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Calcium channel subtypes in porcine adrenal chromaffin cells

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Abstract The effects of nifedipine, ω-conotoxin GVIA (ω-CgTx) and ω-agatoxin IVA (ω-AgTx) on Ca^{2+} currents, a 60-mM-K^+-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and catecholamine secretion were examined to clarify the subtypes of Ca^{2+} channels in cultured adrenal chromaffin cells from the pig. Nifedipine, ω-CgTx, and ω-AgTx inhibited Ca^{2+} currents in a dose-dependent manner, suggesting the presence of L-, N- and P-type Ca^{2+} channels. The maximal doses of nifedipine (10 μM), ω-CgTx (1 μM), and ω-AgTx (0.1 μM) inhibited Ca^{2+} currents to 85%, 22%, and 94% of control currents, respectively. The inhibitory effects of these three blockers were observed in the same cell, indicating that at least three subtypes of Ca^{2+} channels are present in porcine chromaffin cells. The increase in $[Ca^{2+}]_i$ and catecholamine secretion induced by 60 mM K^+ were inhibited equally by nifedipine (10 μM) and ω-CgTx (1 μM), but not by ω-AgTx (0.1 μM). These results suggest that L-, N- and P-type Ca^{2+} channels are present in porcine adrenal chromaffin cells, and that the major pathways of Ca^{2+} entry evoked by a high concentration of K^+ are L- and N-type Ca^{2+} channels.

Key words Adrenal chromaffin cell · Ca^{2+} current · Ca^{2+} channel · Nifedipine · ω-Conotoxin GVIA · ω-Agatoxin IVA

Introduction

Ca^{2+} channels in neuronal cells are classified into several subtypes by their pharmacological and electrophysiological properties [19, 22]. Adrenal chromaffin cells originating in the neural crests have been reported to have only the high voltage-activated type of Ca^{2+} channels [2, 10] which are classified as N-, L-, P-, and Q/O-type channels by use of their selective blockers, i.e., ω-conotoxin GVIA (ω-CgTx), dihydropyridines, ω-agatoxin IVA (ω-AgTx), and ω-conotoxin MVIIC, respectively [2, 4, 5, 12, 17]. It appears that the relative contributions made by currents through the various Ca^{2+} channels to be total Ca^{2+} current and the electrophysiological characteristics of these channels differ among species. In bovine adrenal chromaffin cells, L-type Ca^{2+} channels are normally quiescent and appear only when cells are stimulated by preceding depolarizing pulses or D_1-receptor agonists [3, 5], and N-type Ca^{2+} channels have been proposed as being present heterogeneously and to be absent from some cells [6]. In contrast, in chromaffin cells of the cat and rat, L-type Ca^{2+} channels are reported to be involved in standard Ca^{2+} currents [2, 15].

Ca^{2+} entering into cells through voltage-dependent Ca^{2+} channels is well known to play an essential role in catecholamine release from adrenal chromaffin cells. However, it has been proposed that secretory machinery can discriminate between Ca^{2+} passing through Ca^{2+} channel subtypes. L-Type Ca^{2+} channels or L- and Q-type channels seem to contribute to catecholamine secretion more efficiently than other channel types in adrenal chromaffin cells of the ox and cat [5, 16, 17], but not in the rat [15].

It has been reported that in cultured adrenal chromaffin cells of the pig, high K^+ and nicotine evoke catecholamine secretion depending on the extracellular Ca^{2+} concentration [24]. As to the properties of Ca^{2+} channels in this species, however, there is only one study, which found that one of the L-type Ca^{2+} channel blockers, nifedipine, partially inhibits secretory responses to high K^+ concentrations and nicotine [11]. The possibility exists, therefore, that other subtypes of voltage-dependent Ca^{2+} channels are involved in secretory responses to depolarization in porcine adrenal chromaffin cells.

In the present study, the effects of ω-CgTx, nifedipine, and ω-AgTx on a whole-cell Ca^{2+} current evoked by a depolarizing pulse, the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and catecholamine release in response to a high K^+ concentration were investigated to

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clarify the subtypes of Ca^{2+} channels in porcine adrenal chromaffin cells.

Materials and methods

Cell culture

Porcine adrenal glands were obtained from the local slaughterhouse and were brought back to our laboratory in an ice-cold Ca^{2+}-free physiological salt solution [Ca^{2+}-free PSS, in mM: 154 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 10 glucose, 10 HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid), pH = 7.4 with NaOH]. Porcine adrenal chromaffin cells were isolated from the adrenal medullary tissue by the collagenase digestion method reported by Brooks [9] with some modification. In brief, after the adrenal cortex was removed, adrenal medullae were minced into about 2-mm pieces with scissors in ice-cold Ca^{2+}-free PSS. Minced pieces were incubated in Ca^{2+}-free PSS with 200 U/ml collagenase (type I, Worthington Biochemical) and 0.5% bovine serum albumin (BSA) for 30 min at 37°C with shaking. After the incubation, the cell suspension was agitated gently with a pipette. Collagenase was removed by centrifugation and isolated cells were resuspended in fresh Ca^{2+}-free PSS and then filtered with nylon mesh. Collagenase digestion was repeated five to six times with a solution containing fresh collagenase. The cell suspension from the first and second digestion was discarded, since it included a large amount of cell debris and red blood cells. The cells obtained from later digestions were combined and resuspended in Dulbecco's modified Eagle's Medium (DMEM, Gibco) and cultured on coverslips and in 48-well plates. The cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 in an incubator at 37°C for 2 weeks. DMEM was supplemented with 10% new-born calf serum (Gibco), 100 U/ml penicillin, 100 mg/ml streptomycin, 0.5–2.5 μg/ml fungizone, 10 μM cytosine arabinoside and 25 μM fluorodeoxyuridine. The culture medium was replaced with fresh medium every 2 days.

