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Original Research

Comparison of binding affinities of ω-conotoxin and amlodipine to N-type Ca^{2+} channels in rat brain

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KEY WORDS amlodipine; nifedipine; SM-6586; conotoxin; radioligand assay; calcium channels

AIM: To compare the binding affinities of ω-conotoxin (CTX) and amlodipine to N-type Ca^{2+} channels in rat brains. **METHODS**: Whole rat brains were homogenized in HEPES buffer 50 mmol·L^{-1} (pH 7.4) and centrifuged at 40 000 × *g* to obtain the membrane-entriched fraction. ^{125}I-ω-conotoxin (^{125}I-ω-CTX) was used as a radioligand. Using radioligand binding assay K_d and B_{max} values of the radioligand were determined by Scatchard analysis. The IC_{50} value for each drug was obtained from displacement experiments. **RESULTS**: No differences in B_{max} values of ^{125}I-ω-CTX binding sites between frozen and fresh tissues were observed. Values of K_d and B_{max} of N-type Ca^{2+} channels were 0.02 ± 0.01 nmol·L^{-1} and 1029 ± 108 pmol/g protein, respectively. The pK_i values of ω-CTX and amlodipine were 9.57 and less than 4, respectively. The pK_i values of propranolol, prazosin, atropine, and histamine were very low. **CONCLUSION**: The binding affinity of the L-type Ca^{2+}-antagonist amlodipine to N-type Ca^{2+} channels in the rat brain was very low.

We have previously shown that a novel 1,4-dihydropyrindine derivative amlodipine is a long-lasting Ca^{2+} antagonist for displacing the specific bindings of [^{3}H]isradipine[1-4]. Voltage-dependent calcium channels (VDCC) subtypes, termed L (long lasting), T (transient), N (neither L or T), and P (Purkinje) types, were found in tissues[5]. It is well known that 1,4-dihydropyridine (DHP) Ca^{2+} antagonists interact with L-types, implying that they can block Ca^{2+} influx. On the other hand, N-type Ca^{2+} channels may play an important role in transmitter release from nerve terminals[6], implying that these two subtypes have different functions. Thus, the purpose of this study was to compare the interactions (binding potencies) between ω-conotoxin (ω-CTX) and amlodipine and N-type Ca^{2+} channels in rat brain.

MATERIALS AND METHODS

Preparations of membrane-enriched fractions from rat brain Membrane-enriched fractions were prepared according to previous methods[1-4,7]. The brains from male Wistar rats (weighing 180 – 300 g) were homogenized in HEPES 50 mmol·L^{-1} (pH 7.4) using a Glass-homogenizer and filtered through 4 layers of gauze. The filtrates were centrifuged at 40 000 × *g* for 30 min. The pellets obtained were resuspended in HEPES 50 mmol·L^{-1} (pH 7.4). Protein concentrations were determined using the method of Lowry *et al*[8].

^{125}I-ω-conotoxin binding assay The ^{125}I-ω-CTX (74 TBq·mol^{-1}, Amersham) binding assay was carried out as described by Czyrak *et al*[9]. The incubation mixture (0.5 mL) consisted of 400 μL of assay buffer 50 mmol·L^{-1} HEPES-NaOH buffer (pH 7.4), 50 μL of membrane suspension (approximately 0.5 μg of protein), 5 μL of either assay buffer (total binding) or a solution containing unlabeled ω-CTX (final concentration of 10 mmol·L^{-1} for nonspecific binding) and 45 μL of ^{125}I-ω-CTX. Membranes were preincubated in duplicate in the presence or absence of unlabeled ω-CTX at 25 ℃ for 30 min. The final incubations were initiated by the addition of ^{125}I-ω-CTX and terminated after 15 min at 22 ℃ by rapid filtration through glass-fiber filters (GF/C, Whatman). The filters were immediately rinsed with 1 mL aliquot of ice-cold assay buffer three times. Radioactivity was measured by gamma counting at an efficacy of 50 %. Specific binding of ^{125}I-ω-CTX was defined as the difference between total and nonspecific bindings. For saturation experiments, the binding of increasing concentrations of ^{125}I-ω-CTX (0.01 – 2.0 nmol·L^{-1}) were analyzed as described by Scatchard. The K_d (apparant

