different cell batches. Toxins were present 10–20 min before and during the entire depolarization period. **A:** Toxin effect expressed as a percentage of the protein expression induced by high K^+ in the absence of the blocker (defined as 100%). Data are mean ± SEM (bars) values. The number of experiments done is in parentheses. **B:** Two representative immunoblots of SNAP-25 expression in nondepolarized [control (C)] and in depolarized chromaffin cells (K^+) incubated in the absence or the presence of Ctx-GVIA or Aga-IVA.

interruption of Ca^{2+} entry through N or P/Q channels did not modify SNAP-25 expression induced by depolarization. This contrasts with the sharp reduction in protein expression when both channel types were blocked by ω-conotoxin MVIIC (Fig. 3A) or with a cocktail of 1 μ*M* ω-conotoxin GVIA plus 1 μ*M* ω-agatoxin IVA.

Role of extracellular Ca^{2+} on SNAP-25 expression

Previous data from our group have demonstrated that raising the external Ca^{2+} concentration ($[Ca^{2+}]_o$) enhanced the $[Ca^{2+}]_i$ signal generated by depolarization of chromaffin cells (Montiel et al., 1984; López et al., 1994*a*; Michelena et al., 1995; Lara et al., 1998). Therefore, we performed new experiments to study how the increase of $[Ca^{2+}]_o$ affected the SNAP-25 expression induced by depolarization in the absence (control) or presence of different Ca^{2+} channel blockers.

The SNAP-25 expression induced by depolarization was enhanced 4.0 ± 0.5-fold (n = 4) in 5 m*M* $[Ca^{2+}]_o$. Such an increase was significantly higher ($p < 0.05$) than that obtained in 1.6 m*M* $[Ca^{2+}]_o$. Furthermore, the blockade of SNAP-25 expression by furnidipine in low $[Ca^{2+}]_o$ was partially reversed in 5 m*M* $[Ca^{2+}]_o$. This result is shown in Fig. 5, where the inhibition by furnidipine reached 55.6 ± 6% (n = 4), a value significantly lower ($p < 0.01$) than that obtained in 1.6 m*M* $[Ca^{2+}]_o$ (compare Figs. 3 and 5). Also, simultaneous blockade of L- and N-type Ca^{2+} channels (with furnidipine plus ω-conotoxin GVIA) in high $[Ca^{2+}]_o$ did not prevent completely the activation of protein expression induced by depolarization (Fig. 5). We have not performed experiments with ω-conotoxin MVIIC in high $[Ca^{2+}]_o$ because the binding of this toxin to its receptor has been described as being markedly reduced by high concentrations of divalent cations (Albillos et al., 1996; Lara et al., 1998). It is noteworthy that although the global $[Ca^{2+}]_i$ signal generated by 38 m*M* K^+ in 5 m*M* $[Ca^{2+}]_o$ nearly duplicated the response in 1.6 m*M* $[Ca^{2+}]_o$, the contribution of each Ca^{2+} channel subtype to the global $[Ca^{2+}]_i$ signal induced by depolarization was similar in both experimental situations (data not shown).

SNAP-25 expression as a function of the depolarizing interval

These experiments were designed to determine the minimal duration of the depolarizing stimulus required to trigger SNAP-25 expression. Cells were incubated with 38 m*M* K^+ for different times (10, 30, or 60 min or 24 h). At the end of each depolarizing period, Ca^{2+} entry was immediately stopped by addition of EGTA (3 m*M*) to the incubation medium. Ten minutes later, EGTA was washed out, and the external solution was changed to a fresh normal medium. Then, cells were maintained for an additional interval for a total, in all cases, of 48 h after initiation of K^+ treatment. A significant increase in pro-