Whole-cell current recording

Whole-cell currents were measured with standard whole-cell voltage-clamp techniques [14]. Heat-polished electrodes with 2.5- to 4-MΩ tip resistance were used. The composition of normal extracellular solution was: 134 NaCl, 6 KCl, 1.2 $MgCl_2$, 1.7 $CaCl_2$, 10 glucose and 10 HEPES (in mM, pH = 7.4 adjusted with NaOH). For the measurements of Ca^{2+} currents, 5 or 10 mM $CaCl_2$ was used instead of 1.7 mM $CaCl_2$. To inhibit voltage-dependent Na^+ currents, 0.2 μM tetrodotoxin was added to the extracellular solution. The pipette solution contained: 120 CsCl, 20 TEACl (tetraethylammonium chloride), 1.2 $MgCl_2$, 1.2 ATP (adenosine triphosphate), 0.2 GTP (guanosine triphosphate), 10 HEPES and 10 EGTA [ethyleneglycolbis(aminoethylether)tetra-acetate] (in mM, pH = 7.2 adjusted with CsOH). Whole-cell currents were measured by the patch-clamp amplifier (CEZ-2300, Nihon Koden, Japan) and sampled at 5–40 kHz by an analog/digital converter (MacLab, AD Instruments, Australia) in conjunction with a personal computer (Macintosh, Apple, USA). Stored data were analyzed by the software Igor Pro (Wavemetrics, USA). The liquid junction potential between the pipette solution and the extracellular solution was canceled before forming gigaohm seals. Cells were continuously superfused with normal PSS at a flow rate of 1–2 ml/min through the pipette placed 150 μm from the cells. Drugs were applied using the same pipette. Experiments were carried out at room temperature (20–25°C).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in single porcine adrenal chromaffin cells was measured with the fluorescent Ca^{2+} indicator, fura-2. Chromaffin cells attached to coverslips were incubated in Ca^{2+}-free PSS containing fura-2 acetoxymethyl ester (fura-2/AM, 5 μM) for 45–60 min at 37°C. After the incubation, a coverslip was mounted in a chamber fixed on the stage of a fluorescence microscope (model TMD, Nikon, Japan). High-K^+ solution (in mM: 80 NaCl, 60 KCl, 1.2 $MgCl_2$, 1.7 $CaCl_2$, 10 glucose and 10 HEPES, pH = 7.4 with NaOH) was applied through the pipette connected to a peristaltic pump (minipuls, Gilson, USA) controlled by the personal computer. In this system, the solution around the cell could be changed completely within 200 ms. Changes in $[Ca^{2+}]_i$ were measured by dual excitation microfluorometry using a CAM-200 spectrometer (Jasco, Japan). The fluorescence signal was detected using a Nikon CF UV lens (Fluor, X40 oil-immersion objectives), and the emission light, which was passed through a pinhole diaphragm slightly larger than the cell, was collected by a photomultiplier through a 500-nm filter. Fluorescence intensity was measured at excitation wavelengths of 340 and 380 nm and sampled at 20 Hz by MacLab in conjunction with the personal computer and stored data were analyzed by the software mentioned above. The ratio of fluorescence of a cell excited at 340 nm to that excited at 380 nm was used to calculate $[Ca^{2+}]_i$ [13]. The standard curves were determined using a series of Ca^{2+}-buffered solutions (Molecular Probes). Differences between calculated ratios (F_{340}/F_{380}) obtained in cells loaded with fura-2/AM and the standard curve were corrected [20]. Experiments were done at room temperature (20–25°C).

Catecholamine secretion

Experiments for the measurement of catecholamine secretion were carried out using cells cultured in 48-well plates. First, cells were washed with normal PSS and preincubated with or without Ca^{2+} channel blockers at 37°C for 20 min. Ca^{2+} channel blockers were present during the periods from the latter half of preincubation (10 min) throughout the incubation for the test. After preincubation, the cells were washed with ice-cold PSS three times. Then high-K^+ solution was added, and the cells were incubated for 15 min. Adrenaline and noradrenaline released from cells and remaining in the cells were measured by an electrochemical detector (EC-100, EICOM, Japan) connected to a high-performance liquid chromatography (HPLC) system (Jasco, Japan). The detection limit was 10 nM for both adrenaline and noradrenaline. The treatment of samples for HPLC was carried out according to the method of Salzman and Sellers [21]. The ratio of noradrenaline content to adrenaline content (3 to 25) tended to increase during culture as reported [23]. The release of noradrenaline from cells was therefore considered to be an index of catecholamine release. Catecholamine secretion was expressed as a percentage of the total catecholamine content in the cells.

Drugs

The following drugs were purchased: nifedipine (Wako Pure Chemicals, Japan); ω-conotoxin GVIA and ω-agatoxin IVA (Peptide Institute, Japan). Media containing nifedipine were prepared from a concentrated stock solution (0.1 and 0.01 M) of the drug in dimethyl sulfoxide immediately before use. The stock solution of nifedipine was kept in the dark at –20°C. All other drugs used were of analytical grade.

Statistics

The data are presented as the mean ± SEM (*n* = number of observations). Statistical significance was assessed using Student's *t*-test, and *P* values less than 0.05 were considered significant.

Results

Voltage-current relationship of voltage-dependent Ca^{2+} channel currents

Porcine adrenal chromaffin cells, 8–15 μm in diameter, were used to measure Ca^{2+} currents. Cell capacitance was 5.2 ± 0.2 pF (n = 90). The application of a depolarizing pulse to +15 mV from a holding potential of –80 mV evoked an inward current which was completely blocked by Co^{2+} (0.5 mM), indicating the contribution of voltage-dependent Ca^{2+} channels. Since Ca^{2+} currents increased during culture in the first 3 days, and currents of steady amplitude could be obtained from cells cultured for 4–10 days, the experiments were performed using 4- to 10-day-old cultured cells. After cells were voltage-clamped, the amplitude of Ca^{2+} currents induced by depolarizing pulses was gradually increased and reached a steady level within 3 min. The experiments were therefore started after steady Ca^{2+} currents had been obtained. In the presence of 10 mM Ca^{2+}, the holding current at a holding potential of –80 mV was –11.5 ± 0.9 pA (n = 90), and the peak amplitude of Ca^{2+} currents evoked by a depolarizing pulse to +15 mV for 25 ms was –341 ± 13 pA (n = 90).

Figure 1A, B shows the voltage-current (*V-I*) relationships of Ca^{2+} current responses to various depolarizing pulses from a holding potential of –80 mV in the presence of 5 and 10 mM Ca^{2+}. Ca^{2+} current began to be evoked by a depolarizing pulse of –30 mV, increased in amplitude with an increase in the magnitude of the pulses, and attained a maximum at approximately +10 mV. The potential of pulses which evoked maximal Ca^{2+} currents was slightly higher in the presence of 10 mM Ca^{2+} than 5 mM Ca^{2+}. The reversal potential of Ca^{2+} currents was 74 ± 2 mV (n = 9) in the presence of 10 mM Ca^{2+}. This *V-I* relationship was not influenced by holding potentials of between –100 mV and –60 mV (data not shown). In addition, low-voltage-activated Ca^{2+} currents did not appear in all cells tested. In the following experiments, Ca^{2+} currents were evoked in the presence of external 10 mM Ca^{2+}.

