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Characteristics of specific ^{125}I-ω-conotoxin GVIA binding and ^{125}I-ω-conotoxin GVIA labeling using bifunctional crosslinkers in crude membranes from chick whole brain

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Abstract

Characteristics of specific ^{125}I-ω-conotoxin GVIA (^{125}I-ω-CgTX) binding and ^{125}I-ω-CgTX labeling using bifunctional crosslinkers were systematically investigated in crude membranes from chick whole brain. Aminoglycosides and dynorphine A (1–13) inhibited the specific binding of ^{125}I-ω-CgTX, but not that of the L-type calcium ion channel antagonist [^{3}H](+)PN200–110. It seems likely that the inhibitory effect of dynorphine A (1–13) does not involve κ-opiate receptors, based on results with the opiate receptor antagonist naloxone and the κ-opiate receptor agonist U50488H. Spider venom, Cd^{2+} and La^{3+} inhibited the specific binding of ^{125}I-ω-CgTX, as well as that of [^{3}H](+)PN200–110. Various L-type Ca^{2+} channel antagonists did not affect the specific binding of ^{125}I-ω-CgTX. ^{125}I-ω-CgTX specifically labeled 135 kDa and 215 kDa bands in crude membranes under reduced and non-reduced conditions, respectively. The crosslinker disuccinimidyl suberate (DSS) yielded better ^{125}I-ω-CgTX labeling than the other two crosslinkers tested. We investigated the effect of various Ca^{2+} channel antagonists on ^{125}I-ω-CgTX labeling with DSS in detail, and found that there is a strong correlation between the effects of Ca^{2+} channel antagonists on ^{125}I-ω-CgTX labeling of the 135 kDa band and specific ^{125}I-ω-CgTX binding. These results suggest that aminoglycosides and dynorphine A (1–13) are specific inhibitors of specific ^{125}I-ω-CgTX binding, and that labeling of the 135 kDa band with ^{125}I-ω-CgTX using DSS involves the specific binding sites of ^{125}I-ω-CgTX, perhaps including one of the neuronal N-type Ca^{2+} channel subunits in the crude membranes.

Keywords: ω-Conotoxin binding; Crosslinker; Labeling; Calcium ion channel blocker, N-type; Channel blocker; (Chick brain)

1. Introduction

Voltage-sensitive calcium channels (VSCCs) are widely distributed in excitable cells and play a fundamental role in the regulation of many intracellular processes [1–3]. Electrophysiological and pharmacological studies on embryonic chick dorsal root ganglion cells in culture have revealed the presence of three types of VSCCs in neuronal tissue: i.e., L-, N-, and T-type VSCCs [4]. These three types of VSCCs have different electrophysiological and pharmacological characteristics. It has recently become apparent that there is a fourth type of Ca^{2+} channel, a P-type channel, present in some neurons (mainly in cerebellar Purkinje cells) [5,6].

ω-Conotoxin GVIA (ω-CgTX), which is isolated from the venom of a marine snail, *Conus geographus* [7], specifically blocks calcium currents through neuron-specific VSCCs [8–12]. This toxin appears to act on neuronal N-type VSCCs [9,11,13]. Irreversible and specific binding of ^{125}I-ω-CgTX to chick and rat brain membranes has also been investigated [14–16], and the specific binding of ^{125}I-ω-conotoxin to these brain membranes is affected by aminoglycoside antibiotics, spider venom of *Plectreurys tristes* and dynorphine A (1–13) [16–18]. However, there has been little systematic investigation of the specific inhibitory effects of these blockers on ^{125}I-ω-CgTX binding to N-type VSCCs.

The molecular masses of the ω-CgTX binding sites have been determined to be 310 kDa + 230 kDa + 34 kDa [19], 222 kDa [20] and 210 kDa [21] by photoaffinity labeling in rat brain membranes, and 135 kDa [22] and 170

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kDa/140 kDa + 30 kDa [21] by chemical crosslinking in chick brain membranes. We have also observed that bands of 135 kDa (reduced conditions) and 215 kDa (non-reduced conditions) are selectively labeled with ^{125}I-ω-CgTX using the crosslinker disuccinimidyl suberate (DSS) in chick whole brain, but not in rat whole brain [16]. However, there has been little systematic investigation of the characteristics of ^{125}I-ω-CgTX labeling of its binding sites in chick whole brain using a crosslinker.

We used well known Ca^{2+} channel agonist (Ca^{2+} agonist) and Ca^{2+} channel antagonists (Ca^{2+} antagonist) to identify a specific inhibitor of the specific ^{125}I-ω-CgTX binding to crude membranes from chick whole brain, and we investigated the characteristics of ^{125}I-ω-CgTX labeling in the crude membranes using a crosslinker, and particularly examined whether there is a correlation between the effects of various Ca^{2+} antagonists on such labeling and the effects of these Ca^{2+} antagonists on the specific binding of ^{125}I-ω-CgTX.

2. Materials and methods

2.1. Preparation of crude membranes

Crude membranes were prepared from cardiac, ileal, and skeletal muscle, and whole brain of 5-day-old chicks as described previously [16,23,24]. The concentrations of protein in these crude membrane preparations were 4.42 ± 0.20 (n = 5), 5.30 ± 0.78 (n = 5), 5.16 ± 0.62 (n = 5) and 6.20 ± 0.62 (n = 5) mg protein/ml, respectively.

2.2. Assay of ^{125}I-ω-CgTX binding

Specific binding of ^{125}I-ω-CgTX to the various types of crude membranes was assayed by a modification of the methods of Abe et al. [14], Cruz and Olivera [15] and Ichida et al. [16].

50-μl aliquots of the crude membranes (about 75 μg/tube; the concentrations of these crude membranes were adjusted to 1.5 mg protein/ml) were added to assay medium consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-tris(hydroxymethyl)aminomethane (Tris) (pH 7.4 at 4°C), 0.1% bovine serum albumin (BSA), and various concentrations of ^{125}I-ω-CgTX with or without unlabeled ω-CgTX at 1000-fold the concentration of ^{125}I-ω-CgTX. The concentration of 100 pM ^{125}I-ω-CgTX in the absence and presence of 100 nM unlabeled ω-CgTX was used in the examination of the effects of Ca^{2+} agonist and antagonists. The final volume of the assay system was 200 μl. After incubation for 14 h at 4°C, the mixture was rapidly filtered through a Whatman GF/C glass filter that had been preimmersed in ice-cold washing solution with suction and the filter was washed four times with 2 ml of ice-cold washing solution consisting of 50 mM Hepes-Tris (pH 7.4 at 4°C), 0.1% BSA, 1.5 mM $CaCl_2$, and 160 mM choline chloride. The radioactivity on the filter was counted in a γ-counter (Aloka, JDC-752).

The specific binding of [^{3}H](+)PN200–110 ([^{3}H](+)PN) to crude membranes from rat whole brain was assayed by a modification of a previously reported method [23]. 50-μl aliquots of the crude membranes (about 80 μg/tube; 1.6 mg protein/ml) were added to assay medium consisting of 50 mM Tris-HCl (pH 7.4 at 30°C), 0.5 mM phenylmethylsulfonyl fluoride, 0.15 M NaCl, 1 mM $CaCl_2$, and 0.25 nM [^{3}H](+)PN in the absence and presence of unlabeled 0.25 μM (+)PN. The final volume of the assay mixture was 450 μl. After incubation for 50 min at 30°C, the mixture was rapidly filtered through a Whatman GF/C glass filter with suction and the filter was washed four times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 30°C). The filter was then transferred to a counting vial, 5 ml of Triton/toluene-based scintillation mixture was added, and the radioactivity was counted in a liquid scintillation counter (Packard Tricarb 2050).