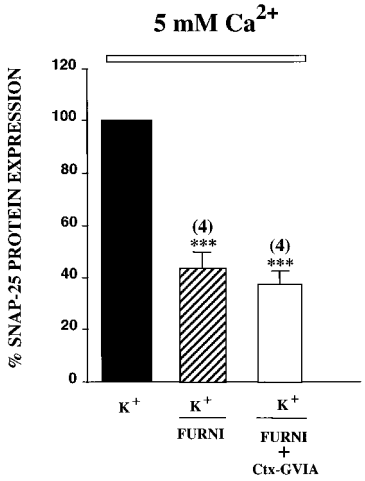

FIG. 5. High-calcium medium partially reversed the blockade by furnidipine of the SNAP-25 expression induced by 38 m*M* K^+. Cells were depolarized in 5 m*M* $[Ca^{2+}]_o$, in the absence (control) or in the presence of furnidipine (FURNI; 3 μ*M*) or FURNI plus ω-conotoxin GVIA (Ctx-GVIA; 1 μ*M*). Protein expression in the presence of the blockers was expressed as a percentage of the control expression induced by high K^+ (defined as 100%). Data are mean ± SEM (bars) values. The number of experiments done is in parentheses. ***$p < 0.001$ compared with K^+ values in the absence of blockers.

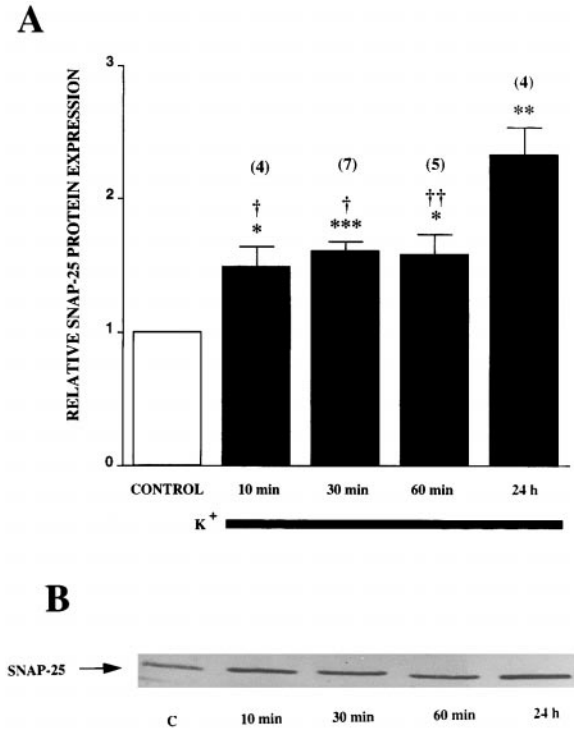

FIG. 6. Time course of expression of SNAP-25 protein. Cells were depolarized with 38 m*M* K^+ for 10, 30, or 60 min or 24 h as described in Results. Protein expression was determined 48 h after addition of K^+ began. **A:** Mean ± SEM (bars) values of protein expression induced by each depolarization period obtained from the number of experiments shown in parentheses. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control, non-depolarized cells; †$p < 0.05$, ††$p < 0.01$ compared with 24 h of K^+ treatment. **B:** A typical immunoblot under these experimental conditions. Control (C) cells were treated with NaCl for 30 min.

tein expression was observed already after a 10-min depolarization (1.49 ± 0.16-fold, n = 4; $p < 0.05$, compared with paired untreated cells). Similar increases were obtained in cells preincubated for 30 or 60 min in high K^+ (1.60 ± 0.08- and 1.58 ± 0.16-fold, respectively). However, a 24-h depolarization caused an additional increase (2.32 ± 0.21-fold, n = 4) that was statistically different from the expression values obtained using 10–60-min depolarizing periods (Fig. 6A) but not from the value obtained on 48 h of high K^+ treatment (Fig. 1B). In Fig. 6B, a representative immunoblot of SNAP-25 expression is shown. Observe the different SNAP-25 expression levels after different periods of K^+ exposure. In this experiment, control cells were treated with high NaCl for 30 min (there were no statistical differences among results of expression in control cells incubated with NaCl for different intervals; data not shown).