Figure 1C shows a steady-state inactivation curve for Ca^{2+} currents. Ca^{2+} currents were repetitively elicited by depolarizing pulses to +15 mV at intervals of 20 s from various holding potentials that were maintained long enough for inactivation to reach a steady state (40–300 s). Ca^{2+} currents were decreased by holding potentials of higher than –60 mV, and half inactivation was obtained at about –40 mV. Ca^{2+} currents were inactivated completely by holding potentials more positive than –10 mV.

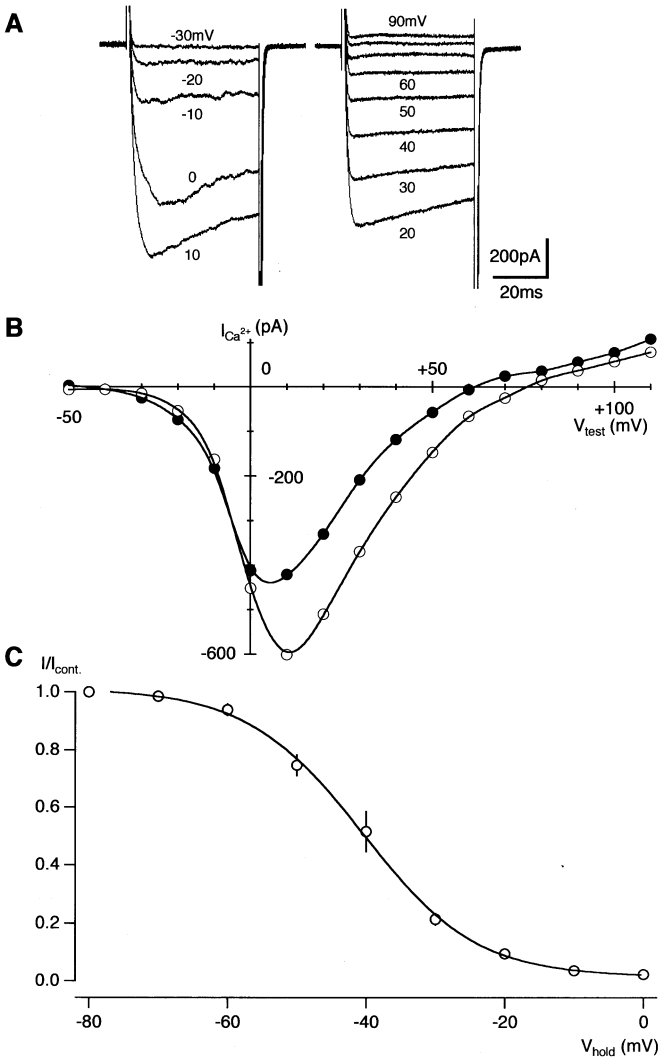

Fig. 1A–C Voltage-current relationships and steady-state inactivation curve of voltage-dependent Ca^{2+} current in porcine adrenal chromaffin cells. **A** Ca^{2+} currents evoked by depolarizing pulses to various voltages indicated from a holding potential of –80 mV in the presence of 10 mM Ca^{2+}. **B** Peak Ca^{2+} currents ($I_{Ca}{}^{2+}$) shown in **A** were plotted against pulse potentials (*open circles*). The voltage-current relationship obtained in the presence of 5 mM Ca^{2+} from the same cell in **A** is also indicated (*solid circles*). Leak currents caused by voltage pulses were subtracted. Similar results were obtained from 4 other cells of two cultures. **C** Steady-state inactivation curve for Ca^{2+} currents evoked by depolarizing pulses to +15 mV from various holding potentials. Ca^{2+} currents relative to those at –80 mV (I/I_{cont}) were plotted against holding potentials (V_{hold}). *Symbols* and *vertical bars* indicate the mean value ± SEM of I/I_{cont} obtained from 4 to 11 experiments. A *smooth curve* derived from the Boltzmann relation is given by $I = I_{max}\{1+\exp[(V_{1/2}-V)/k]\}^{-1}$, where $V_{1/2}$ = –40.6 mV and k = 8.2 mV

Effects of Ca^{2+} channel blockers on Ca^{2+} currents

The inhibitory effects of ω-CgTx, nifedipine and ω-AgTx on Ca^{2+} currents were examined to clarify the Ca^{2+} channel subtypes in porcine adrenal chromaffin cells (Fig. 2). Ca^{2+} currents were evoked by depolarizing pulses to +15 mV from a holding potential of –80 mV at intervals of 20 s. The application of nifedipine (10 μM) caused a rapid decrease of Ca^{2+} currents that reached a steady level within at most 40 s (2nd pulse). ω-AgTx inhibited Ca^{2+} currents gradually, and maximal inhibition was observed within 2 min in each cell. The effect of ω-

