Phoneutria nigriventer ω-Phonetoxin IIA Blocks the Ca_{2,2} Family of Calcium Channels and Interacts with ω-Conotoxin-binding Sites*

Received for publication, December 24, 2001, and in revised form, January 25, 2002
Published JBC Papers in Press, February 4, 2002, DOI 10.1074/jbc.M112348200

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ω-Phonetoxin IIA (ω-PTxIIA), a peptide from spider venom (Phoneutria nigriventer), inhibits high threshold voltage-dependent calcium currents in neurons. To define its pharmacological specificity, we have used patch-clamp methods in cell lines expressing recombinant Ca_{2,1}, Ca_{2,2}, and Ca_{2,3} channels (P/Q-, N-, and R-type currents, respectively). Calcium currents generated by Ca_{2,1} and Ca_{2,2} were blocked almost irreversibly by 3 nM ω-PTxIIA, whereas Ca_{2,3} showed partial and readily reversible inhibition. Binding assays with monol{'125}Iiodo-ω-PTxIIA indicated that membranes expressing recombinant Ca_{2,1} or Ca_{2,2} channels showed a single class of sites with similar affinity (KD ~ 50 pM), whereas low affinity interactions were detectable with Ca_{2,3}. Kinetic, saturation, and displacement assays demonstrated that rat brain synaptosomes displayed multiple classes of binding sites for {125}I-ωPTxIIA. High affinity binding of {125}I-ωPTxIIA was totally displaced by ω-PTxIIA (KD = 100 pM), but only partially by ω-conotoxin GVIA (25% inhibition) and ω-conotoxin MVIIIC (50% inhibition at 0.3 μM). {125}I-ωPTxIIA thus defines a unique high affinity binding site that is predominantly associated with Ca_{2,1} or Ca_{2,2} channels.

Voltage-gated calcium channels play a crucial role in coupling the electrical activity of neurons to a variety of cellular processes, including gene expression, morphological differentiation, and the synaptic release of neurotransmitters. Neurons express multiple types of calcium channels that were initially classified according to the biophysical and pharmacological properties of transmembrane currents. Channels were subsequently characterized in molecular terms as hetero-oligomers composed of α_{1}, α_{2}β, β, and γ subunits and are generally defined by the nature of the pore-forming α_{1} subunit (1–3). The high threshold calcium channels of the Ca_{2,2} family carry P/Q-, N-, or R-type currents and have been designated as Ca_{2,1} (α_{1}A subunits), Ca_{2,2} (α_{1}B subunits), and Ca_{2,3} (α_{1}E subunits), respectively (4). Ca_{2} channels are insensitive to the 1,4-dihydropyridine (DHP) drugs that block high threshold channels of the Ca_{1} (L-type) family. However, a panel of natural peptide antagonists has been identified in animal venoms, providing molecular probes to analyze Ca_{2} channel structure and function. ω-Conotoxins GVIA (ωGVIA) and MVIIA purified from the venoms of the marine mollusks Conus geographus and Conus magus, respectively, are specific ligands for Ca_{2,2} channels (3). Another C. magus peptide, ω-conotoxin MVIIIC (ωMVIC), blocks both Ca_{2,1} and Ca_{2,2} channel currents at micromolar concentrations, whereas at subnanomolar concentrations {125}I-ωMVIIIC constitutes a specific radioligand for Ca_{2,1} channels (5–7). ω-Agatoxin from the spider Agenesolopsis aperta also inhibits currents generated by Ca_{2,2} channels, and blockade by ω-agatoxin (ωAga) IVA is considered as diagnostic for Ca_{2,1} channel activity (8). Differential sensitivity to ω-AgaIVA initially defined the P- and Q-type currents (9), which were subsequently shown to be supported by Ca_{2,1} splice variants (10). A second A. aperta peptide, ω-AgaIIIA, blocks all high threshold calcium channels and provides a broad spectrum antagonist (11, 12). Finally, SNX-482 from the venom of the tarantula Hysterocrates gigas inhibits a subclass of Ca_{2,3} channels (13) initially designated as R-type channels because of their resistance to blockade by DHPs, ωGVIA, and ωAgaIVA.

Thus, peptide antagonists isolated from animal venoms have been instrumental in pharmacologically defining neuronal Ca_{2,2} channels and particularly in demonstrating their role in transmitter and neuropeptide release. Furthermore, radiolabeled derivatives of these molecules are used as ligands to evaluate calcium channel density, to monitor biochemical isolation of channel proteins, and to assay anti-calcium channel antibodies for the diagnosis of human autoimmune disease (14, 15).
Interactions of ωPtxIIA with Ca,2 Channels

15. In this context, we have examined the properties of ω-phonotoxin IIA (ωPtxIIA), a neurotoxic peptide from the South American spider Phoneutria nigriventer. Subnanomolar concentrations of ωPtxIIA have been recently reported to block native N-type currents (Ca,2.2) in rat dorsal root ganglion neurons, but to be inactive on low voltage-activated T-type currents (Ca,3) (16).

