SNX-325, A Novel Calcium Antagonist from the Spider *Segestria florentina*

Robert Newcomb,* Andrew Palma, Jim Fox, Smita Gaur, Kwok Lau, Dave Chung, Ruth Cong, John R. Bell, Bill Horne, Laszlo Nadasdi, and J. Ramachandran

Neurex Corporation, 3760 Haven Avenue, Menlo Park, California 94025-1012

Received February 28, 1995; Revised Manuscript Received May 3, 1995[⊗]

ABSTRACT: A novel selective calcium channel antagonist peptide, SNX-325, has been isolated from the venom of the spider *Segestria florentina*. The peptide was isolated using as bioassays the displacement of radioiodinated omega-conopeptide SNX-230 (MVIIC) from rat brain synaptosomal membranes, as well as the inhibition of the barium current through cloned expressed calcium channels in oocytes. The primary sequence of SNX-325 is GSCIESGKSCTHSRSMKNGLCCPKSRCNCRQIQHRHDYLGKRKY-SCRCS, which is a novel amino acid sequence. Solid-phase synthesis resulted in a peptide that is chromatographically identical with the native peptide and which has the same configuration of cysteine residues as the spider venom peptide omega-Aga-IVa [Mintz, I. M., et al. (1992) *Nature 355*, 827-829]. At micromolar concentrations, SNX-325 is an inhibitor of most calcium, but not sodium or potassium, currents. At nanomolar concentrations, SNX-325 is a selective blocker of the cloned expressed class B (N-type), but not class C (cardiac L), A, or E, calcium channels. SNX-325 is approximately equipotent with the N-channel selective omega-conopeptides (GVIA and MVIIA as well as closely related synthetic derivatives) in blocking the potassium induced release of tritiated norepinephrine from hippocampal slices (IC_{50}s, 0.1−0.5 nM) and in blocking the barium current through cloned expressed N-channels in oocytes (IC_{50}s 3−30 nM). By contrast, SNX-325 is 4−5 orders of magnitude less potent than is SNX-111 (synthetic MVIIA) at displacing radioiodinated SNX-111 from rat brain synaptosomal membranes. SNX-325 will be a useful comparative tool in further defining the function and pharmacology of the N- and possibly other types of high-voltage activated calcium channels.

Calcium channels have recently been categorized into at least five distinct subclasses based on characterization of the channel genes, protein structure, and electrochemical properties (Olivera et al., 1994; Miller et al., 1987; Tsien et al., 1988). Because of the diversity of these ion channels, as well as of the processes which they regulate, calcium channels are attractive targets for the development of novel therapeutic agents. Available calcium channel drugs, such as the dihydropyridines, target primarily the "L" channels of the peripheral cardiovascular system (Janis et al., 1987). However, many of the important calcium channels of the central nervous system are functionally and pharmacologically distinct from the peripheral L channels, and the exploration of their therapeutic modulation is only beginning. Drugs targeted toward these channels would be useful, for example, in minimizing neuronal damage caused by excess calcium entry during periods of ischemia (Choi, 1988; Seisjo & Begtsson, 1989).

Polypeptides from animal venoms interact with subtypes of neuronal calcium channels (Olivera et al., 1994) and are useful models for the development of therapeutics directed toward these ionophores (Miljanich and Ramachandran, 1995). These natural peptides can be used as tools to examine the contribution of calcium channel subtypes to individual physiological processes. The peptides can also be used to identify organic compounds in radioligand binding assays as leads in the development of orally active therapeutics. Additionally, the peptides can be used in disease models to test the therapeutic utility of compounds directed toward particular calcium channel subtypes.

Polypeptide antagonists of calcium channels were first described in the venoms of the predatory snails *Conus geographus* and *Conus magus* (omega-conopeptides GVIA and MVIIA; Olivera et al., 1984, 1987). These peptides are antagonists of the N-type calcium channel (Hirning et al., 1988; Olivera et al., 1994). Cone snail peptides with additional specificity for neuronal calcium channels (omega-conopeptides MVIIC and SVIB) have either been isolated or deduced from cDNA sequences (Hillyard et al., 1992; Ramilo et al., 1992; Ramachandran et al., 1993).

The venoms of various species of spiders have also proven to be sources of peptides with specificity toward diverse calcium channels (Cohen et al., 1992, Lampe et al., 1993, Venema et al., 1992). The spider peptide omega-Aga IVa interacts with the "P"-type calcium channel (Mintz et al., 1992), whereas omega-Aga-IIIa (Venema et al., 1992; Cohen et al., 1992) is a more general inhibitor.

However, none of the isolated calcium antagonist peptides are completely specific. Higher concentrations of GVIA will inhibit a neuronal dihydropyridine sensitive calcium current (Wang et al., 1992) while MVIIC and SVIB interact with both N- and P/Q-type, and possibly other calcium channels (Sather et al., 1993; Ramachandran et al., 1993). Aga-IVa, while being a potent inhibitor of the P-type calcium channel, will also inhibit the Q-type channel (Sather et al., 1993). There is therefore a need for the isolation of additional ligands with unique molecular specificities toward neuronal calcium channels.

* Address correspondence to this author.

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1995.

0006-2960/95/0434-8341$09.00/0

The more specific blockers of N-type calcium channels, for example, the omega-conopeptide SNX-111[1] (synthetic MVIIA), have shown promise as potential therapeutics. SNX-111 is neuroprotective in a rat model of global ischemia (Valentino et al., 1993) and also has analgesic properties in the rat formalin test (Malmberg & Yaksh, 1994). However, this peptide lowers blood pressure (Bowersox et al., 1992) and, at high doses, can produce the "shaker" effect which is characteristic of many omega-conopeptides. Compounds which are directed toward the N-type calcium channel, but with different properties, would potentially be valuable compounds in the development of a pharmacology directed toward the N-channel.

