

# A Toxin Fraction (FTX) from the Funnel-Web Spider Poison Inhibits Dihydropyridine-Insensitive $\text{Ca}^{2+}$ Channels Coupled to Catecholamine Release in Bovine Adrenal Chromaffin Cells

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**Abstract:** In adrenal chromaffin cells, depolarization-evoked  $\text{Ca}^{2+}$  influx and catecholamine release are partially blocked by blockers of L-type voltage-sensitive  $\text{Ca}^{2+}$  channels. We have now evaluated the sensitivity of the dihydropyridine-resistant components of  $\text{Ca}^{2+}$  influx and catecholamine release to a toxin fraction (FTX) from the funnel-web spider poison, which is known to block P-type channels in mammalian neurons. FTX (1:4,000 dilution, with respect to the original fraction) inhibited  $\text{K}^+$ -depolarization-induced  $\text{Ca}^{2+}$  influx by 50%, as monitored with fura-2, whereas nitrendipine (0.1–1  $\mu\text{M}$ ) and FTX (3:3), a synthetic FTX analogue (1 mM), blocked the  $[\text{Ca}^{2+}]_i$  transients by 35 and 30%, respectively. When tested together, FTX and nitrendipine reduced the  $[\text{Ca}^{2+}]_i$  transients by 70%. FTX or nitrendipine reduced adrenaline and noradrenaline release

by ~80 and 70%, respectively, but both substances together abolished the  $\text{K}^+$ -evoked catecholamine release, as measured by HPLC. The  $\omega$ -conotoxin GVIA (0.5  $\mu\text{M}$ ) was without effect on  $\text{K}^+$ -stimulated  $^{45}\text{Ca}^{2+}$  uptake. Our results indicate that FTX blocks dihydropyridine- and  $\omega$ -conotoxin-insensitive  $\text{Ca}^{2+}$  channels that, together with L-type voltage-sensitive  $\text{Ca}^{2+}$  channels, are coupled to catecholamine release. **Key Words:** Potassium depolarization—Calcium channels—Funnel-web spider toxin— $\omega$ -Conotoxin GVIA—Nitrendipine—Catecholamine release—Bovine adrenal chromaffin cells. **Duarte C. B. et al.** A toxin fraction (FTX) from the funnel-web spider poison inhibits dihydropyridine-insensitive  $\text{Ca}^{2+}$  channels coupled to catecholamine release in bovine adrenal chromaffin cells. *J. Neurochem.* **60**, 908–913 (1993).

The acetylcholine released from the splanchnic nerve depolarizes the adrenal medulla chromaffin cells, thus increasing the  $\text{Ca}^{2+}$  influx and the intracellular free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) and evoking the release of catecholamines (Douglas, 1968; Holz et al., 1982; Rosario et al., 1989). Although the precise mechanism for  $\text{Ca}^{2+}$  entry is not completely known,  $\text{Ca}^{2+}$  is thought to enter the cells through voltage-sensitive  $\text{Ca}^{2+}$  channels (VSCCs) and, to a lesser extent, through the acetylcholine receptor-associated channel (Boarder et al., 1987).

The identity of the VSCCs present in bovine chromaffin cells is largely unknown. The dihydropyridine antagonists of L-type  $\text{Ca}^{2+}$  channels block only part of the depolarization-evoked  $[\text{Ca}^{2+}]_i$  transients (Rosario et al., 1989), and some authors have found little or no

effect of  $\omega$ -conotoxin GVIA ( $\omega$ -Cg Tx), a blocker of N-type VSCCs (Fox et al., 1987; McCleskey et al., 1987), on the depolarization-induced  $^{45}\text{Ca}^{2+}$  uptake,  $[\text{Ca}^{2+}]_i$  transients, and catecholamine secretion (Owen et al., 1989; Rosario et al., 1989; Jan et al., 1990). Moreover, T-type VSCCs do not appear to be present in the bovine chromaffin cell (Hans et al., 1990; Artalejo et al., 1991a).