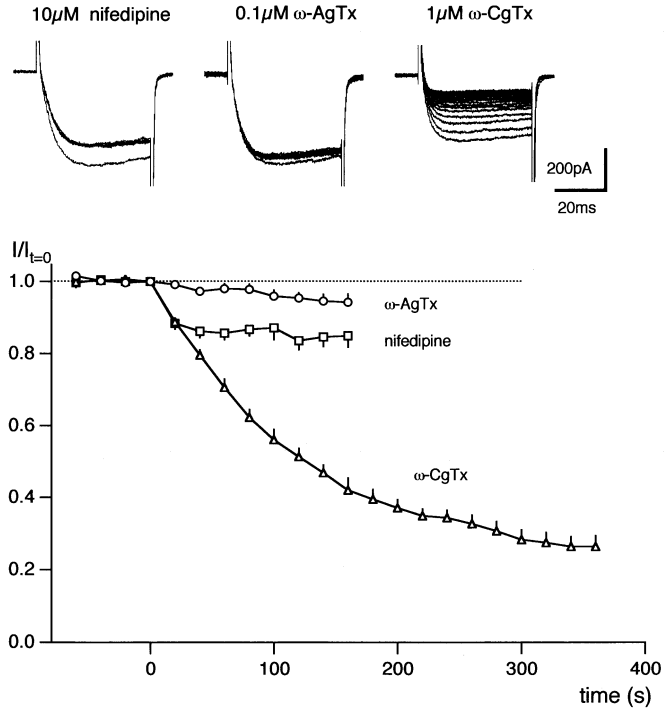

Fig. 2 Time courses of inhibition of Ca^{2+} currents by nifedipine, ω-CgTx and ω-AgTx. Ca^{2+} currents were evoked by the depolarizing pulses to +15 mV from –80 mV at intervals of 20 s in the presence of 10 mM Ca^{2+}. The *upper panel* shows the overlapped traces of the typical Ca^{2+} currents in the presence of nifedipine (10 μM), ω-agatoxin IVA (ω-*AgTx*, 0.1 μM) and ω-conotoxin GVIA (ω-*CgTx*, 1 μM). In the *lower panel*, peak Ca^{2+} currents were normalized to peak currents measured just before application of Ca^{2+} channel blockers. These normalized values of peak currents ($I/I_{t=0}$) in cells treated with 0.1 μM ω-AgTx (*circles*, n = 7), 10 μM nifedipine (*squares*, n = 9) and 1 μM ω-CgTx (*triangles*, n = 5) were plotted against time. *Symbols* and *vertical bars* indicate the mean value ± SEM of $I/I_{t=0}$

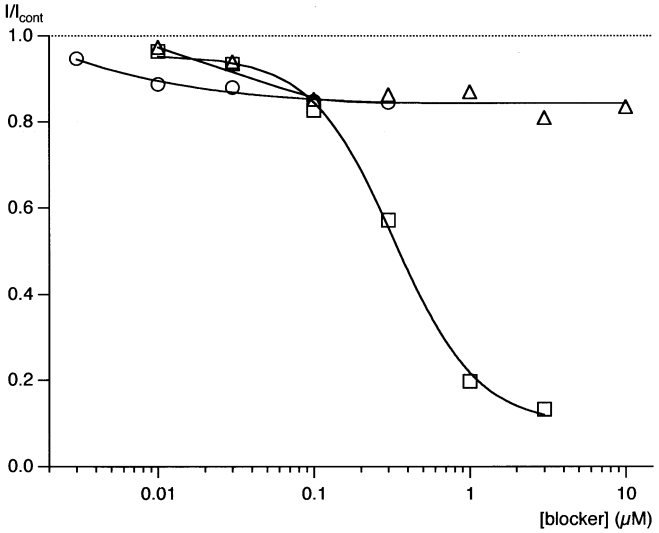

Fig. 3 Dose-dependent inhibition of Ca^{2+} currents by Ca^{2+} channel blockers. Ca^{2+} currents were evoked by depolarizing pulses to +15 mV from –80 mV at intervals of 20 s in the presence of 10 mM Ca^{2+}. Two doses of nifedipine, ω-CgTx or ω-AgTx were applied to each cell. Peak Ca^{2+} currents relative to those measured immediately before application of blockers were plotted against log doses of the drugs. Peak Ca^{2+} currents were plotted 120 s after application of nifedipine (*open triangles*, n = 4–8) and ω-AgTx (*open circles*, n = 4–5), and 240 s after application of ω-CgTx (*open squares*, n = 4–11). *Symbols* indicate the mean values

CgTx (1 μM) on Ca^{2+} currents was characterized by slowly developing inhibition. It took 284 ± 31 s (n = 5) for ω-CgTx to cause maximal inhibition of Ca^{2+} currents. Assuming that the time course of this inhibitory action was single exponential decay, the apparent time constant was 135 ± 17 s (n = 5). The percentages of Ca^{2+} currents in the presence of ω-CgTx (1 μM), nifedipine (10 μM) and ω-AgTx (0.1 μM) were 22 ± 3% (n = 9), 85 ± 4% (n = 5) and 94 ± 3% (n = 7), respectively. The inhibitory effect of nifedipine, but not those of ω-CgTx and ω-AgTx, was partially reversible (data not shown).

Dose-dependent inhibition of Ca^{2+} currents by Ca^{2+} channel blockers

Ca^{2+} currents were evoked by depolarizing pulses to +15 mV for 25 ms from a holding potential of –80 mV at intervals of 20 s. Each concentration of Ca^{2+} channel blockers was applied to a cell until the inhibition attained a steady level. Since repetitive application of depolarizing pulses caused a "rundown" of the Ca^{2+} currents, two concentrations of Ca^{2+} channel blockers were applied to each cell to reduce the influence of the rundown. Peak amplitudes of Ca^{2+} currents relative to those observed immediately before applications of Ca^{2+} channel blockers (I/I_{cont}) were plotted against the log doses of the drugs (Fig. 3).

ω-CgTx inhibited Ca^{2+} currents dose dependently, and maximal inhibition was obtained at 1–3 μM (to 13 ± 3% of control, n = 4 at 3 μM). Nifedipine and ω-AgTx also inhibited Ca^{2+} currents in a dose-dependent manner. These inhibitory effects were much weaker than that of ω-CgTx, though ω-AgTx was more effective at lower concentrations than the other two drugs. The maximal inhibition was attained at 3–10 μM (to 81 ± 2% of control, n = 4 at 10 μM) with nifedipine and at 0.1 μM (to 85 ± 3% of control, n = 5) with ω-AgTx. These values are larger than those obtained from the experiments with the single application of Ca^{2+} channel blockers at maximal doses. These differences seem to be caused by the rundown of Ca^{2+} currents masked by the inhibitory action of Ca^{2+} channel blockers applied over a long period, i.e., the rundown of Ca^{2+} currents might overestimate the inhibitory effects of nifedipine, ω-CgTx and, in particular, ω-AgTx.

The results illustrated in Figs. 2 and 3 suggest that porcine adrenal chromaffin cells have N-, L- and P-type Ca^{2+} channels and the N-type channels contribute to whole-cell currents much more than do the L- and P-type channels.

