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Contribution of Different Types of Ca^{2+} channels to Catecholamine Secretion in Rat Adrenal Chromaffin Cells

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ABSTRACT

Adrenal chromaffin cells secrete catecholamine in response to acetylcholine. The secretory response has absolute requirement for extracellular calcium, indication that Ca^{2+} influx through voltage dependent Ca^{2+} channel (VDCC) is the primary trigger of the secretion cascade. Although the existence of various types of Ca^{2+} channels has been explored using patch clamp technique in adrenal chromaffin cells, the contribution of different types of Ca^{2+} channels to catecholamine secretion remains to be established.

To investigate the quantative contribution of different types of Ca^{2+} channels to catecholamine secretion, Ca^{2+} current(I_{ca}) and the resultant membrane capacitance increment(ΔC_m) were simultaneoulsy measured. Software based phasor detector technique was used to monitor ΔC_m.

After blockade of L type VDCC with nicardipine(1 μM), I_{Ca} was blocked to 43.85± 6.72% (mean± SEM) of control and the resultant ΔC_m was reduced ot 30.10± 16.44% of control. In the presence of nicardipine and ω-conotoxin GVIA(1 μM), an N type VDCC antagonist, I_{Ca} was blocked to 11.62± 2.96% of control and the resultant ΔC_m was reduced to 26.13 ± 8.25% of control. Finally, in the presence of L, N, and P type $Ca^{2\pm}$ channel antagonists(nicardipine, ω–conotoxin GVIA, and ω-agatoxin IVA, respectively), I_{Ca} and resultant ΔC_m were almost completely blocked.

From the observation of parallel effects of Ca^{2+} channel antagonists on I_{Ca} and ΔC_m, it was concluded that L, N, and also P type Ca^{2+} channels served and Ca^{2+} source for exocytosis and no difference was observed in their efficiency to evoke exocytosis amost L, N, and P type Ca^{2+} channels.

INSTRODUCTION

Exocytosis is the process of releasing hormones or neurotransmitters in response ot physiological stimuli. Adrenal chromaffin cells secrete catecholamines in response to the neurotransmitter, acetylcholine(ACh), released from the splanchnic nerve terminal. The removal of extracellular Ca^{2+} abolishes the secretion of catecholamines[1], which implies that the influx of Ca^{2+} into the chromaffin cell is the start point of the secretory process

Key words : Adrenal chromaffin cell, Voltage-dependent Ca^{2+} channel(VDCC), Ca^{2+} current(I_{Ca}), Membrane capacitance increment(ΔCs_m), Ca^{2+} channel antagonist, and Exocytosis.

of catecholamines.

According to generally accepted physiologic paradigm of ionic events leading to Ca^{2+} influx, ACh opens up a non-specific receptor operated cation channel, which in turn depolarizes the membrane and activates voltage dependent calcium channels(VDCC)[2].

While the existence of various types of VDCC has benn relatively weel documented int eh chromaffin cell[(L type[3], N type[4,5], and P type[6]), the contribution of different types of Ca^{2+} channels to catecholamine secretion remains to be established. The biophysical measurements of plasma membrane capacitance(C_m) can be used as a direct probe to exocytosis at very high resolution[7-9]. The method is based on the fact that biological membrane shows a constant capacitance of 1 $\mu F/cm^2$. Therefore, the changes in membrane surface area after exocytosis lead to a proportional increase in C_m.Ca^{2+}

With whole cell configuration f the patch-clamp technique, simultaneously measuring Ca^{2+} current(I_{Ca}) and C_m, Ca^{2+} influx through VDCC and the resultant exocytosis can be correlated in a single cell to figure out each VDCC'S contribution to exocytosis.

MATERIALS AND METHODS

1. Preparation of single adrenal chromaffin cell.

Rat chromaffin cells were obtained by a method modified from Akaike et al[10].Ca^{2+}

Both adrenal glands were dissected from an adult Sprague-Dawley rat(200 gm) by aseptic procedure.

In Hank's balanced salt solution(HBSS) {(mM) NaCl 109.5, KCl 5.36, $NaHCO_3$ 23.8, NaH_2PO4 10.07, HEPES 10, D-glucose 10, penicillin-G 100 IU/ml, streptomycin 100 μg/ml (pH 7.4)}, the capsule and cortex were removed, and the isolated medullae were minced into small pieces.