Recently, a seemingly new class of voltage-sensitive  $\text{Ca}^{2+}$  channels, i.e., P-type channels, was characterized in mammalian neurons and cephalopods (Llinás et al., 1989). These channels were found to be sensitive to the spider toxin FTX, but were otherwise insensitive to dihydropyridines and  $\omega$ -Cg Tx (Llinás et al., 1989). The P channel has been isolated using synthetic FTX (Cherksey et al., 1991) and a channel with

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*Abbreviations used:* BSA, bovine serum albumin;  $[\text{Ca}^{2+}]_i$ , inter-

nal free calcium ion concentration;  $\omega$ -Cg Tx,  $\omega$ -conotoxin GVIA; DMEM, Dulbecco's modified Eagle's medium; F-12, Ham's F-12 medium; FTX, funnel-web spider toxin; FTX (3:3), arginine-polyamine synthetic analogue of FTX; fura-2/AM, acetoxymethyl ester of fura-2; VSCCs, voltage-sensitive calcium channels.

similar characteristics was recently cloned and sequenced (Mori et al., 1991). Our present results indicate that FTX blocks nitrendipine-insensitive  $Ca^{2+}$  channels in bovine adrenal chromaffin cells, and suggest that both FTX-sensitive and nitrendipine-sensitive VSCCs are coupled to catecholamine release.

## MATERIALS AND METHODS

### Materials

The acetoxymethyl ester of fura-2 (fura-2/AM) was purchased from Molecular Probes Inc., Eugene, OR, U.S.A. Nitrendipine was obtained from Sandoz Laboratories, Switzerland. Gadolinium chloride was from Alfa Products, Morton Thiokol, Inc., Denver, MA, U.S.A. The  $\omega$ -Cg Tx was from Peninsula Laboratories, Inc., Belmont, CA, U.S.A. Antibiotics and fetal calf serum were from Biological Industries, Beth Ha Emek, Israel. FTX was purified from the crude venom of American funnel-web spiders (Spider Pharm, Black Canyon, AZ, U.S.A.), as described (Llinás et al., 1989; Cherksey et al., 1991). The synthetic analogue of FTX was prepared as described (Cherksey et al., 1991). All other reagents were from Sigma Chemical Co., St. Louis, MO, U.S.A., or Merck, Darmstadt, F.R.G.

### Preparation and culture of bovine adrenal chromaffin cells

Bovine adrenal glands were obtained from the local slaughterhouse and taken to the laboratory in ice-cold Locke's solution, containing (in mM) 154 NaCl, 5.6 KCl, 5 NaHCO<sub>3</sub>, 5.6 glucose, and 5 HEPES-Na, pH 7.2, in addition to streptomycin (200  $\mu$ g/ml), penicillin (200 U/ml), and amphotericin B (2.5  $\mu$ g/ml). The glands were washed by retrograde perfusion with Locke's solution supplemented with 0.5% bovine serum albumin (BSA), and the cells were dissociated by perfusing the glands three times (15 min each) with the same solution containing 0.13% collagenase B (Boehringer Mannheim Biochemicals) and 0.02% soybean trypsin inhibitor. The medullae were dissected away from the cortex, minced with Locke's solution supplemented with 0.5% BSA, and passed through a 100- $\mu$ m mesh nylon. The filtered cells were washed twice in Locke's solution, and further purified on a self-generated Percoll (Pharmacia LKB, Uppsala, Sweden) gradient, as described (Kuijpers et al., 1989). Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (F-12), buffered with 15 mM HEPES and 26 mM NaHCO<sub>3</sub>, and supplemented with 5% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and amphotericin B (250 ng/ml). For catecholamine-release experiments, the cells were plated on 24-well cluster plates (Nunc 143982) at a density of  $1.0 \times 10^6$  cells/well/1.5 ml, whereas for  $^{45}Ca^{2+}$ -uptake experiments, cells were plated at a density of  $1.5 \times 10^6$  cells/well/2.0 ml on 12-well cluster plates (Costar 3512). For fluorescence experiments, cells were kept in suspension culture in bacterial petri dishes (Nunc 240142) at a density of  $30 \times 10^6$  cells/dish/30 ml. The cells were maintained in culture at 37°C in a humidified atmosphere of 95% air/5% CO<sub>2</sub> for 2–3 days before use.