Binding studies were carried out in duplicate. All assays using derivatives of 1,4-dihydropyridine (DHP) were performed under a safety light. The specific bindings of ^{125}I-ω-CgTX and [^{3}H](+)PN were defined as the differences between the radioactivities bound in the absence (total binding) and presence (nonspecific binding) of unlabeled ω-CgTX and unlabeled (+)PN at 1000-fold the concentration of ^{125}I-ω-CgTX and [^{3}H](+)PN, respectively. The results of binding assays were analyzed by nonlinear least-squares regression using a computer program (SIMPLEX) [25] from the program library of the Computer Center, Osaka University.

2.3. Reaction for crosslinking followed by specific ^{125}I-ω-CgTX binding

Specific binding of ^{125}I-ω-CgTX was assayed as described in Section 2.2 with the following modification.

80-μl aliquots of the crude membranes (about 300 μg protein/tube; the concentrations of these crude membranes were adjusted to about 3.75 mg protein/ml) were added to assay medium consisting of 30 mM Hepes-NaOH (pH 7.4 at 4°C), 0.1% BSA, and 0.1 nM ^{125}I-ω-CgTX with or without unlabeled 0.1 μM ω-CgTX, unless otherwise indicated. The final volume of the assay system was 200 μl.

After specific binding of ^{125}I-ω-CgTX to the crude membranes was carried out for 14 h at 4°C, crosslinking of ^{125}I-ω-CgTX with DSS, disuccinimidyl tartrate (DST) or ethylene glycol bis(succinimidyl succinate) (EGS) to its binding sites was assayed by a modification of the methods of Barhanin et al. [21] and Ichida et al. [16]. The reaction mixture for specific ^{125}I-ω-CgTX binding was centrifuged at 40000 × g for 20 min. The pellets were gently washed with 30 mM Hepes-NaOH (pH 8.5 at 22°C)

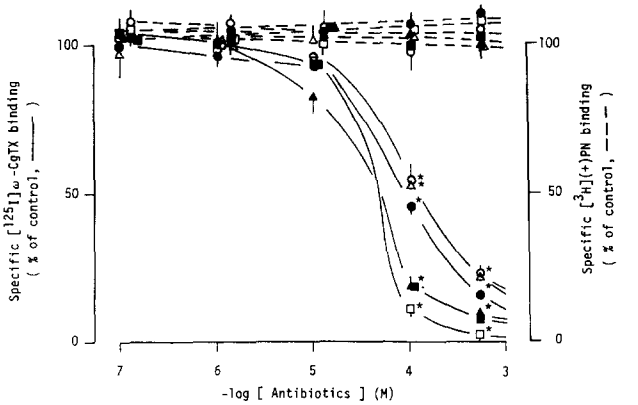

Fig. 1. Effects of aminoglycosides on specific binding of ^{125}I-ω-CgTX (—) and [^{3}H](+)PN(- - -). Symbols (●) Ami, (○) Kan, (▲) Gen, (△) Str, (■) Tob, (□) Neo. Points represent the means of four or five preparations from separate animals. Bars indicate standard errors. * $P < 0.01$ as compared with specific [^{3}H](+)PN binding with an equal concentration of each aminoglycoside.

solution and then suspended with the same solution. The reaction for crosslinking was initiated by addition of the crosslinker DSS (500 μM). The reaction was stopped by addition of 1.2 ml of 100 mM Tris-Cl (pH 8.5 at 22°C) after incubation for 1.5 min at 22°C, unless otherwise indicated, and centrifuged at 40 000 × g for 20 min. The pellets were gently washed once with 100 mM Tris-Cl (pH 8.5 at 22°C) and then solubilized with Laemmli's sample buffer [26] for 30 min at room temperature. The samples (about 100 μg of protein) were then applied to a 5–16% gradient gel and submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [26]. For SDS-PAGE under non-reduced conditions, 20 mM *N*-ethylmaleimide was used in the sample buffer [26] instead of 2% 2-mercaptoethanol. After electrophoresis, the slab gel was dried and then exposed with Du Pont Cronex Lightning Plus Screens to preflashed Kodak XAR-5 film for about 5 days at −75°C.

2.4. Other methods

Protein was measured by the method of Lowry et al. [27] with BSA as a standard.

Statistical analyses were performed using the paired or unpaired Student's *t*-test and the statistical evaluation of multiple groups was performed by a one-way analysis of variance. A *P*-value of less than 0.05 was chosen as statistically significant.

2.5. Materials

^{125}I-ω-CgTX (ω-conotoxin GVIA labeled with ^{125}I in Tyr^{22}, 81.4 TBq/mmol) and (+)[5-*methyl*-^{3}H]PN200–110 (2.59–3.22 TBq/mmol) were purchased from Amersham or New England Nuclear. EGS, DSS and DST were purchased from Pierce (Rockford, IL). Dynorphine (1–13) and ω-CgTX were purchased from the Peptide Institute (Osaka, Japan). Spider venom from *Plectreurys tristes* was purchased from Spider Pharmaceutical (Arizona). Amikacin (Ami), gentamycin (Gen), kanamycin (Kan), neomycin (Neo), streptomycin (Str) and tobramycin (Tob) were purchased from Sigma (St. Louis, MO), Nakarai (Kyoto, Japan) or Wako (Osaka, Japan). Naloxone was purchased from Sigma (St. Louis, MO). Bay K 8644 was a gift from Bayer AG (Leverkusen, Germany), D- and L-diltiazem was from Tanabe Seiyaku (Osaka, Japan), nitrendipine was from Yoshitomi Pharmaceutical (Osaka, Japan), (+) and (−)PN 200–110 was from Sandoz (Basel, Switzerland), U50488H was from Dr. A. Kawabata (Kinki University) and verapamil was from Eisai (Tokyo, Japan). 1,4-Dihydropyridine (DHP) derivatives and ω-CgTX were dissolved in 50% PEG#200 and 0.1% BSA, respectively. All assays using DHP derivatives were performed under a safety light.

3. Results

3.1. Specific binding of ^{125}I-ω-CgTX and [^{3}H](+)PN

Specific binding of ^{125}I-ω-CgTX to crude membranes from chick whole brain was saturable and irreversible (data not shown). A Scatchard plot of the saturation of specific ^{125}I-ω-CgTX binding gave a single linear curve (data not shown). The concentrations for half-maximal saturation of the specific binding of ^{125}I-ω-CgTX, values of K_d and B_{max} from the Scatchard plots, and the Hill coefficient are shown in Table 1. A concentration of 100 pM ^{125}I-ω-CgTX, which was similar to the K_d value from the Scatchard plot, was used in all subsequent experiments on the specific binding of ^{125}I-ω-CgTX to crude membranes from whole brain. The corrected free concentration of ^{125}I-ω-CgTX was calculated to be 33.0 ± 2.2 pM with 74.7 ± 3.8 μg protein of the crude membranes/tube (n = 12). The specific binding of ^{125}I-ω-CgTX at a concentration of 100 pM to the crude membranes was 85–90% of the total binding.

Table 1
Values of K_d and B_{max} for specific binding of ^{125}I-ω-CgTX and [^{3}H](+)PN from Scatchard plots or saturation curves

Specific binding	Concn. for half-maximal saturation (pM)	Amount of ^{125}I-ω-CgTX bound at equilibrium (fmol/mg protein)	K_d value (pM)	B_{max} (fmol/mg protein)	Hill coefficient
^{125}I-ω-CgTX	87.3 ± 3.7	948.0 ± 29.5	101.2 ± 3.2	1024.1 ± 53.2	1.03 ± 0.04
[^{3}H](+)PN	2076.7 ± 54.5	2310.8 ± 64.3	2584.9 ± 87.2	2537.0 ± 76.1	0.99 + 0.02

Values are means ± S.E. for 4–6 separate animals.

