Age-Related Decrease in Omega Conotoxin Binding to Rat Cardiac Synaptosomes (43380)

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Abstract. **A cardiac synaptosomal preparation developed by this laboratory was used to study neuronal calcium channels in aging rat heart. Ca^{2+} channels were quantified by measuring binding of iodinated omega conotoxin, which is reported to specifically block neuronal Ca^{2+} channels. We determined the binding of [^{125}I]-omega conotoxin GVIA to a synaptosomal preparation from the hearts of 6- and 24-month-old male Fisher 344 rats. The maximum number of binding sites (B_{max} ± SD, fmol/mg protein) is lower in preparations from 24-month (2.2 ± 0.6) than from 6-month (3.4 ± 0.7)-old rats. This decrease in number of binding sites suggests an age-related reduction in the number of neuronal calcium channels. Since calcium is essential for exocytotic release of norepinephrine and is made available intracellularly through neuronal calcium channels, the reduction in neuronal calcium channel number may explain, in part, our previous observations of diminished release of norepinephrine in senescent hearts.**

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Release of the neurotransmitter norepinephrine (NE) by neurons in the heart is an important regulator of cardiac function. When these neurons are stimulated to release NE, the rate and force of cardiac contraction increases resulting in increased cardiac output. This provides a homeostatic mechanism for maintaining blood pressure. Aging in humans and laboratory animals is associated with a decrease in the ability of the heart to respond via these mechanisms (see Ref. 1 for references). Previously, we have shown that the age-related reduction in cardiac function in rats is due at least in part to a reduction in the ability of cardiac neurons to release NE (2). To further investigate this phenomenon, we have developed a method to isolate nerve endings (synaptosomes) from adult and senescent rat hearts (3). These cardiac synaptosomes possess several functional characteristics that are similar to the brain synaptosomes, as indicated by the dynamics of the ^{3}H-NE uptake. In brief, the desmethylimipramine (DMI)-sensitive, metanephrine-insensitive ^{3}H-NE uptake by the cardiac synaptosomes is temperature sensitive and is greatly attenuated in hypotonic milieu (3). In addition, the K_m and V_{max} values of ^{3}H-NE uptake by the cardiac synaptosomes closely parallel the values obtained previously using the brain synaptosomes (3). Using this preparation, we find that the cardiac synaptosomes obtained from senescent rats have a substantially diminished capacity to release NE (4). Thus, the age-related reduction in cardiac neuronal NE release seems to be attributable to a defect in the nerve terminal.

Calcium ions are required for the exocytotic release of NE (5, 6), and the influx of extracellular Ca^{2+} into the nerve terminal during depolarization triggers NE release (7). Several previous studies indicated that Ca^{2+} homeostasis may be impaired during aging. Depolarization-induced Ca^{2+} uptake in brain synaptosomes obtained from rats and other rodents decreases during aging (8, 9). Depolarization-induced NE release (a Ca^{2+}-dependent process) is diminished in isolated nerve heart preparations from older rats (2), but tyramine-induced NE release (a Ca^{2+}-independent process) remains unchanged (10). Furthermore, the Ca^{2+} ionophore, ionomycin, ameliorates the effect of age on diminished NE release (11). To further explore the mechanism responsible for decreased NE release in older nerve heart preparations and its relationship to Ca^{2+}, we decided to study the Ca^{2+} channels of sympathetic nerves innervating the heart.

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Omega conotoxin, a neurotoxic peptide obtained from the marine snail *Conus geographus*, is a specific blocker of voltage-sensitive neuronal Ca^{2+} channels (12). Omega conotoxin blocks the release of several neurotransmitters in a dose-dependent manner (13, 14). Omega conotoxin-sensitive Ca^{2+} channels have been reported in neuronal tissues from brain and numerous peripheral sites, such as myenteric plexus of ilium (15, 16), vas deferens and urinary bladder (17), and submandibular parasympathetic ganglia (18). Recently, it was reported that omega conotoxin-binding sites decrease in the striatum and cortex of 18- to 24-month-old rat brains (19, 20).