After washing with HBSS, cells were dissociated by incubation at 37℃ in a Ca^{2+} -free collagenase solution(2 mg/ml Boehringer Mannheim Type A) for 30 min with shaking. The digested tissues were rinsed with HBSS and triturated gently with plastic transfer pipette. The dissociated cells were centrifuged down at 100G-force for 10 min and the supernatant was discarded. The pellet was suspended in media(Dulbecco's Modified Eagle's Medium(DMEM) with supplements : 10% fetal bovine serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml fungizone, 1 mM glutamine, and 1 mM pyruvate). The cell suspension was plated on matrigel(Becton Dickinson) coated glass coverslips. After waiting for about an hour until the settlement of cells on the surface, the coverslips were flooded with media and kept for up to 7 days in a CO_2(5%) incubator at 37℃.

2. Solutions

The tip patch pipette was filled with a solution containing(in mM) 28 Cs_2SO_4, 92 CsCl, 20 TEACL, 0.5 EGTA, 2 $MgCl_2$, and 10 HEPES titrated to pH 7.2 with CsOH. The pipette

was then backfilled with 250 μg/ml nystatin in DMSO.

The bath solution contained (in mM) 145 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 10 $CaCl_2$ 5 glucose, and 10 HEPES titrated to pH 7.2 with NaOH, and 0.5 μM tetrodotoxin(TTX).

Stock solution(1 mM) of nicardipine(Sigma, USA) was prepared in ethanol and diluted in the bath solution to the final concetration(1 μM).

ω-conotoxin(ω-CgTX, fraction GVIA, Sigma, USA) and ω-agatoxin(fraction IVA, Almone labs, Israel) were prepared as a 10 μM and 1 μM stock solutions, respectively in distilled water and kept in aliquots at -20℃ until use. Nicardipine was applied with the continuous bath perfusion method(1~2 ml/min), and stocks of ω-CgTX and ω-agatoxin were added to static constant volumed(0.9 ml) chamber.

3. Simultaneous measurements of Ca^{2+} current (I_{Ca}) and membrane capacitance(C_m)

Membrane currents were measured using the patch-clamp technique in the perforated patch variant of whole cell clamp technique[11, 12].

The experimental set-up is shown in Fig. 1. After giga-sealing, time was taken until

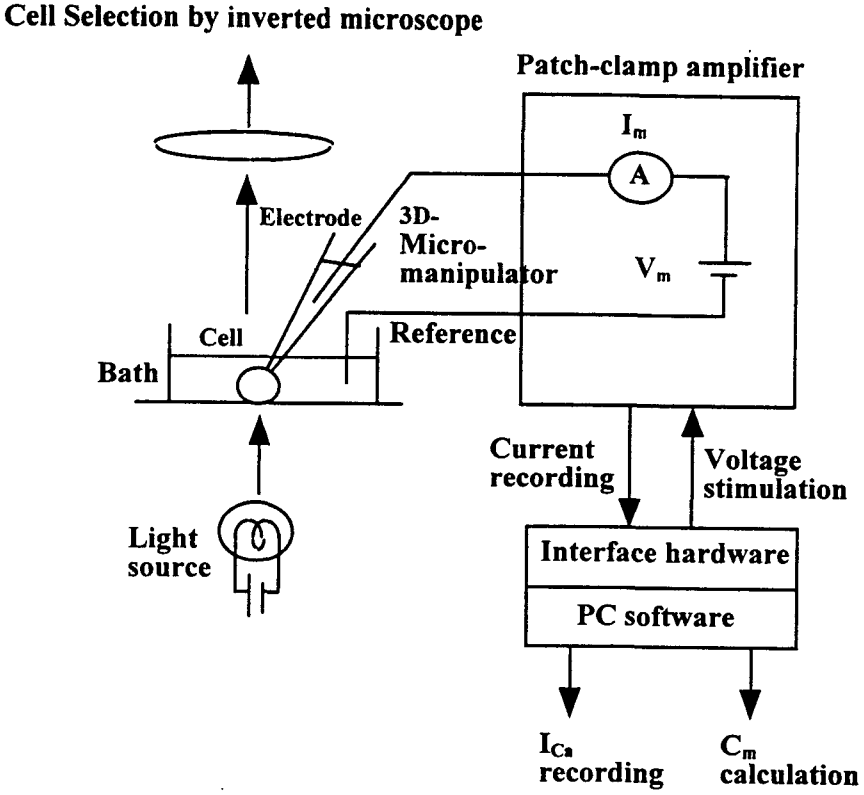


Fig. 1. Experimental set-up

Abbreviations

V_m : membrane potential, I_m : membrane current

I_{Ca} : Ca^{2+} −current, C_{Cm} : plasma membrane capacitance

the access conductance(G_s) of the perforated patch reached 〉 0.03 μS. After capacitive transient neutralization by EPC-7's cancellation circuitry, a 10 mV r.m.s, 1 kHz sinusoidal wave riding on a −70 mV dc offset was applied as a stimulus.

