

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

We have previously shown that a novel 1,4-dihydropyridine derivative amlodipine is a long-lasting  $\text{Ca}^{2+}$  antagonist for displacing the specific bindings of [<sup>3</sup>H]isradipine<sup>[1-4]</sup>. 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<sup>[5]</sup>. It is well known that 1,4-dihydropyridine (DHP)  $\text{Ca}^{2+}$  antagonists interact with L-types, implying that they can block  $\text{Ca}^{2+}$  influx. On the other hand, N-type  $\text{Ca}^{2+}$  channels may play an important role in transmitter release from nerve terminals<sup>[6]</sup>, implying that these two subtypes have different functions. Thus, the purpose of this study was to compare the interactions (binding potencies) between  $\omega$ -conotoxin ( $\omega$ -CTX) and amlodipine and N-type  $\text{Ca}^{2+}$  channels in rat brain.

### MATERIALS AND METHODS

**Preparations of membrane-enriched fractions from rat brain** Membrane-enriched fractions were prepared according to previous methods<sup>[1-4,7]</sup>. The brains from male Wistar rats (weighing 180-300 g) were homogenized in HEPES 50 mmol·L<sup>-1</sup> (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<sup>-1</sup> (pH 7.4). Protein concentrations were determined using the method of Lowry *et al*<sup>[8]</sup>.

**<sup>125</sup>I- $\omega$ -conotoxin binding assay** The <sup>125</sup>I- $\omega$ -CTX (74 TBq·mol<sup>-1</sup>, Amersham) binding assay was carried out as described by Czyrak *et al*<sup>[9]</sup>. The incubation mixture (0.5 mL) consisted of 400  $\mu$ L of assay buffer 50 mmol·L<sup>-1</sup> HEPES-NaOH buffer (pH 7.4), 50  $\mu$ L of membrane suspension (approximately 0.5  $\mu$ g of protein), 5  $\mu$ L of either assay buffer (total binding) or a solution containing unlabeled  $\omega$ -CTX (final concentration of 10 nmol·L<sup>-1</sup> for nonspecific binding) and 45  $\mu$ L of <sup>125</sup>I- $\omega$ -CTX. Membranes were preincubated in duplicate in the presence or absence of unlabeled  $\omega$ -CTX at 25 °C for 30 min. The final incubations were initiated by the addition of <sup>125</sup>I- $\omega$ -CTX and terminated after 15 min at 22 °C 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 <sup>125</sup>I- $\omega$ -CTX was defined as the difference between total and nonspecific bindings. For saturation experiments, the binding of increasing concentrations of <sup>125</sup>I- $\omega$ -CTX (0.01-2.0 nmol·L<sup>-1</sup>) were analyzed as described by Scatchard. The *K<sub>d</sub>* (apparent

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Received 1997-09-15

Accepted 1997-12-03

dissociation constant) and  $B_{max}$  (maximal binding capacity) were determined for each rat brain region.  $^{125}\text{I-}\omega\text{-CTX}$   $0.05 \text{ nmol}\cdot\text{L}^{-1}$  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  $\text{IC}_{50}$ . This value is expressed as  $K_i(-\lg K_i)$ .

**Radioligand and drugs**  $^{125}\text{I-}\omega\text{-CTX}$  ( $74 \text{ TBq}\cdot\text{mol}^{-1}$ ) 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**

