Neuropharmacological Characterization of Voltage-Sensitive Calcium Channels: Possible Existence of Neomycin-Sensitive, \(\omega\)-Conotoxin G Via- and Dihydropyridines-Resistant Calcium Channels in the Rat Brain

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ABSTRACT—We attempted to characterize the functional roles of subtypes of voltage-sensitive calcium channels in the brain. The maximal number of \([^{125}\text{I}]\omega\text{-conotoxin G Via (}\omega\text{-CTX)}\ binding sites in rat brain associated with N-type calcium channels (N-channels) was approximately 10 times more than that of \([^{3}\text{H}]\text{PN200-110}\) associated with L-type calcium channels (L-channels). \([^{125}\text{I}]\omega\text{-CTX binding was inhibited by aminoglycoside antibiotics, neomycin and dynorphin A(1–13), but not by various classes of L-channel antagonists. A 6-hydroxydopamine-induced lesion of the striatum resulted in a marked reduction of both \([^{125}\text{I}]\omega\text{-CTX and}^{[3}\text{H}]\text{PN200-110 binding. Kainic acid-induced lesion of the striatum reduced}^{[3}\text{H}]\text{PN200-110 binding by 57°10, but did not reduce}^{[25]}\text{I}\omega\text{-CTX binding. }\omega\text{-CTX produced a small (18%) but significant reduction of potassium-stimulated }\text{Ca}^{2+}\text{ influx into rat brain synaptosomes, although it produced a concentration-dependent inhibition in chick brain synaptosomes. Neomycin inhibited }\text{Ca}^{2+}\text{ influx in both preparations in a concentration-dependent manner. Both }\omega\text{-CTX and neomycin inhibited potassium-stimulated }^{[3}\text{H}]\text{dopamine (DA) release from rat striatal slices. The L-channel antagonists had no effect on either }\text{Ca}^{2+}\text{ influx or }^{[3}\text{H}]\text{DA release. These results suggest that DA release in the striatum is regulated by }\text{Ca}^{2+}\text{ influx through N-channels located in presynaptic nerve terminals, and that the most of the }\text{Ca}^{2+}\text{ influx in rat brain appears to be governed by neomycin-sensitive, }\omega\text{-CTX- and DHP-resistant calcium channels.}

Keywords: Calcium channel (N-type, L-type), \(\omega\)-Conotoxin G Via, Neomycin, Ca\(^{2+}\) influx
influx and neurotransmitter release (17). In contrast, recent pharmacological studies have indicated that N-channel blockade with \( \omega \)-CTX produced a dose-dependent inhibition of the release of a number of neurotransmitters (18–20). With regard to Ca\(^{2+} \) influx, however, results vary, depending on the source of the brain tissue. For example, \( \omega \)-CTX inhibits potassium-stimulated Ca\(^{2+} \) influx into chick brain synaptosomes in a dose-dependent manner, while much higher concentrations of \( \omega \)-CTX have only a marginal effect on Ca\(^{2+} \) influx into rat brain synaptosomes (21, 22).

In the present study, we characterized N- and L-channels neuropharmacologically by using \([^{125}\text{I}]\omega \)-CTX and \([^{3}\text{H}]\)PN200-110 binding, respectively. We also examined the effects of selective antagonists for each channel on potassium-stimulated Ca\(^{2+} \) influx into both rat and chick brain synaptosomes and the \([^{3}\text{H}]\)dopamine (DA) release from rat striatal slices.

**MATERIALS AND METHODS**

**Materials**

The following materials were obtained from the indicated commercial sources: \([^{125}\text{I}]\omega \)-CTX (2200 Ci/mmol), \([^{3}\text{H}]\)PN200-110 (74 Ci/mol), \( ^{45}\text{CaCl}_2 \) (30.33 mCi/mg) and \([^{3}\text{H}]\)DA (24.1 Ci/mol) (New England Nuclear, Boston, MA, USA); \( \omega \)-CTX, nifedipine, verapamil, diltiazem, flunarizine, amiloride and GBR-12909 (Research Biochemicals, Inc., Natick, MA, USA); dynorphin A(1–13) and dynorphin A(1–8) (Peninsula Laboratories, Inc., Belmont, CA, USA); neomycin (Wako Pure Chemicals, Osaka); streptomycin, gentamicin, erythromycin, kainic acid (KA) and 6-hydroxydopamine (6-OHDA) (Sigma Chemical Co., St. Louis, MO, USA); and Bay K-8644 (Calbiochem Co., La Jolla, CA, USA).