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dissociation constant) and B_{max} (maximal binding capacity) were determined for each rat brain region. ^{125}I-ω-CTX 0.05 nmol·L^{-1} of was used for the assessment of displacement potencies of each drug. In addition, the K_i (inhibition constant) value of each drug was calculated from the apparent IC_{50}. This value is expressed as K_i ($-\lg K_i$).

Radioligand and drugs ^{125}I-ω-CTX (74 TBq·mol^{-1}) was purchased from Amersham International plc (Buck, UK). Amlodipine and (+) SM-6586 (methyl 1,4-dihydro-2,6-dimethyl-3-(3-(*N*-benzyl-*N*-methylaminomethyl)-1,2,4-oxadiazolyl-5-yl)-4-(3-nitrophenyl)pyridine-5-carboxylate) were kindly donated by Sumitomo Pharmaceuticals Co. Japan.

RESULTS

Decreases in total and specific bindings of ^{125}I-ω-CTX to rat brain membranes by addition of BSA were found. However, no significant differences were seen in ^{125}I-ω-CTX bindings between fresh and frozen brain membranes.

The representative saturation experiments for ^{125}I-ω-CTX binding to rat brain are shown in Fig 1. The radioligand interacted with a single population of saturable high affinity sites in rat brain. K_d and B_{max} for ^{125}I-ω-CT bindings were 0.02 ± 0.01 nmol·L^{-1} and 1029 ± 108 pmol/g protein, respectively.

The pK_i values of ω-CTX, amlodipine, and other Ca^{2+} antagonists are shown in Tab 1. Although a high pK_i values for ω-CTX (pK_i values: 9.57) was observed, those of amlodipine and other Ca^{2+} antagonists were very low. The high pK_i values of Ca^{2+} antagonists for the [^{3}H]isradipine binding sites (L-type) are shown in Tab 1.

Tab 1. pK_i values of ^{125}I-ω-CTX (N-type) and [^{3}H]isradipine (L-type) bindings in rat brains to Ca^{2+} antagonists. (Number of experiments) $\bar{x} \pm s$.

Drugs	pK_i values	
	^{125}I-ω-CTX	[^{3}H]isradipine
Nifedipine	<4 (5)	8.33 ± 0.71 (7)
ω-CTX	9.57 (5)	≥9.00
Amoldipine	<4 (4)	7.41 ± 0.22 (4)
Nisodipine	<4 (5)	9.31 ± 0.24 (4)
Benidipine	<4 (5)	8.05 ± 0.22 (4)
Nitrendipine	<4 (5)	8.51 ± 0.56 (7)
Nicardipine	<4 (5)	8.50 ± 0.40 (7)
Nimodipine	<4 (5)	8.93 ± 0.45 (8)
(+)SM-6586	<4 (5)	8.98 ± 0.26 (4)
(−)SM-6586	<4 (5)	7.68 ± 0.34 (6)
(±)SM-6586	<4 (5)	8.57 ± 0.27 (9)
Manidipine	<4 (5)	8.23 ± 0.30 (4)