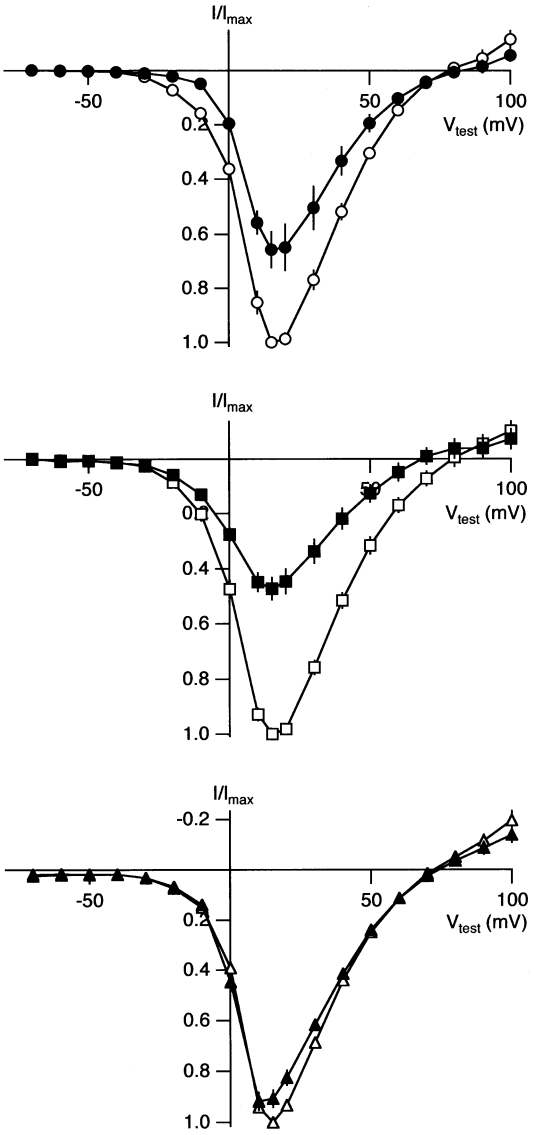

Fig. 4 Effects of nifedipine, ω-CgTx and ω-AgTx on the voltage-current (*V-I*) relationship of Ca^{2+} current. Ca^{2+} currents were evoked by depolarizing pulses for 25 ms to various potentials from a holding potential of –80 mV at intervals of 20 s in the presence of 10 mM Ca^{2+}. Ca^{2+} currents are normalized to the control Ca^{2+} current evoked by the depolarizing pulse to +15 mV in each cell and plotted as I/I_{max}. The effects of nifedipine (*closed circles*, n = 6), ω-AgTx (*closed triangles*, n = 5) and ω-CgTx (*closed squares*, n = 5) on the *V-I* relationship of Ca^{2+} current were examined 2 min, 2 min and 4 min after application of these drugs, respectively. The *V-I* relationship of control Ca^{2+} currents is also illustrated (*open symbols*). *Symbols* and *vertical bars* indicate the mean value ± SEM of I/I_{max}

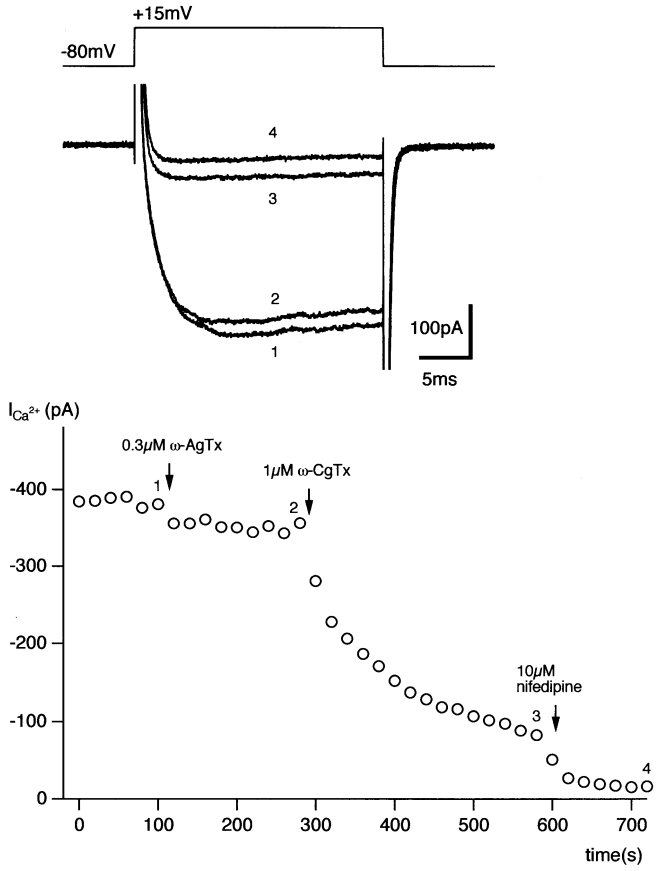

Fig. 5 Effects of sequential application of ω-AgTx, ω-CgTx and nifedipine on voltage-dependent Ca^{2+} currents. Ca^{2+} currents were evoked by depolarizing pulses for 25 ms to +15 mV from a holding potential of –80 mV at intervals of 20 s in the presence of 10 mM Ca^{2+}. The *upper panel* shows Ca^{2+} currents in a cell before (*1*) and after treatment with 0.3 μM ω-AgTx (*2*), 1 μM ω-CgTx (*3*) and 10 μM nifedipine (*4*) sequentially. In the *lower panel*, the peak Ca^{2+} currents ($I_{Ca^{2+}}$) evoked by depolarizing pulses shown in the *upper panel* are plotted against time. Each Ca^{2+} channel blocker was applied as indicated by *arrows*. *Numbers 1–4* correspond to those in *upper traces*

Effects of Ca^{2+} channel blockers on the *V-I* relationship of Ca^{2+} currents

Figure 4 shows the *V-I* relationship of Ca^{2+} currents before and after treatment with 10 μM nifedipine, 1 μM ω-CgTx and 0.1 μM ω-AgTx. Ca^{2+} currents were elicited by depolarizing pulses to various voltages from a holding potential of –80 mV at intervals of 20 s. Ca^{2+} currents were recorded 2 min after application of nifedipine (10 μM) and ω-AgTx (0.1 μM) and 4 min after application of ω-CgTx (1 μM). All these Ca^{2+} channel blockers caused the decrease in amplitudes of Ca^{2+} currents evoked by various steps without large effects on their voltage dependency.

Effects of sequential applications of Ca^{2+} channel blockers on Ca^{2+} currents

To investigate whether these Ca^{2+} channels were all present in the same cells, Ca^{2+} channel blockers were applied sequentially (Fig. 5). ω-AgTx at 0.3 μM caused a slight decrease in Ca^{2+} currents to 91 ± 1% of control currents (n = 6). The 1 μM of ω-CgTx applied subsequently caused a great decrease in Ca^{2+} currents, to 22 ± 2% (n = 4). The remaining Ca^{2+} currents were inhibited by 10 μM nifedipine. Similar results were obtained when

these blockers were applied in different sequences, such as ω-CgTx, ω-AgTx and nifedipine. However, in three out of ten cells, a small inward current (44 ± 11 pA) which was completely blocked by 5 mM Co^{2+} remained even in the presence of these three Ca^{2+} channel blockers.