**Membrane preparation for binding assay**

Male Wistar rats weighing 250–300 g were killed by decapitation. Their brains were quickly removed and homogenized in 10 vol. HEPES buffer (pH 7.4) using a polytron. Homogenates were centrifuged for 15 min at 40,000 \( \times \) g. The pellet was resuspended in 10 mM HEPES buffer containing 10 mM EDTA, incubated at 4 °C for 30 min and then recentrifuged at 40,000 \( \times \) g for 15 min. This pellet was resuspended in Chelex-treated 50 mM HEPES buffer (pH 7.4) and used for \([^{125}\text{I}]\omega \)-CTX binding. For \([^{3}\text{H}]\)PN200-110 binding, EDTA pretreatment was omitted, and the membranes were resuspended in 50 mM HEPES buffer (pH 7.4).

**\([^{125}\text{I}]\omega \)-CTX binding**

\([^{125}\text{I}]\omega \)-CTX binding was performed according to the method of Wagner et al. (23). In a routine assay, membranes were incubated with 5 pM \([^{125}\text{I}]\omega \)-CTX and test drugs at 25 °C for 30 min in 50 mM Chelex-treated HEPES buffer (pH 7.4) containing 0.4% bovine serum albumin (BSA) in a total assay volume of 1 ml. The tissue protein concentration in an assay mixture was approximately 1.5 \( \mu \)g/ml. Non-specific binding was defined as the binding in the presence of 100 nM cold \( \omega \)-CTX. Saturation isotherms were generated by using 0.63–40 pM \([^{125}\text{I}]\omega \)-CTX. Incubation was terminated by vacuum filtration with a Brandel Cell Harvester (Gaithersburg, MD, USA) over glass fiber filters (Whatman GF/C) presoaked with 0.3% polyethyleneimine. The filters were washed with 3–4 ml aliquots of ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 160 mM choline chloride and 0.2% BSA. The radioactivity of the filters was determined by \( \gamma \)-counting (Auto Well Gamma System, ARC-600; Aloka, Tokyo).

**\([^{3}\text{H}]\)PN200-110 binding**

\([^{3}\text{H}]\)PN200-110 binding was performed according to the method for \([^{3}\text{H}]\)nitrendipine binding described by Gould et al. (24), with a minor modification. In brief, membranes were incubated with 0.1 nM \([^{3}\text{H}]\)PN200-110 and test drugs at 25 °C for 90 min in 50 mM HEPES buffer (pH 7.4) in a total assay volume of 1 ml. The protein concentration in an assay mixture was approximately 300 \( \mu \)g/ml. Non-specific binding was defined as the binding in the presence of 10 \( \mu \)M cold nifedipine. Saturation isotherms were generated with 0.03–2.0 nM \([^{3}\text{H}]\)PN200-110. Incubation was terminated by vacuum filtration over glass fiber filters. The filters were washed with 3–4-ml aliquots of ice-cold 50 mM Tris-HCl buffer (pH 7.7), and the radioactivity was determined by liquid scintillation spectrometry (Liquid Scintillation System, LSC-3500; Aloka, Tokyo) at a counting efficiency of 45%.

**Brain lesions**

Injections of KA (0.64 \( \mu \)g/1 \( \mu \)l) and 6-OHDA (8 \( \mu \)g/2 \( \mu \)l) into the right striatum (A: +0.2, L: +3.0, H: −5.5) were performed according to the coordinates in the brain atlas of Paxinos and Watson (25), as described previously (26). Two weeks after the lesions were produced, the rats were killed, and the right striata were dissected. The striatal membranes were prepared as described above and used for the binding assays.