On the other hand, specific binding of [^{3}H](+)PN to crude membranes from chick whole brain was saturable and reversible (data not shown). A Scatchard plot of the saturation of specific [^{3}H](+)PN binding gave a single linear curve (data not shown). The concentration for half-maximal saturation of the specific binding of [^{3}H](+)PN, values of K_d and B_{max} from the Scatchard plots, and the Hill coefficient are shown in Table 1. Although the K_d value for specific [^{3}H](+)PN binding was 2.59 nM (from the Scatchard plot), as shown in Table 1, a concentration of 0.25 nM (250 pM) [^{3}H](+)PN was used in all subsequent studies of the effects of Ca^{2+} antagonists, since we wished to compare these effects with those of various Ca^{2+} antagonists on the specific binding of 100 pM ^{125}I-ω-CgTX.

3.2. Effects of Ca^{2+} agonist and antagonists

The effects of aminoglycoside antibiotics which block N-type Ca^{2+} channels [17], on the specific binding of ^{125}I-ω-CgTX to crude membranes from chick whole brain are shown in Fig. 1. Aminoglycosides including amikacin (Ami), gentamycin (Gen), kanamycin (Kan), neomycin (Neo), streptomycin (Str) and tobramycin (Tob), inhibited the specific binding of ^{125}I-ω-CgTX in a dose-dependent manner. Their IC_{50} values were $9.21 \cdot 10^{-5}$ M, $5.18 \cdot 10^{-5}$ M, $1.18 \cdot 10^{-4}$ M, $5.62 \cdot 10^{-5}$ M, $1.28 \cdot 10^{-4}$ M and $4.77 \cdot 10^{-5}$ M, respectively. However, these aminoglycosides had little effect on the specific binding of 0.25 nM [^{3}H](+)PN. These results suggest that these aminoglycosides specifically inhibited the specific binding of ^{125}I-ω-CgTX to the crude membranes.

ω-CgTX, dynorphine A (1–13) and spider venom from *Plectreurys tristes* inhibited the specific binding of ^{125}I-ω-CgTX to the crude membranes. Their IC_{50} values were $7 \cdot 10^{-10}$ M, $3 \cdot 10^{-5}$ M and $1 \cdot 10^{-5}$ g/ml, respectively (Fig. 2A–C). However, only the spider venom had a marked effect on the specific binding of 0.25 nM [^{3}H](+)PN (Fig. 2C). The IC_{50} value of spider venom for the specific binding of ^{125}I-ω-CgTX was similar to that for the specific binding of [^{3}H](+)PN. These results suggest that ω-CgTX and dynorphine A (1–13) specifically inhibited the specific binding of ^{125}I-ω-CgTX to the crude membranes, but spider venom did not.

To investigate the possibility that the inhibitory effect of dynorphine A (1–13) on the specific binding of ^{125}I-ω-CgTX to the crude membranes is due to participation of κ-opiate receptors, the effects of U50488H (a κ-opiate receptor agonist) and naloxone (a μ-, κ- and δ-opiate receptor antagonist) on the specific binding of ^{125}I-ω-CgTX to the crude membranes were examined (Fig. 3). Neither naloxone (1 μM) nor U50488H (1 μM and 30 μM) affected the specific binding. Furthermore, 1 μM naloxone did not alter the inhibitory effect of 100 μM dynorphine A (1–13) on the specific binding. These results suggest that the inhibitory effect of dynorphine A (1–13) on the specific binding of ^{125}I-ω-CgTX to crude membranes is not due to an opiate-related mechanism involving κ-opiate receptors.

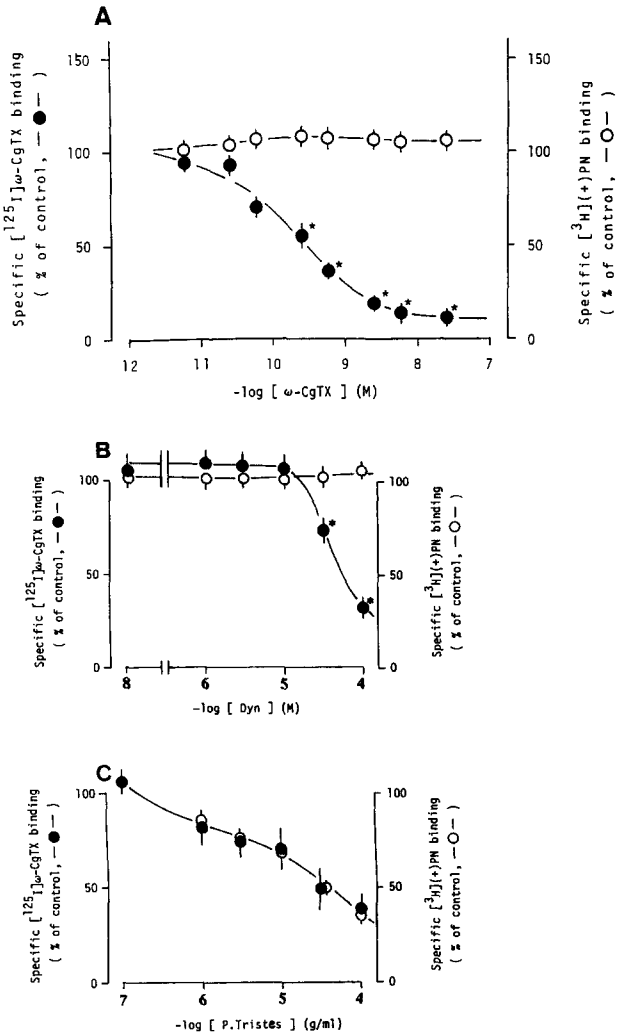

Fig. 2. Effects of ω-CgTX (A), dynorphine A (1–13) (B) and spider venom (C) on specific binding of ^{125}I-ω-CgTX (●) and [^{3}H](+)PN (○). Points represent the means of 4 to 10 preparations from separate animals. Bars indicate standard errors. * $P < 0.01$ as compared with the specific [^{3}H](+)PN binding with an equal concentration of ω-CgTX (A), dynorphine A (1–13) (B) or spider venom (C).

Inorganic calcium antagonists, Cd^{2+} and La^{3+}, inhibited the specific binding of ^{125}I-ω-CgTX to the crude membranes (Fig. 4A and B). The IC_{50} values for Cd^{2+} and La^{3+} were $4.8 \cdot 10^{-4}$ and $2.5 \cdot 10^{-4}$ M, respectively. However, the specific binding of [^{3}H](+)PN was also inhibited by Cd^{2+} and La^{3+}. In this case, the respective IC_{50} values were $1.2 \cdot 10^{-4}$ M and $4.0 \cdot 10^{-4}$ M, and Cd^{2+} and La^{3+} inhibited [^{3}H](+)PN binding and ^{125}I-ω-CgTX binding similarly.

Various Ca^{2+} antagonists and agonist, such as D-diltiazem, L-diltiazem, nitrendipine, (+)PN, (−)PN, verapamil, and Bay K 8644, at concentrations ranging from $1 \cdot 10^{-7}$ to $3 \cdot 10^{-4}$ M did not affect the specific binding of ^{125}I-ω-CgTX to the crude membranes (data not shown).

