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Effect of Ca^{2+}-channel Blockers on Norepinephrine Release in the Rat Hippocampal Slice and Synaptosome

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The aim of this study was to investigate the role of Ca^{2+}-channel blockers in norepinephrine (NE) release from rat hippocampus. Slices and synaptosomes were incubated with [^{3}H]-NE and the releases of the labelled products were evoked by 25 mM KCl stimulation. Nifedipine, diltiazem, nicardipine, flunarizine and pimozide did not affect the evoked and basal release of NE in the slice. But, diltiazem, nicardipine and flunarizine decreased the evoked NE release with a dose-related manner without any change of the basal release from synaptosomes. Also, a large dose of pimozide produced modest decrement of NE release. ω-conotoxin (CTx) GVIA decreased the evoked NE release in a dose-dependent manner without changing the basal release. And ω-CTxMVIIC decreased the evoked NE release in the synaoptosomes without any effect in the slice, but the effect of decrement was far less than that of ω-CTxGVIA. In interaction experiments with ω-CTxGVIA, ω-CTxMVIIC slightly potentiated the effect of ω-CTxGVIA on NE release in the slice and synaptosomal preparations. These results suggest that the NE release in the rat hippocampus is mediated mainly by N-type Ca^{2+}-channels, and that other types such as L-, T- and/or P/Q-type Ca^{2+}-channels could also be participate in this process.

Key Words: Rat, Hippocampus, N-type Ca^{2+}-channels, Synaptosome, Norepinephrine, ω-conotoxin

INTRODUCTION

The exocytotic release of neurotransmitters from presynaptic nerve terminals depends on the Ca^{2+} influx into the cell (Augustine et al, 1988; Linàs et al, 1992). The influx of Ca^{2+} is mediated by the entry of Ca^{2+} through the voltage-sensitive Ca^{2+}-channels (VSCCs) activated upon excitation of the nerve terminal (Tsien et al, 1991). At least seven subtypes of VSCCs (L-, N-, O-, P-, Q-, R- and T-type) have been described by electrophysiological experiments (Olivera et al, 1994; Randall & Tsien, 1995). Although numerous reports have emphasized that Ca^{2+}-mediated neurotransmitter release from nerve terminals is controlled by multiple VSCCs, it has not been clearly elucidated yet that which type of VSCCs is involved in the regulation of neurotransmitter release from presynaptic nerve terminals.

Neurotransmitter release associated with Ca^{2+}-influx in non-mammals (Suszkiw et al, 1987; Lundy et al, 1989) and rat sympathetic neurons (Hirning et al, 1988) appears to be resulted from activation of N-type VSCCs. Also, in mammalian brain N-type VSCCs are responsible for the release of various neurotransmitters (Thomas et al, 1989; Pullar & Findlay, 1992; Clos et al, 1994). However, there are reports that the neurotransmitter release appears to be only partially sensitive to N-type VSCCs and additional activation of other VSCCs such as P-type are needed (Turner et al, 1992; Uchitel et al, 1992; Kimura et al, 1995). Moreover, it has been suggested that the Q-type (Wheeler et al, 1994) and P/Q-type (Grassi et al, 1999) VSCCs modulate synaptic transmission.

On the other hand, it is known that neurotransmitter release in brain is generally not blocked by L-type Ca^{2+}-channel blockers (Miller, 1987). However, Bay K 8644, a L-type channel activator, has been shown to augument evoked release of neurotransmitter in rat hippocampus and this effect is reversed by L-type VSCCs blockers (Middlemiss, 1985; Bostwick et al, 1993). Sabrià et al (1995) reported that presynaptic K^+-evoked norepinephrine release is regulated by L-type in rat cortex and N-type in rat hippocampus. Also, Grassi et al (1999) showed that L-type Ca^{2+}-channel gives the modest contribution to NE release in the rat cortical synaptosome.

From the above reports, it is suspected that the relative contribution of each type varies from region to region and the release of different neurotransmitters is mediated by different combination of VSCCs. The present study, therefore, was designed to compare the effects of various Ca^{2+}-channel blockers on K^+-evoked norepinephrine release from rat hippocampal slice and synaptosome and also to define, if possible, which type of Ca^{2+}-channel is important in the regulation of presynaptic neurotransmitter release in the central nervous system.

