Calcium Channel Subtype in Rat Adrenal Chromaffin Cells

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Adrenal chromaffin cells secrete catecholamine in response to acetylcholine. The secretory response has absolute requirement for extracellular calcium, indicating that Ca^{2+} influx through voltage operated Ca^{2+} channels 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, there is still disagreement with the types of Ca^{2+} channels existed in different species.
Therefore, we have tried to identify several distinct types of Ca^{2+} channels in rat chromaffin cells.

By using nicardipine(L type channel blocker), ω-CgTx GVIA(N type channel blocker), and ω-AgaTx VIA(P type channel blocker), it was identified that L, N, and P type Ca^{2+} channel exist in rat adrenal chromaffin cells and the order of contribution of each channel type to whole cell Ca^{2+} current was L type> N type> P type.

Keyword : Calcium Channel, Adrenal Chromaffin Cells

INTRODUCTION

Exocytosis is the process of releasing hormones or neurotransmitters in response to 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 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 potential and activates voltage dependent calcium channels (VDCC)[2].

At many synapses, when two action potentials separated by a short interval arrive at the presynaptic terminal, the second action potential releases more transmitter than the first. This phenomenon is referred as monosynaptic facilitation of transmitter release, and it represents one of the most common types of synaptic plasticity[3].

Chromaffin cells have two components of whole cell Ca^{2+} current: standard Ca^{2+} currents that are activated by brief depolarizations, and facilitation

This work was supported by the grant No. 951-0708-078-1 from the Korea Science and Engineering Foundation.

Ca^{2+} currents, which are normally quiescent but can be activated by large pre-depolarizations or by repetitive depolarizations to physiological potentials[4].

There has been debate on the types of voltage dependent calcium channels (VDCC) in adrenal chromaffin cell. The existence of L-type[5], N-type[6),7),8)] and P-type[9),10)] calcium channel have been reported while there has been no report of the existence of T-type calcium channel. Since both L and N type calcium channels belong to high voltage activated (HVA) Ca^{2+} channels, it is not easy to separate two channels electrically[11]. Fortunately, pharmacological isolation is possible due to specific channel blockers; dihydropyridine derivatives for L type and ω-CgTX GVIA for N type, and ω-AgaTX IVA for P type[12].

The existence of various types of VDCC has been suggested in different chromaffin cell [(L type[13], N type[6),14)], P type, and facilitation Ca^{2+} channel[4)]] but sometimes different type of VDCC was reported in the chromaffin cell of same species. Therefore, we have tried to identify several distinct types of Ca^{2+} channels in rat chromaffin cells using whole cell patch clamp technique.

MATERIALS AND METHODS

1. Preparation of single adrenal chromaffin cell.

Rat chromaffin cells were obtained by a method modified from Akaike et al[15].

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 of patch pipette was filled with a solution containing (in mM) 120 CsCl, 20 TEACl, 11 EGTA, 2 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES titrated to pH 7.2 with NaOH.

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 concentration (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. Recording of membrane current

Membrane currents were recorded with standard dialyzed cell (i.e. whole-cell) patch clamp procedures[16]. Cells were maintained in primary culture for 1-7 days following dispersion. Currents were recorded with an EPC-7 amplifier (List Electronic, Germany). Micropipettes were prepared from Kimax-51(Kimble products, USA). Pipette resistances ranged from 2-5 MΩ. Electrodes were coated with Sylgard and fire-polished. Voltage commands and acquisition of membrane currents were accomplished with the Clampex program from pCLAMP software package. Current signals were filtered with an 8-pole Bessel filter, sampled at 250-500 μS per point with a 12 bit analog to digital converter (Lab Master board interface, Warner instrument, USA) and stored by PC for later analysis.

All experiments were done at room temperature (20-25 ℃) and in an hour of getting the cells from CO_2 incubator.