**Ca\(^{2+} \) influx into synaptosomes**

Rat cerebral cortices and hippocampi and chick forebrains (white leghorn chicks 2–3 days post-hatching) were used for the Ca\(^{2+} \) influx assay. The tissues were homogenized in 10 volumes of ice-cold 0.32 M sucrose at 4 °C in a teflon glass homogenizer. All further procedures was performed at 4 °C. The homogenate was centrifuged
at 1,000 × g for 10 min. The supernatant was collected and then diluted 1 : 1 with basal buffer of the following composition: 145 mM choline chloride, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂ and 10 mM HEPES, pH adjusted to 7.4 with Tris base, and centrifuged at 10,000 × g for 15 min. The pellet was resuspended in basal buffer and used for the assay.

The synaptosome suspension (200 μl) was preincubated with the test compound for 5 min at 37°C. When the effects of ω-CTX were to be examined, the synaptosome suspension was incubated with ω-CTX at 4°C for 20 min, followed by an additional 5-min preincubation at 37°C. The Ca²⁺ influx assay was initiated by adding 500 μl of pre-warmed basal or high potassium (45 mM)-containing buffer with 45CaCl₂ at 1 μCi/ml and the test drugs. The reaction was terminated after 5 sec by adding 3 ml of ice-cold Ca²⁺-free basal buffer (stop buffer: CaCl₂ was replaced by 3 mM EGTA). The mixture was rapidly filtered under vacuum over GF/C filters, and the filters were washed twice with 4 ml of stop buffer. The radioactivity was determined by liquid scintillation spectrophotometry at a counting efficiency of 90%. Ca²⁺ influx was defined by subtracting the non-specific binding of ⁴⁵Ca²⁺ to the filters. Potassium-stimulated Ca²⁺ influx was defined as the difference between the influx in high-potassium-containing buffer and the basal buffer.

[^1H]DA release from rat striatal slices

Rat striata were dissected and sliced in two directions at a thickness of 300 μm in a McIlwain tissue chopper (27). The slices (total tissue weight of 130–170 mg) were incubated at 37°C for 15 min in 5 ml Krebs-Ringer buffer containing 0.1 μM[^1H]DA and 10 μM pargyline. The composition of the Krebs-Ringer buffer was 125 mM NaCl, 4.8 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.3 mM MgCl₂, 1.2 mM CaCl₂, 10 mM dextrose and 0.57 mM ascorbic acid, gassed with 95% O₂–5% CO₂. After three washes with this buffer, the slices (about 20 mg tissue) were transferred to superfusion chambers and superfused at a rate of 1 ml/min with 1 μM GBR-12909 to prevent the reuptake of DA (28). After 40-min superfusion (t=40 min), 15 successive 5-min fractions were collected (t=40 min to t=115 min). The slices were exposed to two separate 2-min stimuli with Krebs buffer containing 25 mM K⁺ (isomolar replacement of NaCl to KCl) starting at t=50 min (S₁) and t=90 min (S₂). Test drugs were added to the superfusate at t=70 min and were present throughout the remainder of the superfusion period. After the end of superfusion, radioactive material remaining in the tissue slices was extracted with 0.1 M HCl. After radioactivity was determined by liquid scintillation spectrophotometry, the fractional efflux rate of each fraction was determined. To assess the effects of test drugs on potassium-stimulated[^1H]DA release, the magnitude of the efflux evoked by the first stimulus (S₁) was compared with that evoked by the second stimulus (S₂) by measuring the additional efflux evoked by high-potassium-containing Krebs buffer. For the purpose of this calculation, a linear decline of the basal efflux was assumed. The effects on basal release were measured in fractions collected just before each stimulus (Sp₁ and Sp₂).

Protein content was determined according to the method of Lowry et al. (29), utilizing BSA as a standard.

**Statistical analyses**

Statistical significance was assessed by Student's two-tailed t-test (two group comparisons) or one-way analysis of variance followed by the two-tailed Dunnett's test (multiple comparisons). The IC₅₀ values were calculated by log-probit analysis. Results were expressed as the mean±S.E.M. of at least three separate experiments.