Brain synaptosomes have been widely used to examine the properties of voltage-sensitive Ca^{2+} channels (21). Following the method of preparing brain synaptosomes (22), we have developed a method for obtaining synaptosomes from the rat heart (3). Omega conotoxin binding to cardiac synaptosomal preparations obtained from 6-month and 24-month-old male Fisher 344 rats was measured to determine whether a reduction in neuronal Ca^{2+} channels could account for our previous findings of decreased cardiac neuronal NE release during aging.

Materials and Methods

Materials. Iodinated omega conotoxin GVIA (sp act 2200 Ci/mmol) was obtained from NEN Research, Boston, MA. Unlabeled omega conotoxin was obtained from Peninsula Laboratories, Belmont, CA. Collagenase (Type 2) was purchased from Worthington Biochemical Corp., Freehold, NJ. All other chemicals were obtained from Sigma Chemical So., St. Louis, MO. GF/B Whatman filters were obtained from Brandel, Gaithersburg, MD.

Animals. Male Fisher 344 rats at 6 and 24 months of age were used. These rats were maintained under barrier conditions at Harlan Laboratories, Inc. (Indianapolis, IN). In our institution, rats were housed, under barrier conditions, in standard filtered cages (two per cage) in a temperature-regulated environment (21 ± 1°C) on a 12:12-hr light:dark cycle. The animals were fed *ad libitum* on a pasteurized rodent diet (20% protein and 5% fat) and on autoclaved water adjusted to pH 3.

Preparation of Synaptosomes. Cardiac synaptosomes were obtained by a modification (3) of the Gray and Whittaker (22) method.

In brief, 6-month and 24-month-old male Fisher 344 rats were decapitated and the hearts were removed and dissected free of surrounding fat and connective tissue. Only one heart was used for each synaptosomal preparation. Individual heart was minced finely in 0.32 *M* sucrose EGTA (1 m*M*) solution. The mince was digested for 40 min at 37°C with collagenase (75 mg/g wet wt). After digestion, low-speed centrifugation was carried out to remove any remaining collagenase. The resulting pellet was suspended in 10 vol (by weight) of 0.32 *M* sucrose, homogenized using a Teflon glass homogenizer with 20 strokes, and centrifuged for 10 min (650*g* at 4°C). The pellet was resuspended in 5 vol (by weight) of 0.32 *M* sucrose, homogenized with 10 strokes, and centrifuged for 10 min (650*g* at 4°C). The supernatant was combined with the supernatant of the previous centrifugation and recentrifuged for 20 min (21,000*g* at 4°C). The final pellet was suspended in ice-cold HEPES buffer (pH 7.4 at 22°C) containing 50 m*M* HEPES/NaOH, 144 m*M* NaCl, 5 m*M* KCl, and 10 m*M* glucose, and used immediately for radioligand binding studies.

Omega Conotoxin Binding Assay. Iodinated omega conotoxin (10–160 p*M*) was used to perform the radioligand binding assays. The assay volume was 0.3 ml and it contained 50–100 μg of synaptosomal protein and 0.2% bovine serum albumin in HEPES buffer (see above). After 40 min of incubation at 37°C, the reaction was terminated by rapid filtration under vacuum through GF/B filters (Whatman) using a Brandel cell harvester. The filters were washed four times with 2 ml of ice-cold HEPES buffer and were counted using a Beckman gamma counter. Specific binding of omega conotoxin to cardiac synaptosomes was defined as the difference between binding in the presence and in the absence of 0.1 m*M* unlabeled omega conotoxin. The binding studies were carried out simultaneously in a paired manner using the synaptosomal preparations from the 6- and 24-month-old age groups.

Inhibition Studies. Ca^{2+} displacement curves were determined by preincubating cardiac and brain synaptosomes for 10 min in the presence of Ca^{2+} (0.01–10.0 m*M*) at 37°C. Binding assays were then carried out using iodinated omega conotoxin (60 p*M*), as described above. The specific binding of omega conotoxin (as a percentage of control) obtained in the presence of various concentrations of calcium was plotted against the calcium concentration (m*M*) to derive an estimate of the IC_{50} value. Drug inhibition studies using diltiazem and nifedipine were carried out in a similar manner.

Data Analysis. Experimental points were obtained in quadruplicate and a mean value was determined. Student's *t* test (paired, two-tailed) was used to determine the statistical significance of the data and the results were considered significant for the *P*-value of less than 0.05.