In this study, we describe the purification of ωPtxIIA from P. nigriventer venom and evaluation of its activity on calcium currents generated by recombinant Ca,2.1, Ca,2.2, and Ca,2.3 channels expressed in stable baby hamster kidney (BHK) cell lines. Our electrophysiological data indicate that ωPtxIIA is a potent and practically irreversible antagonist of both Ca,2.1 and Ca,2.2, whereas it displays partial and rapidly reversible blockade of Ca,2.3. Recombinant Ca,2.1 and Ca,2.2 channels each constitute a single class of high affinity binding sites for 125I-ωPtxIIA. In contrast, nerve terminals contain multiple classes of binding sites, and competition with ω-conotoxins suggests that a fraction of the high affinity sites are associated with native Ca,2.1 and Ca,2.2 channels.

EXPERIMENTAL PROCEDURES

Purification and Sequencing of ωPtxIIA—P. nigriventer venom was obtained by electrical stimulation of anesthetized spiders. The collection and storage of venom and fraction P24C4 were performed as described by Rezende et al. (17). The pooled fractions of P24C4 (causing facial paralysis) were obtained by reverse chromatography on a Vydac C18 column (0.46 × 25 cm, 5 μm, 300 Å), dissolved in 0.1% (v/v) trifluoroacetic acid, and subjected to reverse-phase HPLC on a Vydac C18 column (218TP54) analytic column (0.46 × 25 cm) equilibrated in the same solvent. The column was eluted (1 ml/min) with a linear gradient of 0–21% acetonitrile in 0.1% trifluoroacetic acid for 15 min, followed by a second linear gradient from 21 to 29.4% for 35 min. Separation was conducted on a Hewlett-Packard HP1100 system coupled to a UV detector while monitoring elution at 215 nm. A second purification step was carried out with a linear gradient of 0–40% acetonitrile in 0.3% trifluoroacetic acid for 80 min on the same column. The toxin eluted in the second step was rechromatographed under the same conditions and sequenced with an Applied Biosystems 476A sequencer using a standard protocol.

Mass spectrometry of the native toxin dissolved in α-cyano-4-hydroxy-cinnamic acid matrix was carried out on a MALDI-TOF mass spectrometer (Voyager DE-RI, Perseptive Biosystems, Framingham, MA) with the linear mode and positive polarity using an internal calibration method with a mixture of bovine insulin (M+H+ = 5734.59 Da, average mass) and horse apomyoglobin (M+2H+ = 8476.6 Da, average mass; HPLC purified). MALDI-TOF mass spectrometry was performed on a Bruker MALDI-TOF spectrometer (Bremen, Germany).

Cell Culture and Electrophysiology—Stable BHK cell lines expressing Ca,2.1 splice variant B1-2 (18), Ca,2.2, or Ca,2.3 splice variant B1-2 (19) with the α,βα,β auxiliary subunits were cultured as previously described (20). Whole cell current traces (with inward currents downward) are shown with leak subtraction, after digitization (4 kHz) and low pass filtering (1 kHz). Dashed lines near the traces indicate zero current level. The voltage stimulation protocol consisted of steps to +10 mV (100 ms) applied every 30 s from a holding potential of −90 mV. In figures, the peak calcium (or sodium in Fig. 2H) current was plotted against episode starting time. The transient sodium current present in some cells (e.g., Fig. 2C) did not impair the measurement of peak calcium currents, performed in the absence of tetrodotoxin. The pipette solution was composed of 130 mm CsCl, 1 mm MgCl2, 10 mm HEPES, 10 mM BAPTA, and 3 mM ATP adjusted to pH 7.2 with CsOH. The extracellular solution contained 150 mm NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES adjusted to pH 7.4 with NaOH. NaOH, and the solutions superfused before or during ωPtxIIA application also contained bovine serum albumin (1 g/liter, fraction V, Roche Molecular Biochemicals). Except during application of LaCl3, a continuous solution solution was applied near the cell using a local multi-way superfusion system (70–100 μm/min). Solution flow was stopped for manual applications of LaCl3 (50 μM, 1 min) near the cell.

Analysis of Electrophysiological Data—We analyzed calcium current inhibition data assuming the channel to be functional when free (R) and blocked when bound to a toxin molecule (RL), with rate constants k1 and k−1 for association and dissociation of the toxin (L)-to-channel reversible binding reaction (R + L → RL). In this model, for a given stimulation protocol (tens of millisecond range), the ratio of peak calcium currents observed in the presence of a given toxin concentration (L) and in the absence of toxin (peak current fraction, fraction A) is linked to the constant toxin concentration (L) by the Michaelis equation, RL + R/L = 1/(Kd + L), where Kd = k−1/k1. Therefore, a measurement of Kd is derived from peak current inhibition data by Kd = L/(fraction remaining/fraction inhibited).

