Toxityping Rat Brain Calcium Channels with ω-Toxins from Spider and Cone Snail Venoms†

Michael E. Adams,*,‡ Richard A. Myers,§ Julita S. Imperial,§ and Baldomero M. Olivera§

Departments of Entomology and Neuroscience, University of California, Riverside, California 92521, and Department of Biology, University of Utah, Salt Lake City, Utah 84112

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ABSTRACT: Different types of voltage-sensitive Ca^{2+} channels in the brain can be defined by specific ligands: L-type Ca^{2+} channels are uniquely sensitive to dihydropyridines, and N-type Ca^{2+} channels are selectively blocked by the *Conus* peptide ω-CTX-GVIA. Cloning data have revealed additional calcium channel types in mammalian brain for which selective ligands would be desirable. We describe binding experiments involving three newer ligands that block dihydropyridine- and ω-CTX-GVIA-resistant Ca channels: ω-Aga-IIIA and ω-Aga-IVA from venom of the spider *Agelenopsis aperta* and ω-CTX-MVIIC from *Conus magus*. [^{125}I]ω-Aga-IVA binds with high affinity (IC_{50} = ~50 nM) to receptors in rat brain which may correspond to P-like calcium channels. A second high-affinity site (IC_{50} = ~1 nM) is defined by [^{125}I]ω-CTX-MVIIC, proposed here to be on an "O"-type calcium channel. [^{125}I]ω-Aga-IIIA targets homologous binding sites present on multiple Ca channel types. The IIIA sites overlap with the binding sites for [^{125}I]ω-CTX-GVIA and [^{125}I]ω-CTX-MVIIC. The IIIA sites do not overlap with the site defined by ω-Aga-IVA. Thus toxin ligands may be highly specific for a particular Ca channel (i.e., GVIA for the N-type channel) or exhibit broader specificity (i.e., ω-Aga-IIIA, which appears to bind L-, N-, P-, and O-type Ca^{2+} channels).

Voltage-sensitive Ca channels regulate the excitability of neurons, modulate synaptic input, and transduce electrical signals into an output biochemical event, neurotransmitter release. These diverse functions are associated with multiple Ca channel types distinguished by biophysical and pharmacological methods (Nowycky et al., 1985; Llinas et al., 1989; Regan et al., 1991; Mintz et al., 1992b) and, more recently, by molecular cloning (Snutch et al., 1990, 1991; Starr et al., 1991; Mori, et al., 1991; Williams et al., 1993; Soong et al., 1993; Ellinor et al., 1993). Molecular cloning indicates that a much higher diversity of Ca channel types occurs in the brain than was previously recognized. Associating structurally diverse Ca channels defined through molecular cloning with those expressed *in situ* by identifiable groups of neurons is an emerging challenge.

The high threshold Ca channels have been differentiated into L, N, and P subtypes (Fox et al., 1987a,b; Llinas et al., 1989; Regan et al., 1991; Regan, 1991; Mogul & Fox, 1991; Mintz et al., 1992a,b). This current classification scheme is largely based on the unique sensitivity of L-type channels to dihydropyridine antagonists (Nowycky et al., 1985; Fox et al., 1987a,b), N-type channels to ω-CTX-GVIA[1] (McCleskey et al., 1987; Williams et al., 1993), and P-type channels to ω-Aga-IVA (Mintz et al., 1992a,b). Additional components of calcium current that are insensitive to agents defining L-, N-, and P-type Ca channels are clearly present in certain types of neurons (Mintz et al., 1992b).

Several calcium channel antagonists with novel selectivities recently have been identified from spider (Adams et al., 1990; Venema et al., 1992; Mintz dt al., 1992a) and cone snail venoms (Hillyard et al., 1992). ω-Aga-IVA, a toxin from the spider *Agelenopsis aperta*, is a high-affinity antagonist of P-type Ca channels in cerebellar Purkinje neurons and also blocks Ca currents resistant to dihydropyridines and ω-conotoxin GVIA in a variety of central and peripheral neurons (Mintz et al., 1992a,b). Another Ca channel antagonist from spider venom is ω-Aga-IIIA, which targets multiple high-threshold channel types (Mintz et al., 1991; Mintz, 1994). ω-Aga-IIIA is a potentially valuable probe, because it appears to recognize a binding domain common to all high-threshold Ca channels. A recently discovered member of the ω-conotoxin family, ω-CTX-MVIIC, also blocks multiple Ca channel types, although its affinity for different subtypes appears to vary (Hillyard et al., 1992).