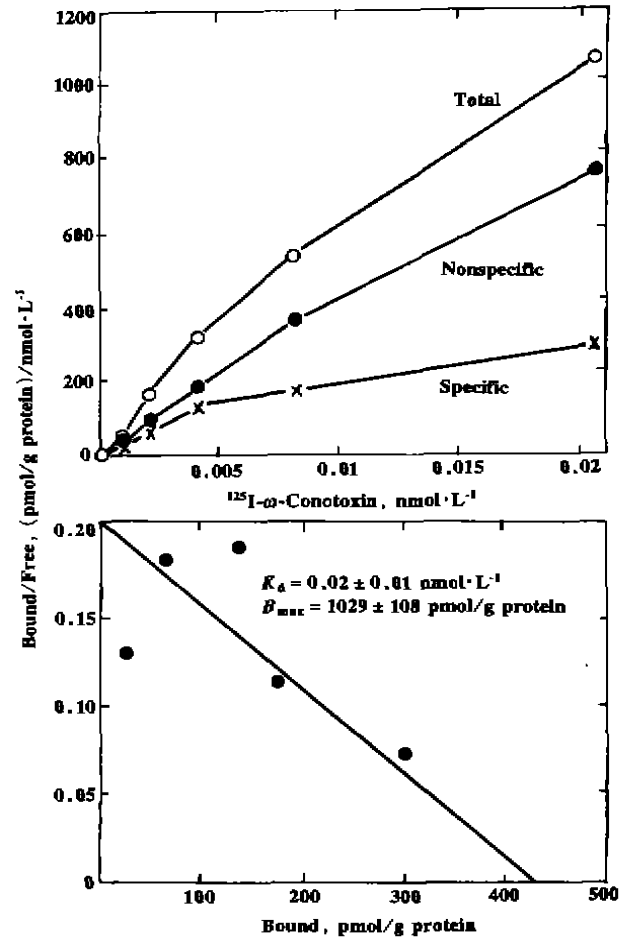
Decreases in total and specific bindings of  $^{125}\text{I-}\omega\text{-CTX}$  to rat brain membranes by addition of BSA were found. However, no significant differences were seen in  $^{125}\text{I-}\omega\text{-CTX}$  bindings between fresh and frozen brain membranes.

The representative saturation experiments for  $^{125}\text{I-}\omega\text{-CTX}$  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  $^{125}\text{I-}\omega\text{-CT}$  bindings were  $0.02 \pm 0.01 \text{ nmol}\cdot\text{L}^{-1}$  and  $1029 \pm 108 \text{ pmol/g protein}$ , respectively.

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

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

| Drugs               | $\text{pK}_i$ values               |                            |
|---------------------|------------------------------------|----------------------------|
|                     | $^{125}\text{I-}\omega\text{-CTX}$ | [ $^3\text{H}$ ]isradipine |
| Nifedipine          | < 4 (5)                            | $8.33 \pm 0.71$ (7)        |
| $\omega\text{-CTX}$ | 9.57 (5)                           | $\geq 9.00$                |
| Amlodipine          | < 4 (4)                            | $7.41 \pm 0.22$ (4)        |
| Nisodipine          | < 4 (5)                            | $9.31 \pm 0.24$ (4)        |
| Benidipine          | < 4 (5)                            | $8.05 \pm 0.22$ (4)        |
| Nitrendipine        | < 4 (5)                            | $8.51 \pm 0.56$ (7)        |
| Nicardipine         | < 4 (5)                            | $8.50 \pm 0.40$ (7)        |
| Nimodipine          | < 4 (5)                            | $8.93 \pm 0.45$ (8)        |
| (+) SM-6586         | < 4 (5)                            | $8.98 \pm 0.26$ (4)        |
| (-) SM-6586         | < 4 (5)                            | $7.68 \pm 0.34$ (6)        |
| (±) SM-6586         | < 4 (5)                            | $8.57 \pm 0.27$ (9)        |
| Manidipine          | < 4 (5)                            | $8.23 \pm 0.30$ (4)        |



**Fig 1. Scatchard analysis of  $^{125}\text{I-}\omega\text{-CTX}$  bindings to rat brain membranes.**

(L-type) are shown in Tab 1.

The  $\text{pK}_i$  value of  $\omega\text{-CTX}$  to L-type  $\text{Ca}^{2+}$  channels was taken from Ertel *et al*<sup>(10)</sup>. The  $\text{pK}_i$  values from other  $\text{Ca}^{2+}$  antagonists were taken from reports previously published<sup>(4,7)</sup>.