RESULTS

1. Characterization of HVA Ca^{2+} Channels in Rat Chromaffin Cells

In cultured rat chromaffin cells, I_{Ca} were elicited by 150 ms depolarizing pulses applied from a holding potential (V_h) of -80 mV in 10 mV steps up to ±60 mV. Other ionic currents were suppressed by dialysing the cells with the Cs^+- and TEA-based intracellular solutions, and by bathing them in a Na^+-based solution containing TTX (0.5 μM). I_{Ca} activated around -30 mV, peaked at +10 mV and showed an apparent reversal potential at +60 mV (Fig. 1). In more than 200 cells, no sign of fast-inactivating, low-voltage-activated (LVA, T-type) Ca^{2+}

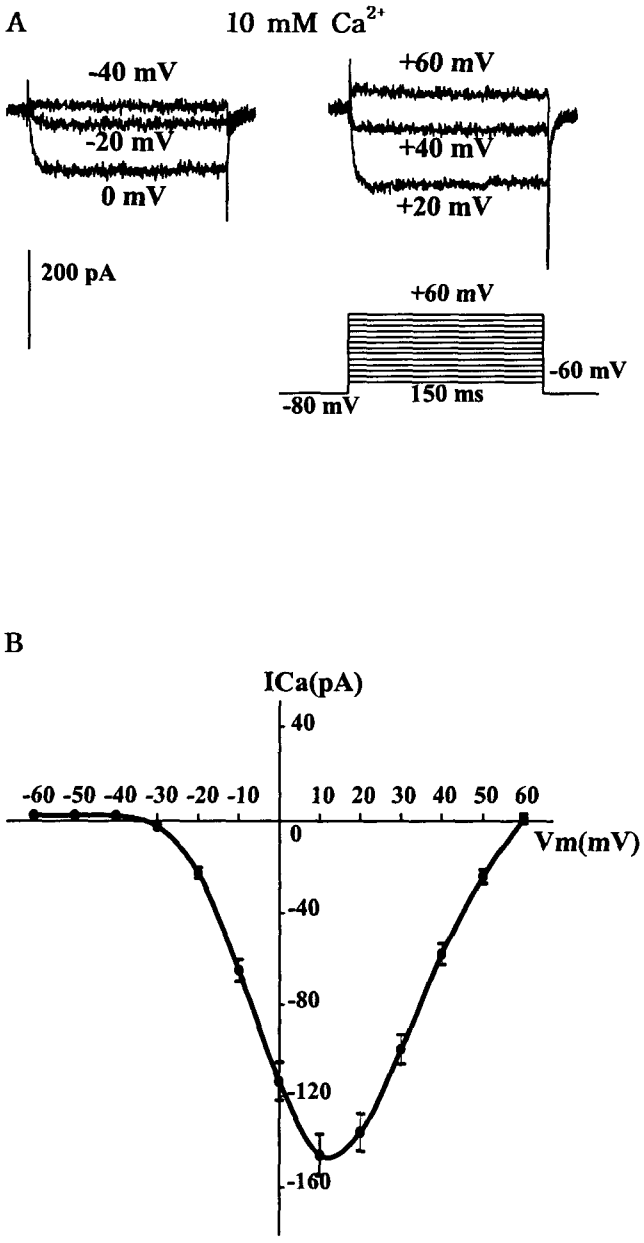
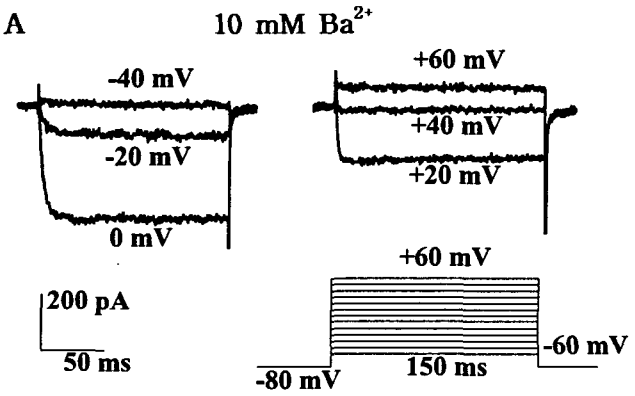


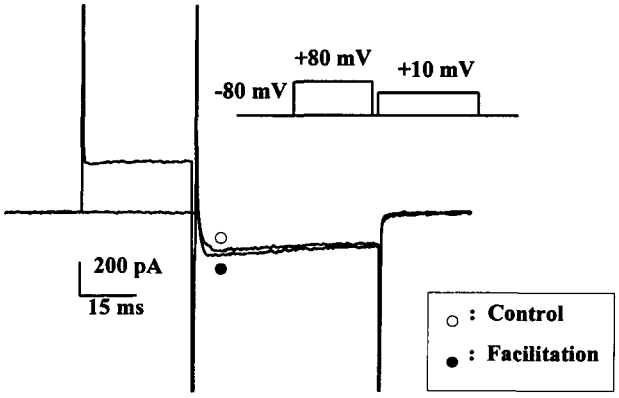
Fig. 1. Ca^{2+} current (I_{Ca}) in rat adrenal chromaffin cell.
A. Ca^{2+} currents were recorded on stepwise depolarization from -60 mV to +60 mV with each increment of 10 mV. Holding potentials (V_h) was -80 mV.
B. Voltage-current relationship of Ca^{2+} current. I_{Ca} was plotted as mean ±S.E.M. for n=69 cells.