In the present work, we describe the preparation and use of [^{125}I]ω-agatoxins and [^{125}I]ω-conotoxins as radioligands to discriminate putative binding sites on distinct Ca channel types.

EXPERIMENTAL PROCEDURES

Materials. Bolton–Hunter reagant and chloramine T were purchased from Sigma; Iodogen was from Pierce; sodium [^{125}]-iodide was from Amersham.

Preparation of [^{125}I]ω-Aga-IVA. [^{125}I]ω-Aga-IVA was synthesized by direct iodination of Tyr 9 using a modification of the Iodogen (Pierce) method. One nanomole of native ω-Aga-IVA dissolved in 0.5 M ammonium acetate (pH 8.0) was transferred to a 1.5-mL polypropylene tube, the inner walls of which were previously coated with Iodogen as described by the manufacturer. To this solution was added 1 mCi of $Na^{125}I$, and the solution was gently vortexed for 5–10 min at room temperature. The reaction was stopped by transferring the solution to a second tube (with a 70-μL water wash), to which an equal volume of 5% acetic acid was added.

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* To whom correspondence should be addressed.

‡ University of California.

§ University of Utah.

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[1] Abbreviations: ω-Aga-IIIA, ω-agatoxin IIIA; ω-Aga-IVA, ω-agatoxin IVA; ω-CTX-MVIIC or MVIIC, ω-conotoxin MVIIC; ω-CTX-GVIA or GVIA, ω-conotoxin GVIA.

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Unincorporated ^{125}I was then removed by two 170-μL ethyl acetate extractions. The aqueous phase was immediately subjected to reversed-phase liquid chromatography (HPLC) on a Vydac C_4 column, and the labeled toxin was eluted with a linear gradient of acetonitrile (25–27% at 0.1%/min) in constant 0.1% trifluoroacetic acid (TFA). Mass analysis of the nonradioactive iodinated toxin ([^{127}I]ω-Aga-IVA) was performed by matrix-assisted laser desorption time-of-flight mass spectrometry on a Finnigan Lasermat instrument at the UC Riverside Biotechnology Facility.

Preparation of [^{125}I]ω-Aga-IIIA. Radioiodination of ω-Aga-IIIA was accomplished using the Bolton–Hunter (BH) reagent in two steps. The first step involved preparation of [^{125}I]BH. Four microliters of sodium [^{125}I]iodide (1 mCi) was added to 11 μL of 0.8 mg of chloramine T/mL in 0.5 M Na_2HPO_4 (pH 7.0). To this mixture was added BH reagent (0.5 nmol, equimolar to ^{125}I). Following a 2-min incubation at room temperature (sufficient for the reaction to essentially reach completion), 150 μL of ethyl acetate and 10% NaCl were added and vigorously mixed, thus extracting the [^{125}I]BH into the ethyl acetate phase while leaving the reactive chloramine T species in the aqueous phase. The ethyl acetate phase was transferred to another vessel, washed with another 150 μL of 10% NaCl, and evaporated under a stream of argon.

For synthesis of [^{125}I]ω-Aga-IIIA, [^{125}I]BH was resuspended in 2 μL of dry dimethyl sulfoxide (DMSO). Two hundred picomoles of ω-Aga-IIIA in 20 μL of 20 mM HEPES buffer (pH 8) was added, and the reaction was allowed to proceed for 20 min on ice. Twenty microliters of 5% acetic acid was added, and the unincorporated [^{125}I]BH was removed by two ethyl acetate extractions. [^{125}I]ω-Aga-IIIA was purified by HPLC on a Vydac C_4 column with a linear gradient of acetonitrile/water in constant 0.1% TFA.

Preparation of ^{125}I-Labeled Derivatives of ω-CTX-GVIA and ω-CTX-MVIIC. [^{125}I]ω-CTX-GVIA was prepared according to the methods of Cruz and Olivera (1986). [^{125}I]ω-CTX-MVIIC was prepared according to Hillyard et al. (1992).