Off Kinetics—Following toxin washout at zero time, the toxin dissociation reaction in the absence of free toxin follows a simple law, dRL(t)/dt = −k1RL(t), leading to RL(t) = RL0*exp(−k1t). Therefore, the toxin dissociation rate constant (k1) is measured directly as the reciprocal of the time constant of calcium current recovery: 1/τoff = k1.

On Kinetics—During superfusion of a constant toxin concentration (L), starting at time zero, the kinetic equation is dRL(t)/dt = k1RL(t) − k−1RL(t), with at any given time, RL(t) = RP0 − RL(t). Therefore, dRL(t)/dt = −(k1 − k−1)RL(t) + k1LR(t) or dRL(t)/dt = (k1 − k−1)RT(t) − k−1LR(t) = (k1 − k−1)R(t) − (k−1)RT(t) or R(t) = R0 + R0*exp(−(k1 − k−1)t). Therefore, the reciprocal of the time constant of calcium blockade or the apparent inhibition time constant (kapp) in kapp = 1/τoff = k1 − k−1, and a plot of kapp = 1/τoff against toxin concentration gives k1 (slope) and k−1 (extrapolation to null toxin concentration) (see Fig. 3C).

Isoindion of ωPtxIIA—ωPtxIIA contains two tyrosine residues. 1 mol of native ωPtxIIA was reacted for 2 min with 0.5 mol of carrier-free Na125I (Amersham Biosciences) in the presence of lactoperoxidase and H2O2 in 50 mM phosphate buffer at pH 7.2. Moniodotoxin analogs were separated by reverse-phase liquid chromatography on a C18 column (0.46 × 25 cm; 5-μm particles). The column was eluted with a linear gradient of 20–53% acetonitrile for 65 min in constant 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Native toxin was eluted after 29 min, and two radioactive peaks (1 and 2) corresponding to the two possible monoiodo-ωPtxIIA derivatives were detected at 27 and 40 min. Mono[125I]iodo-ωPtxIIA from peak II was used in this study, calculating the concentration from the specific radioactivity of 2200 Ci (81.4 TBq/mmol).

Binding Assay—The standard buffer contained 10 mM Tris, 140 mM NaCl (pH 7.4) or 10 mM bovine serum albumin (0.1% v/v) buffer solution (pH 7.4) or 10 mM bovine serum albumin (0.1% v/v) buffer solution. BHK cell lines were washed with 0.2 or 0.3 ml of buffer. Bound ligand was separated by rapid filtration over glass-fiber filters (Whatman GF/C) pretreated with 0.3% polyethyleneimine in aqueous solution. After washing the filters three times with 2 ml of ice-cold binding buffer, bound ligand was measured by γ-counting with 60% efficiency. In all experiments, the level of nonspecific binding (data not shown) was determined in the presence of 50–100 μM unlabeled ωPtxIIA and subtracted from the total binding to yield the specific component. At 0.2 mol 125I-ωPtxIIA, non-specific binding accounted for ~10% (BHK cells) or 30% (synaptosomes) of the total binding. Membrane protein concentrations were chosen so that <5% of the radioligand was bound in the course of the experiment. Under these conditions, the total ligand concentration is considered to be an acceptable approximation of the free ligand concentration.

RESULTS

ωPtxIIA was purified from P. nigriventer venom using a published method with certain modifications (see "Experimental Procedures"). The molecular mass was determined by MALDI-TOF spectrometry using internal calibration. Data analysis yielded a molecular mass of 8364 Da for ion M+H+ and RT, which, together with Edman sequencing of the first 41 residues, was entirely consistent with published data, allowing identification of the purified peptide as ωPtxIIA (Fig. 1).

Voltage-dependent currents were measured in BHK cells expressing Ca,2.1, Ca,2.2, and Ca,2.3 channels using the whole cell configuration of the patch clamp with 2 mM Ca2+ as the charge carrier. Step depolarization from a holding potential of −90 mV to a test potential of +10 mV evoked inward calcium...
currents that were reduced by perfusion of ωPtxIIA (Fig. 2, A, C, and E). Inhibition of Ca v2.1 and Ca v2.2 developed over tens of minutes, reaching >70% blockade at nanomolar concentrations of ωPtxIIA (Fig. 2, A–D). The effect of ωPtxIIA on Ca v2.1 and Ca v2.2 channels was practically irreversible, displaying <15% unblockade after a 30-min washout period (Fig. 2, B and D). In contrast, 17 nM ωPtxIIA inhibited Ca v2.3 channel current only by 20%, with significantly more rapid off rates (Fig. 2, E and F). Some BHK cells, including cells from the recipient cell line tk-ts13 (Fig. 2G), express an endogenous fast, tetradotoxin-sensitive sodium current, which was unaffected by the application of ωPtxIIA (Fig. 2, C and H). Additional experiments with a Chinese hamster ovary cell line expressing the voltage-gated sodium channel Na v1.2 indicated that sodium currents were totally insensitive to ωPtxIIA (data not shown). These results indicate that nanomolar concentrations of ωPtxIIA inhibit the three channels of the Ca v2 family. ωPtxIIA displays a similar high apparent affinity for Ca v2.1 and Ca v2.2, but its action on Ca v2.3 is less potent and more readily reversible.