The resultant currents were amplified for better resolution, and the conductive(G) and capacitive(C_m) components were separated at two orthogonal phase angles, using a software-based phase sensitive detector(PD). The correct angle for the PD was located with the change of 100 fF in C_{slow} of the EPC-7 amplifier to calibrate $\Delta C_m Ca^{2+}$. The correct phase angle was determined by finding the phase angle at which the 100 fF change in the C_m made no change in the G trace. And also 100 fF change in C_m was used as a calibration reference.

4. Statistics

The statistical significance of differences between the control and drug was determined by paired Student's *t*-test. Presence of facilitation was determined by Wilcoxon signed rank test. Kruskal-Wallis test was performed to identify the statistical significance of differences among five trials of depolarization induced capacitance increment(ΔC_m). Comparison between every two trials was made by nonparametric multiple comparison method. P-value of 〈0.05 was considered to indicate statistical significance. Values were expressed in mean± standard error(SEM).

RESULTS

1. I_{Ca} dependent increase in C_m

I_{Ca} was activated by depolarization and I_{Ca} induced C_m increase, which reflects the fustion of secretory granules with plasma membrance, was measured electrically.

In Fig. 2A, the C_m of the first 4.5 sec represents control trace at 10 mV r.m.s, 1 kHz sinusoidal wave riding on a −70 mV dc offset with the 100 fF change delivered to calibrate the C_m change. The sinusoidal stimulus was interrupted to depolarize the membrane potential from +70 mV to +10 mV for 500 ms, during which I_{Ca} was recorded. We applied +10 mV of depolarization as stimulus at which level I_{Ca} usually reached a maximum. IC_a is demonstrated on an expanded time scale and the dotted line indicates zero current level. C_m measurement for 4.5 sec followed each of the five 500 ms depolarizations. The difference of Cm values before and after IC_a activation indicated the amount of exocytosis during stimulations and the constant G levels, which were measured at the orthogonal phase angle of the C_m component current, confirmed that the C_m traces were not biased not biased by G change.

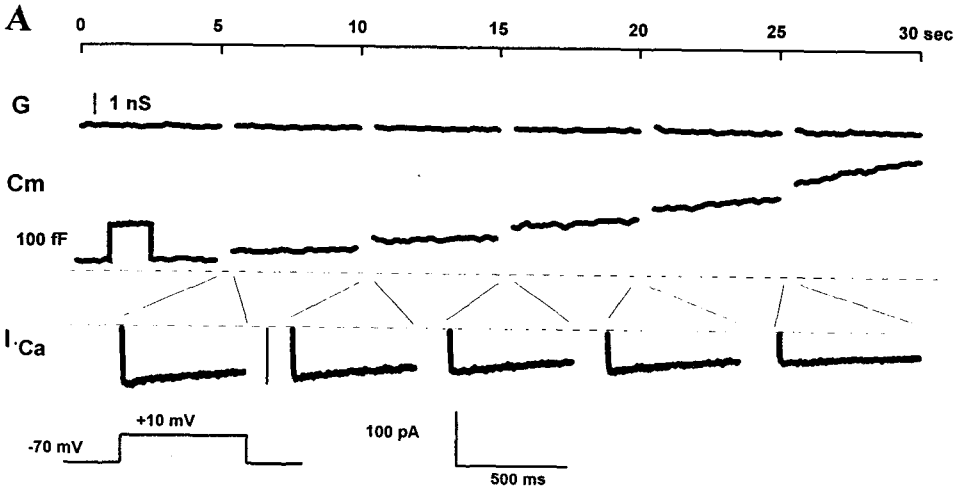
With repetitive-pulse protocols, the amplitude of C_m increment usually peaked at the 3rd pulse(ΔC_m=65.51± 12.17 fF, n=27).

This occurred despite the reduction of peak IC_a to the repetitive pulses.

27 cells examined with the protocol of repetitive 5-pulses showed similar patterns of

facilitation and the plot of averaged C_m increments of each trace showed the largest C_m increase at the 3rd pulse(Fig. 2B).