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ABBREVIATIONS: CTx, ω-conotoxin; VSCCs, voltage-sensitive Ca^{2+}-channels; NE, norepinephrine.

METHODS

Slice preparation

Slices of 2.5~3.0 mg (wt/wt, 400 μm in thickness) were prepared from the hippocampus of male Sprague-Dawley rats weighing 250~300 gm with a Balzers® tissue chopper (Balzer Union, England) and were incubated in 2 ml of modified Krebs-Henseleit medium containing 0.1 μmol/L [^{3}H]norepinephrine (NE) for 30 min at 37°C.

Synaptosomal preparation

Crude synaptosomes were prepared by the method of Raiteri et al (1984) with some modifications. In brief, both hippocampi from 4 male rats were homogenized in 4 ml of 0.32 M sucrose buffered at pH 7.4 with phosphate. The homogenates were centrifuged at 1,000 g for 10 min to remove nuclei and cellular debris, and synaptosomal fractions (P_2) were isolated from the supernatants by centrifugation at 15,000 g for 10 min. The synaptosomal pellets (ca. 2~3 mg/ml protein) were incubated in 4 ml of modified Krebs-Henseleit medium containing 0.1 μmol/L [^{3}H]-NE for 10 min at 37°C. Protein content was determined according to the procedure of Lowry et al (1951).

Release experiment

The [^{3}H]NE-pretreated slices were superfused with medium containing desipramine (1 μM) and yohimbine (10 nM) for 150 min at a rate of 0.5 ml/min. The composition (mM) of superfusion medium was 118 NaCl, 4.8 KCl, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 0.57 ascorbic acid, 0.03 Na_2EDTA, and 11 glucose, and the superfusate was continuously aerated with 95% O_2+5% CO_2, and the pH adjusted to 7.4. In slice experiments, collection of 5 min fractions (2.5 ml) of the superfusate began 50 min after the superfusion. The slices were then depolarized for 2 min by exposure to high K^+ concentration (substituting an equimolar concentration of NaCl) performed at 60 min (S_1) and 125 min (S_2). Drugs to be tested were present from 15 min before the S_2 stimulation onwards. Their effects were evaluated by calculating the ratio (S_2/S_1) of the fractional release in the two stimulation periods. Effect of drugs on basal outflow are expressed as the ratio b_2/b_1 between fractional rates of outflow immediately before S_2 (120~125 min) and before S_1 (55~60 min). At the end of superfusion, the slices were solubilized in 0.5 ml tissue solubilizer (0.5 N quaternary ammonium hydroxide in toluene).

In synaptosomal experiments, collection of the superfusate began 70 min after the superfusion with $CaCl_2$- and Na_2EDTA-free medium. At 90 min, the synaptosomes were depolarized by changing the superfusion fluid for 3 min with a medium containing 100 μM $CaCl_2$ and 25 mM KCl; equimole of NaCl was removed. Drugs were introduced from 10 min before stimulation, and the effects were compared with the corresponding ratio obtained under control conditions (no drug added). At the end of experiment, the synaptosomes in the superfusion chambers were extracted with 1% Triton solution.

The radioactivity in the superfusates, solubilized tissues and extracted synaptosomes were determined by liquid scintillation counter (Beckman LS 5000 TD). The fractional rate of tritium-outflow was calculated as tritium-outflow per 5 min divided by the total tritium content in the slice or synaptosome at the start of the respective 5 min period (Hertting et al, 1980).

All results are given as Mean±SEM. Significance of difference between the groups was determined by ANOVA and subsequently by one-tailed Student's t-test.