We analyzed calcium current inhibition data assuming a simple mechanism, the channel being functional when free and reversible.

To directly address the binding properties of ωPtxIIA, 125I iodoination was performed by the lactoperoxidase method, and a mono-125I-iiodinated derivative was purified by reverse-phase HPLC (Fig. 4). 125I-ωPtxIIA was used to titrate binding sites associated with recombinant Ca v2.1, Ca v2.2, and Ca v2.3 channels using membranes prepared from stable transfected BHK cell lines. 125I-ωPtxIIA displayed saturable binding to both Ca v2.1 and Ca v2.2 as illustrated by plots of the specific binding component (Fig. 5A). Scatchard plots of these data indicate a single class of binding sites for both Ca v2.1 (equilibrium dissociation constant, K D = 160 pm; and binding site capacity, B max = 0.4 pmol/mg of protein) and Ca v2.2 (K D = 50 pm and B max = 1.3 pmol/mg of protein) channels (data not shown). Assays in which the binding of 0.1 nM 125I-ωPtxIIA was displaced by increasing concentrations of unlabeled ωPtxIIA yielded calculated K D values of 40 pm for Ca v2.1 (Fig. 5B) and 50 pm for Ca v2.2 (Fig. 5C). Complete displacement of 125I-ωPtxIIA binding to Ca v2.1 and Ca v2.2 channels was also obtained with 0.1
In contrast, in the same concentration range, binding of 125I-α-PtxIIA to membranes expressing Cav2.3 channels was barely detectable. This is unlikely to be due to the fact that Cav2.3 channel expression is significantly lower, as whole cell currents in the 1-nA range were recorded (Fig. 2E), which were generally comparable or superior to those in Cav2.1 and Cav2.2 cell lines. A small specific binding component was detected at ligand concentrations >0.4 nM, indicative of low affinity binding sites. However, for practical reasons, it was impossible to quantify interactions with Cav2.3 channels using 125I-α-PtxIIA.

The binding properties of 125I-α-PtxIIA were also assessed using rat brain synaptosomes. The association and dissociation

\begin{align*}
\text{FIG. 3. Kinetics of blockade of Ca}_2.1 & \text{ and Ca}_2.2 \text{ calcium currents.} \quad \text{A and B, for each cell, peak calcium currents were normalized to} \\
& \text{the value before toxin application. The fraction of peak current remaining} \\
& \text{is plotted against time for four cells expressing Ca}_2.1 \text{ in the} \\
& \text{presence of 3.5, 10, 17, and 350 nM α-PtxIIA (A) and for four cells} \\
& \text{expressing Ca}_2.2 \text{ in the presence of 2, 3.5, 10, and 350 nM α-PtxIIA (B).} \\
& \text{Each plot was fitted with a monoexponential decay function, superimposed on the plot. C, shown is the graphical determination of} \\
& α\text{-PtxIIA/calcium channel on rate constants for cells expressing Ca}_2.1 \text{ and} \\
& \text{Ca}_2.2 \text{ (see “Experimental Procedures”). The reciprocal of decay time} \\
& \text{constants } (\tau_{\text{rec}}^{-1}) \text{ obtained as described for A was plotted against} \\
& \text{α-PtxIIA concentration for cells expressing Ca}_2.1 \text{ or Ca}_2.2. \text{ Each data} \\
& \text{point represents one or two cells. A linear fit of each series of points is} \\
& \text{shown. The corresponding slopes are as follows: for Ca}_2.1, \\
& k_{\text{off}}(\text{α-PtxIIA}) = 7.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}; \text{ and for Ca}_2.2, k_{\text{off}}(\text{α-PtxIIA}) = 2.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}.
\end{align*}
Interactions of oPtxIIA with Ca\textsubscript{2}\textsubscript{2} Channels

Fig. 6. Kinetics of 125\textsuperscript{I}-oPtxIIA binding to rat brain synaptosomes. A, association kinetics. 125\textsuperscript{I}-oPtxIIA (0.2 nM) was added to rat brain synaptosomes (10 \(\mu\)g of protein), and the membrane-bound radioactivity was determined after the indicated times at 30 °C. Non-specific binding estimated in the presence of 0.1 \(\mu\)M unlabeled oPtxIIA was subtracted. B, dissociation kinetics. At equilibrium, 0.1 \(\mu\)M unlabeled oPtxIIA was added, and the remaining specifically bound radioactivity was measured at the indicated times. Insets, linear semilogarithmic plots of kinetic data. B = specifically bound membrane radioactivity; \(B_{eq}\) = B at equilibrium. The data illustrated are the results of one of two independent experiments that yielded quasi-identical rate constants.