currents were observed. Therefore, Ca^{2+} currents in rat chromaffin cells appeared to be almost exclusively through high-voltage-activated (HVA) Ca^{2+} channels.

When Ca^{2+} was replaced by Ba^{2+} in the external

medium (Fig. 2A), HVA Ba^{2+} currents were activated and reached maximal amplitude at more negative potential (0 mV) than Ca^{2+} current and reversed at between +40 mV and +50 mV (Fig. 2B). I_{Ba} increased by about two times to five times of the size of I_{Ca}.





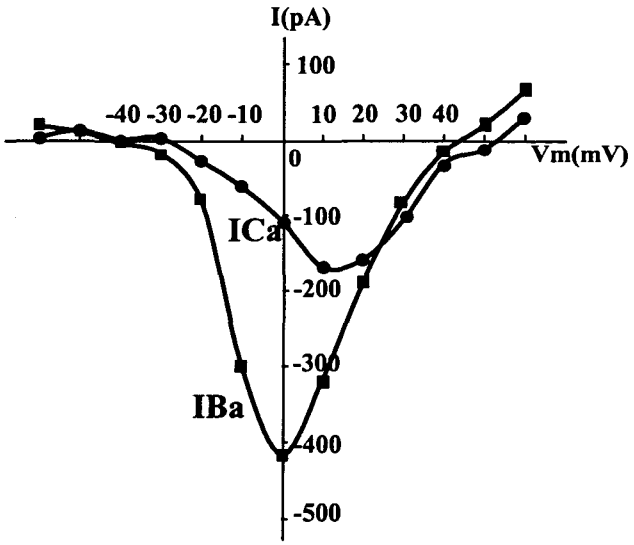


Fig. 2. Activation of Ba^{2+} current (I_{Ba}) in rat chromaffin cell.
A. After replacing 10 mM Ca^{2+} in bath solution with 10 mM Ba^{2+}, Ba^{2+} currents were recorded on stepwise deolarization from -60 mV to +60 mV with each increment of +10 mV in the same cell as in Fig. 1.
B. Voltage-Current relationship of the peak amplitude of Ba^{2+} (g) (Fig. 2A) and Ca^{2+} currents (n) (Fig. 1A).

Fig. 3. Prepulse induced facilitation of Ca^{2+} currents. Ca^{2+} currents were recorded with (n) and without (m) 40 ms prepulses with the voltage level changed from Vh=-80 mV to +80 mV. A 2.5 ms repolarization back to Vh was allowed before applying test pulse to +10 mV.

2. Facilitation of HVA currents by depolarizing prepulses

Facilitation of Ca^{2+} currents by depolarizing prepulses in bovine chromaffin cells has been reported consistently by several groups.[4),5),17),18)] This property was also observed in cat chromaffin cells[14)].

To test whether rat chromaffin cells also exhibit this property, conditioning prepulses of 40 ms duration, +80 mV amplitude preceded the test depolarization to +10 mV (Fig. 3). We used this

pulse protocol since facilitation of Ca^{2+} currents has been reported to be dependent on duration and amplitude of conditioning prepulse[4),17),18),19)] and it peaked with the prepulse of +40 ms duration, +80 mV amplitude[19)].

Conditioning prepulse caused an current increase (facilitation) of 6.17 ± 1.21% (n=26), which showed statistical significance ($P < 0.001$) with Wilcoxon signed rank test.

Our result of facilitation showed big difference with Artalejo's[4)] (average : 60%) but showed similar result with Lim's[19)] (8.8 ± 2.0%, n=8).

Specific D_1 dopamine agonist, SKF-38393 (1 μM) activated the facilitation of Ca^{2+} current in bovine chromaffin cells(182 ± 6% of control, n=11)[20)].

