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Studies on curare-like action of the tripeptide carbobenzoxy-Gly-Gly-Arg-β-naphthylamide in mouse diaphragm

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Abstract

The effects of several protease substrates or protease inhibitors on neuromuscular transmission in the isolated mouse phrenic nerve-diaphragm were studied. *N*-Carbobenzoxy-Gly-Gly-Arg-β-naphthylamide (Z-GGR-N) but none of the other agents inhibited the nerve-evoked muscle contractions. By means of electrophysiological studies, Z-GGR-N was found to inhibit the amplitudes of both end-plate potentials (epps) ($IC_{50} \sim 50$ μM) and miniature end-plate potentials (mepps) but to increase the frequencies of mepps. This tripeptide could protect the nicotinic acetylcholine receptor from the irreversible inhibitory action of α-bungarotoxin on the mouse diaphragm. Similar to D-tubocurarine, Z-GGR-N induced tetanic fading both of nerve-evoked muscle contractions and of the amplitude of epps. Furthermore, Z-GGR-N exhibited a greater depression of the amplitudes of train-epps than those of mepps, similar to that of hexamethonium and D-tubocurarine, indicating an effect on presynaptic autoreceptors. Suramin, which could competitively reverse the inhibitory effects of non-depolarizing relaxants, acted in this study as an antagonist of all the effects of Z-GGR-N, especially those at the presynaptic site. All of these findings suggest that Z-GGR-N is a novel tripeptide possessing curare-like actions at both presynaptic and postsynaptic sites and that these actions are independent of its protease substrate property. © 1998 Elsevier Science B.V.

Keywords: *N*-Carbobenzoxy-Gly-Gly-Arg-β-naphthylamide; Suramin; Nicotinic receptor

1. Introduction

Recent studies of transmitter release have shed light on the synaptic proteins involved in the membrane fusion between the synaptic vesicle and the nerve terminal plasmalemma. Among these processes prior to exocytosis, protease activity, such as that of metalloendoprotease (Barrett, 1977), has been implicated in transmitter release. Thus, protease inhibitors or substrates, such as metalloendoprotease inhibitors, have been reported to affect neurotransmitter release from nerve terminals in the peripheral (Baxter et al., 1983) and central nervous system (Frederick et al., 1984). In the present study, we attempted to examine whether protease inhibitors and substrates e.g. *N*-carbobenzoxy-Gly-Gly-Arg-β-naphthylamide (Z-GGR-N), *N*-carbobenzoxy-Gly-Phe-amide (Z-GF-N), *N*-carbobenzoxy-Pro-Leu-Gly-amide (Z-PLG-N), SCH 39370 (*N*-[*N*-[-1-(*S*)-carboxyl-3-phenylpropyl]-(*S*)-phenyl-alanyl]-(*S*)-isoserine) and aprotinin, exerted an effect on the neuromuscular transmission of the isolated mouse phrenic nerve-diaphragm. It is interesting to note that Z-GGR-N but not other protease substrates and inhibitors, was an antagonist of the nicotinic acetylcholine receptor. The curare-like effect of Z-GGR-N was similar to that previously reported for tripyridine (Lin-Shiau et al., 1992; Hsu et al., 1993a) and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) (Hsu et al., 1993b, 1994).

2. Materials and methods

2.1. Animals and muscle tension recordings

Mice of the ICR strain (18–25 g) were used in the present study. Mice were killed by rapid cervical dislocation to minimize suffering. Phrenic nerve-hemidiaphragm preparations were isolated and suspended in 5–10 ml of oxygenated modified Krebs solution (in mM: NaCl, 131; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 12.5 and glucose, 11) maintained at 37 ± 0.5°C and pH 7.2–7.4. Muscle contractions were elicited by electrical stimulation