Fig. 5 shows the specific binding of ^{125}I-ω-CgTX to crude membranes from various chick organs, including whole brain, and cardiac, ileal, and skeletal muscle. ^{125}I-ω-CgTX was observed to specifically bind to crude membranes from whole brain, but not to crude membranes from the other organs. These results suggest that the specific binding sites of ^{125}I-ω-CgTX exist mainly in whole brain.

3.3. Characteristics of labeling with crosslinkers

Fig. 6A shows the specific labeling of 0.1 nM ^{125}I-ω-CgTX with the bifunctional reagents (500 μM) DSS, DST and EGS in crude membranes from chick whole brain. ^{125}I-ω-CgTX specifically labeled its binding sites in the crude membranes with DSS and EGS. The reaction time for crosslinking by the crosslinkers was 1.5 min at 4°C. The apparent molecular masses of the labeled sites using DSS or EGS under reduced and non-reduced conditions were 135.0 ± 1.6 (n = 12) kDa and 215.3 ± 3.3 (n = 15) kDa, respectively. The specific ^{125}I-ω-CgTX labeling of the 135 kDa and 215 kDa bands by DSS was stronger than that by EGS. DST did not provide specific labeling of the 135 kDa and 215 kDa bands under reduced or non-reduced conditions. Therefore, the crosslinker DSS was used in all subsequent experiments for ^{125}I-ω-CgTX labeling of its binding sites on the crude membranes. When the crosslinking reaction was carried out in the absence of crosslinker, labeling was not observed despite the use of different conditions, such as the absence or presence of unlabeled ω-CgTX and SDS-PAGE under reduced or non-reduced conditions. On the other hand, when the SDS-PAGE gels that had been prepared under various conditions were stained with Coomassie brilliant blue (CBB), the patterns were identical (Fig. 6B), suggesting that the labeling of the 135 kDa or 215 kDa band with ^{125}I-ω-CgTX using DSS was not due to an increase in the aggregation of the protein in the 135 kDa or 215 kDa

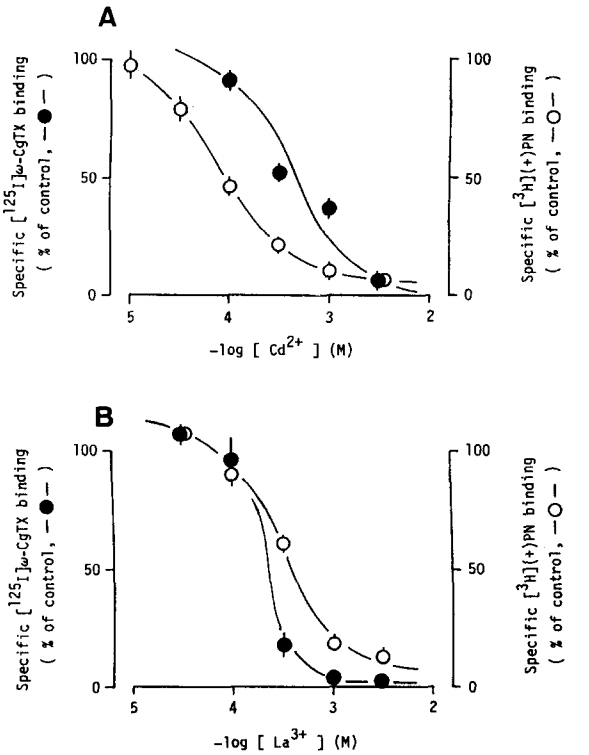

Fig. 4. Effects of Cd^{2+} (A) and La^{3+} (B) on specific binding of ^{125}I-ω-CgTX (●) and [^{3}H](+)PN (○). Points represent the means of 4 to 6 preparations from separate animals. Bars indicate standard errors.

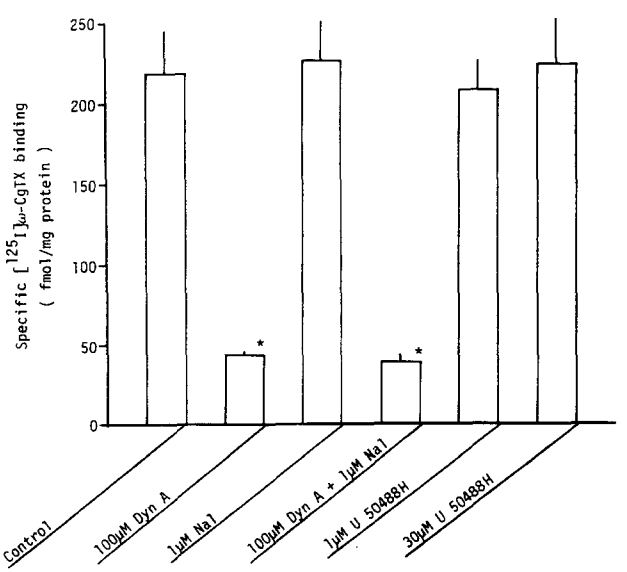

Fig. 3. Effects of dynorphine A (1–13), U50488H and naloxone on specific binding of ^{125}I-ω-CgTX. Columns represent the means of four or five preparations from separate animals. Bars indicate standard errors. * $P < 0.01$ as compared with control.

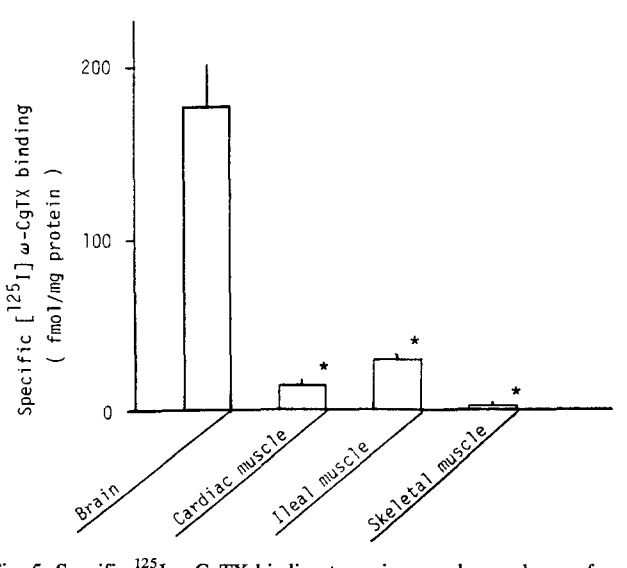

Fig. 5. Specific ^{125}I-ω-CgTX binding to various crude membranes from chick cardiac, ileal, and skeletal muscle and whole brain. Columns represent the means of four preparations for separate animals. Bars indicate standard errors. * $P < 0.005$ as compared with the specific ^{125}I-ω-CgTX binding in whole brain.