In this report, we detail the isolation, characterization, synthesis, and initial study of a novel selective polypeptide antagonist of the N-type calcium channel.

MATERIALS AND METHODS

Starting Material and Extraction. The lyophilized venom of *Segestria florentina* was purchased from Biotox Ukraine through Ameri/Markt Associates, Gates Mills, OH. Venom was dissolved at room temperature in 0.1 N hydrochloric acid at 10 mg/mL. Particulates were removed by centrifugation, and the supernatant was stored at −80 deg °C until use.

Chromatography. Peptide isolation was performed on a Gilson HPLC described in Newcomb et al., (1988). For the isolation of SNX-325, the two-step elution paradigm described in Newcomb (1989) was used. The acidic venom extract (0.5 mL, or 5 mg) was neutralized immediately prior to chromatography by the addition of 200 μL of 3 M sodium acetate. Reversed-phase high-performance liquid chromatography (rpHPLC) was over a 4.5 × 300 mm column (Alltech Associates) packed with the Vydac 10 micron wide pore ("TP") octadecylsilica, and a guard column of 3 × 40 cm which was packed with the same material. A flow rate of 1.0 mL/min was used, at room temperature.

Following application of the crude venom, the column was eluted using a gradient of methanol in 0.025 M sodium phosphate pH 6.2 (0−100% methanol over 2 h). The column was reequilibrated in the aqueous buffer, and this was then changed to 0.1% trifluoroacetic acid (TFA). The remaining proteins and highly basic peptides were then eluted with a second gradient to methanol (0−100% over 1 h). Elution was monitored by absorbance at 220 nm with a Gilson Holochrome detector.

For comparison of the retention times of the native and synthetic SNX-325, a Hewlett Packard model 1050 HPLC was used. Chromatography was over a 4.5 × 250 mm octadecylsilica column packed with 5 μM particles (Vydac "TP"). For elution, the aqueous solvent was 0.1% TFA in water, and the organic solvent was acetonitrile containing 0.1% TFA. The column was equilibrated in the aqueous solvent, and a linear gradient of 0.67% acetonitrile/min was used for elution at 1 mL/min and 30 °C.

Amino Acid Analysis. For the characterization of the natural product, amino acid analysis was performed as previously described (Newcomb 1989) with the *o*-phthaldialdehyde procedure. Hydrolysis was performed for 20, 48, or 72 h at 105 °C. All values were, within the normal error of the analysis, in agreement with the sequence [the serine value obtained with the native peptide was slightly low (2.17 vs 3) but was also obtained with picomole level hydrolysates of the synthetic compound].

For the determination of tryptophan (not present in SNX-325), the chromatography of the crude venom was repeated (as above, but using 1 mg of the venom), and intrinsic fluorescence was monitored using a Hewlett-Packard Model 1046A fluorescence detector, with excitation at 260 nm and emission at 340 nm. Emitted light was passed through a 280 nm cutoff filter.

In the characterization of proteolytic fragments for the assignment of disulfide linkages (below), ion exchange chromatography with post-column ninhydrin derivatization was used for amino acid separation and detection.

Reduction and Alkylation. SNX-325 (2 nmol, from Figure 1) was dried from 200 μL of the HPLC fraction to about 5 μL in a tapered autosampler vial, and the vial was capped with a teflon lined septum. Derivatization with 4-vinylpyridine was performed as described [Hawke & Yuan, 1987; see also Tarr et al. (1983)].

Enzymatic Digestion. For digestion with carboxypeptidase Y, 200 pmol of the pyridethylated SNX-325 was dried and reconstituted in 100 μL of 10 mM sodium phosphate pH 6.2. Carboxypeptidase Y (0.5 μg, Pierce) was added, the digest was incubated at room temperature, and aliquots of 20 μL were removed at various times for analysis.

For digestion with chymotrypsin, 1 nmol of the pyridethylated SNX-325 was dried and reconstituted in 50 μL of 0.1 M potassium phosphate, pH 7.4. Chymotrypsin (1 μg, Worthington) was added, and the digest was incubated at 37 °C for 1 h. The fragments were chromatographed over the column described above (Gilson Instrument), but using a gradient from 0.1% aqueous TFA to methanol containing 0.075% TFA (0−40% over 45 min).

Sequence Analysis. Edman degradation was performed on the alkylated intact or digested peptides with an Applied Biosystems Model 120 Sequenator, using procedures recommended by the manufacturer for chemistry and identification.

Disulfide Assignment. This was performed by tryptic and chymotryptic digestion of the unreduced synthetic peptide as described for the omega-conopeptides (Chung et al., 1995). Fragments were separated by reversed phase liquid chromatography and subjected to amino acid analysis. When necessary, the position of cystine was determined by Edman degradation. Where tyrosine interfered with the determination of cystine, derivatization with iodine was used to modify its retention time.

Electrophoretic Analysis. Gel electrophoresis was performed on 10−20% acrylamide gradient gels using the Tricine buffered system described by Schagger and Jagow (1987). Precast gels and buffers were purchased from Novex, and 2% 2-mercaptoethanol was included in the sample loading buffer. The low molecular weight prestained standards provided by Bethesda Research Labs were used for calibration. Gels were stained with Coomassie blue dye.