The following chemicals were used: l-[7,8-^{3}H]noradrenaline (30~50 Ci mmol^{-1}; Amersham International, Amersham, Buckinghamshire, UK), desipramine HCl, yohimbine HCl, nifedipine, nicardipine, diltiazem, flunarizine, cadmium (Sigma chemical Co; St. Louis, Missouri, USA), ω-conotoxin MVIIC, ω-conotoxin GVIA and (±)-Bay K 8644 (Research Biochemicals Inc; Natick, MA., USA). Drugs were dissolved in the medium except diltiazem and pimozide which were initially dissolved in DMSO, and flunarizine which was initially dissolved in metahnol then diluted with the medium. All the other chemicals were reagent grade and obtained from commercial sources.

RESULTS

Exposure of the slices and synaptosomes to a high K^+ medium increased the rate of [^{3}H]norepinephrine (NE) release that was strongly dependent on the presence of external Ca^{2+} (Fig. 1). The K^+-evoked release of NE (NE release) was inhibited by $CdCl_2$ with concentration-dependent manner (Fig. 2), confirming that this phenomenon is mediated by calcium entry through VSCCs.

To identify the Ca^{2+}-channel subtypes involved in the NE release in the experiment, the influence of various Ca^{2+}-channel inhibitors on NE release were investigated. The selective L-type Ca^{2+}-channel antagonists, nifedipine, nicardipine and diltiazem did not affect the basal and NE release in the slices. Pimozide, a non-selective L- and T-type Ca^{2+}-channel antagonist, and flunarizine, an another T-

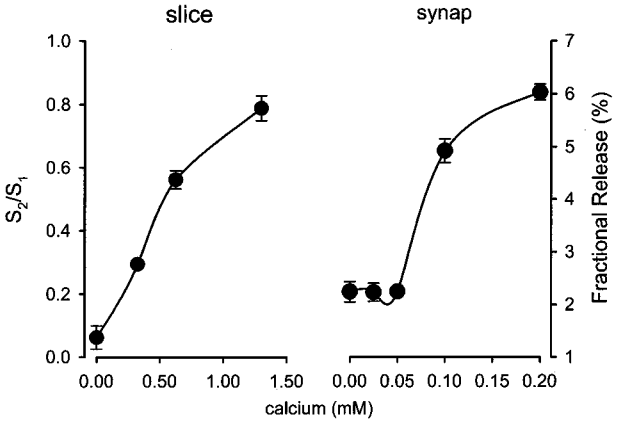

Fig. 1. Effects of various Ca^{2+} concentrations on 25 mM K^+-evoked release of tritiated norepinephrine from the rat hippocampal slice and synaptosome (synap). The slices were preincubated with [^{3}H]norepinephrine for 30 min and were stimulated twice for 2 min each, after 60 and 125 min of superfusion (S_1, S_2). The concentration of Ca^{2+} in the medium was changed 15 min before S_2, and the effect on the stimulation-evoked tritium outflow is expressed by the ratio S_2/S_1. In the synaptosomal experiments, the synaptosomes were preincubated with [^{3}H]norepinephrine for 10 min and were stimulated with Ca^2-containing 25 mM K^+-medium for 3 min after 100 min of superfusion with Ca^{2+}-free medium. Each point denotes mean±SEM from 5~8 experiments per group.

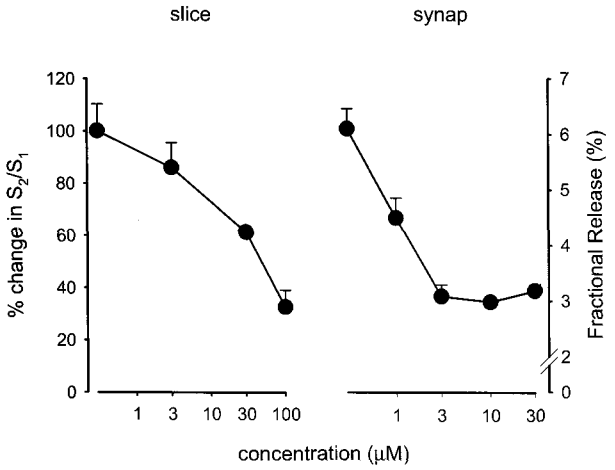

Fig. 2. Effects of $CdCl_2$ on 25 mM K^+-evoked release of tritiated norepinephrine from the rat hippocampal slice and synaptosome (synap). Legends are the same as in Fig. 1.