Preparation of Rat Brain Membranes. The crude membrane fraction used for measurement of [^{125}I]ω-Aga-IIIA binding was prepared from whole brain tissue of 6-month-old Sprague-Dawley rats according to Catterall et al. (1970) with modifications in buffer components as described by Cruz and Olivera (1986).

For measurement of [^{12I}]ω-Aga-IVA binding, a solubilized receptor preparation was used. A portion of the crude rat membrane fraction (260 μg of protein) was pelleted at 16000*g* for 5 min and then resuspended in a solution containing 2% CHAPS in 80 mM sodium phosphate (pH 7.4) with 10 μg/mL each of leupeptin and pepstatin. The mixture was gently shaken at 4 °C for 35 min and sedimented at 16000*g* for 10 min, and the supernatant, diluted 10-fold with 20 mM HEPES/NaOH (pH 7.4), was used in the binding assay.

Radioligand Binding Assays. The binding of [^{125}I]ω-Aga-IIIA to crude rat brain membranes was measured using 100 μL of assay mixture containing the following: 7.2 or 14.4 μg of membrane protein, carrier-free [^{125}I]ω-Aga-IIIA (75 000 dpm), 0.32 M sucrose, 5 mM HEPES/Tris (pH 7.4), 0.2 mg of lysozyme/mL, 25 mM NaCl and 0.25% sodium cholate. Nonspecific or competitive binding was measured by preincubation of the assay mixture with either 50 nM unlabeled ω-Aga-IIIA or appropriate concentrations of other ligands, for 30 min on ice before addition of [^{125}I]ω-Aga-IIIA. The final assay mixture was then incubated at room temperature for 30–40 min and diluted with 3 mL of ice-cold wash medium containing 160 mM choline chloride, 5 mM HEPES/Tris (pH 7.4), 1.5 mM $CaCl_2$, 1 mg of BSA/mL, and 0.05% Tween 20. Membranes were collected on 0.1% or 0.15% poly(ethylenimine)-soaked glass fiber filters (Whatman GF/F) under vacuum and washed three times with 3 mL of wash medium. Radioactivity retained on the filters was determined by γ counting.

Binding of [^{125}I]ω-Aga-IVA was carried out as described above with the following modifications in the assay mixture: 1 μL of solubilized rat membrane fraction, carrier-free [^{125}I]ω-Aga-IVA (230 kdpm), 0.32 M sucrose 5 mM HEPES/Tris (pH 7.4), 0.2 mg of lysozyme/mL, and 50 mM NaCl. Nonspecific binding was measured by preincubating the membrane preparation with 400 nM unlabeled ω-Aga-IVA. Binding curves shown in Figure 2 are averages of from 2 to 12 experiments.

^{45}Ca Uptake into Synaptosomes. Procedures for preparation of synaptosomes from rat brain and their use for measurements of potassium-stimulated ^{45}Ca uptake through synaptosomal Ca channels were as previously described in Hillyard et al. (1992). Briefly, three brains from adult Sprague-Dawley rats were homogenized in 30 mL of 0.32 M sucrose/20 mM HEPES solution (pH 7.4) using a glass Teflon homogenizer. The supernatant from a low-speed centrifugation (3600 rpm, Sorvall SS-34) was centrifuged again at 12 500 rpm. The resulting pellet was resuspended in 9 mL of "low K" saline without calcium (in mM: 5 KCl, 1.4 $MgCl_2$, 1.2 mM NaH_2PO_4, 10 glucose, 145 NaCl, 20 HEPES; pH adjusted to 7.4 with Tris). Twenty microliters of this suspension was added to each incubation tube, followed by addition of 25 μL of low K saline and 5 μL of toxin (ω-Aga-IIIA, ω-Aga-IVA, ω-CTX-GVIA, or ω-CTX-MVIIC) containing 0.5% lysozyme. Synaptosomes were exposed to toxin for 30 min prior to depolarization with "high K" saline containing 137 mM potassium; the final potassium concentration was thus 74 mM. In some instances, synaptosomes were preexposed to ω-Aga-IIIA for 10 min prior to a 30-min exposure to either ω-Aga-IVA or ω-CTX-MVIIC. The depolarization step was terminated by addition of 30 mM EGTA and rapidly filtered using a "Classic" Skatron cell harvester. Filters were dried and subjected to scintillation counting. Percent total ^{45}Ca entry was defined as the difference between counts obtained from high K and low K solutions. Each toxin treatment was performed in duplicate. The results depicted in Figure 3 are from a typical experiment that was performed four times.