Time course of $[Ca^{2+}]_i$ changes on sustained depolarization of indo-1-loaded chromaffin cells

The activation of SNAP-25 expression was already visible after depolarizing challenges as short as 10 min. It was therefore interesting to study the time course of $[Ca^{2+}]_i$ changes along a prolonged depolarizing stimulus, in either the absence or the presence of various Ca^{2+} channel blockers. Figure 7A shows the time course of $[Ca^{2+}]_i$ rises in cells depolarized with 38 m*M* K^+ during 60 min. During the first 10 min, a transient initial Ca^{2+} spike (1,237 n*M*) was followed by a plateau (200 n*M*) that lasted the 60-min exposure to K^+. In the presence of ω-conotoxin MVIIC (1 μ*M*), a similar transient $[Ca^{2+}]_i$ rise followed by a plateau phase was observed. However, the peak amplitude and the area of the $[Ca^{2+}]_i$ transient response during the first 10 min of depolarization were lower (54 and 65%, respectively) than those obtained in the absence of toxin. Another interesting difference found was that the plateau phase only lasted 20 min, declining slowly to reach the basal $[Ca^{2+}]_i$ (Fig. 7B). In the presence of furnidipine plus ω-conotoxin GVIA, either the peak or the area of the $[Ca^{2+}]_i$ signal induced by K^+ during the first 10 min was smaller (27 and 40% of those obtained in cells depolarized in the absence of blockers; Fig. 7C). Finally, in the presence of furnidipine plus ω-agatoxin IVA, the peak and the $[Ca^{2+}]_i$ area during the first 10 min were 33 and 45% of those obtained in depolarized cells in the absence of blockers (Fig. 7D).

Cytosolic $[Ca^{2+}]_i$ rise and SNAP-25 expression induced by caffeine and DMPP

We have also studied the expression of SNAP-25 by raising $[Ca^{2+}]_i$ with caffeine, which induces the release of Ca^{2+} from internal stores, and the nicotinic receptor agonist DMPP, which activates $\alpha_3\beta_4$ and α_7 neuronal nicotinic acetylcholine receptors in chromaffin cells (López et al., 1998). Figure 8 shows two traces of the $[Ca^{2+}]_i$ signals induced by caffeine and DMPP. Treatment of indo-1-loaded cells with caffeine (10 m*M*) for 30 min produced a $[Ca^{2+}]_i$ peak with an amplitude around 350 n*M*. The area under the curve (first 10 min of stimulation) was 27% of that obtained in high K^+-stimulated cells. The response to caffeine declined slowly and reached basal levels in 15 min (Fig. 8A). DMPP (20 μ*M*) evoked a $[Ca^{2+}]_i$ signal whose peak amplitude reached a value close to that obtained in high K^+-treated cells; however, the $[Ca^{2+}]_i$ signal induced by DMPP was transient (lasting only 13 min) and was not followed by a plateau phase as with K^+ (Fig. 8B).

Figure 9A shows mean ± SEM values of SNAP-25 expression induced by caffeine and DMPP. Expression of SNAP-25 in chromaffin cells incubated for 48 h with caffeine (10 m*M*) or DMPP (20 μ*M*) was increased 1.4 ± 0.1- ($p < 0.05$) and 2.6 ± 0.2-fold ($p < 0.005$), respectively, compared with parallel control cells. When the expression induced by DMPP was compared with that produced by 38 m*M* K^+ in cells from the same culture day, no significant differences were found between these two stimuli. However, the protein level induced by caffeine was significantly lower than that evoked by high K^+ in cells belonging to the same culture. Figure 9B shows two typical immunoblots obtained in two different batches of cells treated with caffeine or DMPP for 48 h. For the sake of comparison, two samples