Fig. 7. Interactions between oPtxIIA- and o-conotoxin-binding sites in rat brain synaptosomes. A, 125\textsuperscript{I}-oPtxIIA (0.2 nM) was incubated with membranes (10 \(\mu\)g of protein) in the presence of increasing concentrations of unlabeled oPtxIIA ( ), oGVIA ( ), or oMVIIC ( ) for 60 min at 30 °C. B and C, 125\textsuperscript{I}-oGVIA (0.2 nM) and 125\textsuperscript{I}-oMVIIC (0.2 nM), respectively, were incubated with synaptosomes in the presence of increasing concentrations of unlabeled oPtxIIA (±S.E., n = 6). B = specifically bound radioactivity; \(B_{0}\) = B in the absence of unlabeled toxin. CgTx, conotoxin.

The kinetics of 125\textsuperscript{I}-oPtxIIA with rat brain synaptosomal membranes are shown in Fig. 6. Semilogarithmic plots (Fig. 6A, inset) of the association data indicated the presence of at least two components, suggesting heterogeneity of 125\textsuperscript{I}-oPtxIIA-binding sites. Linearization of the initial time points gave \(k_{app} = 10.4 \times 10^{-4} \text{ s}^{-1}\). Binding was reversible (Fig. 6B), and the semilogarithmic plot (Fig. 6B, inset) yielded the dissociation rate constant \(k_{-1} = 2.2 \times 10^{-7} \text{ s}^{-1}\). The equation \(k_{+1} = (k_{app} - k_{-1})/[125\textsuperscript{I}-oPtxIIA]\) gave the association rate constant \(k_{+1} = 4.1 \times 10^{9} \text{ M}^{-1} \text{ s}^{-1}\). The equilibrium dissociation constant was therefore \(K_{D} = k_{-1}/k_{+1} = 55 \text{ pm}\).

Displacement assays using 0.2 nM 125\textsuperscript{I}-oPtxIIA can be used to evaluate some of the properties of the high affinity binding sites in synaptosomes. Native oPtxIIA competes with 125\textsuperscript{I}-oPtxIIA for interaction with synaptosomal membranes from rat brain (Fig. 7A), displacing 50% of the labeled toxin (IC\textsubscript{50}) at 500 pM. The equation \(IC_{50} = K_{D}(1 + [L]/K_{D})\), where \(K_{D}\) is the equilibrium binding constant of unlabeled toxin, \([L]\) is the concentration of free radioactive toxin, and \(K_{D}\) is the equilibrium binding constant of labeled toxin, yields a \(K_{D}\) of \(~100 \text{ pm}\) for native oPtxIIA, in good agreement with the \(K_{D}\) of 125\textsuperscript{I}-oPtxIIA. Thus, radioiodination does not significantly modify the binding properties of oPtxIIA. To ascertain which fractions of high affinity 125\textsuperscript{I}-oPtxIIA can be attributed to Ca\textsubscript{2}\textsubscript{1} and Ca\textsubscript{2}\textsubscript{2}, displacement assays were performed with oMVIIC (Ca\textsubscript{2}\textsubscript{1} + Ca\textsubscript{2}\textsubscript{2}) and oGVIA (Ca\textsubscript{2}\textsubscript{2}). A concentration of 0.3 \(\mu\)M oMVIIC displaced \(~50\%\) of 125\textsuperscript{I}-oPtxIIA binding (Fig. 7A), suggesting that at least half of the high affinity oPtxIIA-binding sites are associated with Ca\textsubscript{2}\textsubscript{1} and Ca\textsubscript{2}\textsubscript{2} channels. In contrast, similar experiments with oGVIA indicated a maximum of 25% displacement of 125\textsuperscript{I}-oPtxIIA achieved with 1 nM oGVIA (Fig. 7A). Thus, at least 25% of high affinity 125\textsuperscript{I}-oPtxIIA-binding sites in nerve terminals are associated with the Ca\textsubscript{2}\textsubscript{2} channel; and by subtracting oGVIA-sensitive sites (25%) from oMVIIC-sensitive sites (50%), \(~25\%\) are associated with the Ca\textsubscript{2}\textsubscript{1} channel. Thus, a significant residual fraction of high affinity 125\textsuperscript{I}-oPtxIIA-binding sites in synaptosomes (\(~50\%)\) appear to be associated with oMVIIC-insensitive calcium channels.