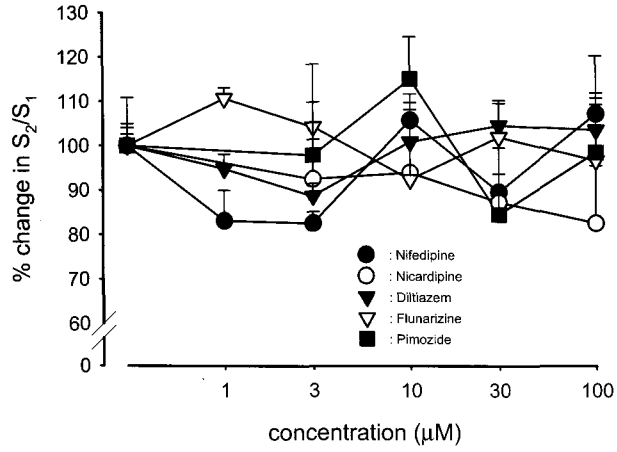

Fig. 3. Effects of various Ca^{2+}-channel blockers on K^+-evoked NE release from the rat hippocampal slice. Each point dednotes mean± SEM from 5~8 experiments per group. Legends are the same as in Fig. 1.

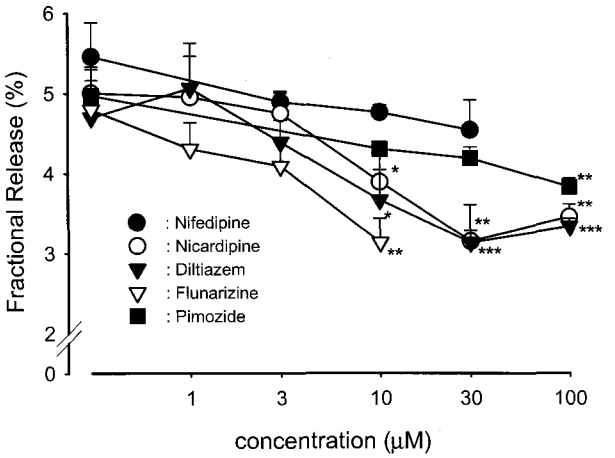

Fig. 4. Effects of various Ca^{2+}-channel blockers on K^+-evoked NE release from the rat hippocampal synaptosome. Asterisks indicate the significant differences from the control (drug free) group (*: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$). Each point dednotes mean± SEM from 5 ~ 8 experiments per group. Other legends are the same as in Fig. 1.

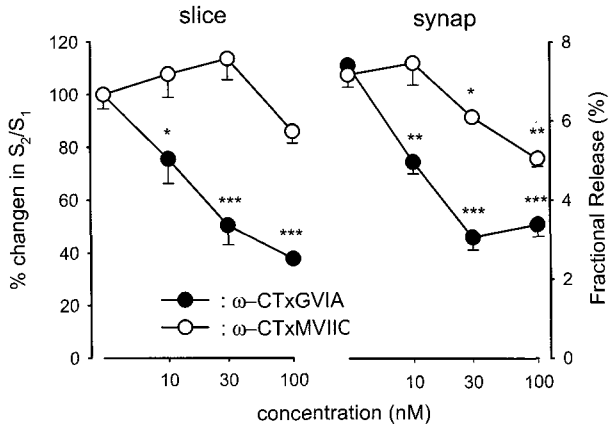

Fig. 5. Effects of ω-conotoxin (CTx) MVIIC and ω-CTxGVIA on K^+-evoked NE release from the rat hippocampal slice and synaptosome (synap). Each point dednotes mean±SEM from 5~8 experiments per group. Legends are the same as in Fig. 4.

type Ca^{2+}-channel antagonist, did not affect the NE relaese as well as basal release (Fig. 3). In order to further quantify the contribution of L- and T-type Ca^{2+}-channels to the NE release, experiments were perfomend in synaptosomes. All of the above drugs did not affect the basal release. Nifedipine did not affect the NE release. Nicardipine and diltiazem induced a dose-dependent reduction of the NE release, and the maximum decrease was observed at 30 μM of nicardipine (37%) and of diltiazem (33%). Also larger dose of pimozide and flunarizine (1~10 μM) decreased the NE release, the maximum effects were 23 and 35%, respectively (Fig. 4). More than 30 μM of flunarizine markedly increased the basal release. Because this phenomenon was thought to be not a physiological status, the data of evoked NE release were not included in this report.