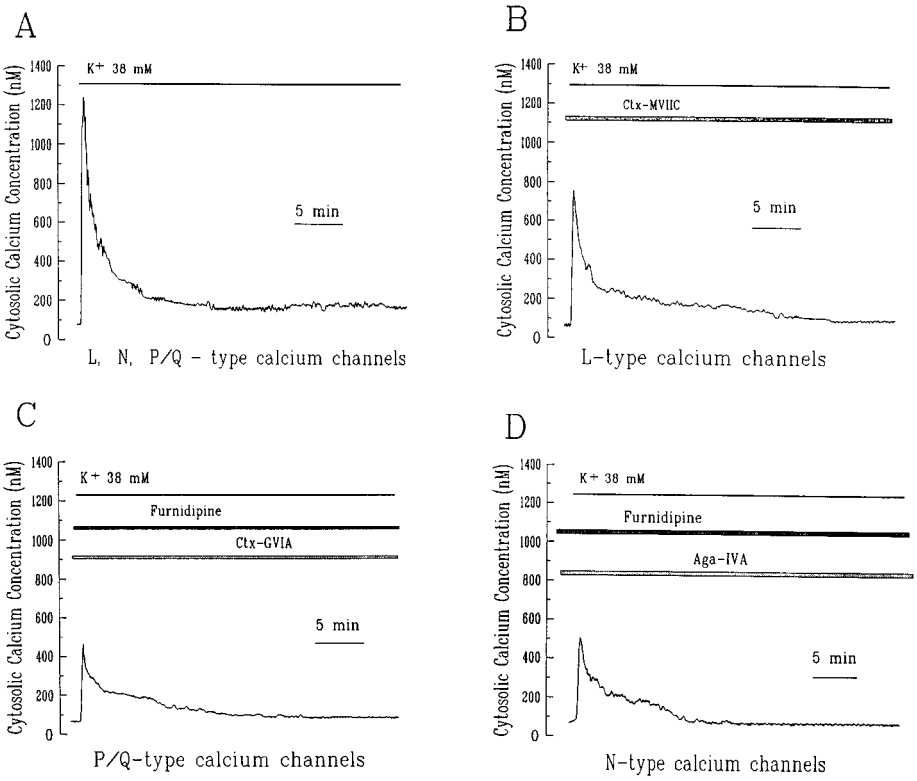

FIG. 7. Increase in $[Ca^{2+}]_i$ levels generated by prolonged exposure of cells to high K^+. Indo-1-loaded chromaffin cells were stimulated with 38 m*M* K^+ for 60 min, (**A**) in the absence of Ca^{2+} channel blockers or in the presence of (**B**) 1 μM ω-conotoxin MVIIC (Ctx-MVIIC), (**C**) 3 μM furnidipine plus 1 μM ω-conotoxin GVIA (Ctx-GVIA), or (**D**) 3 μM furnidipine plus 1 μM ω-agatoxin IVA (Aga-IVA). Blockers were added 10 min before and during all the K^+ stimulation period. Traces are averaged from three different records.

from parallel cells, untreated (control) or treated with 38 m*M* K^+ for 48 h, were also assayed.

DISCUSSION

Exposure of bovine chromaffin cells to a high K^+ medium for different intervals led to an increase of SNAP-25 protein expression. This is a Ca^{2+}-dependent phenomenon because blockade of Ca^{2+} entry during cell depolarization suppressed SNAP-25 expression. Regulation of gene transcription and protein expression triggered by enhanced Ca^{2+} entry and the subsequent increase of $[Ca^{2+}]_i$ is well established in neurons (Murphy et al., 1991; Bading et al., 1993; Bito et al., 1997; Ginty, 1997; Hardingham et al., 1997) and in PC12 cells (Menezes et al., 1996; Sepúlveda et al., 1998). However, it is unclear whether such regulation by Ca^{2+} is selectively coupled to a given Ca^{2+} entry pathway and/or a Ca^{2+} source, i.e., extracellular Ca^{2+} entry versus Ca^{2+} mobilization from intracellular stores.