### Fura-2 loading and $[Ca^{2+}]_i$ measurements

Chromaffin cells ( $30 \times 10^6$  cells/ml) were loaded with the  $Ca^{2+}$ -sensitive fluorescent probe fura-2 (Grynkiewicz et al.,

1985) by incubation with 7.5  $\mu$ M fura-2/AM for 60 min in the original culture medium. The nonhydrolyzed probe was removed by washing the cells twice in Na<sup>+</sup> medium, containing (in mM) 130 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 glucose, and 15 HEPES-Na, pH 7.35, in addition to 1 and 0.2% BSA for the first and second centrifugations, respectively. The final pellet of loaded cells was resuspended at a concentration of  $7.5 \times 10^7$  cells/ml and stored on ice to minimize fura-2 leakage.

Aliquots of chromaffin cells ( $1.5 \times 10^6$  cells/ml, final density) were transferred into cuvettes containing the media indicated in the figure captions and preincubated for 10 min at room temperature. The fura-2 fluorescence was then monitored at 30°C using a computer-assisted Perkin-Elmer LS-5B Luminescence Spectrometer, with excitation at 340 nm and emission at 510 nm, using 5- and 10-nm slits, respectively. The  $[Ca^{2+}]_i$  was calculated as described previously (Rosario et al., 1989), except that  $F_{max}$  was calculated upon addition of 100  $\mu$ M digitonin.

### Catecholamine release

Cells grown in 24-well plates were washed once with Na<sup>+</sup> medium and preincubated with control or test agents for 15 min at 30°C. Stimulation was performed by replacing the medium in each well for a high K<sup>+</sup> solution with or without nitrendipine and/or FTX. The high K<sup>+</sup> solution contained (in mM) 100 NaCl, 35 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 glucose, and 15 HEPES-Na, pH 7.35. Samples were removed after 2-min incubation at 30°C, centrifuged at 8,000 *g* for 1 min, and part of the supernatant was added to 67% acetic acid/2.7 M perchloric acid. These samples were kept at -20°C before being assayed by HPLC for their adrenaline and noradrenaline content (Levine, 1986). The catecholamines remaining in the cells were released by adding 10% acetic acid/0.4 M perchloric acid and kept frozen for later analysis.

### $^{45}Ca^{2+}$ uptake measurements

Cells grown in 12-well plates were washed once and preincubated in DMEM/F-12 medium (nominal calcium concentration, 1.05 mM) with or without  $\omega$ -Cg Tx (0.5  $\mu$ M) for 60 min. The cells were then washed three times with Na<sup>+</sup> medium, after which they were preincubated with a low K<sup>+</sup> solution with or without  $\omega$ -Cg Tx for 5 min. The composition of the low K<sup>+</sup> solution used was (in mM) 134 NaCl, 1 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 glucose, and 15 HEPES-Na, pH 7.35. Stimulation was performed by replacing the medium in each well for a high K<sup>+</sup> solution containing  $^{45}Ca^{2+}$  (2  $\mu$ Ci/ml) with or without  $\omega$ -Cg Tx. The  $^{45}Ca^{2+}$  uptake was stopped by adding to the cells an ice-cold,  $Ca^{2+}$ -free solution supplemented with LaCl<sub>3</sub> (2 mM) 1 min after adding  $^{45}Ca^{2+}$ . The cells were then washed three times using the stop solution (first two steps) and stop solution supplemented with 2 mM EGTA but lacking LaCl<sub>3</sub> (last step). The  $^{45}Ca^{2+}$  retained by the cells was determined by adding 0.1 mM NaOH solution to each well, followed by a freeze-thaw cycle and by a further wash with NaOH solution. Radioactivity was measured using Universol scintillation cocktail (ICN) and a Packard 2000 spectrometer provided with dpm correction. In other experiments, the dependency of the  $\omega$ -Cg Tx effect on extracellular  $Ca^{2+}$  concentration was tested by incubating the cells with the toxin in DMEM/F-12 medium supplemented with 0.95 mM EGTA (final  $Ca^{2+}$  concentration, 0.1 mM; Fabiato and Fabiato, 1979). The cells were then washed and further preincubated with regular Na<sup>+</sup> medium with or without  $\omega$ -Cg Tx for 5 min, after which

they were stimulated with a high  $K^+$  solution containing  $^{45}Ca^{2+}$  as above.