RESULTS

Binding of [^{125}I]ω-Aga-IVA. Direct iodination of ω-Aga-IVA using the iodogen method is described in the Experimental Procedures section. Reversed-phase liquid chromatography purification of the radiolabeled material (Figure 1A,B) yielded approximately 150 μCi of carrier-free monoiodo [^{125}I]ω-Aga-IVA and a smaller amount of the diiodinated material. The identity of this material was verified by preparation of the nonradioactive [^{127}I]ω-Aga-IVA derivative, which gave an average molecular mass of 5329 (expected value = 5330) upon analysis by laser desorption mass spectrometry. The biological activity of the ^{127}I derivative was indistinguishable from that of the native toxin when tested for ability to block potassium-stimulated ^{45}Ca entry into rat brain synaptosomes (data not shown).

[^{125}I]ω-Aga-IVA showed specific, high-affinity binding to the solubilized receptor prepared from rat brain membranes. Preincubation with unlabeled toxin inhibited radioligand binding with an IC_{50} of 50 nM (Figure 2, top left panel). No

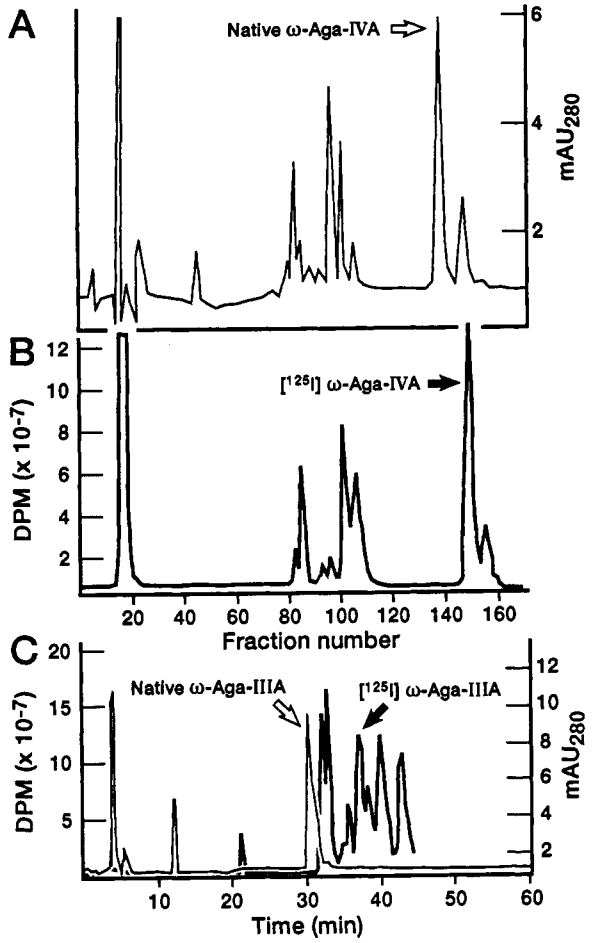

FIGURE 1: (A, B) HPLC Purification of [^{125}I]ω-Aga-IVA. Radioiodination was carried out using the Iodogen method as described under Experimental Procedures. Native toxin (open arrow, panel A) elutes at about 34 min under these solvent conditions, followed by iodinated peaks at 36.7 (monoiodo, solid arrow, panel B) and 38.2 min (diiodo). Monoiodo [^{125}I]ω-Aga-IVA was used throughout the study. Earlier peaks of radioactivity coincide with methionine sulfoxidized forms of the toxin. The fraction number scale 1–160 corresponds to 0–40 min. (C) Reversed-phase elution profiles of native ω-Aga-IIIA and multiple [^{125}I]Bolton–Hunter derivatives. The radiolabeling method is described under Experimental Procedures. The A_{280} native toxin peak is shown superimposed upon the radiochromatogram consisting of seven peaks. The material used in all of the studies described in this paper is shown with the solid arrow as [^{125}I]ω-Aga-IIIA, which elutes at about 37 min. HPLC conditions: Vydac wide-pore C_4 column; acetonitrile/water in constant 0.1% trifluoroacetic acid; flow rate 1.0 mL/min.