Protein content was determined by the method of Bradford (23) using reagents prepared by Biorad (Richmond, CA).

Results

The binding of omega conotoxin to neuronal calcium channels is irreversible (20, 24). Since the application of Scatchard analysis in deriving binding parameters is appropriate only when the ligand-receptor

interaction is reversible (25), its use in omega conotoxin radioligand binding studies is questionable (24). Therefore, to derive the value of B_{max}, we plotted the amount of labeled omega conotoxin specifically bound against the concentration of ligand, and the maximal binding capacity was obtained by extrapolating the plateau of the curve describing the specific binding to the y axis. Figure 1 shows the pooled data for the saturation isotherms of specific binding in two ages. To remove the possibility of bias in the interpretation, the data were analyzed independently by three of the authors (A. M. P., M. D. J., J. R.). In addition, some of the experiments were performed and analyzed "blind" in that the individual performing the study (A. M. P.) had no prior knowledge of the age of the animals from which the synaptosomal preparations were derived. Table I lists the B_{max} values from each individual experiment for 6- and 24-month-old animals. The last two experiments listed in Table I were performed and analyzed blind as discussed above. In cardiac synaptosomes, the specific binding represents 25–45% of the total binding and it saturates at ligand concentrations of 90–120 pM. In four out of seven experiments reported in Table I, specific binding to cardiac synaptosomal preparations from 6-month-old rats appeared to undergo an additional increase at higher ligand concentrations (>130 pM). This was not observed in preparations from 24-month-old rats.

The nonspecific binding of omega conotoxin is relatively high in our cardiac synaptosomal preparation. Using brain synaptosomes, we found lower nonspecific binding (<15%), as has been reported previously by others (24). The high nonspecific binding in cardiac synaptosomes relative to brain synaptosomes is likely attributable to the substantially lower abundance of neuronal tissue in the heart. Electron micrographs of our cardiac synaptosomal preparation clearly reveal the presence of synaptosomes (unpublished observations), but also confirm the presence of a large number of nonsynaptosomal structures to which omega conotoxin may bind nonspecifically.

The number of omega conotoxin binding sites is significantly lower in synaptosomal preparations obtained from 24-month-old hearts than in preparations obtained from 6-month-old hearts (2.2 ± 0.6 and 3.4 ± 0.7 fmol/mg protein (± SD), respectively; $P < 0.01$ by two-tailed paired t test, $n = 7$). We find no change during aging in the number (fmol/mg protein ± SD) of omega conotoxin binding sites in synaptosomal preparations from decerebellate brains (6 months: 427 ± 70, $n = 3$; 24 months: 442 ± 26, $n = 2$). These values for decerebellate brain are similar to the values reported by others (24, 26).

Figure 2 shows the calcium inhibition curves for the cardiac and brain synaptosomes obtained from 6-month-old rats. It appears that Ca^{2+} IC_{50} values for both these types of synaptosomes fall in the range of 1.5–2.5 mM Ca^{2+}, which is similar to the Ca^{2+} IC_{50} value of 1.6 mM obtained using brain synaptosomes by Knaus *et al.* (27). Ca^{2+} in concentrations of 2 mM and above completely inhibited the specific binding of omega conotoxin to the cardiac synaptosome. There was no age difference in Ca^{2+} displacement of omega conotoxin binding to cardiac synaptosomes.

To further characterize omega conotoxin binding to cardiac synaptosomes, the influence of two calcium antagonists, diltiazem and nifedipine, on omega conotoxin binding was tested. We found no effect of diltiazem (1–10 μM) on omega conotoxin binding ($n = 3$), and nifedipine only partially inhibited the binding (19% at 10 μM, $n = 2$).