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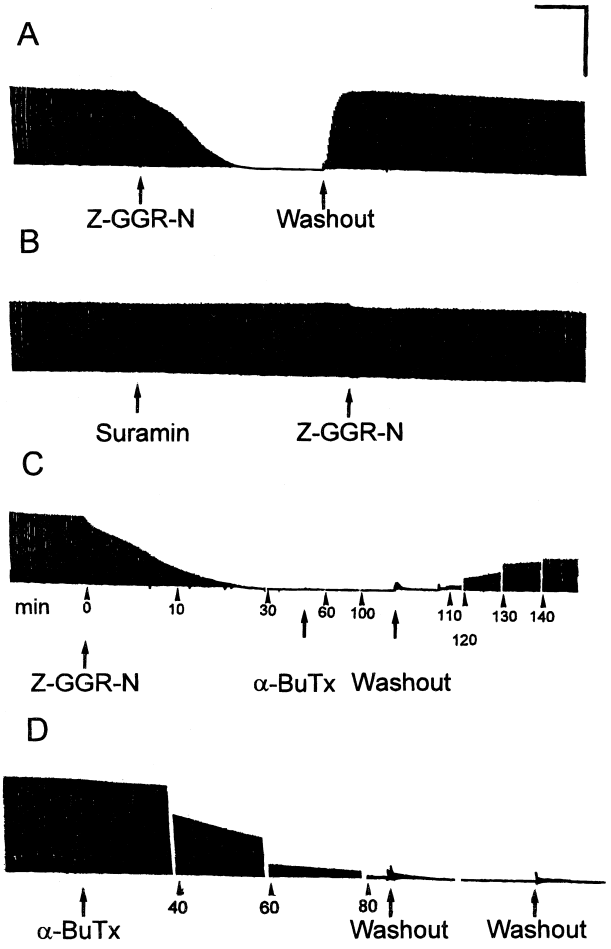

Fig. 1. The inhibitory effects of *N*-carbobenzoxy-Gly-Gly-Arg-β-naphthylamide (Z-GGR-N) on nerve-evoked muscle contractions of mouse diaphragm. Z-GGR-N (0.1 mM) produced a reversible inhibitory effect on nerve-evoked muscle contractions (0.1 Hz) (A) which was prevented by 0.1 mM suramin (B). Pretreatment with Z-GGR-N 0.1 mM (C) prevented the irreversible blocking effect of α-bungarotoxin (2 μg/ml) (D). Calibrations: 3 min and 2 g.

Table 1
Effects of protease substrates or inhibitors on nerve-evoked muscle contractions

Chemical	Muscle contraction (g)		*n*
	control (before drug)	after treatment	
Z-GGR-N (0.1 mM)	1.96 ± 0.50	0.1 ± 0.10[a]	7
Z-PLG-N (0.1 mM)	2.05 ± 0.12	2.18 ± 0.22	5
Z-GF-N (0.1 mM)	1.94 ± 0.32	2.03 ± 0.25	5
SCH 39370 (0.05 mM)	1.78 ± 0.40	1.75 ± 0.40	3
Aprotinin (36 μg/ml)	2.02 ± 0.17	1.96 ± 0.16	5

The muscle contractions of the isolated mouse phrenic nerve-diaphragm evoked by electrical stimulation of the nerve were recorded 20 min after chemical treatment and compared with those recorded prior to the treatment (paired comparison). Data are presented as means ± S.E.
[a] $P < 0.001$ as compared with control (before drug).

of the phrenic nerve with supramaximal rectangular pulses of 0.05 ms duration at 0.1 or 20 Hz and recorded via an isometric transducer (Grass FT. 03) on a Grass Model 7E polygraph. The muscle resting tension was adjusted to 1 g and the preparation was then allowed to stand for 20–30 min before starting the experimental protocol.

2.2. Intracellular recordings

The isolated nerve-diaphragm muscle was pinned out on the Sylgard-coating bottom of the recording chamber containing Krebs solution. End-plate potentials (epps) and miniature end-plate potentials (mepps) were measured by an intracellular glass microelectrode with a high-impedance amplifier (Axoclamp 2A; Axon Instruments, Burlingame, CA) in bridge mode and series connected to a FL4 four-pole bessel filter (Dagan, Minneapolis, MA). Microelectrodes were filled with 3 M KCl and had a resistance of 5–15 MΩ. Epps were evoked by stimulating the phrenic nerve at a frequency of 0.1 or 20 Hz with 0.02 ms supramaximal rectangular pulses; the diaphragm was immobilized by the cut muscle method (Barstad and Lilleheil, 1968). The signals of intracellular recordings were displayed on an oscilloscope (Tektronix 2221A; Beaverton, OR) and stored in a videotape recorder (Neuro-Corder Recording Unit; Neuro Data Instruments Corp., NY). The signal was then played back onto a waveform analyzer Data Precision (DATA 6000 with a Plug-In Model 610; Data Precision, Danvers, MA) for analysis and was plotted by an *X–Y* or HC plotter (Tektronix).