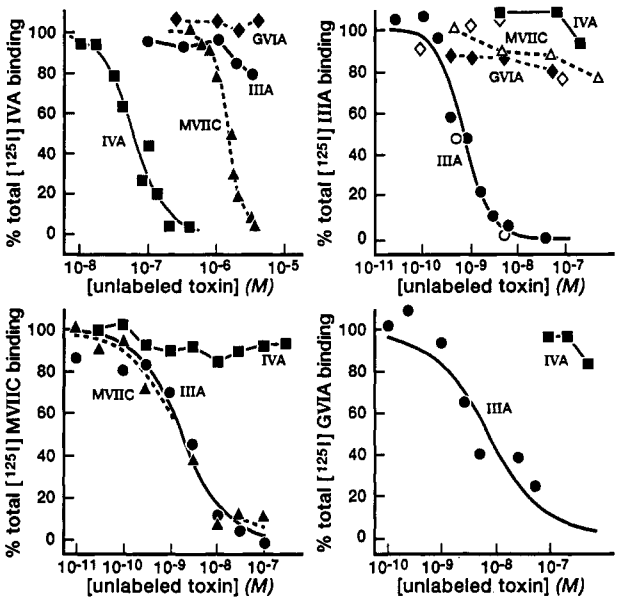

FIGURE 2: Competition binding curves. The following radiolabeled ligands were used: top left panel, [^{125}I]ω-Aga-IVA; top right panel, [^{125}I]ω-Aga-IIIA; bottom left panel, [^{125}I]ω-CTX-MVIIC; bottom right panel, [^{125}I]ω-CTX-GVIA. In each panel, displacement by unlabeled ligands is shown using the following symbols: squares, ω-Aga-IVA; circles, ω-Aga-IIIA; diamonds, ω-CgTx-GVIA; triangles, ω-CmTx-MVIIC. The binding assays were carried out as described under Experimental Procedures. For the experiment using radiolabeled ω-Aga-IIIA, the open symbols are displacement experiments done in the absence of NaCl. For the [^{125}I]ω-conotoxins, not all displacement curves are shown, since in many cases these were previously published (Hillyard et al., 1992).

significant inhibition of [^{125}I]ω-Aga-IVA binding was observed after preincubation of membranes with up to 1 μM concentrations of ω-Aga-IIIA or ω-CTX-GVIA. Higher concentrations of ω-Aga-IIIA (>1 μM) showed small but reproducible levels of binding inhibition (up to 20%) of specific [^{125}I]ω-Aga-IVA binding (Figure 2).

Inhibition of [^{125}I]ω-Aga-IVA binding to the solubilized receptor was observed following preexposure of receptor to relatively high concentrations of ω-CTX-MVIIC (IC_{50} ~ 1 μM). This concentration of ω-CTX-MVIIC was about 1000-fold higher than that needed to inhibit the high-affinity binding of [^{125}I]ω-CTX-MVIIC (Figure 2, lower left). However, the converse did not prove to be true: preexposure of membranes to ω-Aga-IVA at concentrations up to 500 nM did not inhibit binding of [^{125}I]ω-CTX-MVIIC to its high-affinity site (Figure 2).

Binding of [^{125}I]ω-Aga-IIIA. The methods for making [^{125}I]ω-Aga-IIIA by the Bolton–Hunter procedure are described under Experimental Procedures. Using this procedure, multiple radioiodinated peaks were obtained, all of which eluted later than the native toxin (Figure 1C). In all of the binding studies described below, we have used the [^{125}I]ω-Aga-IIIA peak shown by the solid arrow in Figure 1C. This material, in its nonradioactive state ([^{127}I]ω-Aga-IIIA), was shown to be active in a competition binding assay against [^{125}I]ω-CTX-GVIA binding to synaptosomal membranes (data not shown).