Synthesis. SNX-325 was synthesized by solid-phase synthesis starting from Boc-Ser(Bzl)-Pam resin using "Boc" chemistry on an ABI Model 430A automated peptide synthesizer using procedures standardized for the synthesis of omega-conopeptides (Nadasdi et al., 1995). The peptide

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperizine-*N'*-(2-ethanesulfonic acid); rpHPLC, reversed-phase high performance liquid chromatography; SNX-230, synthetic omega-conopeptide MVIIC; SNX-111, synthetic MVIIA; TFA, trifluoroacetic acid.

was deblocked and cleaved off the resin with liquid HF, and the crude linear product was submitted to "air oxidation" in a dilute and buffered solution (pH 8) at 4 °C. A single major product was obtained which was subsequently purified by size exclusion chromatography (Sephadex G-25) and two systems of rpHPLC (using aqueous buffers of 10 mM potassium phosphate, pH 3.0, and 0.1% TFA).

Binding Assay. Native and synthetic SNX-325 were assayed for the inhibition of the binding of radioiodinated omega-conopeptides to rat brain synaptosomes as described (Ramachandran et al., 1993; Kristipati et al., 1994).

Oocyte Assay. The cRNAs from calcium channel α-1 subunit cDNAs described in Ellinor et al., (1993) (DOE1), Ellinor et al., (1994) (class B), Mori et al., (1991) (class A), Mikami et al., (1989) (class C), and Palma et al., (1994) (class E) were expressed in *Xenopus* oocytes by standard methods (Goldin, 1992). For expression, mRNA for the α-1 subunit was coinjected with cRNA for the rabbit skeletal $\alpha_2\delta$ (Ellis et al., 1988), and rabbit β (Ruth et al., 1989; class A, B, and C) or human β (Williams et al., 1992; class E) subunits in equimolar ratios. Approximately 50 nl was injected per oocyte, with the total cRNA concentration between 0.15 and 1.4 μg/μL.

Currents were recorded by two-electrode voltage clamp using a solution of 1 mM barium hydroxide, 40 mM triethylamine hydroxide, 2 mM potassium hydroxide, 5 mM HEPES, 0.1 mg/mL cytochrome *c*, and 115 mM sucrose which was adjusted to pH 7.4 with methane sulfonic acid. The recording solution also contained 0.1 mg/mL cytochrome *c*. Data were sampled at 4 kHz and filtered at 0.8 kHz utilizing an Axoclamp 2A amplifier (Axon Instruments) interfaced to PCLAMP software (Axon Instruments) for acquisition and analysis. Leak and capacitance currents were subtracted on-line by a P/4 protocol. Step pulses of from 90 to 0 mV were used to elicit currents every 15 s.

Sodium and Potassium Channels. Human neuroblastoma cells (IMR-32) were maintained in culture by standard methods. Periodically, the cells were plated and differentiated by the addition of 1 mM dibutyryl cyclic-AMP and 25 μM 5-bromodeoxyuridine. Cells were studied 4−13 days after differentiation.

Whole cell patch clamp analysis was performed using a bath solution of 150 mM sodium chloride, 5 mM potassium chloride, 2.6 mM potassium chloride, 2.6 mM calcium chloride, 10 mM glucose, and 10 mM HEPES, pH 7.3, and an internal solution containing 80 mM potassium chloride, 50 mM asparatic acid, 10 mM HEPES, pH 7.3, 10 mM EGTA, 2 mM ATP, and 2 mM magnesium chloride.

For peptide application, cells were placed in a flow through chamber (0.5−1 mL/min). SNX-325 was applied in saline with 0.1 mg/mL cytochrome *c*, through a small tube placed directly adjacent to the cell.

Sodium currents were elicited under voltage clamp conditions by a step from −80 to −10 mV, and potassium currents were elicited by a step from −80 to +50 mV.

Transmitter Release. Transmitter release assays were performed as described (Gaur et al., 1994). Briefly, hippocampal slices of 250−275 μM were incubated in saline containing 129 mM sodium chloride, 3.3 mM potassium chloride, 0.6 mM magnesium chloride, 0.6 mM calcium chloride, 1.2 mM sodium dihydrogen phosphate, 22 mM sodium bicarbonate, and 11 mM glucose. All solutions were saturated with 95% oxygen/5% carbon dioxide. For stimulation, 30 mM potassium chloride was substituted for sodium. Slices were incubated under oxygen/carbon dioxide in microtiter plates with 100 μL of saline containing 3 μCi of radiolabeled norepinephrine [levo (ring-2,4,6-^{3}H), with or without SNX-325, for 40 min]. Slices were washed and incubated in basal buffer and the stimulation buffers, with varying concentrations of SNX-325, for 0.5 min. The tritium content of the basal and stimulation incubations were then determined.

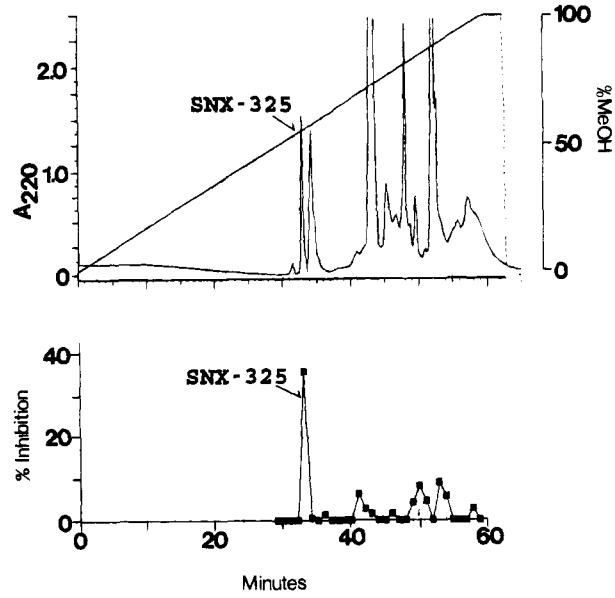

FIGURE 1: Purification of SNX-325. The upper figure shows the separation of those components in the crude venom which elute from the reverse-phase support with an aqueous buffer of 0.1% trifluoroacetic acid, but not with 0.025 M sodium phosphate, pH 6.2. The lower figure shows SNX-230 displacement activity in this chromatogram. For this experiment, 25 μL of the HPLC fraction was added to the incubation buffer. Control experiments showed that the solvents in the fractions had no significant effect on the binding. Values are the mean of duplicate determinations.