Effects of Ca^{2+} channel blockers on Ca^{2+} currents induced by long depolarizing pulses

The effects of maximal doses of ω-CgTx (1 μM), nifedipine (10 μM) and ω-AgTx (0.1 μM) on Ca^{2+} currents evoked by depolarizing pulses of 1 s duration were examined (Fig. 6). Ca^{2+} currents evoked by depolarizing pulses to +15 mV from the holding potential of –80 mV gradually decreased during the 1-s depolarization. The time course of time-dependent inactivation of Ca^{2+} currents fits the double exponential decay curve well and its time constants were 96 ± 25 ms and 793 ± 110 ms (n = 19). Nifedipine and ω-AgTx inhibited the amplitude of the ends of the Ca^{2+} currents induced by depolarizing pulses for 1 s much more strongly than the peak Ca^{2+} currents. The inhibitory rates of the peaks and the ends of Ca^{2+} currents evoked by 1-s depolarizing pulses were 15 ± 2% and 52 ± 3% (n = 6) with nifedipine, and 3.4 ± 0.5% and 31 ± 3% (n = 6) with ω-AgTx. ω-CgTx inhibited the peaks and the ends of Ca^{2+} currents equally, i.e., by 65 ± 4% and 61 ± 3% (n = 7), respectively. As indicated in Fig. 6, only the ω-CgTx-sensitive component of the Ca^{2+} current decreased during the 1-s depolarization. Assuming that the time course of inactivation of ω-CgTx-sensitive component was a double exponential decay, time constants were estimated to be 74 ms and 732 ms. On the other hand, nifedipine and ω-AgTx-sensitive components of Ca^{2+} currents were slowly activated and no inactivation was observed during the 1-s depolarizing pulse. If porcine chromaffin cells had three types of Ca^{2+} channels, the nifedipine-sensitive current was expected to be same as the ω-AgTx- and ω-CgTx-resistant current. Unlike the nifedipine-sensitive current, however, the ω-CgTx- and ω-AgTx-resistant current decreased gradually during the 1-s depolarization.

Effects of Ca^{2+} channel blockers on the high-K^+-evoked increase in $[Ca^{2+}]_i$

Solution containing 60 mM K^+ was applied to cells loaded with fura-2 for 5 s at intervals of 7 min. Representative data are shown in Fig. 7. $[Ca^{2+}]_i$ rose during the 5-s application of high-K^+ solution and began to fall when K^+ concentrations returned to 6 mM. The resting $[Ca^{2+}]_i$ in porcine adrenal chromaffin cells was 34 ± 2 nM (n = 26), and the first application of 60 mM K^+ for 5 s raised $[Ca^{2+}]_i$ to 314 ± 32 nM (n = 26). $[Ca^{2+}]_i$ responses to 60 mM K^+ were gradually decreased by the repetitive application, to 80–90% of each preceding response (Table 1). Therefore, Ca^{2+} channel blockers were applied to cells after two control responses were observed. Then,

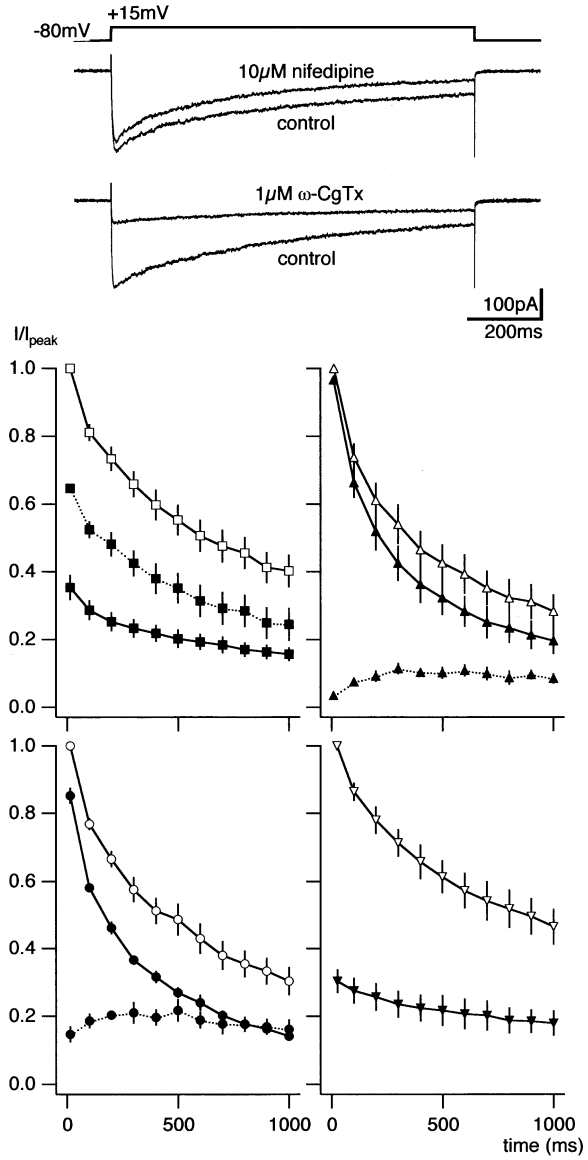

Fig. 6 Effects of nifedipine, ω-CgTx, and ω-AgTx on Ca^{2+} currents by long depolarization. Ca^{2+} currents were evoked by depolarizing pulses for 1 s to +15 mV from the holding potential of –80 mV in the presence of 10 mM Ca^{2+}. Ca^{2+} currents were recorded 180 s after application of 10 μM nifedipine and 0.1 μM ω-AgTx and 240 s after application of 1 μM ω-CgTx. *Upper panels* indicate the overlapped traces of typical Ca^{2+} currents in the presence and absence (control) of nifedipine and ω-CgTx. In the *lower panels*, relative Ca^{2+} currents to peak amplitudes of control currents in each cell were plotted against time. *Open symbols* indicate the control currents, and *closed symbols* with *continuous lines* indicate Ca^{2+} currents after treatment with nifedipine (*circles*, n = 6), ω-CgTx (*squares*, n = 7) and ω-AgTx (*triangles*, n = 5) and ω-CgTx and ω-AgTx in combination (*reversed triangles*, n = 3). *Closed symbols* with *dashed lines* indicate Ca^{2+} currents sensitive to blocker, calculated as the difference of Ca^{2+} currents with and without each Ca^{2+} channel blocker. *Symbols* and *vertical bars* indicate the mean values ± SEM

responses were evoked again after washing out the Ca^{2+} channel blockers. ω-AgTx failed to inhibit the $[Ca^{2+}]_i$ responses to 60 mM K^+. However, nifedipine and ω-CgTx were effective in decreasing $[Ca^{2+}]_i$ responses to 60 mM K^+. The ratio of the third responses to the first response