Binding of [^{125}I]ω-Aga-IIIA was abolished by preincubation with unlabeled ω-Aga-IIIA with an IC_{50} ~ 0.5 nM (Figure 2, top right). ω-Aga-IVA showed no significant inhibition of [^{125}]ω-Aga-IIIA binding at concentrations up to 200 nM, indicating that the two ω-agatoxins define nonoverlapping binding sites. ω-CTX-GVIA and ω-CTX-MVIIC produced slight but significant inhibition of [^{125}I]ω-Aga-IIIA binding (~20%) at relatively low concentrations (~5 nM). However, this inhibition did not increase at 10-fold higher concentrations of the ω-conotoxins.

In reciprocal binding experiments, preexposure of membanes to ω-Aga-IIIA completely inhibited specific binding of both [^{125}I]ω-CTX-MVIIC and [^{125}I]ω-CTX-GVIA (Figure 2). Thus, ω-Aga-IIIA completely occludes the binding of both ω-conotoxins, but a nonreciprocal relationship exists between the two types of toxins; the ω-conotoxins inhibit only a small amount of specific [^{125}I]ω-Aga-IIIA binding.

ω-Aga-IIIA Selectively Occludes ω-CTX-MVIIC Block of Synaptosomal Ca Flux. The foregoing data on radioligand binding suggest that ω-Aga-IIIA and ω-Aga-IVA define nonoverlapping binding sites on P-type Ca channels, whereas overlapping binding sites are indicated for ω-Aga-IIIA and ω-CTX-MVIIC. The binding data lead to the prediction that pretreatment with ω-Aga-IIIA should prevent the binding of ω-CTX-MVIIC to P-type Ca channels and hence occlude its

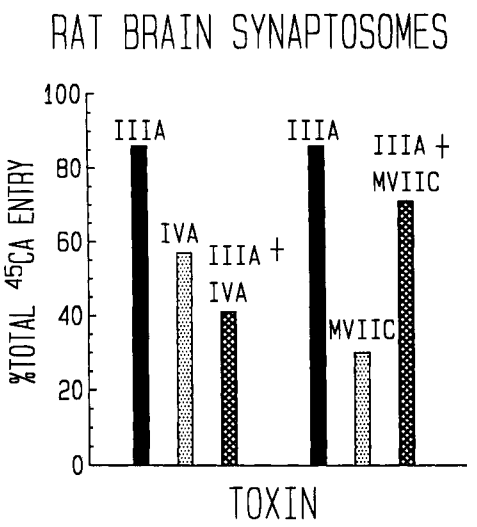

FIGURE 3: Block of rat brain synaptosomal Ca channels by ω-agatoxins and ω-conotoxins. Potassium-stimulated ^{45}Ca uptake was measured as described under Experimental Procedures and normalized to the amount of ^{45}Ca entry upon depolarization in the absence of toxins. The first two bars (left to right) indicate inhibition by a 30-min exposure to ω-Aga-IIIA (200 nM) or ω-Aga-IVA (200 nM) alone. In the third experiment, synaptosomes were exposed to ω-Aga-IIIA for 10 min prior to a 30-min ω-Aga-IVA exposure. In this case, the effects of the toxins seem to be roughly additive. In the last three bars, the effects of ω-Aga-IIIA (200 nM) and ω-CTX-MVIIC (5 μM) are shown. The last bar is an experiment in which synaptosomes were exposed to ω-Aga-IIIA for 10 min prior to ω-CTX MVIIC application. It is clear that prior addition of ω-Aga-IIIA prevents ω-CTX-MVIIC from inhibiting ^{45}Ca entry into rat brain synaptosomes. These results are taken from one experiment that was performed four times.

ability to block potassium-stimulated Ca entry in synaptosomes. Since ω-Aga-IIIA does not inhibit the binding of ω-Aga-IVA, such a preexposure should not show occlusion of such block by ω-Aga-IVA. This "order of addition" type of experiment is particularly well suited for ω-Aga-IIIA, since it is only a partial blocker of Ca channels. Previous data showed that saturating concentrations of this toxin block only 70% of potassium-stimulated ^{45}Ca entry into chick brain synaptosomes (Venema et al., 1992) and 40% of P-type Ca current in rat brain cerebellar Purkinje neurons (Mintz, 1994). In order to test this hypothesis on functional Ca channels, we exposed rat brain synaptosomes to ω-Aga-IIIA (200 nM) prior to treatment with either ω-Aga-IVA (200 nM) or ω-CTX-MVIIC (5 μM), both of which block potassium-stimulated Ca entry (Mintz et al., 1992a; Hillyard et al., 1992).