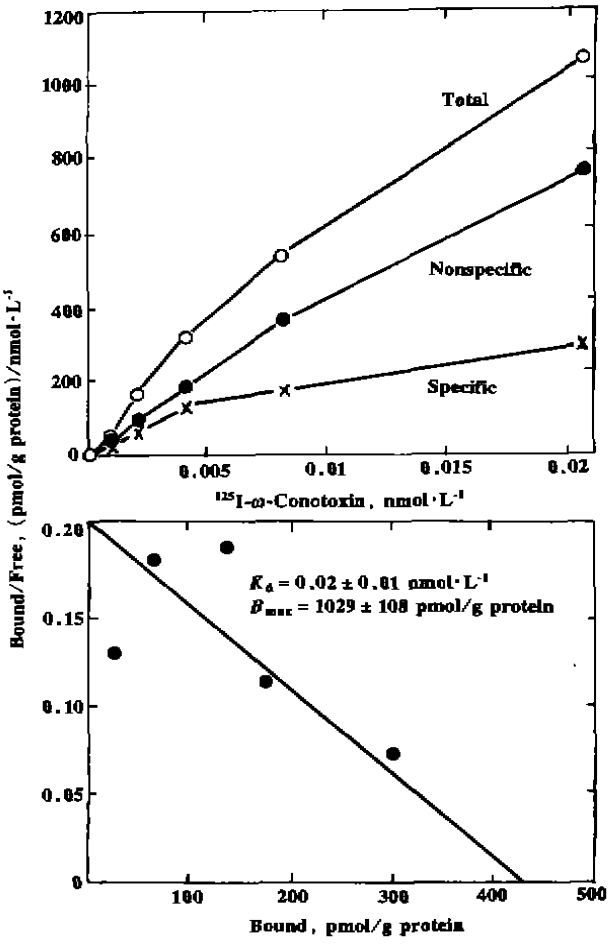

Fig 1. Scatchard analysis of ^{125}I-ω-CTX bindings to rat brain membranes.

The pK_i value of ω-CTX to L-type Ca^{2+} channelswas taken from Ertel *et al*[10]. The pK_i values from other Ca^{2+} antagonists were taken from reports previously published[4,7].

DISCUSSION

The clear results from preliminary experiments for the membrane preparation of brains and the radioligand binding assay were obtained from the present study. Our previous reports[1-3] showed that membrane preparations from rat brains and the radioligand binding assay for the assessment of displacemental potencies of L-type Ca^{2+} antagonists needed to carry out in the same day because the affinity of this channel to rat brains was not stable. The present study showed that either fresh or fronze membrane preparations of rat

brains could be used for the determination of N-type channels using the radioligand binding assay method. Furthermore, the addition of bovine serum albumin to the incubation medium induced the decrease of numbers in specific binding of N-type channels of rat brains, although several papers reported that the serum bovine albumine was needed in incubation medium[9,10]. The present paper suggests that (1) membrane fractions prepared from either fresh or frozen brain tissues can be used for the determination of the N-type Ca^{2+} channels and (2) bovine serum albumine in the incubation medium is not for this determination.

Cruz and Olivera[11] reported that iodine labeled toxins like ω-CTX were shown to bind specifically to high affinity sites on chick brain synaptosomes. The present study revealed that rat brain membranes bound ω-CTX with a high affinity (K_d value: 0.02 nmol·L^{-1}). Results coincide with studies done on rabbit[12] and frog[13] brains. In addition, previous studies have shown ω-CTX distinguished between both the verapamil and 1, 4-DPH Ca^{2+} antagonist target sites[11]. Therefore, the present study shows that this radioligand can be used to assess the displacement potencies of chemical compounds to N-type ω-CTX binding sites.

ω-CTX is a compound that can block N- and L-type Ca^{2+} channels with a high affinity ($IC_{50} \leqslant 1$ nmol·L^{-1})[10]. However, small interactions between 1,4-DPH antagonists like amlodipine and N-type Ca^{2+} channles were observed, suggesting that the 1,4-DPH Ca^{2+} antagonist amlodipine do not affect neurotransmitter release in the brain synaptosomes. Although the present study also assessed many other 1,4-DPH Ca^{2+} antagonists like nifedipine, nisoldipine, benidipine, nitrendipine, and nicardipine, these compounds had low displacement potencies to ω-CTX binding sties in rat brains. Thus, we conclude that although ω-CTX showed high affinities to both L- and N-type Ca^{2+} channels, there is no close interaction between the 1, 4-DPH Ca^2 antagonist amlodipine and N-type Ca^{2+} channels.