As far as the Ca^{2+} entry pathway is concerned, we found that using a mild depolarization stimulus (38 m*M* K^+ in 1.6 m*M* $[Ca^{2+}]_o$), the selective blockade of L-type Ca^{2+} channels suppressed SNAP-25 expression, whereas the separate blockade of N- or P/Q-type channels did not. A priori, this observation might suggest that L-type Ca^{2+} channels are tightly coupled to the protein expression, whereas N- or P/Q-type channels are not. However, the fact that simultaneous blockade of N- plus P/Q-type channels markedly reduced the protein expression to an

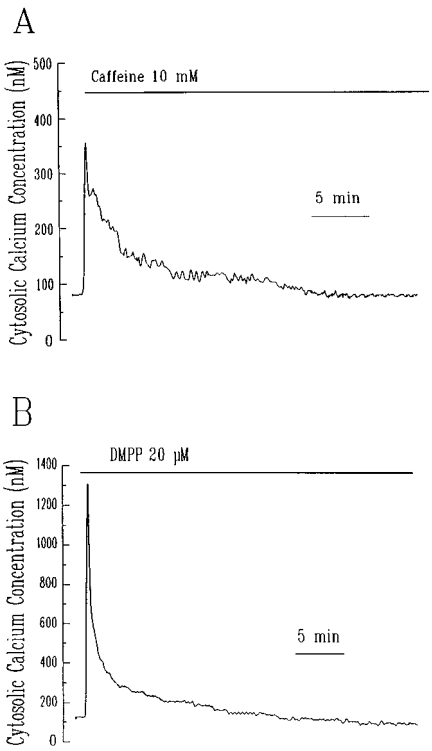

FIG. 8. Cytosolic $[Ca^{2+}]_i$ rise generated by prolonged stimulation with (**A**) caffeine (10 m*M*, 30 min) or (**B**) DMPP (20 μM, 30 min) added to single indo-1-loaded chromaffin cells. Traces are averages of four different records.

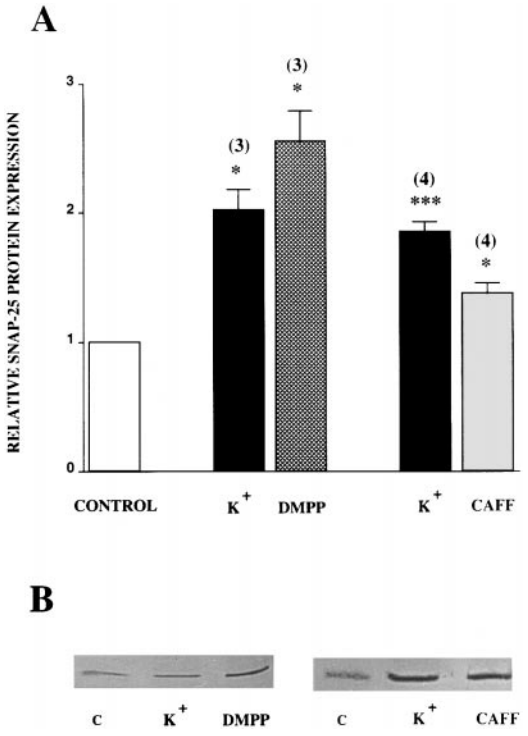

FIG. 9. Expression of SNAP-25 protein in cells stimulated with caffeine (CAFF) or DMPP. Effects of CAFF (10 m*M,* 48 h) or DMPP (20 μM, 48 h) on protein expression were assayed in separate batches of cells. In parallel with each of these stimuli, expression induced by 38 m*M* K^+ was also determined. **A:** Mean ± SEM (bars) relative values obtained in high K^+-, DMPP-, and CAFF-treated cells compared with parallel nontreated cells (control). $^*p < 0.05$, $^{***}p < 0.001$ compared with control. **B:** Two typical immunoblots of the CAFF or DMPP effects. C, control.

extent similar to that obtained on blocking of L-type channels (78% vs. 86%) casts doubts to this hypothesis.