Statistical difference among ΔC_m in each depolarization was determined by Kruskal-Wallis test($P>0.05$). With nonparametric multiple comparison method, we found that ΔC_m induced



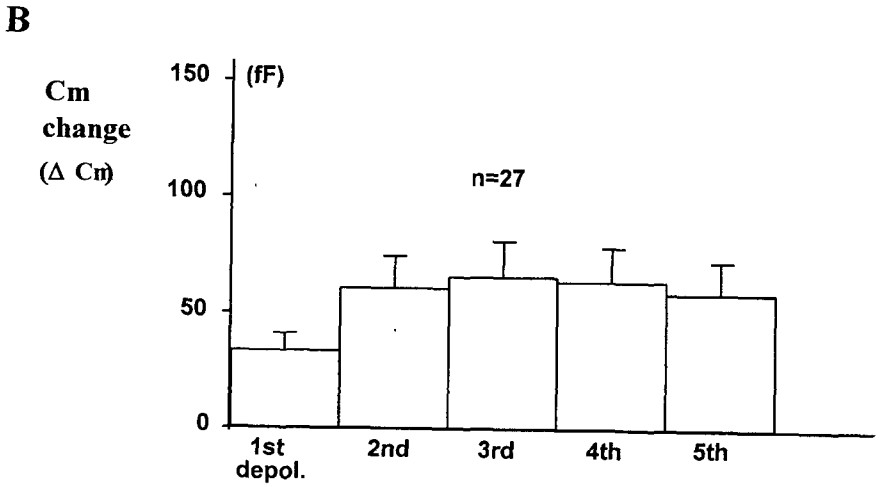


Fig. 2. Ca^{2+} influxes through ICa induce exocytosis

A. At the right phase angle, the 100 fF change in Cs(membrane capacitance after slow compensation) only affected the membrane capacitance(Cm) while the resistance component(G) remained constant.
Cm increased after ICa, evoked by 500 ms depolarization from a holding potential of −70 mV to the test potential of +10 mV. Five same depolarizations induced other Cm increments. The dotted lines indicate zero current and zero Cm level, respectively. The square deflection in the middle of the first Cm trace indicates a calibration of 100 fF.

B. Mean Cm increases were plotted against each depolariation trial. The values were obtained from 27 cells(n=27) and the error bar indicates standard error(S.E.M.)

by the 2nd to 5th depolarizations was larger than the 1st depolarization induced ΔC_m ($P<0.05$)

2. Effects of L type Ca^{2+} channel antagonist on I_{Ca} and exocytosis

We specifically blocked the L type VDCC with a dihydropyridine-derivative VDCC antagonist, nicardipine(1 μM), to examine the role of the L-type VDCC in exocytosis.

In Fig. 3A, the blocking effect of nicardipine is observed after 3 min perfusion of drug.