Exposure to saturating concentrations of ω-Aga-IIIA blocks only 10–15% of potassium-stimulated Ca entry (Figure 3). Exposure of synaptosomes to ω-Aga-IVA (200 nM) alone blocks about 40% of the flux response, while exposure to ω-CTX-MVIIC (5 μM) blocks about 70% of the response. However, whereas preexposure of synaptosomes to ω-Aga-IIIA showed no occlusion of ω-Aga-IVA block, it reduced block by ω-CmTx-MVIIC from 70% to about 30%. In summary, ω-Aga-IIIA occludes block of P-like Ca channels by ω-CmTx-MVIIC but not by ω-Aga-IVA.

DISCUSSION

Toxityping Ca Channels. The data presented suggest that the two ω-agatoxins, ω-Aga-IIIA and ω-Aga-IVA, used here as radioligands for the first time, target distinct, high-affinity binding sites on voltage-activated Ca channels. A third high-affinity binding site is identified by ω-CTX-MVIIC. The binding and competition characteristics of these ligands, and their correspondence to pharmacological effects on functional Ca channels, suggest that they distinguish at least three distinctive sites on dihydropyridine-resistant high-threshold calcium channels:

(1) ω-CTX-GVIA defines a high-affinity site on the N-type calcium channel. This site is also a target for ω-CTX-MVIIC (Hillyard et al., 1992) but not for ω-Aga-IVA (this work). Access to this site is prevented by ω-Aga-IIIA binding.

(2) ω-Aga-IVA defines a high-affinity site on P-like channels. The binding of [^{125}I]ω-Aga-IVA is resistant to ω-CTX-GVIA but is inhibited by micromolar levels of ω-CTX-MVIIC. The high-affinity binding of ω-Aga-IVA (IC_{50} ~50 nM) and apparent low-affinity ω-CTX-MVIIC binding (IC_{50} ~1 μM) parallel the concentrations of the two toxins required to rapidly inhibit Ca channels in cerebellar Purkinje cells and rat brain synaptosomes, that is, nanomolar concentrations of ω-Aga-IVA and micromolar concentrations of ω-CTX-MVIIC. Thus, the binding characteristics correspond to our present understanding of P-type channel pharmacology. Together, the binding studies on [^{125}I]ω-Aga-IVA and [^{125}I]ω-CTX-MVIIC indicate that occupancy of the MVIIC site which prevents [^{125}I]ω-Aga-IVA binding (with IC_{50} ~1 μM) is *not* identical to the high-affinity MVIIC site.

(3) The high-affinity ω-CTX-MVIIC site (IC_{50} ~1 nM) is proposed here to define an "O"-type Ca channel. This binding site is distinct from both the ω-CTX-GVIA site on the N-type channel and the ω-CTX-MVIIC site on a P-like channel. Of the presently studied ligands, neither ω-CTX-GVIA nor ω-Aga-IVA will displace ω-CTX-MVIIC binding under physiological conditions, but access to this site is blocked by ω-Aga-IIIA. Although the O-site is, at present, only biochemically defined as a distinctive binding site characterized by high-affinity MVIIC binding, there are functional effects that correspond to this site, i.e., the ω-conotoxin GVIA-resistant fraction of norepinephrine release in hippocampal slices (Gaur et al., 1992). It seems reasonable as a working hypothesis to postulate that the O-site corresponds to an O-type calcium channel, distinct from the L-, N- or P-types. Thus, the high-affinity binding sites of ω-CTX-GVIA, ω-CTX-MVIIC, and ω-Aga-IVA are distinct from each other.