When we evoked facilitation of Ca^{2+} current in the presence of SKF-38393 (10 μM) in rat chromaffin cells, it was 7.50 ± 2.59% (n=11). There was no statistical difference ($P > 0.05$) between facilitation without SKF-38393 (6.17 ± 1.21%, n=26) and that with SKF-38393 (7.50 ± 2.59%, n=11).

These results suggest that Ca^{2+} currents through facilitation Ca^{2+} channels are very minute in rat chromaffin cells.

3. Sensitivity of I_{Ba} to BayK 8644

Effects of the DHP agonist BayK 8644 on HVA I_{Ba} were tested by applying a series of 150 ms depolarizing pulses of increasing amplitude to rat chromaffin cells held at a V_h of -80 mV. The depolarizing protocol was applied before (control) and during the superfusion of the cells with a solution containing 1 μM BayK 8644. As in bovine chromaffin cells[4),8),21)] and in cat chromaffin cells[14)]. BayK 8644 also produced a marked potentiation of HVA currents in rat chromaffin cells. The current increase was more evident at low membrane potentials (maximum increase: at -40 mV) where BayK 8644 increased the size of the current by about four fold to five fold (Fig. 4).

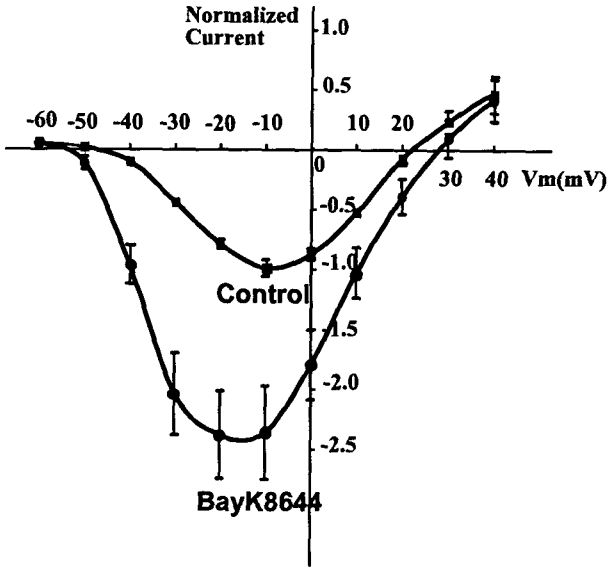


Fig. 4. Drastic potentiation of Ba^{2+} currents (I_{Ba}) by BayK 8644. Averaged peak current-voltage (I-V) relationship for I_{Ba} recorded before (control) and during application of 1 mM BayK 8644. Data were normalized as the percentage of the peak current in control conditions at -10 mV and plotted as means±S.E.M. of 5 cells bathed in 10 mM Ba^{2+}.

4. Effects of DHP antagonist

To test the effects of DHP antagonists on I_{Ba}, nicardipine, a novel DHP derivative which blocks L-type Ca^{2+} channels in bovine chromaffin cells[8)] was selected as a representative of this group of Ca^{2+} channel blockers.

Nicardipine (1 μM) blocked 56 ± 0.05% (n=11) of I_{Ba} recorded at +10 mV. Nicardipine blocked I_{Ba} maximally at +10 mV and gradually less at higher voltages (Fig. 5A, B). Nicardipine blocked I_{Ba} reversibly - the nicardipine block required 30~60 sec to be completed and a washing period of 3~4 min for the cells to attain full recovery (Fig. 5C).

5. Effects of ω-conotoxin (ω-CgTX)

The effects of ω-CgTX (Conus toxin GVIA) on I_{Ba} was tested (Fig. 6.)

In 7 cells, 1 μM ω-CgTX blocked 38 ± 0.08% of I_{Ba} recorded at +10 mV. ω-CgTX blocked I_{Ba} at higher voltages than -10 mV and showed