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ω-Conotoxin 及氨氯地平与大鼠脑内 N 型钙通道亲和性的比较

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关键词 氨氯地平；硝苯地平；SM-6586；conotoxin；放射配位体测定；钙通道

目的：用大鼠脑对 ω-conotoxin (ω-CTX)及氨氯地平与N型钙通道的内在关系进行分析. **方法**：将大鼠全脑匀浆于 HEPES 50 mmol·L^{-1}(pH 7.4)缓冲液中，经40 000 × *g* 离心后，收集膜区域. 以 ^{125}I-ω-conotoxin (CTX)作为放射配体测定. **结果**：^{125}I-ω-CTX与冷冻标本及新鲜标本结合的 B_{max} 无区别. N型钙通道的 K_d 和 B_{max} 值分为 0.02 ± 0.01 mmol·L^{-1}和 1029 ± 108 pmol/g 蛋白质. ω-CTX 及氨氯地平的 pK_i 值分别为 9.57 以及 < 4，普萘洛尔、哌唑嗪、阿托品、组胺的 pK_i 值也非常低. **结论**：L型钙离子拮抗剂氨氯地平与N离子通道的亲和性很低.

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Enhancement of (−)-stepholidine on protein phosphorylation of a dopamine- and cAMP-regulated phosphoprotein in denervated striatum of oxidopamine-lesioned rats[1]

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KEY WORDS stepholidine; phosphoproteins; phosphorylation; dopamine D_1 receptors; oxidopamine; corpus striatum; SK&F-38393; Sch-23390

AIM: To study effects of (−)-stepholidine (SPD) on the phosphorylation of a dopamine- and cAMP-regulated phosphoprotein (DARPP-32) in the striatum of oxidopamine-lesioned rats. **METHODS**: The amount of dephospho-DARPP-32 was measured by a back-phosphorylation assay. **RESULTS**: In the striatum of control rats, SPD *per se* had no effect on the phosphorylation of DARPP-32, but it antagonized the decrease by 28 % of dephospho-DARPP-32 induced by the D_1 agonist SK&F-38393. In the denervated striatum of oxidopamine-lesioned rats, SPD decreased the amount of dephospho-DARPP-32 by 44 %. The effect of SPD was completely counteracted by the concomitant administration of the D_1 antagonist Sch-23390. **CONCLUSION**: SPD exhibits D_1 agonistic action on DARPP-32 phosphorylation in the denervated striatum of oxidopamine-lesioned rats, but it acts as a D_1 antagonist in normal striatum.

(−)-Stepholidine (SPD), an alkaloid isolated from Chinese herb *Stephania intermedia* Lo, is a tetrahydroprotoberberine. SPD has high affinities for both dopamine (DA) D_1 and D_2 receptors with a preference for D_1 receptors, and low affinities for non-DA receptors[1]. SPD possesses the characteristics of a D_2 antagonist[2, 3].

As for D_1 action of SPD, previous studies reported controversial observations. In rats with 6-d reserpine treatment, SPD reduced D_1 agonist SK&F-38393-induced inhibition of firing activity of nigral DA cells although SPD *per se* had no action, indicating a D_1 antagonistic action[4]. In rats with unilateral nigral lesions by oxidopamine, SPD induced a contralateral rotation in the manner similar to SK&F-38393, indicating a D_1 agonistic action[5]. SPD bound to high and low affinity sites (R_H and R_L) of D_1 receptors and the R_H could be regulated by GTP, indicating an intrinsic activity to D_1 receptors[6]. After blockade of D_2 receptors, SPD stimulated striatal cAMP formation[7]. In nigral lesioned rats, SPD induced a firing inhibition of substantia nigra pars reticular

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