RESULTS

Isolation of SNX-325. We first performed preliminary experiments in which we looked for spider venoms that blocked calcium channel activity and which also contained, by polyacrylamide gel electrophoresis, a predominance of low molecular weight peptides. By these criteria, the venom of *S. florentina* proved to be an attractive starting material.

A two-step elution protocol was used to fractionate 5 mg of the dissolved venom on rpHPLC. The majority of the venom peptides were first eluted by a gradient to methanol using a near neutral pH sodium phosphate aqueous buffer. Following this, the proteins and highly basic peptides which did not elute under these conditions were eluted using an acidic aqueous buffer containing 0.1% TFA. Individual fractions were screened for their ability to displace the binding of radioiodinated SNX-230 (synthetic omega-conopeptide MVIIC) to rat brain synaptosomes. A peptide was localized in the acidic pH elution of the crude venom that significantly displaced SNX-230 from rat brain synaptosomes at nanomolar concentrations (Figure 1). Pooled fractions from this region of the chromatogram (30−35 min in Figure 1) also produced partial block of the cloned class A and DOE-1 calcium channel expressed in oocytes (approximate SNX-325 concentration 200 nM, data not il-

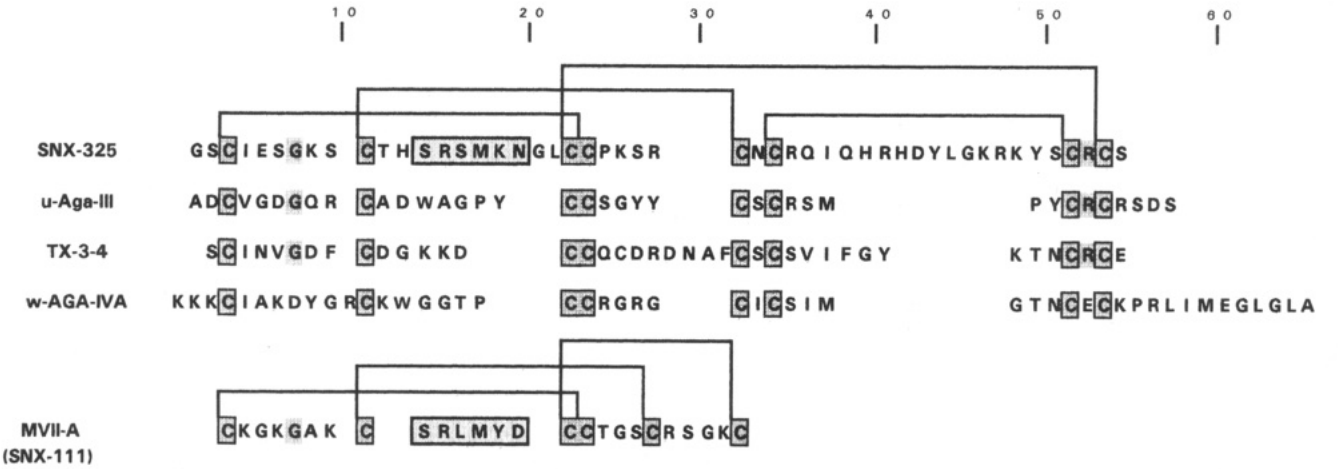

FIGURE 2: Amino acid sequence of SNX-325 and a comparison to some other venom peptides. Included in the comparison are a paralytic insectotoxin from the venoms of the spiders *Agelenopsis aperta* and *Hololena curta* [μ-Aga-III (Skinner et al., 1989); identical to CT-III (Stapleton et al., 1990)], a peptide active on the mammalian central nervous system from the spider *Phoneutra nigraventer* (Cordiero et al., 1993) and the calcium channel blocker ω-Aga-IVA (Mintz et al., 1992). The disulfide bridging patterns for synthetic SNX-325 and SNX-111 (synthetic omega-conopeptide MVIIA) are also illustrated. A glycine residue common to four of these peptides is shaded, as is an arginine residue common to three of the spider peptides. A box encloses a possible homology between SNX-325 and the contact region of MVIIA (SNX-111).

lustrated). Amino acid analysis of the SNX-230 displacing fraction characterized a peptide of 40−50 residues present at 2 nmol per mg of the dried venom, an amount comparable to the other predominant peptides of the venom. The peptide was assigned the SNX number (Neurex compound number) 325.

For the determination of the primary structure of SNX-325, 2.6 nmol of the peptide from the fractionation illustrated in Figure 1 was reduced, and the cysteine side chains were reacted with 4-vinylpyridine. The purified derivative was subjected to Edman degradation, resulting in the assignment of the sequence up to residue number 47. Digestion of the 4-vinylpyridine reacted peptide with carboxypeptidase Y followed by amino acid analysis with the *o*-phthaldialdehyde reagent showed the liberation of the free carboxyl form of serine, followed by arginine and tyrosine.

These results predicted a chymotryptic cleavage site near the carboxyl terminus of the peptide. The reduced and alkylated peptide was digested with chymotrypsin, and the fragments subjected to the Edman degradation. A fragment, SCRCS, was sequenced as a mixture with the peptide CCPK (one of two peptides containing tryptic cleavages which were recovered from the digest). The remainder of the digest fragments confirmed the results of degradation from the amino terminus and showed no additional sequence. On the basis of these results, and the results of the carboxypeptidase digestion, the C-terminal amino acid was assigned as the free carboxyl form of serine. The sequence of SNX-325 is illustrated at the top of Figure 2.