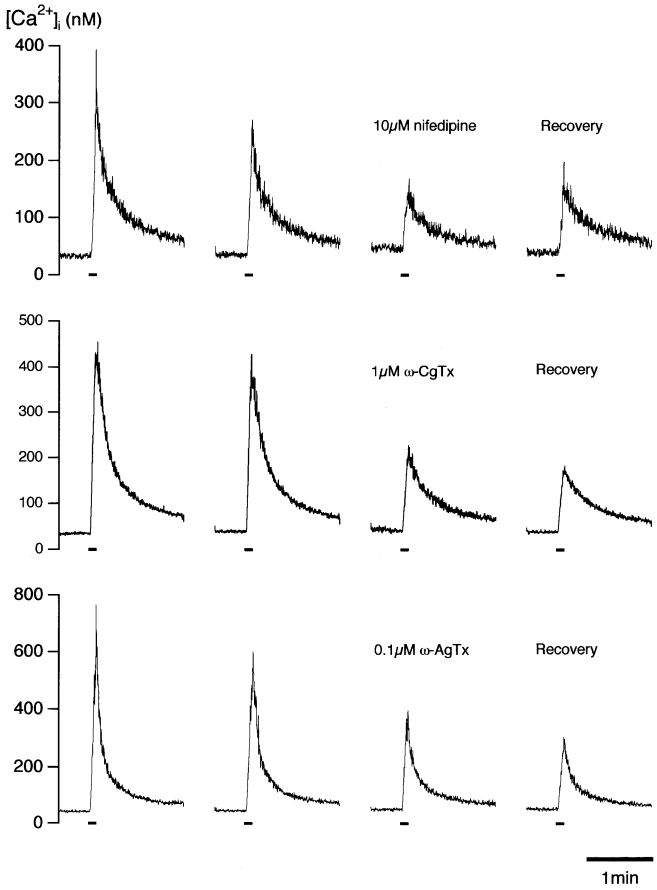

Fig. 7 Effects of nifedipine, ω-CgTx and ω-AgTx on the 60-mM-K^+-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in single porcine adrenal chromaffin cells loaded with fura-2. Cells were stimulated with a solution containing 60 mM K^+ for 5 s (*solid bar*) at intervals of 7 min. *Each panel* represents typical responses to 60 mM K^+ in the same cell. The *third response* in each *panel* was evoked in the presence of 10 μM nifedipine, 1 μM ω-CgTx or 0.1 μM ω-AgTx. Nifedipine and ω-AgTx were applied 3 min before and during stimulation with 60 mM K^+. ω-CgTx was applied 5 min prior to stimulation

in control and in the presence of nifedipine, ω-CgTx, ω-AgTx were 65%, 31%, 32%, and 67%, respectively. If the decrease in the $[Ca^{2+}]_i$ responses to repetitive stimuli with high K^+ is taken into account, nifedipine and ω-CgTx inhibit about 50% of control responses. The inhibitory effect of nifedipine but not that of ω-CgTx was partially reversible, similar to their effects on Ca^{2+} currents evoked by depolarizing pulses.

Effects of Ca^{2+} channel blockers on high-K^+-evoked catecholamine secretion

Cultured porcine adrenal chromaffin cells incubated in normal PSS at 37°C for 15 min spontaneously released 2.4 ± 0.3% (n = 20) of the total content of catecholamine (Fig. 8). This spontaneous release of catecholamine was not affected by any Ca^{2+} channel blocker used. Significant catecholamine release was evoked by 60 mM K^+, which was inhibited by nifedipine (10 μM) and ω-CgTx (1 μM) but not by ω-AgTx (0.1 μM). Nifedipine (10 μM) and ω-CgTx (1 μM) decreased catecholamine release by 53 ± 9% and 48 ± 12%, respectively.

Table 1 Effects of Ca^{2+} channel blockers on 60-mM K^+-induced increase in intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$, nM). High K^+ (60 mM) solution was appplied for 5 s sequentially at intervals of 7 min. The 3rd responses were observed in the absence (control) and presence of 10 μM nifedipine, 1 μM ω-CgTx and 0.1 μM ω-AgTx. Resting intracellular Ca^{2+} concentrations were subtracted. Mean values ± SEM are indicated. (*ω-CgTx* ω-conotoxin GVIA, *ω-AgTx* ω-agatoxin IVA)

Conditions	Response number				
	1st	2nd	3rd	4th	*n*
Control	351±59	279±50	231±37		14
Nifedipine	367±57	318±40	115±25**	167±25	10
ω-CgTx	324±87	285±61	106±28*	97±22	5
ω-AgTx	275±69	255±66	186±54	149±39	10