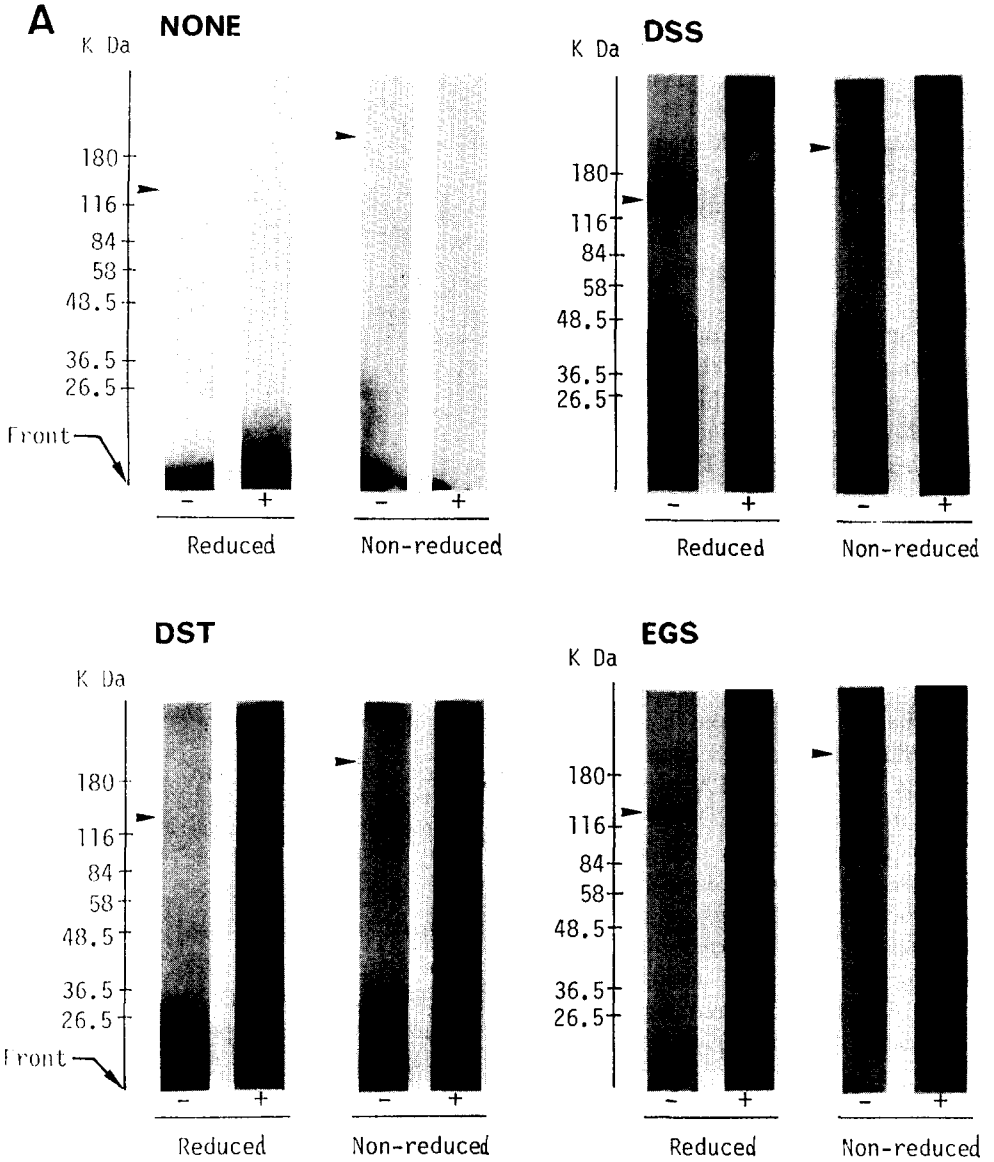

Fig. 6. Autoradiographic (A) and CBB-stained (B) patterns of ^{125}I-ω-CgTX labeling with the cross linkers DSS, DST and EGS under reduced and non-reduced conditions. The amount of protein applied per lane was about 120 μg (A) and 15 μg (B), respectively. The symbols (−) and (+) indicate the absence and presence of unlabeled ω-CgTX, at 1000-fold the concentration of ^{125}I-ω-CgTX, on the reaction medium, respectively. The arrow heads in panels A and B indicate the 135 and 215 kDa bands, respectively.

band. SDS-PAGE was carried out under reduced conditions in all subsequent experiments, unless otherwise indicated.

The crosslinking reaction with DSS showed time (12 s–15 min)- and dose (62.5–1000 μM)-dependence (data not shown). Based on the results of the preliminary experiments, a reaction time of 1.5 min and a concentration of 500 μM were used for the crosslinking with DSS, unless otherwise indicated.

The labeling depended on the initial concentration of ^{125}I-ω-CgTX (0.025–0.4 nM), as shown in Fig. 7. The extent of labeling of the 135 kDa band gradually increased as the concentration of ^{125}I-ω-CgTX increased. A ^{125}I-ω-CgTX concentration of 0.1 nM resulted in the best labeling, since non-specific labeling, which reduced the difference between total and non-specific radioactivity in the 135 kDa band, was greatest at ^{125}I-ω-CgTX concentrations of ≥ 0.2 nM. Accordingly, a ^{125}I-ω-CgTX concentration of 0.1 nM was used for labeling in all subsequent experiments, unless otherwise indicated.

^{125}I-ω-CgTX labeling of the 135 kDa band also depended on the incubation time in the ^{125}I-ω-CgTX binding reaction (Fig. 8). Although labeling had apparently reached a steady-state at an incubation time of 8 h, an incubation time of 14 h was used for labeling in all subsequent experiments, unless otherwise indicated, to ensure that a steady state had, in fact, been reached.

The results in Figs. 7 and 8 were consistent with those for the dose- and time-dependence of the specific binding (data not shown) of ^{125}I-ω-CgTX to the crude membranes.

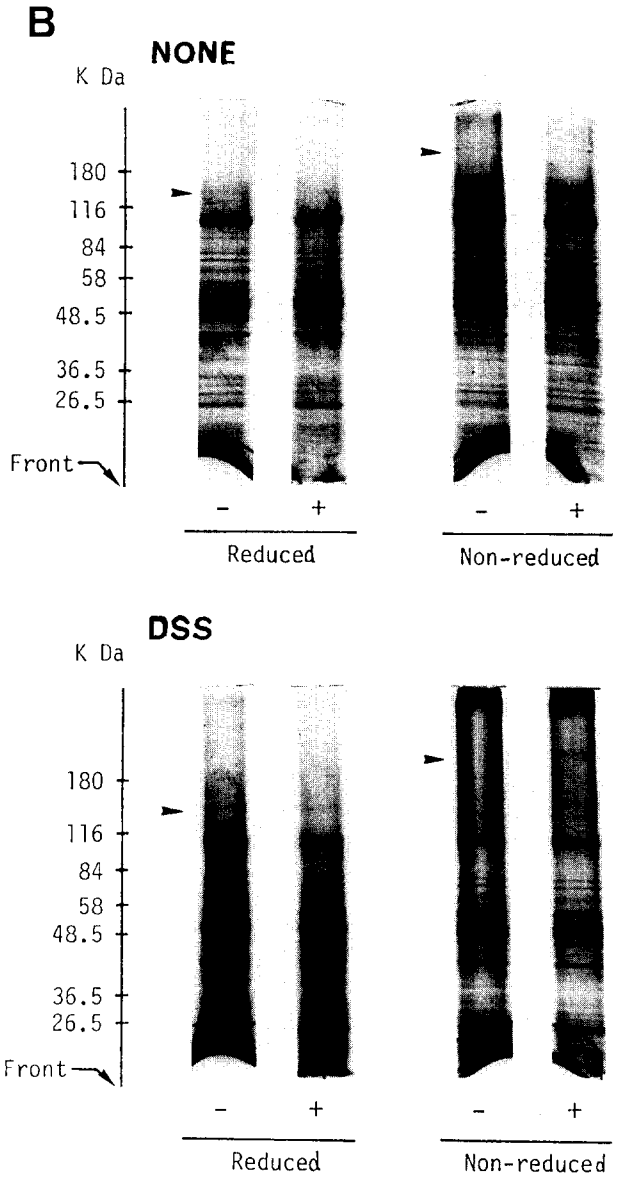

Fig. 6 (continued).