2.3. Chemicals

N-Carbobenzoxy-Gly-Gly-Arg-β-naphthylamide (Z-GGR-N), *N*-carbobenzoxy-Gly-Phe-amide (Z-GF-N), *N*-carbobenzoxy-Pro-Leu-Gly-amide (Z-PLG-N), aprotinin, D-tubocurarine and hexamethonium chloride were obtained from Sigma (St. Louis, MO). α-Bungarotoxin was ob-

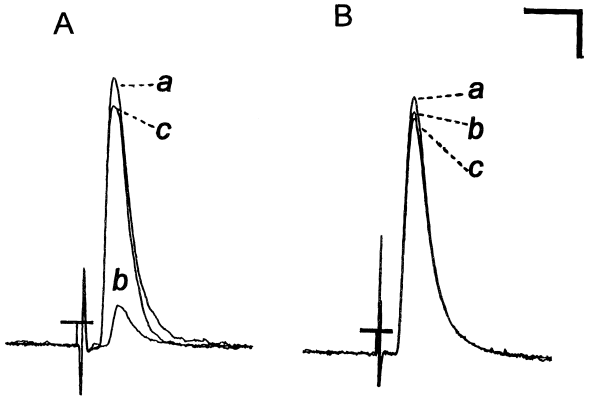

Fig. 2. The inhibitory effect of *N*-carbobenzoxy-Gly-Gly-Arg-β-naphthylamide (Z-GGR-N) on end-plate potentials (epps) of the isolated mouse phrenic nerve-diaphragm. (A) *a*, control epp; *b*, Z-GGR-N 0.1 mM for 15–20 min and *c*, 15–20 min after washout. (B) *a*, control epp; *b*, pretreatment with 0.1 mM suramin for 15–20 min and *c*, 0.1 mM Z-GGR-N for 20 min after *b*. Calibrations: 5 ms and 2 mV.

Table 2
Reversal by suramin of the inhibitory effects of Z-GGR-N on nerve-evoked diaphragm muscle contractions

Treatment	Muscle contraction (g)	
	low frequency (0.1 Hz)	high frequency (20 Hz) (fading index)[a]
Control	1.96 ± 0.50	1.24 ± 0.05
Z-GGR-N	0.10 ± 0.09[b]	0.25 ± 0.05[b]
Suramin	1.88 ± 0.07	0.91 ± 0.03
Suramin + Z-GGR-N	1.75 ± 0.08	0.91 ± 0.03

The isolated mouse phrenic nerve-diaphragm was pretreated with 0.1 mM suramin for 20 min prior to the addition of 0.1 mM Z-GGR-N and the muscle contractions were recorded 20 min after Z-GGR-N. Data are presented as means ± S.E. obtained from 4–7 preparations.
[a] Fading index = last peak tension/initial peak tension.
[b] $P < 0.001$ as compared with that of control.

tained from Biomol (Biomol Research Laboratories, Plymouth Meeting, PA). SCH 39370 (*N*-[*N*-[-1-(*S*)-carboxyl-3-phenylpropyl]-(*S*)-phenyl-alanyl]-(*S*)-isoserine) was kindly supplied by Dr. C.H. Chiu (Sherring Co., NJ).

2.4. Statistics

The results are given as means ± S.E. The significance of differences was evaluated by paired or unpaired Student's *t*-test. When more than one group was compared with one control, significance was evaluated according to a one-way analysis of variance (ANOVA). Probability values (P) of less than 0.05 were considered to be significant.