In order to confirm the sequence and the assignment of biological activity, SNX-325 was chemically synthesized. The synthetic peptide had the identical rpHPLC retention time as the native material (Figure 3). The disulfide pattern of the synthetic peptide was determined to be that shown in Figure 2 (see Materials and Methods).

Characterization of the Activity of SNX-325. Synthetic SNX-325 was subsequently used for the characterization of the biological properties of the peptide. In order to look at the specificity of its interactions with calcium channel subtypes, SNX-325 was assayed for block of barium currents

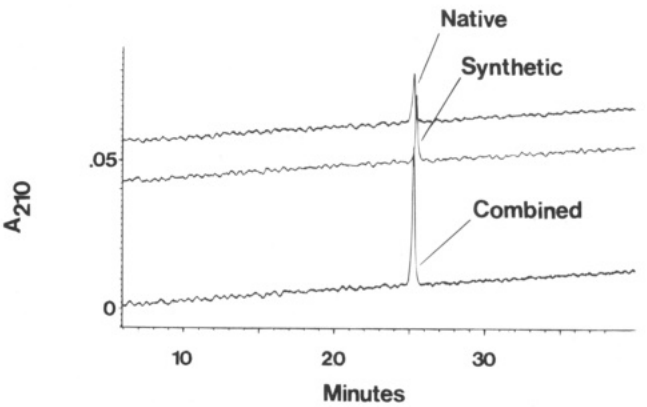

FIGURE 3: Coelution of native with synthetic SNX-325. The top chromatogram shows the elution of 0.3 μg of native SNX-325. The center chromatogram is 0.5 μg of the synthetic material. Coelution is shown in the bottom chromatogram. Elution conditions are in Materials and Methods.

through cloned calcium channels using the *Xenopus* oocyte expression system (Figure 4). At a concentration of 2 μM, SNX-325 blocked the barium current through class B (neuronal N) and class A, as well as class C (cardiac L-type channel), but had only a minor effect on the class E channel. At a concentration of 20 nM SNX-325, only the neuronal N type calcium current was affected. The extent of block of the N-channel at this concentration was similar to that observed at 2 μM.

To test the specificity of the interactions of SNX-325 with diverse cation channels, its effects were tested on sodium and potassium currents in cultured human neuroblastoma cells (IMR-32 cells). No effect of SNX-325 was noted at a concentration of 2 μM (three independent experiments, not shown).

These results defined SNX-325 as a specific blocker of the N-type calcium channel. Subsequent experiments compared the effects of SNX-325 with those of selective N-type calcium antagonists from cone snail venoms, as well as closely related synthetic derivatives, in several in vitro assays. Figure 5 illustrates the comparison of SNX-325 to SNX-111 (synthetic omega-conopeptide MVII-A; Olivera et al.,

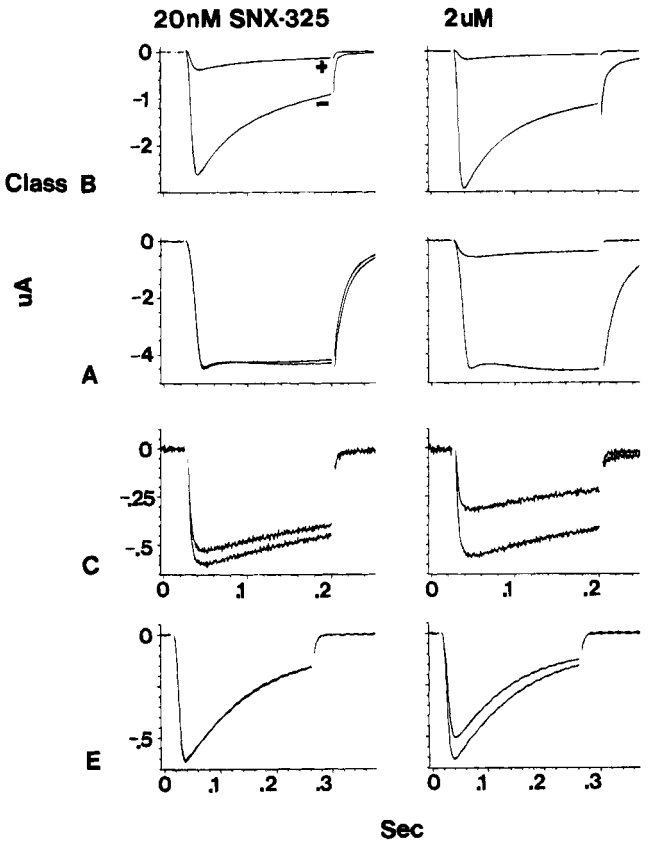

FIGURE 4: Electrophysiological characterization of the effects of synthetic SNX-325 on cloned calcium channels expressed in *Xenopus* oocytes. The class of calcium channel is indicated at the left of the figure. The left hand column illustrates the effects of 20 nM SNX-325, and the effects of 2 μM SNX-325 are indicated in the right hand column. In each panel, the trace with the lesser current is with peptide pesent (+), and the other trace (−) was taken preceding the application of peptide. All experiments were performed in triplicate, with similar results. The difference in currents at 20 nM SNX-325 with the class C type channel reflects rundown of the current.

1987), as well as SNX-194 (the Met-12 to Norleu-12 derivative of SNX-111): SNX-194 behaves, within experimental variation, identically to SNX-111 in the various in vitro assays, as well as in an in vivo neuroprotection assay [as described in Valentino et al. (1993)].