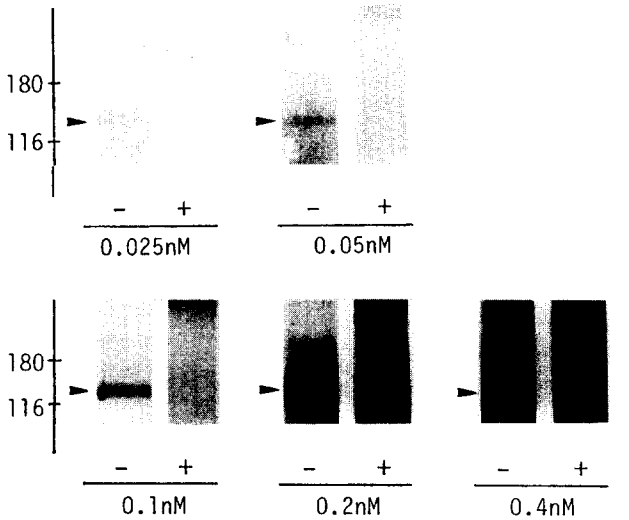

Fig. 7. Autoradiographic patterns of ^{125}I-ω-CgTX labeling with DSS as a function of the concentration of ^{125}I-ω-CgTX (0.025–0.4 nM). The symbols (−) and (+) indicate the absence and presence of unlabeled ω-CgTX, at 1000-fold the concentration of ^{125}I-ω-CgTX, on the reaction medium, respectively. The arrow heads indicate the 135 kDa band.

3.4. Effects of various reagents on labeling

We investigated the effects of various Ca^{2+} antagonists, such as aminoglycoside antibiotics (Ami, Gen, Kan, Neo, Str. and Tob), ω-CgTX, Cd^{2+}, La^{3+}, dynorphine A (1–13) and spider venom (Fig. 9A), on the ^{125}I-ω-CgTX labeling of the 135 kDa band in crude membranes from chick whole brain. 100 μM of each of the aminoglycosides, 10 nM ω-CgTX and 100 μM dynorphine A (1–13) specifically inhibited labeling of the 135 kDa band, and did not inhibit either the labeling of other bands in the absence of unlabeled ω-CgTX or non-specific labeling in the presence of unlabeled ω-CgTX. On the other hand, 3 mM Cd^{2+}, 3 mM La^{3+}, and 50 μg/ml spider venom inhibited not only the specific labeling of the 135 kDa band, but also both the labeling of other bands in the absence of unlabeled ω-CgTX and non-specific labeling in the presence of unlabeled ω-CgTX. These results are consistent with the inhibitory effects of these Ca^{2+} antagonists on the specific binding of ^{125}I-ω-CgTX and [^{3}H](+)PN to crude membranes as shown in Figs. 1, 2 and 4: i.e., 100 μM of each of the aminoglycosides, 10 nM ω-CgTX and 100 μM dynorphine A (1–13) inhibited the specific binding of ^{125}I-ω-CgTX, but not that of [^{3}H](+)PN. On the other hand, 3 mM Cd^{2+}, 3 mM La^{3+} and 50 μg/ml spider venom inhibited the specific binding of both ^{125}I-ω-CgTX and [^{3}H](+)PN.

The effects of Ca^{2+} agonist and various Ca^{2+} antagonists for L-type VSCCs, such as the DHP derivatives, Dil,

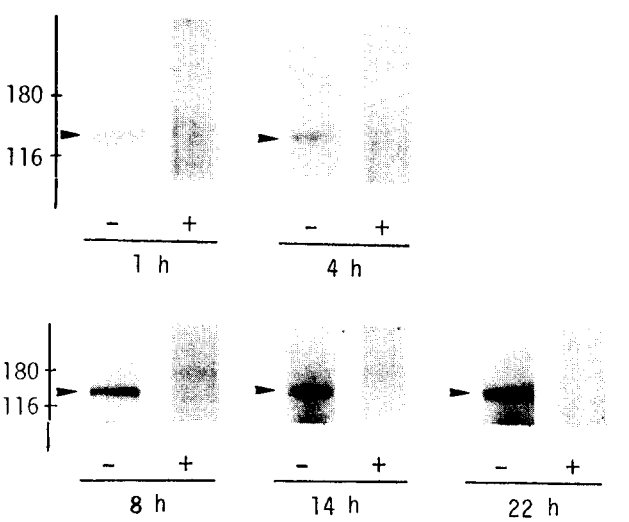

Fig. 8. Autoradiographic patterns of ^{125}I-ω-CgTX labeling with DSS as a function of time (1–22 h) in a ^{125}I-ω-CgTX specific-binding reaction. The symbols (−) and (+) indicate the absence and presence of unlabeled ω-CgTX, at 1000-fold the concentration of ^{125}I-ω-CgTX, on the reaction medium, respectively. The arrow heads indicate the 135 kDa band.

Bay, Nit, (+)PN, (−)PN and Ver, were investigated (Fig. 9B). Bay, Nit, (+)PN and (−)PN at concentrations of 100 μM slightly increased labeling of the 135 kDa band, but D-Dil, L-Dil and Ver (100 μM) had no effect. These results were also consistent with the effects of Ca^{2+} agonist and antagonists on the specific binding of ^{125}I-ω-CgTX to crude membranes (data not shown).

Fig. 10 shows ^{125}I-ω-CgTX labeling of the 135 kDa band in crude membranes from various chick organs, including whole brain, and cardiac, ileal and skeletal muscle. Specific labeling was only observed in crude membranes from chick whole brain, although non-specific labeling was observed in the crude membranes from cardiac and skeletal muscle. In this study, we used a standard concentration of protein for the crude membranes from the different organs in SDS-PAGE (about 330 μg protein/lane). This result was also consistent with the specific binding of ^{125}I-ω-CgTX to these crude membranes as shown in Fig. 5.