![Fig. 1. Scatchard plots of the specific binding of[^125I]ω-CTX (upper) and[^3H]PN200-110 (lower) in rat brain membranes. This figure represents the typical result obtained in three separate experiments each performed in duplicate.](image-url)
RESULTS

Characterization of $[\text{125I}]\omega$-CTX and $[\text{3H}]\text{PN200-110}$ binding

Since previous studies have indicated that the cerebral cortex and hippocampus in rats contain high levels of specific binding sites for $[\text{125I}]\omega$-CTX and $[\text{3H}]\text{PN200-110}$, we used these brain areas combined for a routine assay. As shown in Fig. 1, Scatchard analysis revealed a single class of non-interacting sites with an apparent dissociation constant ($K_d$) of $5.60 \pm 0.76$ pM and a maximal number of binding sites ($B_{\text{max}}$) of $1.32 \pm 0.10$ pmol/mg protein for $[\text{125I}]\omega$-CTX binding ($n=3$); for $[\text{3H}]\text{PN200-110}$ binding ($n=3$), the values were: $K_d$, $0.44 \pm 0.07$ nM and $B_{\text{max}}$, $158 \pm 8$ fmol/mg protein.

The brain regional distribution of $[\text{125I}]\omega$-CTX binding differed from that of $[\text{3H}]\text{PN200-110}$ binding (Fig. 2). The highest number of $[\text{125I}]\omega$-CTX binding sites was found in the cerebral cortex, followed by the striatum and hippocampus. The thalamus/hypothalamus and cerebellum had intermediate levels, while the medulla/pons had the lowest levels of these sites. In contrast, the highest level of $[\text{3H}]\text{PN200-110}$ binding sites was found in the hippocampus, followed by the cerebral cortex, striatum, thalamus/hypothalamus, cerebellum and medulla/pons.

Specific binding of $[\text{125I}]\omega$-CTX was not detected in non-neuronal tissues, i.e., heart, hindleg skeletal muscle, lung, kidney and liver, while high levels of $[\text{3H}]\text{PN200-110}$ binding were detected in the heart and skeletal muscle (data not shown).

The effects of various compounds on the specific binding of $[\text{125I}]\omega$-CTX and $[\text{3H}]\text{PN200-110}$ are summarized in Table 1. $\omega$-CTX was the most potent inhibitor of $[\text{125I}]\omega$-CTX, with an apparent $IC_{50}$ of 7.7 pM, while it had little effect on $[\text{3H}]\text{PN200-110}$ binding up to 10 nM. The aminoglycoside antibiotics, neomycin, gentamicin, and other compounds showed variable effects on both binding sites.

Table 1. Effects of various compounds on $[\text{125I}]\omega$-CTX and $[\text{3H}]\text{PN200-110}$ binding in rat brain membrane

| Compound          | $[\text{125I}]\omega$-CTX IC$_{50}$ (nM) | $[\text{3H}]\text{PN200-110}$ IC$_{50}$ (nM) |
|-------------------|----------------------------------------|---------------------------------------------|
| $\omega$-CTX      | 0.0077                                  | $>10$                                       |
| Neomycin          | 580                                     | $>10,000$                                  |
| Gentamicin        | 2,200                                   | $>10,000$                                  |
| Streptomycin      | 7,200                                   | $>10,000$                                  |
| Erythromycin      | $>10,000$                               | $>10,000$                                  |
| Dynorphin A(1–13) | 2,300                                   | $>10,000$                                  |
| Dynorphin A(1–8)  | $>10,000$                               | $>10,000$                                  |
| Nifedipine        | $>10,000$                               | 2.4                                        |
| Bay K-8644        | $>10,000$                               | 10.9                                       |
| Verapamil         | $>10,000$                               | partial inhibition                         |
| Diltiazem         | $>10,000$                               | $>10,000$                                  |
| Flunarazine       | $>10,000$                               | 470                                        |
| CdCl$_2$          | 420,000                                 | 80,000                                      |
| NiCl$_2$          | 1,040,000                               | 38,900,000                                 |
| LaCl$_3$          | 90,000                                  | 1,590,000                                  |
| GdCl$_3$          | 100,000                                 | 640,000                                     |