Functional Correlates of Radioligand Binding. The [^{125}I]ω-Aga-IVA binding site exhibits features consistent with effects of the toxin on functional Ca channels in neurons. ω-Aga-IVA inhibits potassium-stimulated Ca entry into synaptosomes with an IC_{50} of 20–30 nM (Mintz et al., 1992a), which is in good agreement with the IC_{50} shown here (50 nM) for [^{125}I]ω-Aga-IVA binding to rat brain membranes. However, inhibition of P-type current in cerebellar Purkinje neurons occurs at significantly lower ω-Aga-IVA concentrations (K_d ~ 1–2 nM). This discrepancy could result from decreased affinity of ω-Aga-IVA for its binding site at depolarized membrane potentials (Mintz et al., 1992b); it would be expected that membrane potentials would be depolarized in the ^{45}Ca flux assay as well as in the binding assay. Another likely possibility is that [^{125}I]ω-Aga-IVA binds to a heterogeneous population of non-L-, non-N-type Ca channels with differing binding affinities. Consequently, it seems appropriate to refer to this population of binding sites identified by [^{125}I]ω-Aga-IVA as "P-like".

The ability of ω-CTX-MVIIC to block [^{125}I]ω-Aga-IVA binding occurs only at much higher concentrations (>1 μM) than the unlabeled toxin concentrations necessary to block homologous [^{125}I]ω-CTX-MVIIC binding (~1 nM). The higher displacement concentrations of ω-CTX-MVIIC cor-

relate well with those used to functionally block Ca channels in Purkinje cells and in synaptosomes (Hillyard et al., 1992). However, ω-Aga-IIIA occludes both binding and functional block of Ca channels by MVIIC but *not* by ω-Aga-IVA. Thus ω-CTX-MVIIC may inhibit [^{125}I]ω-Aga-IVA binding to P-like channels by binding to a site distinct from the ω-Aga-IVA site, rather than by a direct competitive inhibition. ω-CTX-MVIIC binding could alter the conformation of the ω-Aga-IVA binding site.

A high-affinity ω-Aga-IIIA binding site appears to be generally present on different types of voltage-sensitive Ca channels. Evidence for this comes from this work, showing that ω-Aga-IIIA blocks the binding of both [^{125}I]ω-CTX-GVIA (N-type antagonist) and [^{125}I]ω-CTX-MVIIC (O-type antagonist) to rat brain membranes. However, whereas ω-Aga-IIIA binds with high affinity to multiple Ca channel types, electrophysiological studies have shown that the degree of block depends on the type of Ca channel bound. L-, N-, and P-type channels are blocked 100%, 70%, and 40%, respectively (Mintz et al., 1991; Mintz, 1994). In the present studies, Ca entry into rat brain synaptosomes was blocked only 15–20% by ω-Aga-IIIA; nevertheless, pretreatment with ω-Aga-IIIA occluded the effect of ω-CTX-MVIIC. Similar results were obtained in studies of chick brain synaptosomes, where ω-Aga-IIIA occluded block by ω-CTX-GVIA. Thus ω-Aga-IIIA acts as a high-affinity partial antagonist, which is capable of preventing access of both ω-conotoxins to their binding sites.

In contrast, ω-Aga-IIIA inhibits neither the binding of [^{125}I]ω-Aga-IVA to rat brain membranes nor its ability to block synaptosomal Ca channels (Figure 3). Furthermore, electrophysiological experiments show that ω-Aga-IIIA does not occlude block of P-type channels in Purkinje neurons by ω-Aga-IVA (Mintz, 1994). These results strengthen the notion that the two ω-agatoxins target separate and distinct binding sites on P-like channels.

The use of toxins with differing selectivities to discern Ca channel diversity (i.e., "toxityping") may be an effective strategy for classifying the many high-threshold calcium channels emerging from molecular cloning studies. The current challenge is to define the correspondence between the pharmacological classes defined by toxins and the molecular classes defined by cloning. In addition to ω-conotoxins and ω-agatoxins described here, additional conotoxins and spider toxins that target high-threshold calcium channels are also emerging; the specificity of these is presently being investigated. Any toxin with the ability to target with high affinity to a particular class of calcium channel has the potential for being developed into a discriminating ligand for pharmacologically defining the ever-lengthening list of calcium channel types.

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