Reciprocal experiments were performed to confirm the binding of oPtxIIA to native Ca\textsubscript{2}\textsubscript{1} and Ca\textsubscript{2}\textsubscript{2} channels in synaptosomes using 125\textsuperscript{I}-oMVIIC and 125\textsuperscript{I}-oGVIA. Total displacement of 125\textsuperscript{I}-oMVIIC and 125\textsuperscript{I}-oGVIA was achieved with calculated \(K_{D}\) values of 100 and 80 pm, respectively (Fig. 7, B)
TABLE I
Summary of the binding parameters of \( \omega \)-phontoxin IIA

|           | Ca\(_{2,1}\) | Ca\(_{2,2}\) | Ca\(_{2,3}\) | Synaptosomes |
|-----------|-------------|-------------|-------------|-------------|
| \( k_{-1} \) (s\(^{-1}\)) | \( <3 \times 10^{-4} \) | \( <10^{-3} \) | \( 1.2 \times 10^{-2} \) | \( 2.2 \times 10^{-4} \) |
| \( k_{1} \) (M\(^{-1}\) s\(^{-1}\)) | \( 7.1 \times 10^{-10} \) | \( 2.6 \times 10^{-9} \) | \( 1.8 \times 10^{-9} \) | \( 4.1 \times 10^{-9} \) |
| \( K_c^\ast \) (nM) | 0.04 | 0.05 | ND | 0.05 |
| \( K_D \) (nM) | <4 | <4 | 67 | ND |

The rate constants \((k_{+}, k_{-})\) and dissociation constants at equilibrium \((K_D, K_{c}^\ast)\) for the interaction of \( \omega \)-PtxIIA with recombinant calcium channels (Ca\(_{2,1}\), Ca\(_{2,2}\), Ca\(_{2,3}\), and Ca\(_{2,3}\)) expressed in BHK cells or native calcium channels in rat brain synaptosomes were determined. The constants indicated in boldface were derived from radioligand binding assays. Other constants were calculated from the kinetics of blockade during toxin application and relief from blockade upon washout (see “Experimental Procedures”), determined by patch-clamp recording of calcium currents. Constants derived from electrophysiological data at equilibrium are not included. ND, not determined.

and \( C \). Thus, \( \omega \)-PtxIIA completely occludes the \( \omega \)-conotoxin-binding sites associated with Ca\(_{2,1}\) and Ca\(_{2,2}\) channels. 125I-\( \omega \)-GVIA and 125I-\( \omega \)-MVIIC binding is known to be inhibited by millimolar concentrations of Ca\(^{2+}\) ions (21, 22). The fact that a fraction of \( \omega \)-PtxIIA-binding sites in synaptosomes overlap with \( \omega \)-conotoxin-binding sites led us to examine the effect of Ca\(^{2+}\); however, the presence of 1.5 mM EGTA or 1.5 mM CaCl\(_2\) did not modify 125I-\( \omega \)-PtxIIA binding (data not shown).

Finally, equilibrium binding experiments were also performed in which increasing concentrations of 125I-\( \omega \)-PtxIIA were added to rat brain synaptosomes in the absence or presence of unlabeled \( \omega \)-PtxIIA. Although specific binding increased with increasing ligand concentration, saturation was not achieved at concentrations approximately six times that of the \( K_D \) calculated from binding kinetics (data not shown). Thus, Scatchard analysis was not performed, and the binding site capacity in synaptosomes cannot be accurately determined.

These results suggest that rat brain nerve terminals express multiple classes of 125I-\( \omega \)-PtxIIA-binding sites. They include high affinity sites associated with Ca\(_{2,1}\) and Ca\(_{2,2}\) that are occupied at subnanomolar concentrations and additional lower affinity sites that saturate at nanomolar concentration ranges. A reasonable interpretation, consistent with binding assays performed on recombinant channels, would be that at least some of the low affinity sites correspond to native Ca\(_{2,3}\) channels.

Table I summarizes the binding parameters of \( \omega \)-PtxIIA, indicating that results from radioligand binding assays were generally consistent with patch-clamp recording data. The only discrepancy concerned the fact that the dissociation of 125I-\( \omega \)-PtxIIA from synaptosomes was significantly more rapid than relief from toxin blockade in BHK cells expressing Ca\(_{2,1}\) and Ca\(_{2,2}\). This may be due to differences in methodology (binding assays \textit{versus} electrophysiology) or in the molecular composition of channels (native presynaptic channels \textit{versus} heterologously expressed channels).