IC$_{50}$ values were determined by using 3–5 different concentrations of each compound and 5 pM $[\text{125I}]\omega$-CTX or 0.1 nM $[\text{3H}]\text{PN200-110}$. Each value represents the mean of three separate experiments performed in duplicate.
streptomycin, inhibited \([^{125}I]\omega\text{-CTX}\) binding in a concentration-dependent manner (Fig. 3), the rank order of potency being neomycin > gentamicin > streptomycin. Dynorphin A(1-13), but not dynorphin A(1-8), was also a potent inhibitor of \([^{125}I]\omega\text{-CTX}\) (Fig. 3). The aminoglycoside antibiotics and dynorphin A(1-13) had no effect on \([^3H]\text{PN}200-110\) binding up to 10 pM (Table 1). Scatchard analysis revealed non-competitive inhibition by neomycin of the specific binding of \([^{125}I]\omega\text{-CTX}\) (Fig. 4). The calcium antagonists, nifedipine, verapamil, diltiazem, and flunarizine, which act on L-channels, had little effect on \([^{125}I]\omega\text{-CTX}\) binding up to 10 pM (Table 1).

As shown in Fig. 3, nifedipine and Bay K-8644, a DHP antagonist and agonist, respectively, as well as flunarizine, a diphenylalkylamine derivative, inhibited \([^3H]\text{PN}200-110\) binding in a concentration-dependent manner. Verapamil produced a partial inhibition of \([^3H]\text{PN}200-110\) binding, with a maximal inhibition of 20% at 10 \(\mu\text{M}\). Diltiazem failed to modify \([^3H]\text{PN}200-110\) binding (Fig. 3). The effects of cations on \([^{125}I]\omega\text{-CTX}\) binding were different from their effects on \([^3H]\text{PN}200-110\) binding, the rank order of potency being \(\text{La}^{3+} = \text{Gd}^{3+} > \text{Cd}^{2+} > \text{Ni}^{2+}\) for \([^{125}I]\omega\text{-CTX}\) binding, and \(\text{Cd}^{2+} > \text{Gd}^{3+} > \text{La}^{3+} > \text{Ni}^{2+}\) for \([^3H]\text{PN}200-110\) binding (Table 1).

To determine the cellular location of the specific binding sites of \([^{125}I]\omega\text{-CTX}\) and \([^3H]\text{PN}200-110\), we examined the effects of two different kinds of lesions (Table 2). The 6-OHDA lesion of the striatum resulted in a 28% reduction of \([^{125}I]\omega\text{-CTX}\) binding and a 19% reduction of \([^3H]\text{PN}200-110\) binding (Table 2). The 6-OHDA lesion of the striatum resulted in a 28% reduction of \([^{125}I]\omega\text{-CTX}\) binding and a 19% reduction of \([^3H]\text{PN}200-110\) binding (Table 2).
PN200-110 binding. The KA-induced lesion of the striatum reduced the specific binding of \(^{[3]H}\)PN200-110 to 43% of the sham-operated control level, while it had little effect on \(^{[125]}I\)w-CTX binding.

**Effects of N- and L-channel antagonists on Ca\(^{2+}\) influx**

As shown in Fig. 5, w-CTX caused a small (18%) but significant reduction of potassium-stimulated Ca\(^{2+}\) influx into rat brain synaptosomes at a concentration of 300 nM, while it inhibited Ca\(^{2+}\) influx into chick brain synaptosomes in a concentration-dependent manner. In contrast, neomycin inhibited Ca\(^{2+}\) influx into both rat and chick synaptosomes in a concentration-dependent manner, while Ca\(^{2+}\) influx into rat brain synaptosomes appeared to be more sensitive than that into chick brain synaptosomes.

**Table 3. Effects of L-channel antagonists on Ca\(^{2+}\) influx into rat and chick brain synaptosomes**

| Compound     | Conc. (nM) | Rat (nmol/5 sec/mg protein) | Chick (nmol/5 sec/mg protein) |
|--------------|------------|----------------------------|------------------------------|
| Control      | N.D.       | 2.18±0.11                  | 1.36±0.14                    |
| Nifedipine   | 1          | 2.09±0.09                  | 1.21±0.11                    |
|              | 10         | N.D.                       | 1.30±0.13                    |
| Verapamil    | 1          | 2.13±0.06                  | N.D.                         |
| Diltiazem    | 1          | 1.67±0.21                  | 1.44±0.10                    |
|              | 10         | 1.98±0.11                  | 1.25±0.11                    |
| Flunarizine  | 1          | 2.28±0.42                  | N.D.                         |