An IC_{50} of 75 nM was obtained for the displacement of radiolabeled SNX-111 from rat brain synaptosomal membranes (Figure 5A). In this assay, SNX-111 has an IC_{50} of about 2 pM (also Figure 5A), while values obtained for GVIA and SNX-194 are comparable (Kristipati et al., 1994; also unpublished data).

SNX-325 produced a potent inhibition of the potassium induced release of tritiated norepinephrine from hippocampal slices, with half-maximal block occurring at about 0.4 nM (Figure 5B). SNX-325 is about equipotent with SNX-111 in this assay. The slope of the dose−response curve for SNX-325 is much shallower than that obtained with SNX-111. The IC_{50}s for SNX-194 and GVIA in this assay are 0.1−0.2 nM (Gaur et al., 1994; also unpublished data).

Quantitative comparison of the effects of the SNX-325 and SNX-194 on the cloned expressed class B (N-type) calcium channels also resulted in similar potencies (Figure 5C). An IC_{50} of 10 nM was obtained for SNX-325, and an IC_{50} of 3 nM was obtained for SNX-194, while GVIA had an IC_{50} of 30 nM (Palma et al., 1994).

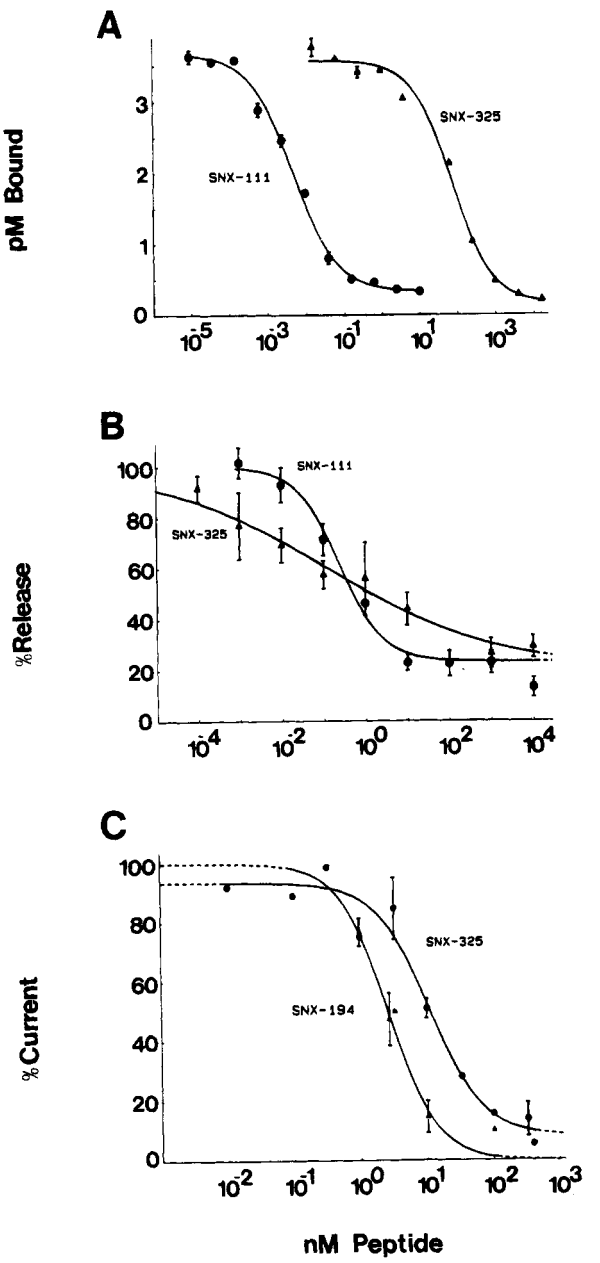

FIGURE 5: Comparison of the effects of SNX-325 to those of SNX-111 or its Met-12 to Norleu analog (SNX-194). (A) Displacement of radioiodinated SNX-111 from rat brain synaptosomal membranes by SNX-111 and SNX-325. Error bars are the standard deviations for duplicate determinations. (B) Inhibition of the potassium induced release of hippocampal norepinephrine by SNX-111 and SNX-325. Data points are the mean and standard error for points obtained in one experiments with $n = 6$ at each concentration. Essentially identical results were obtained in three independent experiments. (C) Inhibition of the barium current through the cloned expressed class B (N-type) calcium channel by SNX-325 and SNX-194. Three independent experiments were performed with both peptides. Data points are either the mean and standard deviations of results from three experiments or are single determinations.

DISCUSSION

The cysteine pattern of SNX-325 is common to a variety of bioactive peptides found in spider venoms (Figure 2). In this large group of peptides (Cordiero et al., 1993; Mintz et al., 1992; Quistad et al., 1991; Skinner et al., 1989; Stapleton et al., 1990), the third and fourth cysteine residues are adjacent, and a single amino acid separates the fifth and sixth, as well as the seventh and eighth cysteine residues. This

framework of cysteine residues is similar to the "3-loop" cysteine framework of the "omega" peptides from cone snail venoms [also illustrated in Figure 2; see Olivera et al. (1994) for review]. The cysteine pattern of the spider peptides is related to that of the omega-conopeptides by the insertion two additional cysteine residues in the region of the spider peptides that corresponds to the third "loop" of the cone snail peptides.