Rather than a specialized Ca^{2+} entry route, our results suggest that Ca^{2+}-activated SNAP-25 expression in chromaffin cells seems to be related more to quantitative aspects of the $[Ca^{2+}]_i$ signals generated on depolarization. Thus, a threshold value of $[Ca^{2+}]_i$ seems to be the critical signal to trigger SNAP-25 expression; under this value there is no activation of protein synthesis. This view is supported by two findings: (a) the need of blocking >45–50% of the Ca^{2+} entry, either with furnidipine (L-type Ca^{2+} channels blockade) or with ω-conotoxin MVIIC (N- plus P/Q-type channels blockade), to inhibit the protein expression; and (b) the absence of an effect on the SNAP-25 expression of the separate blockade of N channels with ω-conotoxin GVIA or P/Q channels with ω-agatoxin IVA. These last maneuvers did not prevent the $[Ca^{2+}]_i$ signal from reaching the critical value necessary to activate the protein expression. In contrast, the threshold $[Ca^{2+}]_i$ required to induce the protein expression was not reached when both non–L-type Ca^{2+} channels were simultaneously inhibited with ω-conotoxin MVIIC or when Ca^{2+} influx through L-type Ca^{2+} channels was interrupted with furnidipine. That the effect of ω-conotoxin MVIIC on SNAP-25 expression was the result of a specific blockade of non–L-type Ca^{2+} channels, and not the consequence of a cumulative effect on L-type channels, could be proved by the fact that ω-conotoxin GVIA plus ω-agatoxin IVA, added simultaneously to block non–L-type Ca^{2+} channels, also produced the same effect as that observed with ω-conotoxin MVIIC.

Our finding of the greater coupling between L-type Ca^{2+} channels and SNAP-25 expression might be the result of two facts: (a) These channels contribute by 50% to the global $[Ca^{2+}]_i$ rise induced by 38 m*M* K^+, and (b) L-type Ca^{2+} channels suffer less inactivation than non–L-type channels, as we have recently demonstrated on prolonged depolarization of bovine chromaffin cells (Villarroya et al., 1999). Thus, it is understandable that under the present experimental conditions (cell depolarization lasting minutes to hours), Ca^{2+} entry through noninactivating L-type Ca^{2+} channels might provide a better signal for gene expression than Ca^{2+} entry through inactivating N- or P/Q-type channels.

According to our hypothesis, the only requirement to trigger the Ca^{2+}-dependent SNAP-25 expression in chromaffin cells is that the Ca^{2+} influx through a given Ca^{2+} channel provides a sufficient threshold $[Ca^{2+}]_i$ signal. If this is true, then the blockade of protein expression observed in our experiments, when L-type channels were blocked in 1.6 m*M* $[Ca^{2+}]_o$, should be reversed by a higher $[Ca^{2+}]_o$ and the consequent increase of Ca^{2+} influx through the other two channel subtypes (N and P/Q). In fact, this is what happened when cells were treated with 38 m*M* K^+ in 5 m*M* $[Ca^{2+}]_o$; under these experimental conditions the blockade of protein expression by furnidipine in low $[Ca^{2+}]_o$ was partially reversed (compare Figs. 3 and 5). Because the $[Ca^{2+}]_i$ signal and protein expression evoked by 38 m*M* K^+ in high $[Ca^{2+}]_o$ almost doubled those obtained in low $[Ca^{2+}]_o$, this last maneuver allows, in the presence of furnidipine, a larger Ca^{2+} influx through non–L-type channels. Another interesting finding was that the Ca^{2+} entry through P/Q-type channels (on blockade of L- and N-channels) was still able to activate the protein expression in high $[Ca^{2+}]_o$ (see Fig. 5). That the Ca^{2+} entry pathway or Ca^{2+} source does not condition the Ca^{2+}-dependent SNAP-25 expression is further supported by the caffeine experiments; just by promoting Ca^{2+} release from intracellular Ca^{2+} stores, caffeine was able to enhance SNAP-25 expression.