### Other methods

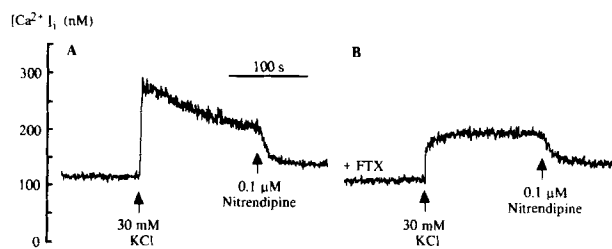
Results are presented as the mean  $\pm$  SEM of the number of experiments indicated. Statistical significance was evaluated using the two-tailed Student's *t* test.

## RESULTS

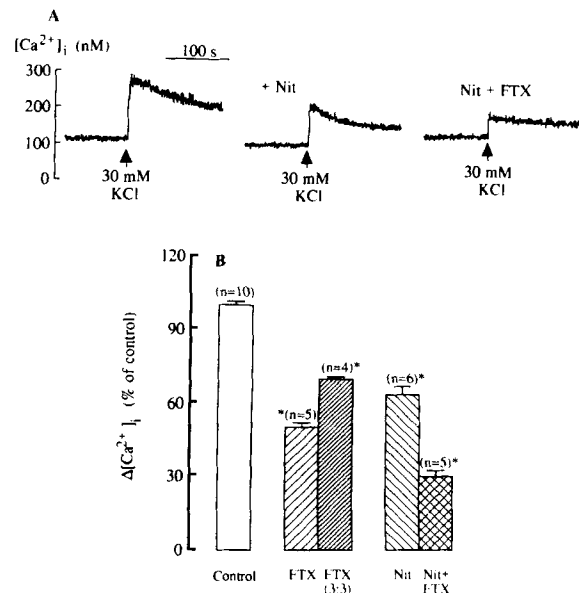
### Effect of FTX and nitrendipine on the $[Ca^{2+}]_i$ response to $K^+$ depolarization

Depolarization of cultured bovine adrenal chromaffin cells with 30 mM KCl rapidly increased the  $[Ca^{2+}]_i$  (Fig. 1A) from  $106.4 \pm 2.1$  to  $289.8 \pm 12.4$  nM ( $n = 10$ ). After the initial peak, the  $[Ca^{2+}]_i$  decreased toward a plateau at  $218.9 \pm 5.9$  nM (66% of the initial  $[Ca^{2+}]_i$  increment). Addition of 0.1  $\mu$ M nitrendipine, a dihydropyridine blocker of L-type VSCCs, further decreased the  $[Ca^{2+}]_i$  by  $142.4 \pm 3.9$  nM ( $n = 7$ ), which corresponded to  $42.1 \pm 2.6\%$  of the initial  $[Ca^{2+}]_i$  increase evoked by  $K^+$ . When tested at 1  $\mu$ M, nitrendipine did not cause further inhibition of the  $[Ca^{2+}]_i$  transients (data not shown).

The toxin fraction (FTX) from the funnel-web spider poison has been reported to block the dihydropyridine- and  $\omega$ -Cg Tx-insensitive P-type channels (Llinás et al., 1989). When the chromaffin cells were depolarized after 15-min preincubation with the toxin, the increase at the onset of the  $[Ca^{2+}]_i$  transient was profoundly reduced (Fig. 1B). Control experiments indicated that maximal inhibitory effect occurred at the toxin concentration used in the experiment of Fig. 1B. Thus, depolarization with 30 mM KCl in the presence of FTX produced a sustained  $[Ca^{2+}]_i$  increase from  $109.2 \pm 5.3$  to  $204.1 \pm 6.7$  nM ( $n = 5$ ), corresponding to  $50.4 \pm 1.6\%$  of control response. Because the  $[Ca^{2+}]_i$  recorded in the presence of the toxin was not statistically different ( $p > 0.05$ ) from the steady-state plateau reached during  $K^+$  depolarization in control conditions (Fig. 1A), FTX



**FIG. 1.** Effect of FTX on the  $[Ca^{2+}]_i$  response to  $K^+$  depolarization. Two different samples from the same batch of fura-2-loaded cells were stimulated with 30 mM KCl (final concentration, 35 mM) as indicated by the arrows, in the absence (A) or in the presence (B) of FTX (1:4,000 dilution, with respect to the original fraction). The cells were preincubated for 10 min at room temperature with or without FTX, and further incubated for 5 min at 30°C, before  $K^+$  depolarization. Where indicated, nitrendipine (final concentration, 0.1  $\mu$ M) was added. KCl was added from a 3.5 M stock solution.