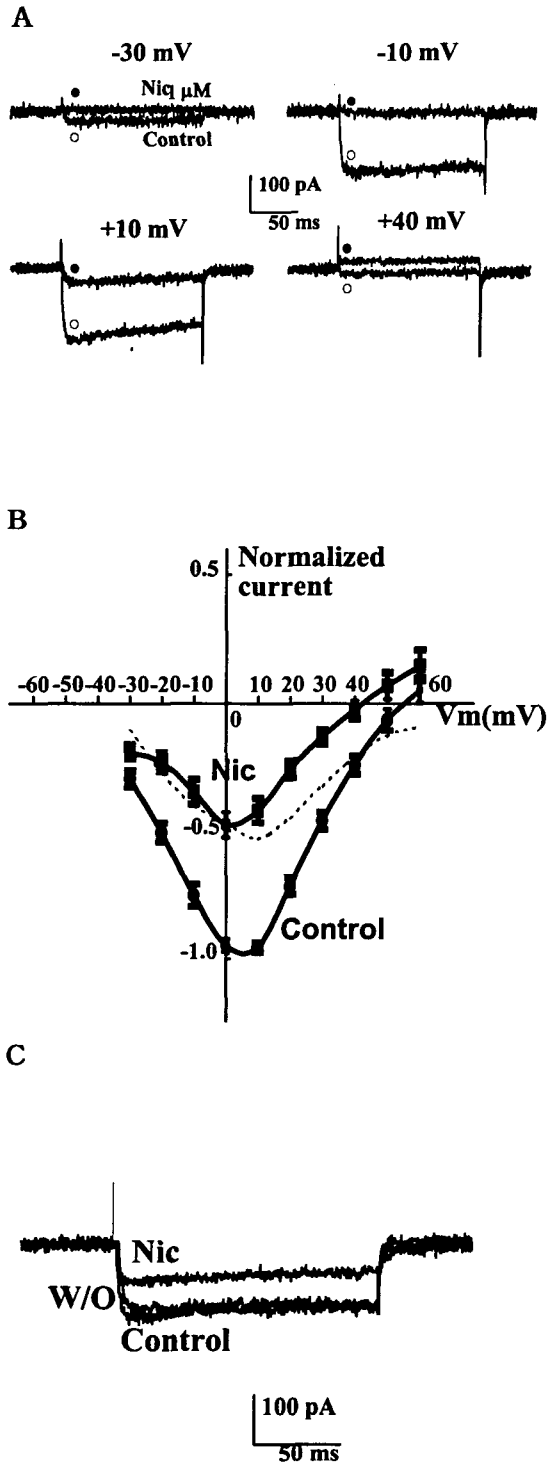


Fig. 5. Voltage dependency of nicardipine effects on Ca^{2+} currents.

A. Ca^{2+} currents were recorded before (m) and during (I) the application of 1 mM nicardipine at the potentials indicated. Notice a marked block of Ca^{2+} currents at -10 mV and +10 mV. V_h was -80 mV.

B. I-V plot at control (I) and in the presence of 1 mM nicardipine (n). Data represent normalized peak Ca^{2+} currents. They are plotted as mean±S.E.M. from 11 cells. The dashed curve represents the I-V relationship of nicardipine-sensitive currents obtained after substracting nicardipine-resistant (n) from control (I) currents.

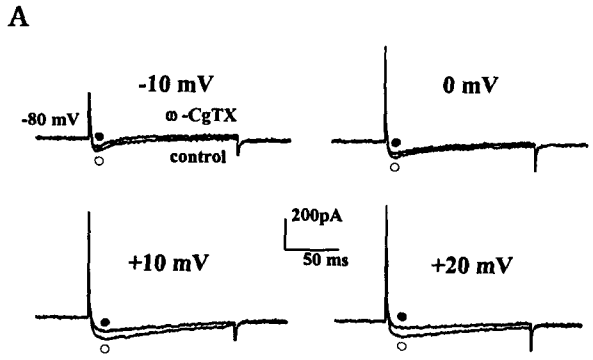
C. Three 150 ms test depolarization to +10 mV, from a V_h of -80 mV, were applied sequentially. The Ca^{2+} current traces were recorded before (control), during (Nic) and after (W/O) the application of 1 mM nicardipine, respectively.

remarkable block at +10 mV and +20 mV. The blocking effects were irreversible upon washing out the toxin from the extracellular medium as reported[14),22)]. Although the blocking effects of ω-CgTX have been considered to be specific for N type Ca^{2+} channels, such blocking effects could also affect L type Ca^{2+} channels in neural tissue[15)]. In order to test such a possibility, effects of nicardipine were also tested after the irreversible blockade of ω-CgTX was achieved. The amount of I_{Ba} blocked by nicardipine is nearly preserved after ω-CgTX application (data not shown). This result suggests that both types of blockers were acting different at different Ca^{2+} channels.

6. Combined effect of nicardipine and ω-CgTX (Fig. 7.)

To identify non-L, non-N type Ca^{2+} channel, nicardipine (1 μM) and also ω-CgTX (1 μM) were applied. With nicardipine, peak current and end current (at the end of 150 ms depolarizing pulse) were blocked to 74 % and 59 % of control, respectively. With additional application of ω-CgTX, peak current and end current were blocked to 19 % and 42 % of control, respectively.