Because the SNAP-25 expression evoked by depolarization of chromaffin cells seems to be a Ca^{2+}-activated process, we tried to correlate the amplitude and duration of the $[Ca^{2+}]_i$ signal generated by a prolonged depolarizing stimulus with the time course of the protein expression levels. Our results show that 38 m*M* K^+, applied for 1 h, evoked a biphasic $[Ca^{2+}]_i$ response that consisted of an initial vigorous peak that subsequently declined over 10 min to a sustained low plateau during the entire stimulation period. The time course of SNAP-25 expression revealed two peaks according with the duration of

the depolarizing stimulus: The first expression peak was seen after depolarization periods of 10–60 min, whereas the second peak was observed on longer K^+ exposure periods (24–48 h). Because the first protein peak was already obtained with a depolarization period of 10 min, most probably it corresponds with the activation of a signaling pathway that would require a high threshold of $[Ca^{2+}]_i$; this level could be reached during the transient $[Ca^{2+}]_i$ phase induced by depolarization. The second peak of SNAP-25 expression would be the result of the activation of a second signaling pathway by lower and prolonged $[Ca^{2+}]_i$ levels, at >1 h, which could be provided by the plateau phase of the late $[Ca^{2+}]_i$ response. In fact, a similar observation has been described in B lymphocytes, where two phases (transient and plateau) in the $[Ca^{2+}]_i$ response induced by chicken egg lysozyme antigen were able to control, differentially, the activation of several Ca^{2+}-sensitive proinflammatory transcriptional regulators (Dolmetsch et al., 1997). However, we cannot exclude that the second peak of SNAP-25 expression observed on prolonged depolarization of chromaffin cells may be the result of the inhibition of physiological mechanisms, i.e., dephosphorylation of transcription factors, that down-regulate gene transcription, as proposed after prolonged periods of synaptic input in neurons (Bito et al., 1997).

In this context, we believe that the regulation by Ca^{2+} of SNAP-25 expression must surely take place at the transcription level because the SNAP-25 mRNA level was also increased on depolarization. However, the transcriptional pathway that, when activated by the Ca^{2+} entry, conveys the signal to the nucleus to stimulate the SNAP-25 gene transcription in depolarized chromaffin cells remains to be elucidated. Furthermore, our results suggest that the increase of SNAP-25 protein levels by Ca^{2+} could be physiologically relevant because a selective agonist for nicotinic receptors, DMPP, which mimics the effect of endogenously released acetylcholine on splanchnic nerve stimulation in the intact adrenal gland, also augmented SNAP-25 expression. As SNAP-25 is a critical protein of the exocytotic machinery in bovine chromaffin cells (Gutiérrez et al., 1995, 1997; Foran et al., 1996; Lawrence et al., 1997), its induction during cell activation might have important implications for the regulation of the rate and extent of exocytosis. This result adds a new member to the list of proteins implicated in catecholamine release that are up-regulated on activation of secretion in PC12 and chromaffin cells. These proteins include not only the granule-secreted proteins chromogranin A (Tang et al., 1996) and proenkephalin A (Stachowiak et al., 1990), but also the catecholamine biosynthetic enzymes such as tyrosine hydroxylase, phenylethanolamine-*N*-methyltransferase, and dopamine-β-hydroxylase (Kilbourne et al., 1992; Hiremagalur et al., 1993; Nankova et al., 1993).

In conclusion, the results of this study demonstrate that on depolarization of chromaffin cells, Ca^{2+} entry through different voltage-dependent Ca^{2+} channel subtypes was able to regulate the expression of SNAP-25 protein. In these cells, the amount of Ca^{2+} entering the cell at a given time, rather than a specialized Ca^{2+} channel subtype or Ca^{2+} source, seems to be the critical signal to trigger the expression of SNAP-25. This is the first time that Ca^{2+} channels of the P/Q and N subtypes have been involved in the regulation of gene transcription. At this point, an interesting question is whether the increase of SNAP-25 protein content following depolarization of chromaffin cells modifies the late functional efficacy of the secretory machinery.

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