**FIG. 2.** Effect of nitrendipine, FTX (3:3), and FTX on the  $[Ca^{2+}]_i$  response to  $K^+$  depolarization. **A:** Three different samples from the same batch of fura-2-loaded cells were stimulated with 30 mM KCl (final concentration, 35 mM), as indicated by the arrows, in the absence (left trace) or in the presence of 0.1  $\mu$ M nitrendipine (middle trace), or 0.1  $\mu$ M nitrendipine + FTX at 1:4,000 dilution with respect to the original fraction (right trace). The cells were preincubated for 10 min at room temperature in the presence of the indicated test agents, and were further incubated for 5 min at 30°C, before  $K^+$  depolarization. **B:** Experiments similar to those depicted in A were analyzed for the effects of FTX (1:4,000 dilution, with respect to the original fraction), FTX (3:3) (1 mM), 0.1  $\mu$ M nitrendipine, and 0.1  $\mu$ M nitrendipine + FTX. Control stands for stimulation performed in the absence of any added drug.  $\Delta[Ca^{2+}]_i$  stands for the  $[Ca^{2+}]_i$  increase (difference between maximal and basal  $[Ca^{2+}]_i$ ) obtained immediately after  $K^+$  depolarization. Results are expressed as the means  $\pm$  SEM of four to 10 measurements performed in four to five different preparations. \*Significantly lower than control,  $p < 0.05$ .

blocked preferentially the decaying component of the  $[Ca^{2+}]_i$  transient. It is also apparent from Fig. 1B that addition of nitrendipine in the presence of FTX decreased the  $[Ca^{2+}]_i$  further by  $55.4 \pm 4.9$  nM ( $n = 5$ ) (Fig. 1B).

Figure 2 shows the effects of preincubating the cells with nitrendipine, FTX, and the two drugs together on the initial increase of the  $K^+$ -evoked  $[Ca^{2+}]_i$  response. Representative examples of original experiments are also shown in Fig. 2A. When the cells were preincubated with nitrendipine, the  $[Ca^{2+}]_i$  response decreased to  $63.8 \pm 3.1\%$  of the control response. This was similar to the effect of nitrendipine on the  $[Ca^{2+}]_i$  when the drug was added 2.5 min after  $K^+$  depolarization (see above). Comparison of the  $[Ca^{2+}]_i$  responses obtained in the presence of separately applied FTX and nitrendipine indicates that FTX is more effective than nitrendipine in blocking the initial increase of the  $[Ca^{2+}]_i$ . It is also apparent from Fig. 2A (right trace) and 2B that simultaneous preincubations with nitrendipine and FTX caused a further inhibition of

the  $[Ca^{2+}]_i$  transients to  $29.6 \pm 2.8\%$  of the control response.

FTX (3:3) is an arginine-polyamine synthetic analogue of FTX, in which each of the terminal nitrogens of the polyamine is separated from the central nitrogen by three methylene groups (Cherksey et al., 1991). Figure 2B shows that this analogue is far less potent than FTX in blocking the  $K^+$ -evoked  $[Ca^{2+}]_i$  increase.