Brain synaptosomes were preincubated with each compound for 5 min; Ca\(^{2+}\) influx was then measured for 5 sec. Basal Ca\(^{2+}\) influx in rat and chick brain synaptosomes in the control was 1.96±0.18 and 3.20±0.30 nmol/5 sec/mg protein, respectively; this was not affected by any of the compounds tested. N.D.: not determined. Each value represents the mean±S.E.M. (n=3–4).

**Table 4. Effects of various classes of calcium channel antagonists on \(^{[3]H}\)DA release from rat striatal slices in vitro**

| Compound       | Conc. (µM) | S\(_{P2}/S_{P1}\) | S\(_{S2}/S_{S1}\) |
|----------------|------------|-------------------|-------------------|
| Control        | N.D.       | 0.99±0.03         | 0.79±0.05         |
| w-CTX          | 0.003      | 0.94±0.04         | 0.73±0.05         |
|                | 0.01       | 0.97±0.05         | 0.60±0.01*        |
|                | 0.03       | 0.90±0.03         | 0.52±0.02**       |
|                | 0.1        | 0.87±0.05         | 0.48±0.02**       |
| Neomycin       | 10         | 0.95±0.03         | 0.67±0.05*        |
|                | 100        | 0.98±0.05         | 0.50±0.03**       |
| Control (0.1% DMSO) | 1.12±0.07 | 0.81±0.05         |
| Nifedipine     | 1.0        | 1.03±0.09         | 0.75±0.02         |
| Verapamil      | 1.0        | 1.09±0.13         | 0.71±0.04         |
| Diltiazem      | 1.0        | 1.10±0.12         | 0.78±0.01         |
| Flunarizine    | 1.0        | 0.93±0.05         | 0.77±0.08         |

Each compound was added to the superfusion medium from 20 min before S\(_{2}\) and was present throughout the remainder of the superfusion period. S\(_{P1}\) and S\(_{S1}\) values in the control were 3.1±0.3 and 20.3±1.6% of the total radioactivity, respectively. Each value represents the mean±S.E.M. (n=3–4). *P<0.05, **P<0.01 vs. control.
aptosomes (Fig. 6). Neither ω-CTX nor neomycin had an effect on basal Ca\(^{2+}\) influx in either preparation (data not shown). The L-channel antagonists failed to modify Ca\(^{2+}\) influx in either preparation (Table 3).

**Effects of N- and L-channel antagonists on \(^{[3]H}\)DA release from striatal slices**

Both ω-CTX and neomycin reduced the potassium-stimulated \(^{[3]}\)HDA release from rat striatal slices in a concentration-dependent manner, while they had no effect on basal release. The L-channel antagonists, nifedipine, verapamil, diltiazem, and flunarizine, however, had little effect on potassium-stimulated \(^{[3]}\)HDA release (Table 4).

**DISCUSSION**

The characterization of the specific binding of \(^{[125]}\)Iω-CTX and \(^{[3]}\)H]PN200-110 described in the present study is generally consistent with that described in previous studies (23, 24, 30, 31). The differences in regional distribution in the brain, tissue distribution and pharmacology of \(^{[125]}\)Iω-CTX and \(^{[3]}\)H]PN200-110 binding suggest that these ligands bind to different molecular entities (N- and L-channels, respectively). N-channels appear to be approximately 10 times more numerous than L-channels in rat brain, based on the \(B_{\text{max}}\) of the specific binding of \(^{[125]}\)Iω-CTX and \(^{[3]}\)H]PN200-110. Furthermore, the tissue distribution of \(^{[125]}\)Iω-CTX binding suggests that N-channels are highly expressed in the nervous system; Especially, the brain regions are enriched in synaptic connections, although recent studies have also indicated the presence of these channels outside the nervous system (32, 33).