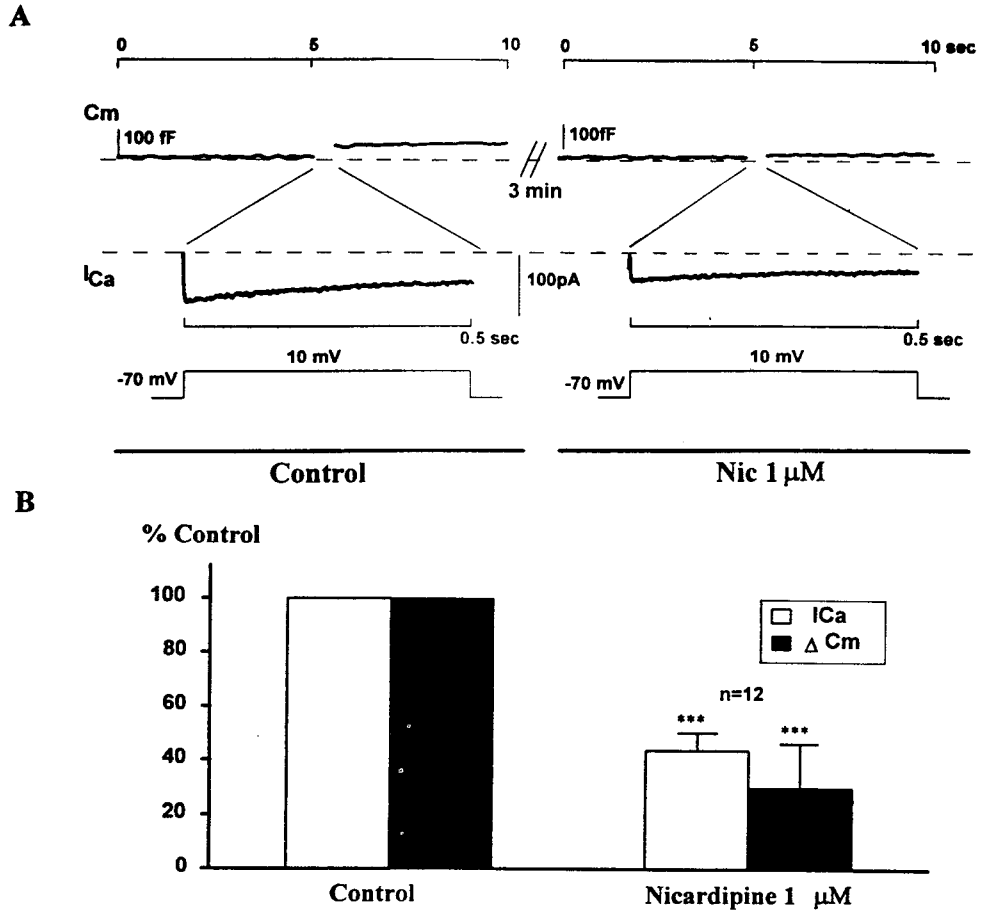


Fig. 3. Effects of nicardipine on ICa and exocytosis

A. L-type ICa was blocked by 1 μM of nicardipine, perfused for 3 min befor stimulation, in perforated patch configuration.
The drug effect was parallel on the resultant exocytosis, Cm change(ΔCm). The value under the time break indicates the interval between the voltage stimulations(500 ms depolarization from a holding potential of -70 mV to the test potential of +10mV). Dotted lines indicate an arbitray zero for Cm comparison and zero level for current trace, respectively.

B. Data represent the averaged percent to control(mean± S.E.M) of 12 cells (n=12), obtained using the protocol of A.
***$P<0.001$ with respect to control

The partial blockade of the I_{Ca} was accompanied by parallel changes of the C_m increment. Nicardipine reduced the I_{Ca} to 43.85± 6.72% of control accompanying the C_m increment by 30.10± 16.44%(n=12) of control with statistical significance(P<0.001).

3. Effects of N type Ca^{2+} channel antagonist on I_{Ca} and exocytosis

To determine the role the N type VDCC during exocytosis, we blocked the N type VDCC specifically with 1 μM of ω-CgTX, perfused for 6 min before stimulation. Peak I_{Ca} was reduced to 56.13± 6.40% of control, and ΔC_m was reduced to 56.66± 9.49% of control(n=9) with statistical significance(P<0.05)(Fig. 4).

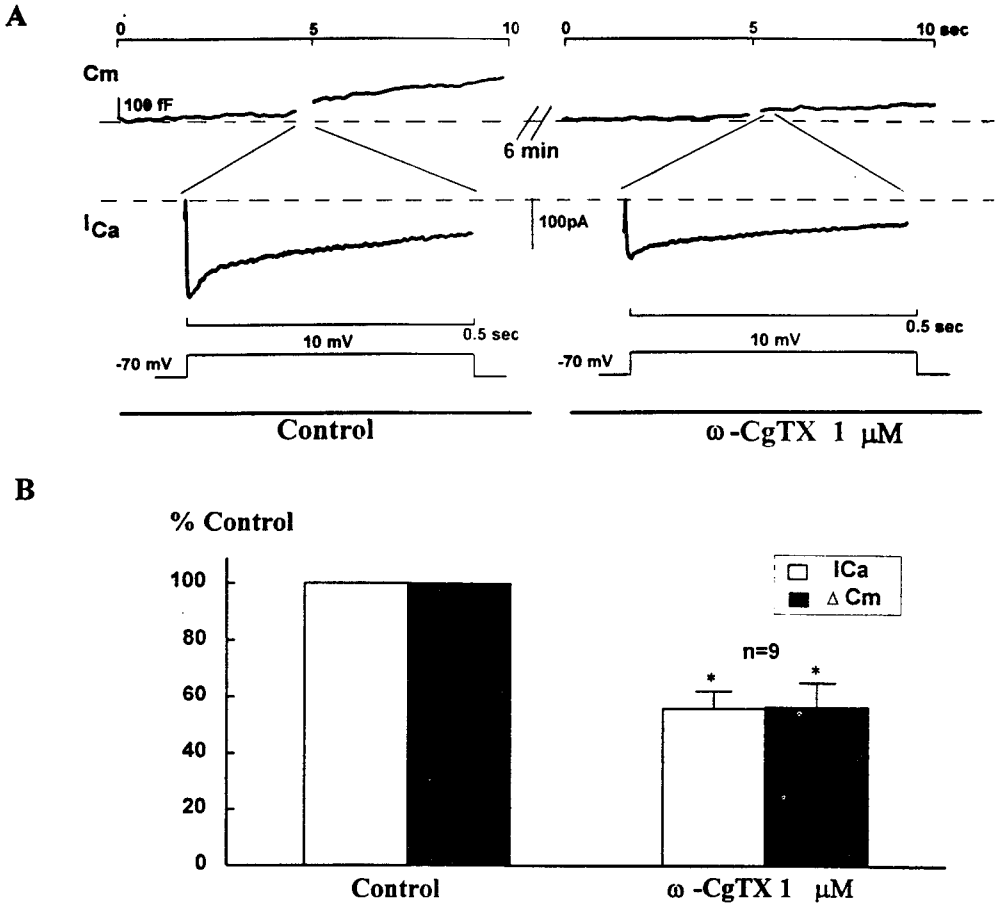


Fig. 4. Effects of ω-CgTX on I_{Ca} and exocytosis.