**DISCUSSION**

\textit{P. nigritventer}, the South American “armed” spider, is an aggressive species frequently responsible for human envenomation in Brazil. In 1993, Cordeiro et al. (23) reported purification and partial sequencing of several neurotoxic peptides from \textit{P. nigritventer} venom, including a 40-amino acid N-terminal sequence designated as \( \text{Tx3-4} \). \( \text{Tx3-4} \) was subsequently shown to inhibit \( 45^\text{Ca}^{2+} \) influx into rat brain synaptosomes (24). \( \text{Tx3-4} \) displays identity at 38 positions to the 40-amino acid N-terminal sequence of \( \omega \)-PtxIIA recently described by Cassola et al. (16). The peptide that we have characterized pharmacologically displays molecular mass and sequence identical to those of \( \omega \)-PtxIIA; and thus, we have maintained this nomenclature. \( \omega \)-PtxIIA has significant homology to \( \omega \)-AgaIIIA and \( \omega \)-AgaIIIB from \textit{A. aperta} (11). The three sequences display 49% homology when aligned with two gaps, although two substitutions suggest that \( \omega \)-PtxIIA may be more similar to \( \omega \)-AgaIIIB (16).

An initial report on the activity of \( \omega \)-PtxIIA demonstrated that it blocks high threshold DHP-resistant calcium current in dorsal root ganglion neurons, but spares low voltage-activated T-type current (16). This suggests that \( \omega \)-PtxIIA inhibits channels of the Ca\(_{2,3}\) family that generate P/Q-, N-, and R-type currents, but does not allow a more precise definition of pharmacological specificity. We therefore evaluated the effects of \( \omega \)-PtxIIA on whole cell calcium currents in stable BHK cell lines expressing recombinant Ca\(_{2,1}\) (\( \alpha_{1A} \) subunits), Ca\(_{2,2}\) (\( \alpha_{1B} \) subunits), or Ca\(_{2,3}\) (\( \alpha_{1E} \) subunits) channels, associated with the same auxiliary subunits. \( \omega \)-PtxIIA in the 10 nM range produced total and practically irreversible blockade of Ca\(_{2,1}\) and Ca\(_{2,2}\), whereas Ca\(_{2,3}\) was only partially inhibited in a rapidly reversible manner. We have not examined whether \( \omega \)-PtxIIA inhibits recombinant channels of the DHP-sensitive Ca\(_{1}\) family. Relatively high concentrations of \( \omega \)-PtxIIA have been shown to block a component of high voltage-activated calcium current in pancreatic \( \beta \) cells that express a major DHP-sensitive current component (16). \( \omega \)-PtxIIA may thus block some Ca\(_{1}\) channels with lower affinity than Ca\(_{2,1}\) and Ca\(_{2,2}\) channels, but additional studies will be required to resolve this issue. The pharmacological profile of \( \omega \)-PtxIIA is emerging from electrophysiological studies suggests that it represents an intermediate between \( \omega \)-AgaIIIA, which inhibits all classes of high threshold calcium channels with equivalent potency, and peptides such as \( \omega \)-AgaIVA and \( \omega \)-GVIA that are diagnostic for single channel subtypes. In keeping with the structural homology discussed above, \( \omega \)-PtxIIA may resemble \( \omega \)-AgaIIIB, which inhibits N-type (Ca\(_{2,2}\)) current more potently than L-type (Ca\(_{1,2}\)) current (11).

125I-\( \omega \)-PtxIIA displayed specific high affinity binding to membranes containing recombinant calcium channels and to rat brain synaptosomes. Results with BHK cell membranes heterologously expressing Ca\(_{2}\) channels were consistent with electrophysiological analysis of channel blockade. Ca\(_{2,1}\) and Ca\(_{2,2}\) channels each constitute a single class of high affinity sites with practically identical \( K_D \) values, indicating that \( \omega \)-PtxIIA does not distinguish between these two channel subtypes. Although cells expressing Ca\(_{2,3}\) subunits displayed robust calcium currents in the 1-NA range, the specific binding of 125I-\( \omega \)-PtxIIA to Ca\(_{2,3}\) was barely detectable, and the low affinity of this interaction precluded further binding studies.

125I-\( \omega \)-PtxIIA displayed specific binding to rat brain synaptosomes, but complex kinetics, partial displacement by other antagonists, and lack of saturation revealed multiple classes of sites. This heterogeneity is unlikely to result from the nature of the ligand or other aspects of methodology. A purified monoiodinated radioligand was used, and saturable binding to a single class of sites was demonstrated when membranes expressed a homogeneous population of recombinant channels. Thus, binding site heterogeneity in synaptosomes is likely to result from the expression of multiple types of calcium channel.