A discrepancy between the present and previous studies was observed in regard to the effects of dynorphin A-(1–13) on \(^{[125]}\)Iω-CTX binding. We observed concentration-dependent inhibition of \(^{[125]}\)Iω-CTX binding by dynorphin A(1–13), although Feigenbaum et al. (34) had reported dynorphin A(1–13) to have a stimulatory effect of on this binding. These authors have also reported that neomycin inhibits \(^{[125]}\)Iω-CTX binding in a competitive manner, while a non-competitive inhibition was shown in both the present study and in that of Wagner et al. (23). The reason for these discrepancies is not clear, but they are probably due to the different methods used for membrane preparation: we used a fresh membrane preparation without freezing for the binding assay, while they used a preparation after a freezing/thawing procedure. However, in any case, dynorphin A(1–13) appears to modulate N-channel activity (35) by a non-opioid mechanism, since neither dynorphin A(1–8) (present study) nor dynorphin A(1–5) (34) was found to change \(^{[125]}\)Iω-CTX binding.

To determine the cellular location of the specific binding sites of \(^{[125]}\)Iω-CTX and \(^{[3]}\)H]PN200-110, we examined the effects of two different kinds of lesions. The injection of 6-OHDA, an agent which causes degeneration of catecholamine-containing afferents in the rat striatum (36), resulted in a significant reduction of the specific binding of both \(^{[125]}\)Iω-CTX and \(^{[3]}\)H]PN200-110, the magnitude of the reduction of \(^{[125]}\)Iω-CTX binding being greater than that of \(^{[3]}\)H]PN200-110. In contrast, the injection of KA, an agent which produces axon-sparing perikaryon lesions (37), caused a large reduction of \(^{[3]}\)H]PN200-110 binding, but had little effect on \(^{[125]}\)Iω-CTX binding. Similarly, Sanna et al. (38) have reported that there were no changes in \(^{[3]}\)Hnitrendipine binding when dopaminergic or serotonergic nerve terminals were destroyed, whereas there was a large reduction in this binding when KA lesions were produced. In addition, Westenbroek et al. (39) have demonstrated that L-channels, visualized using a monoclonal antibody, were located in the cell bodies and proximal dendrites of hippocampal pyramidal cells and were clustered at a high density, at the base of major dendrites. Taken together, all this evidence suggests that \(^{[125]}\)Iω-CTX binding associated with N-channels is localized in nerve terminals, whereas L-channels may be preferentially localized in the cell bodies and proximal dendrites.

To assess the functional role of N- and L-channels in the brain, we determined the effects of selective antagonists for each channel on Ca\(^{2+}\) influx into rat and chick brain synaptosomes and on \(^{[3]}\)HDA release from rat striatal slices. Since different assay conditions, including tissue preparation, incubation time of compounds and potassium concentration for the tissue stimulation, were utilized in the experiments of Ca\(^{2+}\) influx and \(^{[3]}\)HDA release, we carefully compared the effects of each channel antagonist in these two experiments. The L-channel antagonists had little effect on either Ca\(^{2+}\) influx or \(^{[3]}\)HDA release, these findings being in agreement with previous studies (17, 40). These results suggest that the role of L-channels in the brain appears to be other than the regulation of neurotransmitter release from presynaptic nerve terminals. Since L-channels are considered to be localized in the cell body, as noted above (38, 39), it appears that these channels regulate Ca\(^{2+}\) influx into neuronal cell bodies, a process that may regulate many different activities, including protein phosphorylation and gene expression.

In contrast to the effects of L-channel antagonists, the effects of the N-channel antagonists were complicated. Ca\(^{2+}\) influx into rat brain synaptosomes stimulated by potassium depolarization was inhibited by ω-CTX at 300 nM to 82% of the control level, whereas \(^{[3]}\)HDA release from striatal slices was inhibited by relatively low concentrations of ω-CTX, in a concentration-dependent man-
nner. Furthermore, there were species differences in the sensitivity of synaptosomal Ca\(^{2+}\) influx to \(\omega\)-CTX, as noted previously (21). Dissociation between the inhibitory effects of \(\omega\)-CTX on Ca\(^{2+}\) influx and neurotransmitter release has been reported previously in rats (22, 41). For example, it has been demonstrated that \(\omega\)-CTX is a potent inhibitor of acetylcholine release, but is an inactive or a weak blocker of Ca\(^{2+}\) influx in rats (22). In this regard, it is proposed that \(\omega\)-CTX blocks a small amount of total Ca\(^{2+}\) influx in mammalian brain at discrete sites, the so-called hot spots on membranes that are intimately associated with neurotransmitter release (16, 22). This hypothesis seems likely in the light of the biochemical and pharmacological effects of \(\omega\)-CTX demonstrated in this study as well as in others (22, 41).