A. The N-type I_{Ca} was blocked by 1 μM of ω-CgTX, perfused for 6 min before stimulation, in perforated patch configuration. The drug effect was also parallel on the resultant exocytosis, Cm change(ΔCm). The value under the time break indicate the interval between the voltage stimulation(500 ms depolarization from a holding potential). Dotted lines indicate an arbitrary zero for Cm comparison and zero level for current trace, respectively.

B. Data represent the averaged percent to control(mean± S.E.M) of 9 cells, obtained using the protocol of A. *P<0.05 with respect to control.

4. Combine effects of nicardipine and ω-CgTX on I_{Ca} and exocytosis

To determine the role of any other types of VDCC but L and N types, we treated L and N type antagonists concomitantly. With nicardipine(1 μM), I_{Ca} was reduced to 57% of control, and ΔC_m was reduced to 30% of control. With additionl treatment of ω-CgTX(1 μM), both of I_{Ca} and ΔC_m were further reduced to 19% and 14% of control, respectively (Fig. 5A).

The bars of Fig. 5B represent I_{Ca} and C_m changes averaged over 5 cells. After concomitant treatment of nicardipine and ω-CgTX, I_{Ca} was reduced to 11.62± 2.96% of control, and ΔC_m was reduced to 26.13± 8.26% of control.

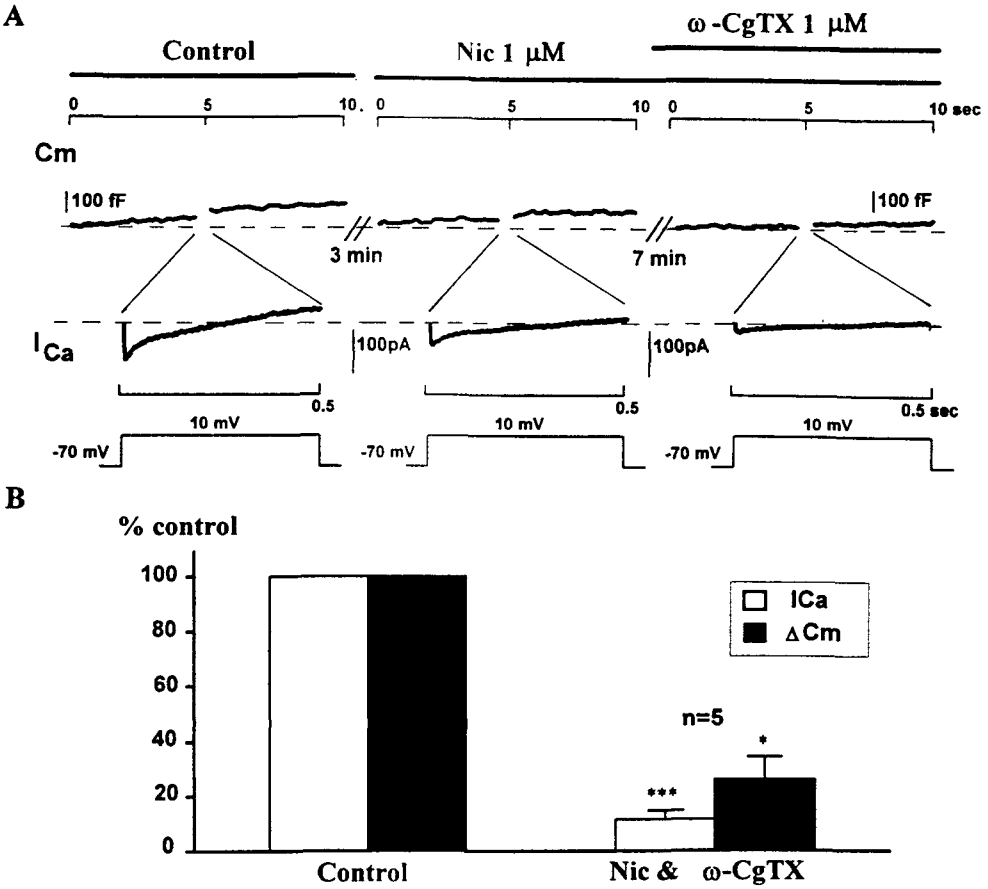


Fig. 5. Combined effects of nicardipine and ω-CgTX on I_{Ca} and exocytosis.