7. Blocking action of sequentially applied ω-AgaTX, ω-CgTX, and nicardipine (Fig. 8.)



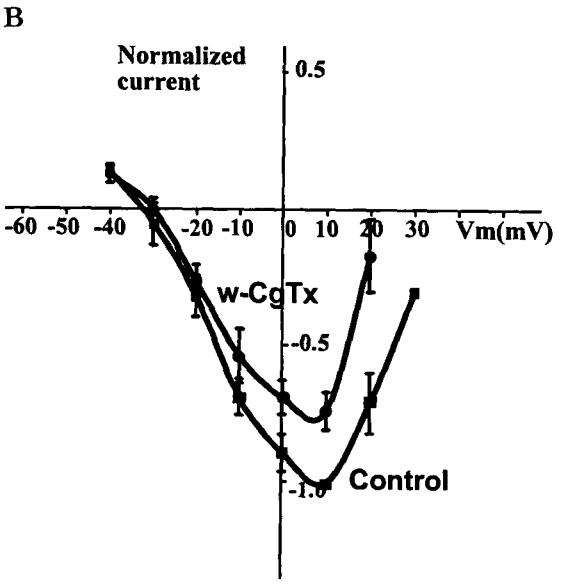


Fig. 6. Voltage dependency of w-CgTx effects on Ca^{2+} currents.

A. Ca^{2+} currents were recorded before(m) and during(l) the application of 1 mM w-CgTX at the potentials indicated. Notice a marked block of Ca^{2+} currents at +10 mV and +20 mV. V_h was -80 mV.

B. I-V plot at control(l) and in the presence of 1 mM of w-CgTX(n). Data represent normalized peak Ca^{2+} currents plotted as mean±S.E.M. from 7 cells.

Since there was 19 % of current left even after the application of nicardipine and also ω-CgTX, we applied P type specific blocker, ω-Aga-IVA (venom from the funnel web spider, *Agelenopsis aperta*) (0.1 μM)[12].

When the 3 Ca^{2+} channel blockers were applied (ω-AgaTX + ω-CgTX + nicardipine), Ca^{2+} current was blocked completely.

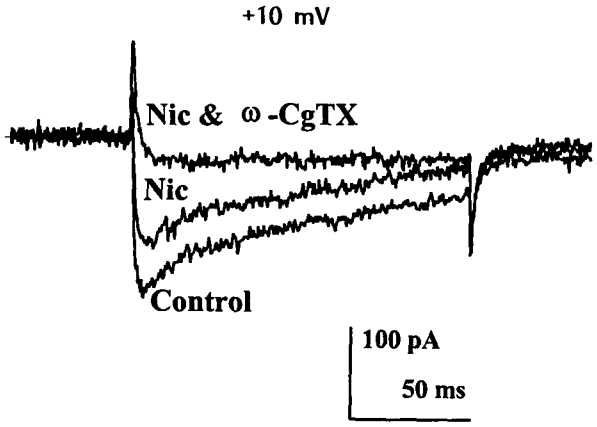


Fig. 7. Blocking action of sequentially applied nicardipine and w-CgTX on Ca^{2+} currents. Test potentials to +10 mV (from V_h of -80 mV) were applied before (control) and during the application of 1 mM nicardipine (middle trace). Late current (at the end of 150 ms depolarizing pulse) was blocked more than the peak current by nicardipine. Upper trace shows the effects of w-CgTX on nicardipine resistant current. w-CgTX induced marked blockade on the peak current rather than the late current.