* $P < 0.05$ and ** $P < 0.01$ compared with the preceding responses

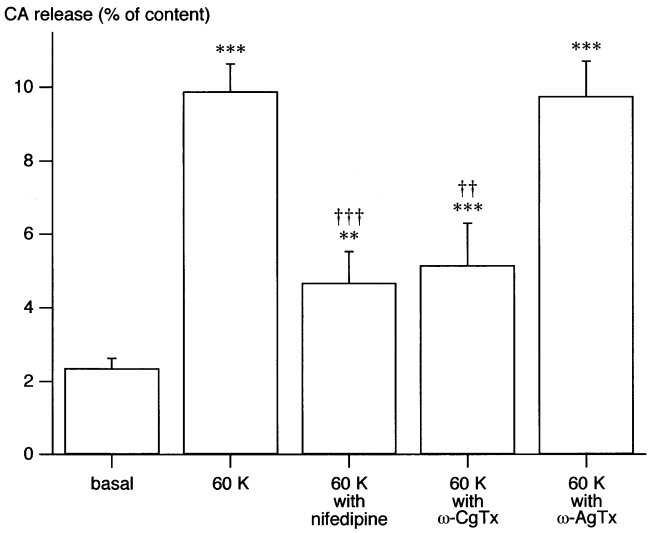

Fig. 8 Effects of nifedipine, ω-CgTx and ω-AgTx on 60-mM-K^+-induced catecholamine (*CA*) release. The catecholamine release from cells during the 15-min incubation at 37°C was examined. Catecholamine leakage from cells incubated for 15 min at 4°C was subtracted. Ca^{2+} channel blockers were applied 5 min before and during stimulation with 60 mM K^+. Results are expressed as the released catecholamine as a percentage of the total catecholamine content in the cells. *Columns* and *vertical bars* indicate mean values ± SEM. From *left* to *right*, *columns* indicate the *basal* (n = 20) level and responses to 60 mM K^+ without (n = 9) and with 10 μM nifedipine, (n = 6) 1 μM ω-CgTx (n = 6) and 0.1 μM ω-AgTx (n = 9), respectively. Experiments were carried out with four independent preparations. **P<0.01 and ***P<0.001, when compared with basal release. ††P<0.01 and †††P<0.001, when compared with 60-mM-K^+-induced release in the absence of blockers

Discussion

The results of the present experiments indicate that only the high-voltage-activated type of Ca^{2+} channels is present in porcine adrenal chromaffin cells. The maximal

doses of ω-CgTx, nifedipine and ω-AgTx decreased Ca^{2+} currents to 78%, 15%, and 6% of control, respectively. This finding suggests that the L-, N- and P-types of Ca^{2+} channels exist in the porcine adrenal chromaffin cells similar to those of the ox [1, 7] and cat [2], because these Ca^{2+} channel subtypes are commonly defined as Ca^{2+} channels sensitive to 1,4-dihydropyridine derivatives, ω-conotoxin GVIA and ω-agatoxin IVA, respectively [19]. Non of the three Ca^{2+} channel blockers changed the *V-I* relationship of Ca^{2+} current, suggesting that each subtype of Ca^{2+} channel is activated by a similar range of pulse voltages.

In bovine adrenal chromaffin cells, however, Ca^{2+} currents have been shown to be sensitive to ω-CgTx in some cells but not in others, suggesting the heterogeneous existence of N-type Ca^{2+} channels [6]; L-type Ca^{2+} channels have been reported not to be involved in standard Ca^{2+} currents but to be unmasked by preceding depolarization or by stimulation of D_1 dopamine receptors [3, 5]. These findings are not similar to the present observations of porcine adrenal chromaffin cells, because Ca^{2+} currents were inhibited stepwise by these Ca^{2+} channel blockers sequentially applied to the same cells. In three of ten cells examined, however, a small inward current still remained in the presence of all these blockers, as was also reported to occur in bovine chromaffin cells [1].

In bovine [6] and feline [2] adrenal chromaffin cells, only 2 min of exposure to ω-CgTx was sufficient to cause maximal inhibition of Ba^{2+} currents. In porcine adrenal chromaffin cells, however, the inhibitory effects of ω-CgTx on Ca^{2+} currents developed much more slowly in the presence of 10 mM Ca^{2+}, though the solution surrounding the cell could be changed completely within 200 ms. The differences in the time course of inhibition by ω-CgTx cannot be explained by Ca^{2+}-dependent inhibition of ω-CgTx binding as reported in bovine chromaffin cells [8], because the inhibitory action of ω-CgTx also developed slowly when Ba^{2+} was used as a charge carrier.

ω-CgTx-sensitive Ca^{2+} currents decreased during a 1-s depolarization, similar to the control current. However, nifedipine- and ω-AgTx-sensitive Ca^{2+} currents were not inactivated during the 1-s depolarizing pulse. These results suggest that ω-CgTx-sensitive N-type Ca^{2+} channels inactivate faster than nifedipine-sensitive L- and ω-AgTx-sensitive P-type channels as reported to be the case in neuronal cells [18, 22]. However, as the inactivation of Ca^{2+} current tended to be accelerated by repetitive application of 1-s depolarizing pulses (data not shown), the time courses of Ca^{2+} current inactivation may be underestimated. Dihydropyridine-type Ca^{2+} channel blockers have been generally known to inhibit Ca^{2+} channels in a manner that is dependent on the membrane potential [22]. When the nifedipine-sensitive current was compared with the ω-CgTx and ω-AgTx-resistant current, there was a great difference in L-type Ca^{2+} channel current. The former had no inactivation phase but the latter had a rapid activation phase and an inactivation phase during a 1-s depolarization to +15 mV, suggesting a time-dependent inhibition of L-type Ca^{2+} current by nifedipine during a 1-s depolarization.

Unlike the current responses, the increase in $[Ca^{2+}]_i$ evoked by 60 mM K^+ was inhibited equally by ω-CgTx and nifedipine but not by ω-AgTx at maximal doses. The failure of ω-AgTx to inhibit the high-K^+-induced increase in $[Ca^{2+}]_i$ is probably related to the finding that the inhibitory action of ω-AgTx on Ca^{2+} currents is the smallest of these three Ca^{2+} channel blockers. The application of nifedipine and ω-CgTx in combination inhibited the high-K^+-evoked increase in $[Ca^{2+}]_i$ to 8 ± 2% (n = 4) of the preceding responses. This result indicates that the major pathways of Ca^{2+} entry in response to high-K^+ stimulation are both L- and N-type Ca^{2+} channels. Although there are considerable differences between the experimental conditions for current and $[Ca^{2+}]_i$ measurements, the difference in the inhibitory efficacy of Ca^{2+} channel blockers for Ca^{2+} currents and for high-K^+-induced increases in $[Ca^{2+}]_i$ may be attributable to the differences in time-dependent inactivation of Ca^{2+} currents. It seems likely that application of a high concentration of K^+ for 5 s caused a greater inactivation of N-type channels than of L-type Ca^{2+} channels. Similar to the inhibitory effects on $[Ca^{2+}]_i$ responses to high K^+, nifedipine and ω-CgTx equally inhibited catecholamine release evoked by 60 mM K^+, but ω-AgTx had little effect on it. These results suggest that ω-CgTx-sensitive N-type and nifedipine-sensitive L-type Ca^{2+} channels are the main pathways by which Ca^{2+} evokes catecholamine release induced by a high K^+ concentration.

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