**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<sup>[1-3]</sup> showed that membrane preparations from rat brains and the radioligand binding assay for the assessment of displacement potencies of L-type  $\text{Ca}^{2+}$  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 frozen 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<sup>(9,10)</sup>. 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<sup>(11)</sup> reported that iodine labeled toxins like  $\omega$ -CTX were shown to bind specifically to high affinity sites on chick brain synaptosomes. The present study revealed that rat brain membranes bound  $\omega$ -CTX with a high affinity ( $K_d$  value:  $0.02 \text{ nmol} \cdot \text{L}^{-1}$ ). Results coincide with studies done on rabbit<sup>(12)</sup> and frog<sup>(13)</sup> brains. In addition, previous studies have shown  $\omega$ -CTX distinguished between both the verapamil and 1,4-DPH  $Ca^{2+}$  antagonist target sites<sup>(11)</sup>. Therefore, the present study shows that this radioligand can be used to assess the displacement potencies of chemical compounds to N-type  $\omega$ -CTX binding sites.

$\omega$ -CTX is a compound that can block N- and L-type  $Ca^{2+}$  channels with a high affinity ( $IC_{50} \leq 1 \text{ nmol} \cdot \text{L}^{-1}$ )<sup>(10)</sup>. However, small interactions between 1,4-DPH antagonists like amlodipine and N-type  $Ca^{2+}$  channels 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  $\omega$ -CTX binding sites in rat brains. Thus, we conclude that although  $\omega$ -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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## 98, 19(2) 97-100 $\omega$ -Conotoxin 及 氟氯地平 与 大鼠 脑内 N 型 钙通道 亲和性的 比较

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

目的: 用大鼠脑对  $\omega$ -conotoxin ( $\omega$ -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  $50 \text{ mmol} \cdot \text{L}^{-1}$  (pH 7.4) 缓冲液中, 经  $40\,000 \times g$  离心后, 收集膜区域. 以  $^{125}\text{I}$ - $\omega$ -conotoxin (CTX) 作为放射配体测定. 结果:  $^{125}\text{I}$ - $\omega$ -CTX 与冷冻标本及新鲜标本结合的  $B_{\text{max}}$  无

区别. N 型钙通道的  $K_d$  和  $B_{\text{max}}$  值分为  $0.02 \pm 0.01 \text{ mmol} \cdot \text{L}^{-1}$  和  $1029 \pm 108 \text{ pmol/g}$  蛋白质.  $\omega$ -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 离子通道的亲和力很低.

## Enhancement of ( - )-stepholidine on protein phosphorylation of a dopamine- and cAMP-regulated phosphoprotein in denervated striatum of oxidopamine-lesioned rats<sup>1</sup>

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**KEY WORDS** stepholidine; phosphoproteins; phosphorylation; dopamine D<sub>1</sub> receptors; oxidopamine; corpus striatum; SK&F-38393; Sch-23390

phosphorylation in the denervated striatum of oxidopamine-lesioned rats, but it acts as a D<sub>1</sub> antagonist in normal striatum.

**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<sub>1</sub> 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<sub>1</sub> antagonist Sch-23390. **CONCLUSION:** SPD exhibits D<sub>1</sub> agonistic action on DARPP-32

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

As for D<sub>1</sub> action of SPD, previous studies reported controversial observations. In rats with 6-d reserpine treatment, SPD reduced D<sub>1</sub> agonist SK&F-38393-induced inhibition of firing activity of nigral DA cells although SPD *per se* had no action, indicating a D<sub>1</sub> antagonistic action<sup>[4]</sup>. In rats with unilateral nigral lesions by oxidopamine, SPD induced a contralateral rotation in the manner similar to SK&F-38393, indicating a D<sub>1</sub> agonistic action<sup>[5]</sup>. SPD bound to high and low affinity sites (R<sub>H</sub> and R<sub>L</sub>) of D<sub>1</sub> receptors and the R<sub>H</sub> could be regulated by GTP, indicating an intrinsic activity to D<sub>1</sub> receptors<sup>[6]</sup>. After blockade of D<sub>2</sub> receptors, SPD stimulated striatal cAMP formation<sup>[7]</sup>. In nigral lesioned rats, SPD induced a firing inhibition of substantia nigra pars reticular

<sup>1</sup> Project supported by the National Natural Science Foundation of China, No 39130091, 39600179; the Foundation of State Key Laboratory of Nuclear Medicine, No 9403; and the Foundation of State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Science, No K016.

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Received 1997-04-23

Accepted 1997-11-27