ω-conotoxin (CTx) GVIA, a specific N-type Ca^{2+}-channel blocker, decreased the NE release in a dose-dependent manner without any change of basal release in slice and synaptosome. The blockade of P/Q-type Ca^{2+}-channel with ω-CTx MVIIC induced a concentration-dependent reduction in evoked NE release from synaptosome, although neither had any effect in the slice experiment. To ascertain the interaction between ω-CTx MVIIC and ω-CTx GVIA, the effects of ω-CTx MVIIC were studied in the presense of 30 nM ω-CTx GVIA (maximum effect dose in synaptosome). As depicted in Fig. 6, when simultaneously treated with two toxins, the decrement of NE release by 30 nM ω-CTx GVIA was potentiated by about 30%, while the decrement was 29% by 100 nM ω-CTx MVIIC alone in the slice experiment. In the synaptosomal experiments, the effect of 30 nM ω-CTx GVIA was potentiated by 100 nM ω-CTx MVIIC to about 12% as in the slice.

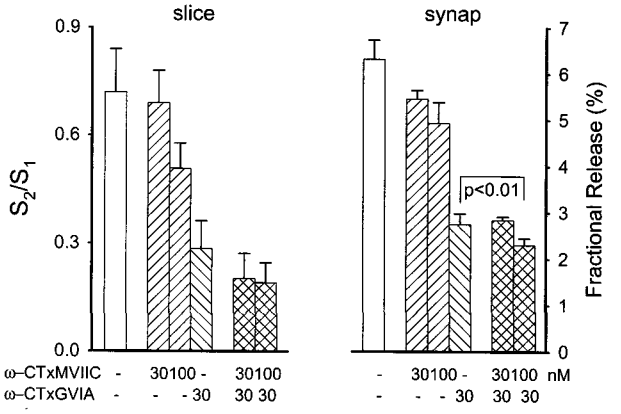

Fig. 6. Interaction between ω-conotoxin (CTx) MVIIC and ω-CTxGVIA on K^+-evoked NE release from the rat hippocampal slice and synaptosome (synap). Each bar dednotes mean±SEM from 5~8 experiments per group. Legends are the same as in Fig. 1.

DISCUSSION

In the present study, the K^+-evoked NE release was concentration-dependently decreased by removing calcium or adding $CdCl_2$ to the medium from the rat hippocampal slices and synaptosomes. These results were in accordance with other reports to indicate that acetylcholine release from the hippocampal slice was calcium dependent (Saydoff & Zaczeck, 1966), and NE release from parietotemporal cortical synaptosomes was inhibited by $CdCl_2$ (Grassi et al, 1999). These facts, in conjuction with the fact that the neurotransmitters release from the presynaptic nerve terminals are mediated by the activation of calcium-channels in the cellular membrane (Jessel & Kandel, 1993), confirmed that the NE release in this experiment is indeed mediated by calcium entry through calcium channels.