#### Effect of FTX and nitrendipine on the $K^+$ -evoked catecholamine release

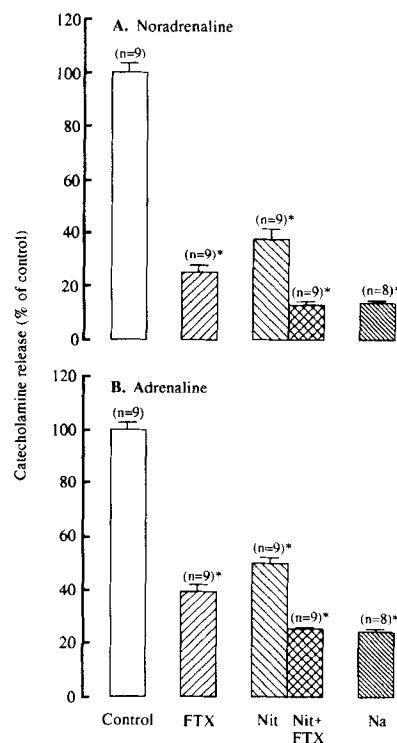
Because nitrendipine and FTX block different  $Ca^{2+}$  influx pathways in Purkinje cells (Llinás et al., 1989), we examined the relative contributions of nitrendipine- and FTX-sensitive VSCCs to catecholamine secretion by depolarizing the chromaffin cells in the presence of these drugs. Figure 3A and B show that FTX decreased the  $K^+$ -evoked noradrenaline and adrenaline release to  $25.0 \pm 2.4$  and  $39.1 \pm 2.5\%$  of the control, more than the inhibition observed in the presence of  $0.1 \mu M$  nitrendipine ( $37.2 \pm 3.8$  and  $49.4 \pm 2.4\%$  for noradrenaline and adrenaline release, respectively) ( $p < 0.001$ ). The release measured in both cases was significantly different from the basal release measured in  $Na^+$  medium. Simultaneous incubation with FTX and nitrendipine further decreased  $K^+$ -evoked noradrenaline and adrenaline release to  $12.9 \pm 1.0$  and  $24.6 \pm 1.1\%$  of the control, respectively, which are not significantly different from the respective basal catecholamine release ( $p > 0.001$ ). Neither nitrendipine nor FTX affected basal catecholamine release (not shown).

#### Effect of $\omega$ -Cg Tx on $K^+$ -evoked $^{45}Ca^{2+}$ uptake

$\omega$ -Cg Tx has been reported to inhibit partially  $K^+$ -evoked  $^{45}Ca^{2+}$  uptake when exposed to cells concomitantly hyperpolarized by low  $K^+$  solutions (Ballesta et al., 1989). However, using similar experimental conditions and a higher toxin concentration ( $0.5 \mu M$ ), we found that the extent of  $^{45}Ca^{2+}$  uptake was not significantly different from control (Fig. 4). Using the Goldman-Hodgkin-Katz equation and published values for the Na/K permeability ratio and the intracellular  $Na^+$  and  $K^+$  concentrations (Rosario et al., 1989), the low  $K^+$  solution used can be shown to hyperpolarize the cells by  $\sim 8$  mV from the resting level of  $\sim -51$  mV. Also shown in Fig. 4 is the effect of incubating the cells with  $\omega$ -Cg Tx in low calcium ( $0.1$  mM), a condition that increases toxin binding to chromaffin cell membranes (Ballesta et al., 1989). Again, no inhibitory effect of  $\omega$ -Cg Tx was found. In contrast, the nonspecific  $Ca^{2+}$  channel blocker  $Gd^{3+}$  (Bourne and Trifaro, 1982) profoundly inhibited the uptake by  $\sim 74\%$  (Fig. 4).

## DISCUSSION

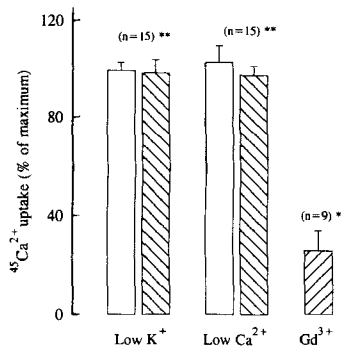
In bovine adrenal chromaffin cells, depolarization-evoked calcium influx and catecholamine release are only partly sensitive to dihydropyridine blockers of



**FIG. 3.** Effect of FTX and nitrendipine on  $K^+$ -evoked catecholamine release. The cells were preincubated for 15 min at  $30^\circ C$  in the presence or in the absence of FTX (1:4,000 dilution, with respect to the original fraction) and/or nitrendipine ( $0.1 \mu M$ ). The cells were subsequently depolarized with  $30$  mM KCl (final concentration,  $35$  mM) for  $2$  min, and catecholamine release was measured as described in Materials and Methods. Noradrenaline (A) and adrenaline (B) release are expressed as percentages of the release measured in the absence of any drug. A:  $100\% = 6.3 \pm 0.3\%$  of the total noradrenaline content, corresponding to  $1.66 \pm 0.07$  nmol. B:  $100\% = 3.3 \pm 0.1\%$  of the total adrenaline content, corresponding to  $2.97 \pm 0.29$  nmol. Results are expressed as the means  $\pm$  SEM of eight to nine measurements performed in three different preparations. The column labeled Na represents basal release in  $Na^+$  medium. \*Significantly lower than control,  $p < 0.05$ .