A. I_{Ca} activated by the voltage protocol(500 ms depolarization from a holding potential of -70 mV to the test potential of +10mV) evoked Cm increment in the perforated patch configuration. The application of nicardipine(1 μM) led to the parallel and partial blockade of I_{Ca}(57% of control) and Cm increment(ΔCm, 32% of control). More blockade of I_{Ca}(19% of control) and ΔCm(14% of control) were induced by an additional application of ω-CgTX(1 μM)

B. Data represent the averaged percent to control(mean± S.E.M) of 5 cells, obtained using the protocol of A. $^{*}P<0.05$ $^{***}P<0.001$ with respect to control.

5. Effects of the sequential addition of ω-Agatoxin, ω-CgTX, and nicardipine on I_{Ca} and Exocytosis

The role of P type VDCC on exocytosis was studied by using ω-agatoxin, the novel toxin isolated from the venom of the spider *Agelenopsis aperta* IVA, that has been recently reported to selectively block P-type Ca^{2+} channels in neurons[13, 14].

With ω-agatoxin(0.1 μM), partial blockade of I_{Ca}(91.3% of control) and ΔC_m(75.2% of control) were induced. Additional application of ω-CgTX(1 μM) led to further blockade of I_{Ca}(79.6% of control) and C_m (61.6% of control). With nicardipine(1 μM) application, remnant I_{Ca} and ΔC_m were blocked completely.

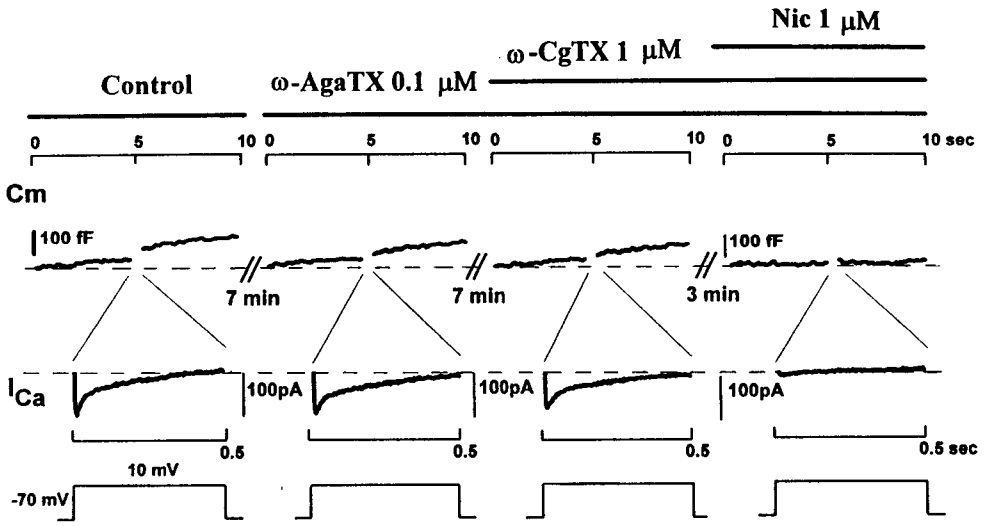


Fig. 6. Effects of the sequential addition of ω-Agatoxin, ω-CgTX, and nicardipine on I_{Ca} and exocytosis.
With ω-Agatoxin(0.1 μM), partial blockade of I_{Ca}(91.3% of control) and ΔCm(75.2% of control) were induced. Additional application of μ-CgTX(1 μM) led to more blockade of I_{Ca}(79.6% of control) and ΔCm(61.6% of control). With nicardipine(1 μM) application, remnent I_{Ca} and ΔCm were blocked almost completely.

In 5 out of 6 cells treated by the above protocol, the similar complete blockade of I_{Ca} and ΔC_m were observed.

DISCUSSION

The results of the present study can be summarized as follows :

(1) Ca^{2+} influx throught VDCC induced exocytosis

(2) L, N, and P type VDCC served as Ca^{2+} source for exocytosis.

(3) In the presence of L, N, and P type VDCC antagonists, the reduction in ΔC_m was

parallel with the blocking effect on each type of I_{Ca}. This fact implies no difference in the efficiency of Ca^{2+} channel type to induce exocytosis.

In rat adrenal chromaffin cells, we approved the comprehending paradigm that plasma membrane depolarization causes exocytosis by opening VDCC and thereby increasing $[Ca^{2\times}]_i$. The low sensitivity of exocytosis to I_{Ca} at high EGTA(11 mM) condition confirmed that exocytosis is also dependent on the elevation of free $[Ca^{2+}]_i$ and not simply on increases in total $[Ca^{2+}]_i$ (data not shown).