Calcium entry into the cells across the cellular membrane via VSCCs is a prerequisite for the stimulus-evoked neurotransmitters release. Based on this fact, it might be anticipitated the calcium antagonists would inhibit this process. Nevertheless, numerous previous studies have failed to demonstrate any effect of these drugs at pharmacologically relevant concentrations on neurotransmitters release induced by K^+- or electrical stimulation (Schoffelmeeer et al, 1981; Starke et al, 1984; Middelmiss, 1985). Multiple Ca^{2+}-channel types are defined by their selectivity to specific antagonists. One of these, 1,4-dihydropyridine sensitive L-type channels are present in cortex, hippocampus, striatum and thalamus (Quirion, 1983; Suszkiw et al, 1987), and preferentially associated with certain areas rich in synaptic contacts (Skattebol & Trigle, 1987). Also several reports also show that L-type Ca^{2+}-channel is involved in neorotransmitters release (Nowycky et al, 1985; Skattebol & Trigle, 1987; Gandhi & Jones, 1992). However, there are reports that following stimulation with dihydropyridine Ca^{2+}-channel activator, Bay K 8644, augments the evoked neurotransmitter release and this additional release is sensitive to blockade by Ca^{2+}-channel antagonists (Middlemiss, 1985; Skattebol & Trigle, 1987; Spedding et al, 1989). In the present study, all the L-type channel blockers did not affect the NE release in the slice. And Bay K 8644 have not any effect of NE relaese in slices and synaptosomes (data are not shown). This result is in accordance with other report that L-type Ca^{2+}-channel is not involved in the acetylcholine release from hippocampus (Fredholm, 1993). While, nicardipine and diltiazem significantly reduced NE release and nifedipine produced modest reduction in the synaptosomes. These findings, in conjuction with other reports that nimodipine and nifedipine reduced NE release from the cortical synaptosome (Sabrià et al, 1995; Grassi et al 1999), suggested that the L-type Ca^{2+}-channel was involved in the NE release in hippocampus, but it gives a modest contribution in this experimental model. However, further studies are required to clarify discrepancies between the results with the slice and synaptosomes.

On the other hand, it has been hypothetized that T-type Ca^{2+}-channels are involved also in neurotransmitter release from presynaptic nerve endings. There are reports that flunarizine, a specific T-type Ca^{2+}-channel blocker, increased the dopamine release in the basal ganglia (Pani et al, 1990) and the cocaine-induced dopamine release in the striatum (Devoto et al, 1993). And Bostwick (1993) showed that the flunarizine inhibited the increment of high potassium-induced ACh release by Bay K 8644. In the present synaptosomal experiments, flunarizine and pimozide reduced evoked NE release. From the above results, it is also suggested that T-type Ca^{2+}-channel could participated in evoked NE release.

ω-CTx GVIA is a toxin purified from Conus geographus (Olivera et al, 1984), and is well known to be a selective N-type Ca^{2+}-channel blocker (Kasai et al, 1987). In the present study, ω-CTx GVIA decreased the NE release significantly in the slices and synaptosomes, and the effects in synaptosomes was far greater. Based on these results, it is suggested that the N-type Ca^{2+}-channel is important in the regulation of the NE release in the hippocampus. This is in accordance with other reports that N-type Ca^{2+}-channels appear to play a major role in ACh (Clos et al, 1994) or NE release (Sabria et al, 1995) in hippocampus and cortical synaptosome (Grassi et al, 1999). However, it has been repeatedly established that other types such as P- and/or Q-type Ca^{2+}-channels are important in neurotransmitter release in the central nervous system. Kimura et al (1995) insisteed that P- or Q-type Ca^{2+}-channel plays a major role in neurotransmitter release in CNS, and Saydoff & Zaczeck (1996) reported that N- and Q-type Ca^{2+}-channels are involved in ACh release in hippocampus. More recently, Grassi et al (1999) have observed that K^+-evoked NE release in cortical synaptosome is mediated by activation of P/Q- and N-type Ca^{2+}-channels. Therfore, in order to confirm the above results, ω-CTxMVIIC was employed in the present study. ω-CTxMVIIC decreased the NE relase in a dose-dependent manner in the synaptosome without any effect in the slice, however, the effect of decrement was much less than that of ω-CTxGVIA. And, in interaction with ω-CTxGVIA, ω-CTxMVIIC slightly potentiated the effect of ω-CTxGVIA in the slice and synaptosomal experiments. This fact led us to suggest that P/Q-type Ca^{2+}-channels appear to play a minor role in the NE release in rat hippocampus.

Overall, from the results obtained from the present study, it is suggested that the NE release in rat hippocampus is mediated by N-type Ca^{2+}-channels, but other types such as L-, T- and/or P/Q-type Ca^{2+}-channels could participate in the process.

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