We also examined the effects of neomycin, since it inhibited the specific binding of \(^{125}\)I\(\omega\)-CTX but not \(^{3}H\)PN200-110. It has been also reported that aminoglycoside antibiotics, including neomycin, inhibited \(^{125}\)I\(\omega\)-CTX binding, but had no effect on either \(^{3}H\)PN200-110 or \(^{3}H\)desmethoxyverapamil binding in guinea pig brain (31). Unlike \(\omega\)-CTX, neomycin caused a concentration-dependent inhibition of Ca\(^{2+}\) influx into both rat and chick brain synaptosomes, this inhibition being associated with an inhibition of \(^{3}H\)DA release. To explain the difference in sensitivity of synaptosomal Ca\(^{2+}\) influx to \(\omega\)-CTX and neomycin, several possibilities may be considered.

While \(\omega\)-CTX inactivates N-channels at the outer membrane of the cell by direct interaction (7), the site required for inhibition by neomycin is unclear. It is suggested, however, that inhibition of \(^{125}\)I\(\omega\)-CTX binding by neomycin may be mediated by a GTP binding protein (42). Although these results indicate that different mechanisms may operate for N-channel inhibition by \(\omega\)-CTX and neomycin, it is difficult to explain the different actions of these compounds on Ca\(^{2+}\) influx. Alternatively, it is possible that the inhibitory effects of neomycin on Ca\(^{2+}\) influx and \(^{3}H\)DA release may be attributed, in part, to mechanisms other than N-channel blockade such as inhibition of phospholipase C (43). Another explanation, which we support, is the existence of a neomycin-sensitive, \(\omega\)-CTX and DHP-resistant calcium channels. Neomycin-sensitive, \(\omega\)-CTX- and DHP-resistant calcium channels have recently been proposed to mediate the depolarization-induced increase in internal calcium levels in cortical slices of immature rat brain (44). In addition, an electrophysiological study has indicated the existence of high-threshold calcium channels that were resistant to both \(\omega\)-CTX and DHP in the rat brain (45). The existence of calcium channels that are resistant to both \(\omega\)-CTX and DHP may be due to a heterogeneous family of calcium channels in rat brain (46). Accordingly, it is conceivable that neomycin may block two such pharmacologically distinct calcium channels as the \(\omega\)-CTX-sensitive N-channels and the neomycin-sensitive, \(\omega\)-CTX- and DHP-resistant calcium channels. The former seems to regulate neurotransmitter release, whereas the latter may mediate the majority of the depolarization-stimulated Ca\(^{2+}\) influx into rat brain synaptosomes. Alternatively, neomycin-sensitive, \(\omega\)-CTX- and DHP-resistant calcium channels might be the same as or closely associated with P-channels, since it has been shown that Ca\(^{2+}\) influx into rat brain synaptosomes was greatly diminished by the P-channel antagonists FTX and \(\omega\)-Aga-IVA (15, 47). In order to characterize the neomycin-sensitive, \(\omega\)-CTX- and DHP-resistant calcium channels in the brain, further studies will be required to determine the optimal conditions, including pretreatment time of neomycin and stimulus conditions of brain tissue, that can separate neomycin's action on the channels.

In conclusion, the present study provides further support for the concept that N-channels regulate Ca\(^{2+}\) influx into presynaptic nerve terminals, which is the trigger for neurotransmitter release. The L-channels are considered to be localized in the cell bodies and proximal dendrites, where they may regulate many different activities, including phosphorylation and gene expression. Furthermore, it is suggested that the majority of depolarization-stimulated Ca\(^{2+}\) influx in rat brain is mediated by neomycin-sensitive, \(\omega\)-CTX- and DHP-resistant channels.

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