L-type VSCCs (Ballesta et al., 1989; Owen et al., 1989; Rosario et al., 1989), suggesting that these cells are equipped with dihydropyridine-insensitive  $Ca^{2+}$  channels. In this study, we show that the funnel-web spider toxin FTX is a potent inhibitor of depolarization-evoked  $[Ca^{2+}]_i$  transients and catecholamine release. FTX appears to block specifically P-type VSCCs in Purkinje cells and in the squid giant synapse (Llinás et al., 1989), which differ pharmacologically from L- and N-type VSCCs in that they are insensitive to dihydropyridines and  $\omega$ -Cg Tx (Llinás et al., 1989). Thus, our data suggest that chromaffin cells are also endowed with heretofore undetected P-type  $Ca^{2+}$  channels.

We have also found that simultaneous incubations with FTX and nitrendipine block the  $K^+$ -stimulated  $[Ca^{2+}]_i$  transients more extensively than each drug alone (Figs. 1 and 2), indicating that FTX blocks a dihydropyridine-insensitive component of  $Ca^{2+}$  in-



**FIG. 4.** Effect of  $\omega$ -Cg Tx on  $K^+$ -induced  $^{45}\text{Ca}^{2+}$  uptake. Two left-most columns (low  $K^+$ ): Effects of incubating the cells with  $\omega$ -Cg Tx in a hyperpolarizing (low  $K^+$ ) solution. The cells were preincubated in DMEM/F-12 medium with (hatched columns) or without (open columns)  $\omega$ -Cg Tx ( $0.5 \mu\text{M}$ ) for 60 min, washed, further preincubated with low  $K^+$  ( $1 \text{ mM}$ ) solution with or without  $\omega$ -Cg Tx for 5 min, and finally stimulated with a high  $K^+$  ( $30 \text{ mM}$ ) solution with or without  $\omega$ -Cg Tx for 1 min, as explained in Materials and Methods. Two center columns (low  $\text{Ca}^{2+}$ ): Effect of incubating the cells with  $\omega$ -Cg Tx in low  $\text{Ca}^{2+}$ . The cells were preincubated for 60 min in DMEM/F-12 medium supplemented with  $0.95 \text{ mM}$  EGTA (free  $\text{Ca}^{2+}$  concentration,  $0.1 \text{ mM}$ ), with (hatched column) or without (open column)  $\omega$ -Cg Tx ( $0.5 \mu\text{M}$ ), washed, further preincubated with regular  $\text{Na}^+$  solution with or without  $\omega$ -Cg Tx for 5 min, and finally stimulated as above. Right-most column: Effect of  $\text{Gd}^{3+}$ , as for the two left-most columns, with the difference being that the low  $K^+$  solution used for the 5-min preincubation contained  $40 \mu\text{M}$   $\text{Gd}^{3+}$ . The  $^{45}\text{Ca}^{2+}$  uptake is expressed as a percentage of the control uptake (difference between stimulated and basal uptake) measured in the absence of any drug. Low  $K^+$ :  $100\% = 0.96 \pm 0.06 \text{ nmol of } ^{45}\text{Ca}^{2+}/10^6 \text{ cells/min}$ ; basal uptake =  $0.61 \pm 0.05 \text{ nmol}/10^6 \text{ cells/min}$ . Low  $\text{Ca}^{2+}$ :  $100\% = 0.82 \pm 0.04 \text{ nmol of } ^{45}\text{Ca}^{2+}/10^6 \text{ cells/min}$ ; basal uptake =  $0.62 \pm 0.04 \text{ nmol}/10^6 \text{ cells/min}$ . Results are given as the means  $\pm$  SEM of nine to 15 measurements performed in three ( $\text{Gd}^{3+}$ ) or five ( $\omega$ -Cg Tx) different preparations. \*Significantly lower than control;  $p < 0.05$ , \*\* $p > 0.05$ , compared with control.