During the five successive depolarizations as stimuli to exocytosis, ΔC_m induced by the 2nd to 5th depolarizations was larger than induced by the 1st depolarization (nonparametric multiple comparison method, $P<0.005$), although peak I_{Ca} reduced to repetitive pulses. This phenomenon resembles the facilitation in neurons, in which a second action potential[15] or depolarization[16, 17] causes more exocvtosis than one given shortly before. This ob served facilitation could result from a build-up in the concentration of free $[Ca^{2+}]_i$ [15, 18] or a shift of secretory vesicles from depot pool to ready to releasable pool the 1st Ca^{2+} flux[19].

The existence of multiple subtypes of VDCC(L, N, and P type) in rat adrenal chromaffin cells raises the question of what their role might be in regulation $Ca^{2\times}$ entry into the cell, and whether Ca^{2+} entering through different channel pathways is equally efficient in triggering exocytosis.

In bovine chromaffin cells, Artalejo[20] suggested that facilitaion of Ca^{2+} channels had higher efficiency to trigger exocytosis than N and P type Ca^{2+} channels. Also Q type Ca^{2+} channels have been recently reported to be related directly to the secretory process[21]. In cat chromaffin cells, only L type $Ca^{2\times}$ channels were predominantly coupled to the secretory machinery despite the presence of L, N, and probably P. type Ca^{2+} channels[22].

However, in rat chromaffin cells, our results showed no difference in efficency to evoke exocytosis among L, N, and P type VDCC.

The Ca^{2+} channel subtype dominating the control of catecholamine release under stressful conditions might vary from species to species. From the standpoint of comparative physiology, this variability could represent the existence of different forms of "fight or flight" response to the same stressful conditions in different animal species.

Further studies are needed to elucidate possible functional features of these multiple subtypes of Ca^{2+} channels found in rat chromaffin cells, for instance how they are regulated by phosphorylation/dephosphorylation processes, selective modulation by neurotransmitters acting through regulatory G-proteins or various intracellular messengers.

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부신수질 Chromaffin 세포의 Ca^{2+} 통로유형이 카테콜아민 분비에 미치는 영향에 관한 정량적 연구

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초 록

부신수질 chromaffin 세포는 생리적 상태에서 acetylcholine에 반응하여 카테콜아민을 세포외분비(exocytosis)하는 바 이 과정에서 막전압의존성 Ca^{2+} 통로를 통한 Ca^{2+}의 세포내유입이 카테콜아민 분비의 시발점임이 알려져있다. 막전압의존성 Ca^{2+} 통로에 대한 연구가 활발히 진행되어 여러가지 유형의 Ca^{2+} 통로가 존재함이 보고되었으나, 아직 부신수질 chromaffin 세포에 존재하는 개개의 Ca^{2+} 통로가 실제로 카테콜아민분비에 기여하는 정도에 관한 연구는 미미할 실정이다.

그러므로 본 연구에서는 stimulus-secretion coupling 과정을 연구하는데 좋은 모델이 되고있는 흰쥐 부신수질 chromaffin 세포를 대상으로하여 개개의 Ca^{2+} 통로를 통한 Ca^{2+} 전류가 카테콜아민 분비에 미치는 기여도를 정량적으로 규명하고자 하였다.

개개의 Ca^{2+} 통로를 통한 Ca^{2+}의 세포내 유입(Ca^{2+} 전류)이 카테콜아민의 세포외분비에 미치는 영향을 규명하기 위하여 저분극자극을 주어 Ca^{2+} 통로를 활성화함과 동시에 이의 결과를 나타나는 막캐패시턴스변화량(ΔC_m)을 직접 측정하였다. ΔC_m의 측정은 software based phase detector technique을 사용하였다. Nicardipine, ω-CgTX, ω-AgaTX을 사용하여 이들 약물을 처치한 군에서 Ca^{2+} 전류의 억제도(% control) ΔC_m의 억제도(% control)를 측정한 결과 약물처치군에서는 항상 비슷한 정도의 Ca^{2+} 전류의 억제 및 ΔC_m의 억제를 보였다. 즉 카테콜아민 분비에 L형, N형, P형 Ca^{2+} 통로 모두 기여하며 그 상대적인 기여도는 L형 > N형 > P형 순이었다. 다른 종에서의 보고와는 달리 흰쥐 부신수질 chromaffin 세포의 Ca^{2+} 통로는 각 유형별로 동일한 효율(efficiency)로 카테콜아민분비에 기여함을 확인하였다.