Interactions between \( \omega \)-conotoxins and \( \omega \)-PtxIIA indicated that Ca\(_{2,1}\) and Ca\(_{2,2}\) account for at least 50% of the high affinity sites in synaptosomes. \( \omega \)-PtxIIA totally occluded 125I-\( \omega \)-GVIA and 125I-\( \omega \)-MVIIC binding, in a manner consistent with competitive inhibition, indicating that it binds to native Ca\(_{2,1}\) and Ca\(_{2,2}\) channels. Furthermore, binding experiments with recombinant Ca\(_{2,1}\) and Ca\(_{2,2}\) channels indicated that submicromolar concentrations of \( \omega \)-conotoxins totally displaced 125I-\( \omega \)-PtxIIA. In synaptosomes, however, displacement of 125I-
\(\omega\)PtxIIA by increasing concentrations of \(\omega\)GVIA was only partial and reached a distinct plateau, suggesting that Ca\(_{\text{v2.2}}\) accounts for \(\sim 25\%\) of high affinity \(\omega\)PtxIIA-binding sites. Displacement by \(\omega\)MVIIA, which binds to Ca\(_{\text{v2.2}}\) but also occludes Ca\(_{\text{v2.2}}\) at higher concentrations, reached 50\%, but did not attain a distinct plateau. Subtraction of the Ca\(_{\text{v2.2}}\) component indicates that Ca\(_{\text{v2.1}}\) may contribute at least 25\% of sites, but this may be an underestimation. These results are comparable to data recently reported using \(125^I\)-\(\omega\)AgAIII, a ligand that labels all high threshold calcium channels in synaptosomes and that is also partially displaced by \(\omega\)-conotoxins (12). The fraction of \(125^I\)-\(\omega\)AgAIII binding to synaptosomes displaced by \(\omega\)-conotoxins is smaller \((\sim 40\%\) with \(\omega\)MVIIA\)) consistent with the idea that \(125^I\)-\(\omega\)AgAIII labels a wider spectrum of calcium channels than \(125^I\)-\(\omega\)PtxIIA.

Which high threshold calcium channels account for the significant fraction of \(125^I\)-\(\omega\)PtxIIA binding that is not inhibited by \(\omega\)-conotoxins? It is unlikely that DHP-sensitive channels of the Ca\(_{\text{v1.1}}\) family provide a major contribution, as channel blockade occurs only at relatively high concentrations of \(\omega\)PtxIIA (16). A comparison of the binding capacities of synaptosomes for \(125^I\)-\(\omega\)GVIA and \([3H]\)DHP in our laboratory has indicated a ratio of about six Ca\(_{\text{v2.2}}\)/one Ca\(_{\text{v1.1}}\) (21, 25). If Ca\(_{\text{v2.2}}\) constitutes \(25\%\) of high affinity sites that appear as low affinity sites that appear as “nonsaturable” binding at higher concentrations of \(125^I\)-\(\omega\)MVIIA. The most parsimonious explanation for the residual sites is that \(\omega\)MVIIIC receptor occupation is incomplete, as suggested by the lack of plateau in the displacement curve. Our experiments were performed at relatively high ionic strength, which decreases the affinity of \(\omega\)MVIIA for its binding sites (5). Thus, part of the residual \(125^I\)-\(\omega\)PtxIIA binding may involve Ca\(_{\text{v2.1}}\) channel variants with a low sensitivity to \(\omega\)MVIIIC. Further work will be necessary to unequivocally identify this channel population.

In conclusion, \(\omega\)PtxIIA is a potent blocker of Ca\(_{\text{v2.1}}\) and Ca\(_{\text{v2.2}}\) channels, although it interacts with Ca\(_{\text{v2.3}}\) and possibly certain channels of the Ca\(_{\text{v1.1}}\) family at higher concentrations. At subnanomolar concentrations, \(125^I\)-\(\omega\)PtxIIA simultaneously labels Ca\(_{\text{v2.1}}\) and Ca\(_{\text{v2.2}}\) channels, the major channel subtypes implicated in neurotransmitter release. These channels are the target for autoantibodies in Lambert-Eaton myasthenic syndrome, a human autoimmune disease affecting neurotransmission. Current antibody assay protocols used for diagnosis involve immunoprecipitation of native calcium channels labeled with \(125^I\)-\(\omega\)MVIIIC and/or \(125^I\)-\(\omega\)GVIA. The specificity and stability of \(125^I\)-\(\omega\)PtxIIA suggest that it may provide a useful alternative reagent.

Acknowledgments—We thank Adriano Monteiro de Castro Pimenta and Razika Oufrar for technical support.

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Phoneutria nigriventer ω-Phonotoxin IIA Blocks the Ca_{\text{\textasciicircum}2} Family of Calcium Channels and Interacts with ω-Conotoxin-binding Sites

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J. Biol. Chem. 2002, 277:13856-13862.
doi: 10.1074/jbc.M112348200 originally published online February 4, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M112348200

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