The pattern of cysteine residues forms a relatively constant template, and evolution of peptide specificity occurs largely through the variation of the intervening amino acids (Woodward et al., 1992). Although there are perhaps a few conserved features among SNX-325 and several spider peptides, there is no significant homology in this group other than the cysteine residues. Interestingly, the sequence of SNX-325 is similar to SNX-111 at a sequence (SRLMYD in SNX-111) which has been found to be an important determinant of calcium channel specificity among the cone snail omega-conopeptides (Ramachandran et al., 1993). This putative homology may be important in the similar specificity for the N-type calcium channel which is observed with SNX-111 and SNX-325.

SNX-325 is selective for calcium currents as opposed to sodium or potassium currents. Within the various cloned and expressed calcium channels tested here, SNX-325 has a broad spectrum of activity at higher concentrations but is selective for the N-type channel at lower concentrations. The potency of SNX-325 in in vitro assays for N-channel activity was equal to those of the omega-conopeptides which have been characterized from cone snail venoms. Consistent with its specificity for N-type calcium channels, SNX-325 at micromolar concentrations had no effect on the potassium induced release of D-aspartic acid from hippocampal slices (not illustrated). Likewise, SNX-325 was comparatively weak at displacing SNX-230 from rat brain synaptosomal membranes (IC_{50} 720−790 nM, not illustrated). Both of these assays are thought to reflect binding to the P/Q class of calcium channels (Ramachandran et al., 1993; Kristipati et al., 1994; Hillyard et al., 1992; Gaur et al., 1994).

The data suggest several potential uses for SNX-325. The peptide should be a useful complement to the omega-conopeptides as a tool for defining the function of the N-type calcium channel. This is important since all calcium antagonist peptides have absolute specificity only at low concentrations, and the concentration at which interaction occurs with additional subtypes is generally not determined. SNX-325, being a structurally unique N-channel antagonist, should have a distinct pattern of lower affinity interactions with other calcium channel subtypes, and this property should aid in the identification of N-channel involvement in different assay systems.

SNX-325 was close to equipotent with the omega-conopeptides in blocking the barium current through the cloned expressed class B channel. This result indicates that the two peptides interact with similar potencies with the cloned expressed N-type calcium channel. This conclusion is verified by the similar potencies of the two peptides in blocking the potassium induced release of hippocampal norepinephrine. In this latter assay, the inhibition caused by SNX-325 occurs over a broad concentration range, which might indicate multiple components to the interactions which lead to blockade of the release. Antagonists of the P/Q types of calcium channels are surprisingly potent in blocking hippocampal norepinepherine release (Gaur et al., 1994), and it is possible that some interaction of SNX-325 with these, or perhaps other, types of calcium channels is being observed in this assay.

In contrast, SNX-325 is considerably less potent than is SNX-111 at displacing radioiodinated SNX-111 from rat brain synaptosomal membranes. Several possibilities can explain these data, including differences in the channel tertiary structure in the two assays, differences in the ionic conditions of the assay, or possible microheterogeneity of the sequences of the channels used in the two assays. Alternatively, the sites of interaction of SNX-325 with the N-type calcium channel may be different in the SNX-111 displacement assay and the functional assays.

Regardless, the data suggest that SNX-325 interacts in a novel but relatively specific way with the N-type calcium channel. As such, SNX-325 provides a useful complement to the various omega-conopeptides as a tool for developing an understanding of structural features of specific interactions with the N- and possibly other types of high voltage activated calcium channels.

ACKNOWLEDGMENT

We thank our many colleagues at Neurex who contributed their insight and efforts to different aspects of this project. This work was supported by Neurex Corporation.

REFERENCES

Bowersox, S. S., Singh, T., Nadasdi, L., Zukowaska-Grojec, Z, Valentino, K., & Hoffman, B. B. (1992) *J. Cardiovasc. Pharmacol. 20*, 756−764.

Choi, D. W. (1988) *Trends Neurosci. 11*, 465−469.

Chung, D., Gaur, S., Bell, J. R., Ramachandran, J., & Nadasdi, L. (1995) *Int. J. Pept. Protein Res.* (in press).

Cohen, C. J., Ertel, E. A., Smith, M. M., Venema, V. J., Adams, M. E., & Leibowitz, M. D. (1992) *Mol. Pharmacol. 42*, 947−951.

Cordiero, M. N., Figueiredo, S. G., Valentim, A. C., Diniz, C. R., Eickstedt, V. R., Gilroy, J., & Richardson, M. (1993) *Toxicon 31*, 35−42.

Ellinor, P. T., Zhang, J.-F., Randall, A. D., Zhou, M., Schwarz, T. L., Tsien, R. W., & Horne, W. A. (1993) *Nature 363*, 455−458.

Ellinor, P. T., Zhang, J. F., Horne, W. A., & Tsien, R. W. (1994) *Nature 372*, 272−275.

Ellis, S. B., Williams, M. E., Brenner, R., Sharp, A. H., Leung, A. T., Campell, K. P., McKenna, E., Koch, W. J., Hui, A., Schwartz, A., & Harpold, M. M. (1988) *Science 241*, 1661−1664.

Gaur, S., Newcomb, R., Rivnay, B., Bell, J. R., Yamashiro, D., Ramachandran, J., & Miljanich, G. (1994) *Neuropharmacology 33*, 1211−1219.

Goldin, A. L. (1992) *Methods Enzymol. 207*, 266−279.

Hawke, D., & Yuan, P. (1987) *Appl. Biosystems User Bull. 28*, 1−8.

Hess, P. (1990) *Annu. Rev. Neurosci. 13*, 337−356.

Hillyard, D. R., Monje, V. D., Gaur, S., Mintz, I. M., Bean, B. P., Nadasdi, L., Ramachandran, J., Miljanich, G., Azimi-Zoonooz, A., McIntosh, J. M., Cruz, L., Imperial, J. S., & Olivera, B. M. (1992) *Neuron 9*, 67−77.