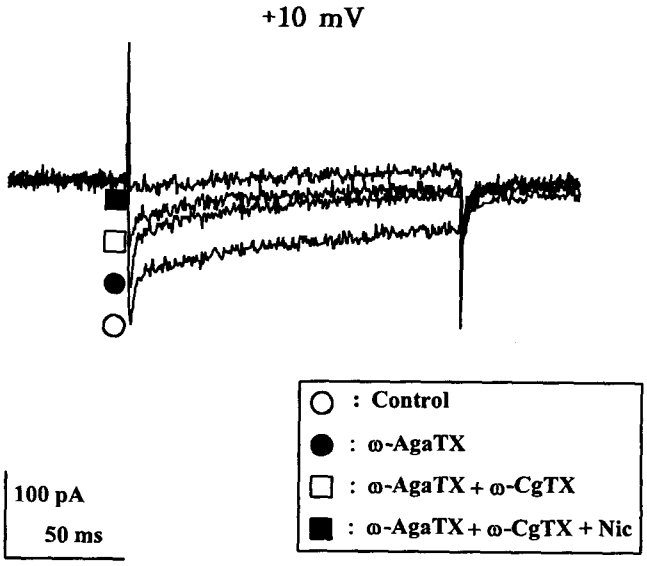


Fig. 8. Blocking action of sequentially applied w-AgaTX, w-CgTX, and nicardipine on Ca^{2+} currents. Ca^{2+} currents were recorded at +10 mV (from V_h=-80 mV). Application of w-AgaTX (0.1 mM) blocked both the peak and late Ca^{2+} currents. Additional application of w-CgTX (1 mM) induced further blockade on Ca^{2+} current. Nicardipine (1 mM) blockade all the remaining current.

Discussion

The major findings of the present study are following;

1. Facilitation Ca^{2+} current was activated by large depolarizing prepulse (6.17 ± 1.21 %, n=26).
2. Nicardipine (1 μM) blocked Ca^{2+} current to 43.8 ± 0.05 % of control (n=11).
3. ω-CgTX GVIA (1 μM) blocked Ca^{2+} current to 62 ± 0.08 % of control (n=17).
4. Combined application of Nicardipine and ω-CgTX GVIA blocked Ca^{2+} current to 19 % of control peak current and 42 % of control late current.
5. With 3 Ca^{2+} channel blockers (ω-AgaTX VIA + ω-CgTX GVIA + nicardipine), Ca^{2+} current was blocked completely.

Facilitation of Ca^{2+} currents

Ca^{2+} currents in bovine chromaffin cells were facilitated to 25-35 % with large depolarizing prepulse[5]. Hoshi et al[17] confirmed this observation and showed that the facilitation of Ca^{2+} currents is a voltage-dependent phenomenon independent of Ca^{2+} entry. Albillos et al[14] reported that depolarizing prepulses of 40 ms to +70 mV induced a 37 % facilitation of Ba^{2+} currents in feline chromaffin cells. In our present study we observed 6.17 ± 1.21 % (n=26) facilitation of Ca^{2+} currents and the statistical significance was confirmed with Wilcoxon signed rank test ($p<0.001$). Thus, the facilitation of Ca^{2+} currents seems to be a common phenomenon of chromaffin cells in various animal species.

But the kinetics showed differences. In bovine chromaffin cells, Artalejo et al [4),7),20)] interpreted the phenomenon of facilitation in terms of the recruitment, by prepulses, of a current associated with a 27 pS L-type Ca^{2+} channel that is superimposed on a standard current. The standard current is an HVA current, evoked without prepulses, flowing through neither L- nor classical N-type channels. The arguments for this are that ① the facilitated current is suppressed selectively by 1 μM nisoldipine ② this suppression is absent in the presence of 1 μM BayK 8644[20]. In addition, facilitation appears to be derived from a fast voltage-dependent phosphorylation process that is independent of the activation of G-proteins[24].

At variance with this, the facilitation in cat chromaffin cells exhibits a strong sensitivity to ω-CgTX, is weakly affected by 3 μM nisoldipine and BayK 8644, and is regulated by G-protein activation[14]. Thus, at least in the cat, facilitation seems to be derived from a recruitment of ω-CgTX-sensitive channels that are partly inhibited at rest.

In the present study of rat chromaffin cells, there are some differences with the results of bovine chromaffin cells[25]. First, nicardipine (1 μM) blocked facilitation completely (data not shown) but the block still happened in control condition without prepulse. Second, facilitation was not affected with BayK 8644.

In our study, 40 ms prepulse duration showed maximal effect on facilitation. This finding is not consistent with that in bovine chromaffin cell, either, (70 ms;[17], 200 ms;[4]) but it is compatible with that in cat chromaffin cell (40 ms;[14]). And it was found that facilitation in rat chromaffin cell is G-protein mediated process[26].

Facilitation process in rat chromaffin cells shows similarities with that in cat chromaffin cells but it shows differences with that in bovine chromaffin cells.

There are arguments against the results of Artalejo's (personal communication with Stanley Misler of Washington University).