flux. This is unlikely to be due to the nitrendipine concentrations used ( $0.1$ – $1 \mu\text{M}$ ), which are  $\sim 100$ – $1,000$ -fold higher than the  $K_D$  for nitrendipine binding to bovine adrenomedullary membrane fragments (Garcia et al., 1984; Ballesta et al., 1989). Furthermore, because under our experimental conditions  $\omega$ -conotoxin is without effect on depolarization-evoked  $^{45}\text{Ca}^{2+}$  uptake (Fig. 4), the dihydropyridine-insensitive component of  $^{45}\text{Ca}^{2+}$  influx blocked by FTX does not result from activation of  $\omega$ -conotoxin-sensitive N-type  $\text{Ca}^{2+}$  channels.

The insensitivity to  $\omega$ -Cg Tx found in this study is in agreement with the previously reported lack of effect of the toxin on  $\text{Ca}^{2+}$  influx,  $[\text{Ca}^{2+}]_i$  transients, and catecholamine secretion (Owen et al., 1989; Rosario et al., 1989; Jan et al., 1990), but is in apparent disagreement with the partial sensitivity to the toxin reported by others (Hans et al., 1990; Bossu et al., 1991; Artalejo et al., 1991b). Although we have no immediate explanation for this discrepancy, it is possible that the more negative holding potentials generally used in the electrophysiological experiments may either en-

hance toxin binding to a specific  $\text{Ca}^{2+}$  channel type or, alternatively, remove the inactivation of a low-threshold,  $\omega$ -Cg Tx-sensitive  $\text{Ca}^{2+}$  channel. In keeping with this possibility, Ballesta et al. (1989) have previously reported enhanced toxin sensitivity when the cells were exposed to  $\omega$ -Cg Tx in hyperpolarizing low  $K^+$  solutions. However, using similar conditions, we failed to uncover an  $\omega$ -Cg Tx effect (Fig. 4).

Our results also show that  $K^+$  depolarization in the presence of FTX produces a sustained  $[\text{Ca}^{2+}]_i$  increase that is similar to the steady-state  $[\text{Ca}^{2+}]_i$  plateau recorded in the absence of the toxin. Furthermore, although FTX had practically no effect when added 2 min after  $K^+$  depolarization (not shown), nitrendipine was apparently equally effective in inhibiting the early (Fig. 2) and the late (Fig. 1) phases of the  $[\text{Ca}^{2+}]_i$  transients. Thus, although the FTX-sensitive  $\text{Ca}^{2+}$  channel appears to contribute preferentially, if not exclusively, to the decaying phase of the  $[\text{Ca}^{2+}]_i$  transient, the dihydropyridine-sensitive channel appears to contribute significantly to both the early and the late phases of the transient. This is consistent with FTX-sensitive channels inactivating due to elevated  $[\text{Ca}^{2+}]_i$  and/or membrane depolarization, as has been proposed for other systems where VSCCs are not of the L and N types (Nachshen, 1985; Carvalho et al., 1986; Duarte et al., 1991; Lundy et al., 1991; Regan et al., 1991).

Depolarization-evoked catecholamine secretion was potently inhibited by separate exposures to FTX and nitrendipine. Furthermore, the secretion was totally blocked by simultaneous exposure to the drugs (Fig. 3). Thus, the results indicate that both FTX-sensitive and nitrendipine-sensitive  $\text{Ca}^{2+}$  channels are functionally involved in depolarization-evoked catecholamine secretion. It is interesting to note that FTX was somewhat more effective than nitrendipine in inhibiting release, but the conclusion that the FTX-sensitive channel might be more efficient in driving secretion cannot be warranted at the present owing to the possibility that the  $\text{Ca}^{2+}$  influxes brought about by activation of both channel types might synergize to cause secretion. Clearly, further studies are necessary to investigate the relative role of different  $\text{Ca}^{2+}$  channel types in catecholamine secretion.

In conclusion, we show here that chromaffin cells are endowed with FTX-sensitive, nitrendipine-insensitive  $\text{Ca}^{2+}$  channels, in addition to L-type VSCCs. Both channel types are coupled to catecholamine secretion.

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