All of these results regarding ^{125}I-ω-CgTX labeling of

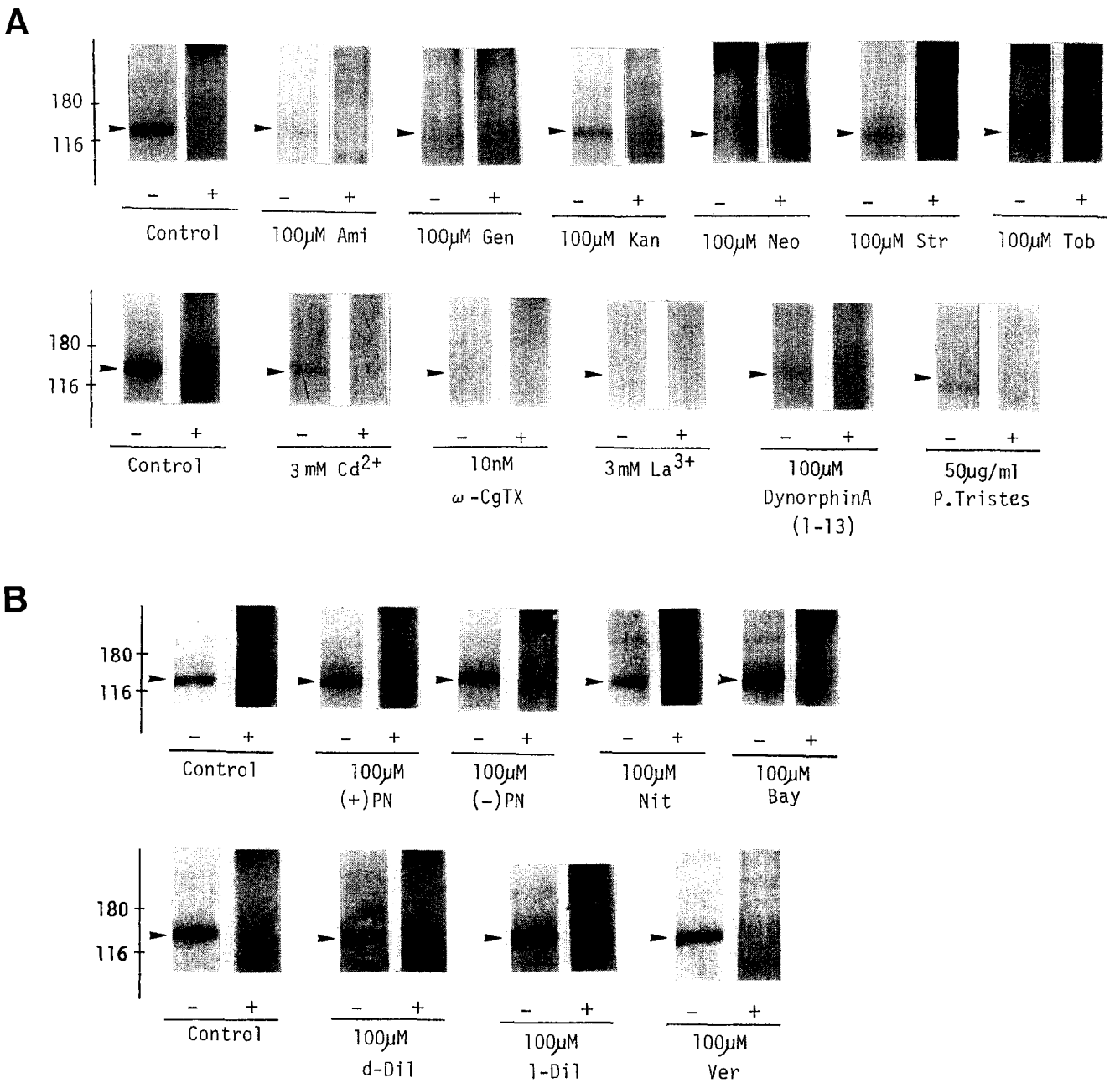

Fig. 9. Effects of various aminoglycoside antibiotics, Cd^{2+}, ω-CgTX, La^{3+}, dynorphine A (1–13) and spider venom (panel A), and various DHP derivatives, D-diltiazem, L-diltiazem and verapamil (panel B) on ^{125}I-ω-CgTX labeling of the 135 kDa band. The symbols (−) and (+) indicate the absence and presence of unlabeled ω-CgTX, at 1000-fold the concentration of ^{125}I-ω-CgTX, on the reaction medium, respectively. The arrow heads indicate the 135 kDa band.

the 135 kDa band with DSS were similar to those on the specific binding of ^{125}I-ω-CgTX, which suggests that ^{125}I-ω-CgTX labeling of the 135 kDa band may involve specific binding sites of ^{125}I-ω-CgTX in crude membranes from chick whole brain.

4. Discussion

4.1. Characteristics of specific ^{125}I-ω-CgTX binding

K_d and B_{max} values for specific binding of ^{125}I-ω-CgTX to crude membranes from chick whole brain were comparable to those reported by other authors [14–16,18,20,21]. ^{125}I-ω-CgTX was observed to specifically bind to crude membranes from chick whole brain, but not to crude membranes from other organs, suggesting that the specific binding sites of ^{125}I-ω-CgTX exist only in whole brain, and that these binding sites are N-type VSCCs. Therefore, the characteristics of the specific binding sites in crude membranes from chick whole brain appear to be similar to those reported by other authors [14–16,18,20–22].

To identify a selective antagonist of ^{125}I-ω-CgTX binding sites in crude membranes from chick whole brain, various types of reagents were investigated with regard to specific binding of both ^{125}I-ω-CgTX and [^{3}H](+)PN. Aminoglycoside antibiotics, such as Ami, Gen, Kan, Neo, Str, and Tob (which block N-type VSCCs [17]) and dynorphine A (1–13) (which blocks neuronal Ca^{2+} channels [28–30]) inhibited the specific binding of ^{125}I-ω-CgTX,

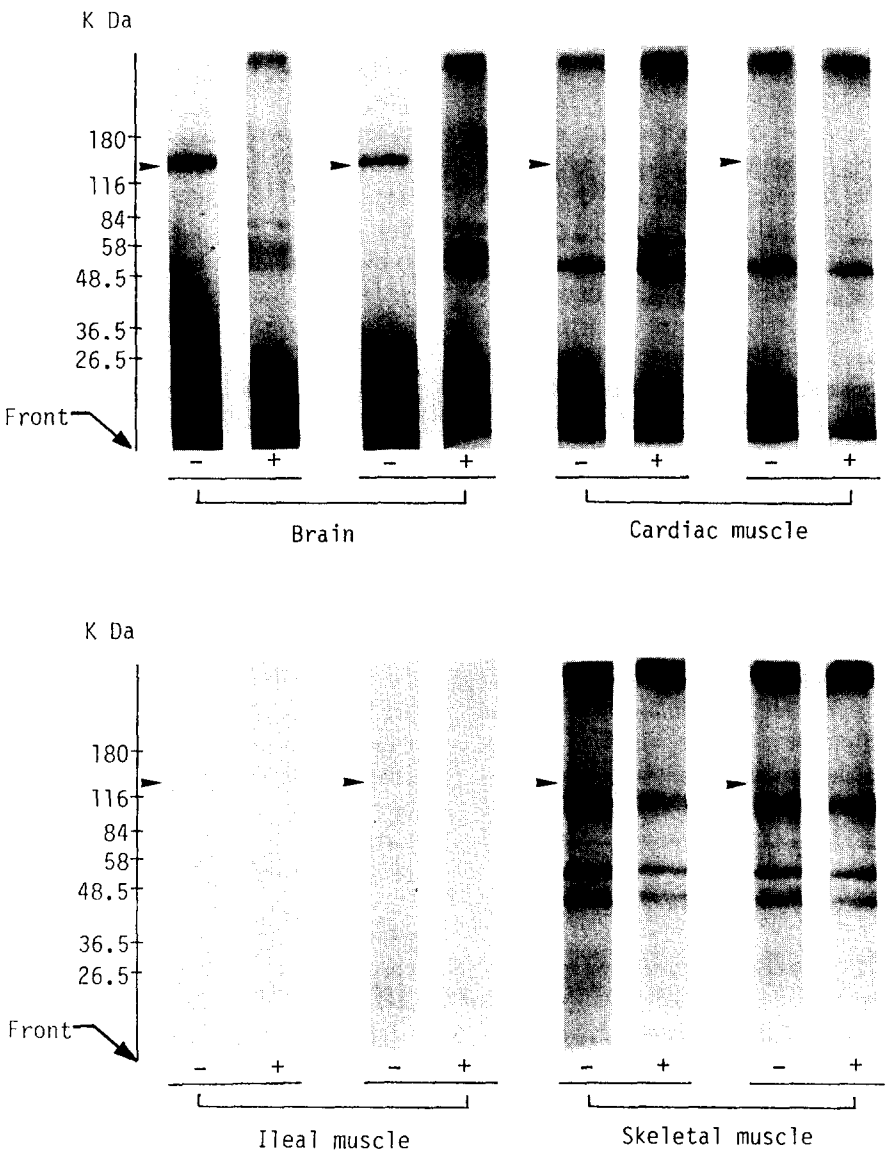

Fig. 10. Autoradiographic patterns of ^{125}I-ω-CgTX labeling with DSS of crude membranes from brain, cardiac muscle, ileal muscle and skeletal muscle. Two samples from each type of membrane are shown. The concentration of protein for each crude membrane applied in SDS-PAGE was almost the same (about 120 μg protein/lane). The symbols (−) and (+) indicate the absence and the presence of unlabeled ω-CgTX, at 1000-fold the concentration of ^{125}I-ω-CgTX, on the reaction medium, respectively. The arrow heads indicate the 135 kDa band.

but not that of [^{3}H](+)PN. On the other hand, crude spider venom from *Plectreurys tristes* (which interferes with transmitter release by inhibiting presynaptic Ca^{2+} entry [31]) and inorganic divalent cations (Cd^{2+} and La^{3+}) inhibited the specific bindings of both ^{125}I-ω-CgTX and [^{3}H](+)PN with equal potency. These results suggest that aminoglycosides and dynorphine A (1–13) specifically block specific ^{125}I-ω-CgTX binding, but spider venom, Cd^{2+} and La^{3+} do not. The effects of aminoglycosides were comparable to those reported by Knaus et al. [17] using guinea pig cerebral cortex membranes.