First, they used calf chromaffin cells instead of adult bovine chromaffin cells. When other groups repeated same experiment with adult bovine chromaffin cells, Artalejo's findings were not

observed, which suggests that Artalejo's findings[20),25)] may not be generalized to be the characteristics of adult bovine chromaffin cells.

Second, they used D1 dopamine agonist, SKF 38393 (10 μM) for the experiment of the effect of facilitation Ca^{2+} channel on catecholamine secretion. With SKF 38393, the vesicles became ready to be released, which is not physiological condition.

In our study we found that SKF 38393 (10 μM) made no effects on facilitation (facilitation of control: 6.17 $\pm$ 1.21 % vs facilitation with SKF: 7.50 $\pm$ 2.59 %, $p>0.05$).

Therefore, before making any conclusion related with species difference, Artalejo's experiments should be redone in well controlled experimental condition.

DHP-sensitive channels

Bossu et al[8)] reported that a marked reduction of Ca^{2+} currents by nicardipine (1 μM) in bovine chromaffin cells. However, higher doses (10 ~ 30 μM) of nisoldipine was required to block 20 % of the currents in bovine chromaffin cells[10)]. The action of nisoldipine (3 μM) in cat chromaffin cells appears to be particularly potent at low membrane potential (75 % at -30 mV) and it was gradually less at higher voltages than -30 mV[14)].

In our study blocking effect of nicardipine (1 μM) was maximal at +10 mV (56 $\pm$ 0.05 %, n=11) and it was gradually less at higher voltages than +10 mV. The probable reason for the different blocking effect of DHP antagonists between species is that bovine chromaffin cells express a lower density of L-type channels than both cat and rat chromaffin cells.

ω-CgTX sensitive channels

The coexistence of ω-CgTX-sensitive channels and L-type channels has been reported consistently in bovine chromaffin cells. In bovine cells, the block of HVA currents by ω-CgTX was largely irreversible and varied from cell to cell (on average: ~50 %)[6),10),24)].

In cat chromaffin cells, the action of ω-CgTX varied from cell to cell, average blocked fraction was about 46 % and the block by ω-CgTX was partly reversible[14)].

Our present data shows that the blocked fraction by ω-CgTX (1 μM) is less than that in bovine and cat chromaffin cells (38 % of Ca^{2+} currents at +10 mV). Given the additive action of DHPs and ω-CgTX (Fig. 7.), the only conclusion to be drawn from our present data is that the irreversibly blocked ω-CgTX-sensitive channels are pharmacologically distinct from L-type channels in rat chromaffin cells and that the two sets of channels contribute to the majority of the control currents (81 %). The limited size of residual currents in our cells suggest contribution of P-type channels and it was confirmed by the use of ω-AgaTX showing that the residual currents were completely blocked by ω-AgaTX (0.1 μM). And this result is compatible with the report of Gandia et al[22)] where ω-AgaTX blocks Ba^{2+} currents in cat chromaffin cells by only 10-15 %.

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흰쥐 부신수질 크로마핀세포의 칼슘통로 유형

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부신수질 크로마핀세포는 아세틸콜린에 반응하여 카테콜아민을 분비한다. 카테콜아민이 분비되기 위하여는 세포외 칼슘이 절대적으로 필요한데 이는 막전압 의존성 칼슘통로를 통하여 칼슘이 세포 속으로 유입되어야 분비기전이 시작됨을 시사한다. 부신수질 크로마핀 세포를 단일세포로 분리한 후 패치클람프 테크닉을 적용하여 여러 종류의 칼슘통로가 존재한다는 것이 알려져 있으나 아직 종이 달라짐에 따라 다른 칼슘통로가 존재하는 지 여부가 확실하지 않다.

그러므로 본 연구에서는 흰쥐 부신수질 크로마핀 세포를 대상으로 하여 단일 세포 패치클람프 테크닉을 적용하여 이 세포에 존재하는 다양한 칼슘통로의 존재를 확인하고자 하였다.

L형 칼슘통로 억제제인 nicardipine, N형 칼슘통로 억제제인 ω-CgTx GVIA, P형 칼슘통로 억제제인 ω-AgaTx IVA를 사용하여 L형, N형, P형 칼슘통로가 흰쥐 부신수질 세포에 존재함을 확인하였고 개개의 칼슘통로가 전체 칼슘전류에 기여하는 정도는 L형 >N형> P형이었다.

중요단어: 칼슘통로, 부신수질 크로마핀세포