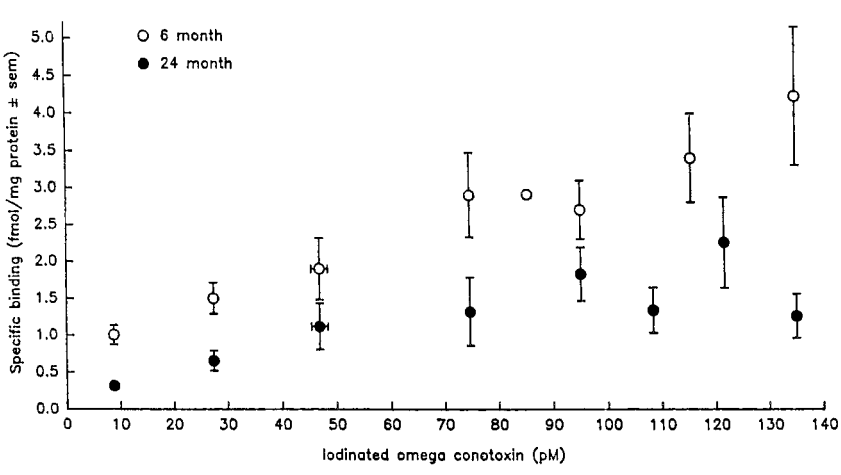

Figure 1. Specific binding of omega conotoxin to cardiac synaptosomes obtained from 6- and 24-month-old rats ($n = 7$). Synaptosomal protein (50–100 μg) was incubated at 37°C for 40 min with the indicated concentration of the ligand and the reaction was terminated by rapid filtration. The specific binding was obtained by subtracting nonspecific binding (obtained in the presence of 0.1 μM unlabeled omega conotoxin) from the total binding (obtained in the absence of unlabeled omega conotoxin). Each point represents mean (±SE) value derived from seven experiments (four determinations per ligand concentration for each experiment) in each age group.

Table I. Omega Conotoxin Binding in Cardiac Synaptosomes Obtained from 6- and 24-month-old Fisher 344 Rats[a]

Experiment	Maximal binding (fmol/mg protein)	
	6 month	24 month[b]
1	2.2	1.3
2	3.9	2.2
3	2.6	1.8
4	3.9	2.6
5	3.0	2.0
6	3.4	3.0
7	3.8	2.2

[a] The maximal binding of omega conotoxin in cardiac synaptosomes was derived as explained in the Methods section. The experiments in 6- and 24-month-old rats were performed in a paired manner. In addition, the last two experiments were performed blind as described in the Results section. The values in the 24-month age group are significantly less than the corresponding values in the 6-month age group.
[b] $P < 0.05$; Student's paired t test.

Discussion

The amount of specific binding of omega conotoxin to cardiac synaptosomes is relatively small when compared with the specific binding of omega conotoxin to brain synaptosomes. This suggests that the cardiac synaptosomal preparation may contain a lower concentration of synaptosomes than the brain synaptosomal preparation.

Our results demonstrate that calcium inhibits omega conotoxin binding in a concentration-dependent manner. Moreover, there is no effect of diltiazem on omega conotoxin binding and, in view of the evidence that diltiazem does not block neuronal calcium channels (12), our studies suggest that omega conotoxin binds to neuronal calcium channels in the cardiac synaptosomal preparation.

The maximum capacity of omega conotoxin binding is significantly diminished in cardiac synaptosomes obtained from older rats. Since omega conotoxin binds specifically to neuronal calcium channels, the above finding suggests that there is an age-related decrease in cardiac neuronal calcium channels. Previously, this laboratory has demonstrated age-related reductions in the release of NE from intact heart nerve preparations (2) and from cardiac synaptosomal preparations (4). Our recent studies indicate the K^+-stimulated (60 mM) ^{3}H-NE release from cardiac synaptosomes is reduced by at least 30% in the presence of 10 nM omega conotoxin, and synaptosomal K^+-stimulated $^{45}Ca^{2+}$ uptake is reduced by 39% in the presence of 100 nM omega conotoxin (unpublished observations). Therefore, we believe that omega conotoxin-sensitive neuronal Ca^{2+} channels are involved in NE release from cardiac sympathetic neurons and that a reduction in channel number in old age leads to decreased entry of calcium ions upon depolarization and subsequent diminished release of the neurotransmitter NE from the neuronal cell.