Feigenbaum et al. [18] have reported that dynorphine A (1–13) stimulates ^{125}I-ω-CgTX binding to synaptic membranes from rat brain by increasing toxin affinity through a non-opiate allosteric mechanism. The stimulating effect of dynorphine A (1–13), as reported by Feigenbaum et al. [18] contrasts with the results of the present study and with those of a previous study using crude membranes from rat whole brain [16]. However, the cause of this difference in the observed effects of dynorphine A (1–13) is unknown. In our experiment, the opiate-receptor antagonist naloxone (1 μM) and the κ-receptor agonist U50488H (1 and 30 μM) did not affect the specific binding of ^{125}I-ω-CgTX (Fig. 3). Therefore, it seems likely that the inhibitory effect of dynorphine A (1–13) is not due to an opiate-related mechanism involving κ-opiate receptors. Our findings regarding the inhibitory effect of dynorphine A (1–13) were comparable with those from electrophysiological experiments in which dynorphine A blocked neuronal Ca^{2+} channels [28–30].

Various types of Ca^{2+} agonist and antagonists, such as Bay K 8644, D-diltiazem, L-diltiazem, nitrendipine, (+)PN, (−)PN and verapamil, had no effect on the specific binding of ^{125}I-ω-CgTX (data not shown). These results were generally comparable with those reported by other laboratories [14–16,21], however, the IC_{50} value for crude spider venom on specific binding differed from that reported by Feigenbaum et al. [18].

To clarify the selective effect of aminoglycosides and dynorphine A (1–13) on the specific binding sites of ^{125}I-ω-CgTX in chick whole brain, we must further investigate the effect of these agents on Ca^{2+} influx via N-type VSCCs in chick brain.

4.2. Labeling of ^{125}I-ω-CgTX with crosslinker

We found that ^{125}I-ω-CgTX specifically labeled its binding site in crude membranes from chick whole brain with DSS under conditions similar to those in which the specific binding of ^{125}I-ω-CgTX to the crude membranes occurred. The apparent molecular mass of the labeled site by SDS-PAGE under reduced and non-reduced conditions was 135 kDa and 215 kDa, respectively. The results were in part similar to those published by Cruz et al. [22] and Barhanin et al. [21]. The results by Barhanin et al. [21], in which autoradiography by SDS-PAGE under reduced and non-reduced conditions showed labeling of 140 and 170 kDa bands in chick brain, respectively, were particularly comparable with those in the present study.

The results of the present study showed that the extent of ^{125}I-ω-CgTX labeling of its binding site in the crude membranes depended on the bifunctional cross-linker used: i.e., DSS, DST or EGS (Fig. 6). These reagents differ only in the length of the active portion of the bifunctional molecule in the crosslinker: the lengths for DSS, DST and EGS are 11.4, 6.4 and 16.1 Å, respectively. The rank order of specific labeling of both the 135 and 215 kDa bands was DST ≪ EGS < DSS. Therefore, these results suggested that DSS was the best crosslinker for ^{125}I-ω-CgTX labeling of the 135 and 215 kDa bands in the crude membranes under our experimental conditions.

On the other hand, labeling with ^{125}I-ω-CgTX using DSS in rat brain has not yet been observed [16,21], although photoaffinity labeling with azido-derivatives of ^{125}I-ω-CgTX of the 310 kDa + 230 kDa + 34 kDa, 210 kDa + 36 kDa and 222 kDa bands in rat brain has been reported by Abe and Saitsu [19], Barhanin et al. [21] and Marqueze et al. [20], respectively. The cause of this difference in labeling using DSS between rat and chick brain is not yet clear. However, it seems likely that this difference is due to the amount of specific ^{125}I-ω-CgTX binding in rat and chick brain, since the amount of specific binding in chick brain is about 5-times greater than that in rat brain [16,21].

Biochemical studies have shown that DHP-sensitive Ca^{2+} channels from skeletal muscle T-tubule membranes (L-type VSCCs) consist of four distinct subunits, α_1 (170 kDa), α_2/δ (175 kDa), β (52 kDa), and γ (32 kDa) [32,33]. The δ-subunit can be released from the α_2/δ-protein upon reduction of the disulfide bonds [32,33]. In the present study, we showed that while the 215 kDa band was labeled under non-reduced conditions, the 135 kDa band was labeled under reduced conditions. Therefore, we believe that the sites at which the 135 kDa/215 kDa bands are labeled under reduced/non-reduced conditions in crude membranes from chick brain are closely related to the characteristics of the α_2 subunit in skeletal L-type VSCCs or neuronal VSCCs, although the α_1 subunit is believed to provide the binding sites for Ca^{2+} antagonists, DHPs and phenylalkylamines [34,35].

We investigated the effects of various Ca^{2+} agonist and antagonists on labeling with ^{125}I-ω-CgTX using DSS. A correlation was observed between the effects on labeling and the effects on the specific binding of ^{125}I-ω-CgTX. Common findings between the two studies are as follows:

(a) Time and dose of ^{125}I-ω-CgTX dependence were observed in both studies, and both the pattern of the time-course and the ED_{50} value for ^{125}I-ω-CgTX labeling were similar to those for the specific binding of ^{125}I-ω-CgTX (Figs. 7 and 8, and Table 1).

(b) Labeling of the 135 kDa band was selectively inhibited by aminoglycosides, ω-CgTX, and dynorphine A

(1–13). In contrast, Cd^{2+}, La^{3+} and spider venom inhibited both the labeling of the 135 kDa band and non-specific labeling in the presence of unlabeled ω-CgTX (Figs. 1, 2A, 2B, 2C, 4 and 9A). Moreover, the concentration used for the Ca^{2+} agonist and antagonists were almost the same in both the labeling and binding studies.

(c) ^{125}I-ω-CgTX selectively labeled the 135 kDa band in the brain, but not in the other organs examined (Fig. 10). This result was also consistent with that in ^{125}I-ω-CgTX binding (Fig. 5). Based on these similar findings, it seems likely that the 135 kDa band that is labeled with ^{125}I-ω-CgTX by DSS in crude membranes from chick whole brain is closely related to the specific binding sites of ^{125}I-ω-CgTX, probably α_2-subunits in neuronal VSCCs of the crude membranes, although it was reported that the Ca^{2+} current of cloned N-type Ca^{2+} channel α_1-subunit showed ω-CgTX sensitivity by coexpression with β-subunit but without α_2-subunit, in mammalian HEK293 cells [36] or frog oocyte expression studies [37].

Further study is required to clarify the characteristics of the 135 kDa band in crude membranes from chick brain.

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