Hirning, L. D., Fox, A. P., McClesky, E. W., Olivera, B. M., Thayer, S. A., Miller, R., & Tsien, R. (1988) *Science 239*, 57−61.

Janis, R. A., Silver, P. J., & Triggle, D. J. (1987) *Adv. Drug Res. 18*, 309−537.

Kristipati, R., Nadasdi, L., Tarczy-Hornoch, K., Lau, K., Miljanich, G. P., Ramachandran, J., & Bell, J. R. (1994) *Mol. Cell. Neurosci. 5*, 219−228.

Lampe, R. A., Defeo, P., Davison, M. D., Young, J., Herman, J. L., Spreen, R. C., Horn, M. B., Mangano, T. J., & Keith, R. A. (1993) *Mol. Pharmacol. 44*, 451−460.

Malmberg, A. B., & Yaksh, T. L. (1994) *J. Neurosci. 14*, 4882−4890.
Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumiya, S., & Numa, S. (1989) *Nature 340*, 230−233.
Miller, R. J. (1987) *Science 235*, 46−52.
Miljanich, G. P., & Ramachandran, J. (1995) *Annu. Rev. Pharmacol. Toxicol. 35*, 707−734.
Mintz, I. M., Venema, V. J., Swiderek, K. M., Lee, T. D., Bean, B. P., & Adams, M. (1992) *Nature 355*, 827−829.
Mori, Y., Friedrich, T., Kim, M.-S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hoffman, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T., & Numa, S. (1991) *Nature 350*, 398−402.
Nadasdi, L., Yamashiro, D., Chung, D., Tarczy-Hornoch, K., Adriensen, P., & Ramachandran, J. (1995) *Biochemistry* (in press).
Newcomb, R. (1989) *LC-GC 7*, 570−578.
Newcomb, R., Fisher, J., & Scheller, R. H. (1988) *J. Biol. Chem. 263*, 12514−12521.
Olivera, B. M., McIntosh, J. M., Cruz, L. J., Luque, F. A., & Gray, W. R. (1984) *Biochemistry 23*, 5087−5090.
Olivera, B. M., Cruz, L., Santos, V., LeCheminant, G. W., Griffin, D., Zeikus, R., McIntosh, J. M., Galyean, R., Varga, J., Gray, W. R., & Rivier, J. (1987) *Biochemistry 26*, 2086−2090.
Olivera, B. M., Miljanich, G. P., Ramachandran, J., & Adams, M. E. (1994) *Annu. Rev. Biochem. 63*, 823−867.
Palma, A., Ruby, R., Zhou, M., Hashimoto, C., Rock, D., Brogley, L., & Horne, W. (1994) *Soc. Neurosci. Abstr.* No. 34.7.
Quistad, G. B., Reuter, C. C., Skinner, W. S., Dennis, P. A., Suwanrumpha, S., & Fu, E. W. (1991) *Toxicon 29*, 329−336.
Ramachandran, J., Nadasdi, L., Gohil, K., Kristipati, R., Tarczy-Hornoch, K., Gaur, S., Singh, T., Bell, J., & Miljanich, G. (1993) in *Perspectives in Medicinal Chemistry* (Testa, B., Kyburz, E., Fuhrer, W., & Geiger, R., Eds.) pp 378−388, VCH Publishers, New York.
Ramilo, C. A., Zafarella, G. C., Nadasdi, L., Hammerland, L. G., Yoshikami, D., Gray, W. R., Kristipati, R., Ramachandran, J., Miljanich, G., Olivera, B. M., & Cruz, L. J. (1992) *Biochemistry 31*, 9919−9926.
Ruth, P., Rohrkasten, A., Biel, M., Bosse, E., Regulla, S., Meyer, H. E., Flockerzi, V., & Hoffmann, F. (1989) *Science 245*, 1115−1118.
Sather, W. A., Tanabe, T., Zhang, J.-F., Mori, Y., Adams, M. E., & Tsien, R. W. (1993) *Neuron 11*, 291−303.
Schagger, H., & Jagow, G. V. (1987) *Anal. Biochem. 166*, 368−379.
Siesjo, B. K., & Bengtsson, F. (1989) *J. Cereb. Blood Flow Metab. 9*, 127−140.
Skinner, W. S., Adams, M. E., Quistad, G. B., Kataoka, H., Cesarin, B. J., Enderlin, F. E., & Schooley, D. A. (1989) *J. Biol. Chem. 264*, 2150−2155.
Stapleton, A., Blankenship, D. T., Ackermann, B. L., Chen, T.-M., Gorder, G. W., Manley, G. D., Palfreyman, M. G., Coutant, J. E., & Cardin, A. D. (1990) *J. Biol. Chem. 265*, 2054−2059.
Tarr, G. E., Black, S. D., Fujita, V. S., & Coon, M. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6552−6556.
Tsien, R. W., Lipscombe, D., Madison, D. V., Bley, K. R., & Fox, A. P. (1988) *Trends Neurosci. 11*, 431−438.
Wang, X., Treistman, S. N., & Lemos, J. (1992) *J. Physiol. 445*, 181−199.
Valentino, K. V., Newcomb, R., Gadbois, T., Singh, T., Bowersox, S., Bitner, S., Justice, A., Yamashiro, D., Hoffmann, B. B., Ciaranello, R., Miljanich, G., & Ramachandran, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7894−789.
Venema, V., J., Swiderek, K. M., Lee, T. D., Hathaway, G. M., & Adams, M. E. (1992) *J. Biol. Chem. 267*, 2610−2615.
Woodward, S. R., Cruz, L. J., Olivera, B. M., & Hillyard, D. R. (1990) *EMBO J. 9*, 1015−1020.

BI950468S