The age-related reduction in omega conotoxin

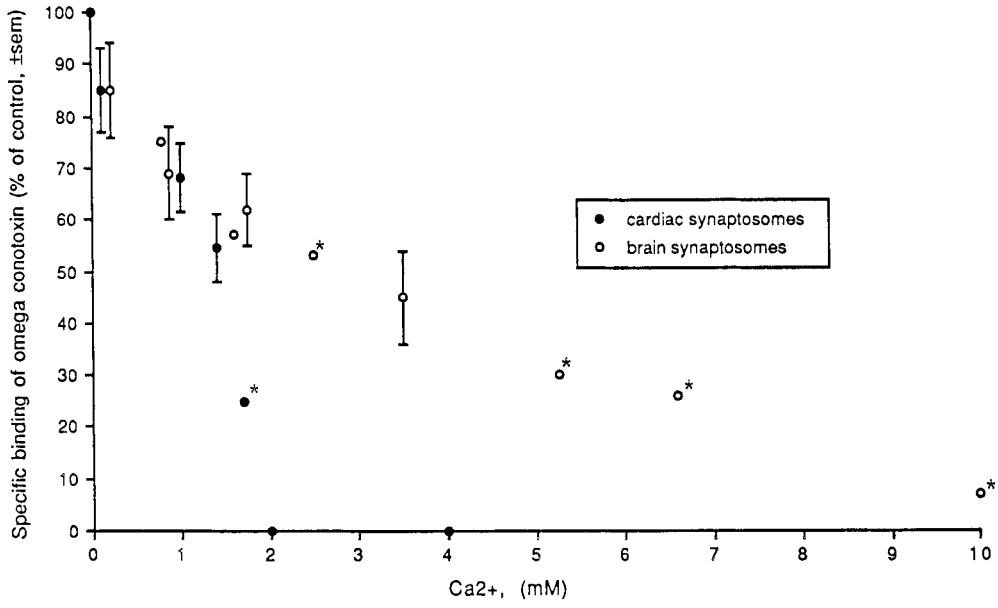

Figure 2. Calcium inhibition curves for omega conotoxin binding to cardiac and brain synaptosomes. The synaptosomes were preincubated for 10 min at 37°C with the indicated concentration of calcium before adding labeled (60 pM) or unlabeled (0.1 μM) omega conotoxin. The binding assays were then carried out as described in the Methods section (also see Fig. 1). Each point (except those marked by an asterisk) represents the mean (±SE) from seven (cardiac) and three (brain) separate experiments. Asterisk represents a mean value from four determinations from a single experiment.

binding could be due to either a decrease in the number of omega conotoxin-sensitive neuronal calcium channels per neuron or a decrease in the number of neurons. We believe that the reduction in the number of neuronal calcium channels per neuron is responsible for the diminution in NE secretion in older hearts, because the calcium ionophore ionomycin stimulates equivalent NE secretion in older and younger hearts (11). Nevertheless, McLean *et al.* (28) showed an increased degeneration of adrenergic neurons in old heart; therefore, reduced neuronal number may contribute to reduced omega conotoxin binding. Further experiments are needed to resolve these possibilities.

Although our studies using ionomycin indicate that a defect in calcium transport rather than in responsiveness to Ca^{2+} occurs with increasing age (11), there may be additional factors that contribute to diminished NE secretion. In rat brain synaptosomes, omega conotoxin produces only partial blockade (40–50%) of K^+-stimulated Ca^{2+} uptake (29, 30), and in our preliminary studies using cardiac synaptosomes, omega conotoxin (10 n*M*) diminished K^+-stimulated (60 m*M*) ^{3}H-NE release by only 30%. Thus, alterations at other sites may also contribute to reduced NE release.

For example, calcium ions regulate the activity of protein kinase C (PKC), which is an important phosphorylating enzyme with a major role in neurotransmitter release (31). Thus, alterations in phosphorylation by PKC may contribute to the decrease in calcium-dependent release of NE with age. PKC activity decreases during aging in blood vessels and in brain (32, 33). Additionally, the ability of PKC activation to stimulate blood vessel contraction and to potentiate serotonin release is reduced during aging (32, 33). Similar alterations may exist in aging cardiac sympathetic neurons.

Our data indicate that it would be extremely important to develop a novel pharmaceutical agent to "bypass" the compromised calcium channels in aging sympathetic neurons and carry calcium ions inside the neuronal cells via an alternate route, thus reestablishing the normal neurosecretory process. Calcium ionophores have this property and are used experimentally. It is possible that in the future, similar drugs with selective actions on peripheral neural tissues may be useful clinically for treatment of certain geriatric cardiovascular disorders.

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