3. Results

3.1. Effect of protease substrates and aprotinin on the nerve-evoked muscle contractions

N-Carbobenzoxy-Gly-Gly-Arg-β-naphthylamide (Z-GGR-N), a protease substrate, produced a novel neuromuscular-blocking effect on the muscle contraction of mouse diaphragm evoked by 0.1 Hz nerve stimulation. The muscle tension after treatment with Z-GGR-N (0.1 mM) for 20–30 min was 0.1 ± 0.1 g as compared with the control tension prior to Z-GGR-N of 1.96 ± 0.50 g ($n = 7$, $P < 0.001$). This inhibitory effect was reversible by washout (Fig. 1A). Other protease substrates, such as 0.1 mM Z-GF-N, 0.1 mM Z-PLG-N and the protease inhibitors, 0.05 mM SCH 39370 and 36 μg/ml aprotinin, had no significant effect on the nerve-evoked muscle contractions (Table 1). α-Bungarotoxin (2 μg/ml) irreversibly blocked the nerve-evoked muscle contractions (Fig. 1D); the time to complete blockade was 76 ± 5 min ($n = 3$). Pretreatment with Z-GGR-N (0.1 mM) prevented the irreversible blockade elicited by α-bungarotoxin of nerve-evoked muscle contractions (Fig. 1C). The resting membrane potentials of mouse diaphragms after treatment with 0.1 mM Z-GGR-N for 20 min remained unchanged (control, −79.4 ± 0.9 mV, $n = 14$; treated with Z-GGR-N, −79.1 ± 0.7 mV, $n = 14$).

Table 3
Effect of Z-GGR-N on epp and mepp amplitudes of mouse diaphragm

Treatment	EPP (mV) ($n = 4$)	MEPP (mV) ($n = 12$)
Control	9.0 ± 0.7	1.15 ± 0.06
Z-GGR-N	1.2 ± 0.4[a]	0.22 ± 0.05[a]
Suramin	8.7 ± 0.8	1.03 ± 0.05
Suramin + Z-GGR-N	8.2 ± 0.8	0.93 ± 0.05

The isolated mouse phrenic nerve-diaphragm was pretreated with 0.1 mM suramin for 20 min prior to the addition of 0.1 mM Z-GGR-N. Epps and mepps were recorded 20 min after Z-GGR-N. Data are presented as means ± S.E.
[a] $P < 0.001$ as compared with that of the control.

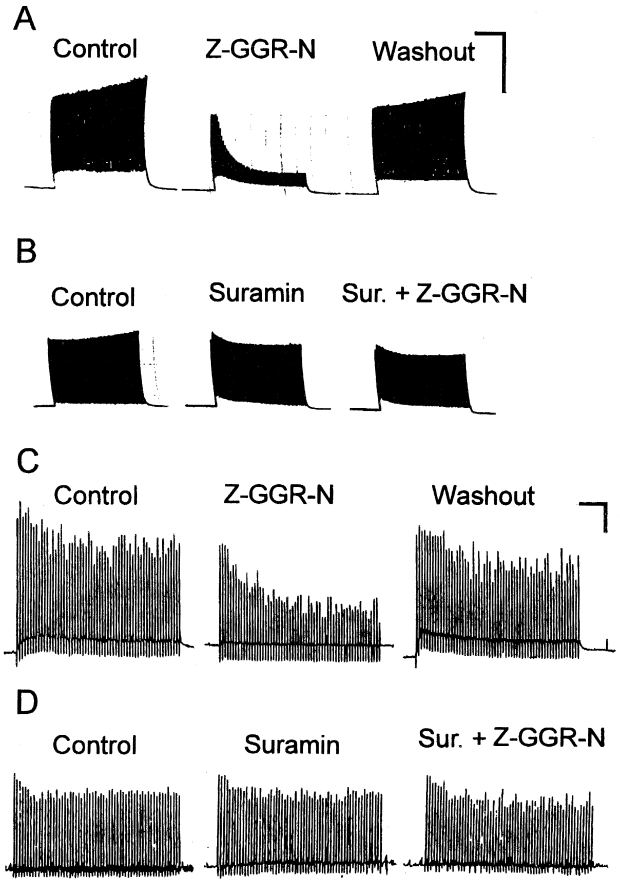

Fig. 3. Prevention by suramin of tetanic fading of nerve-evoked muscle contractions and end-plate potentials (epps) induced by Z-GGR-N in the isolated mouse phrenic nerve- diaphragm. Z-GGR-N (0.05 mM) can produce tetanic fading of nerve-evoked muscle contractions (A) and epps (C) elicited by nerve stimulation at 20 Hz with 3 s train duration. Pretreatment with suramin (0.1 mM) prevented the tetanic fading of nerve-evoked muscle contractions (B) and epps (D) induced by Z-GGR-N. Calibrations: 1 s and 2 g (A–B); 0.5 s and 2 mV (C–D).

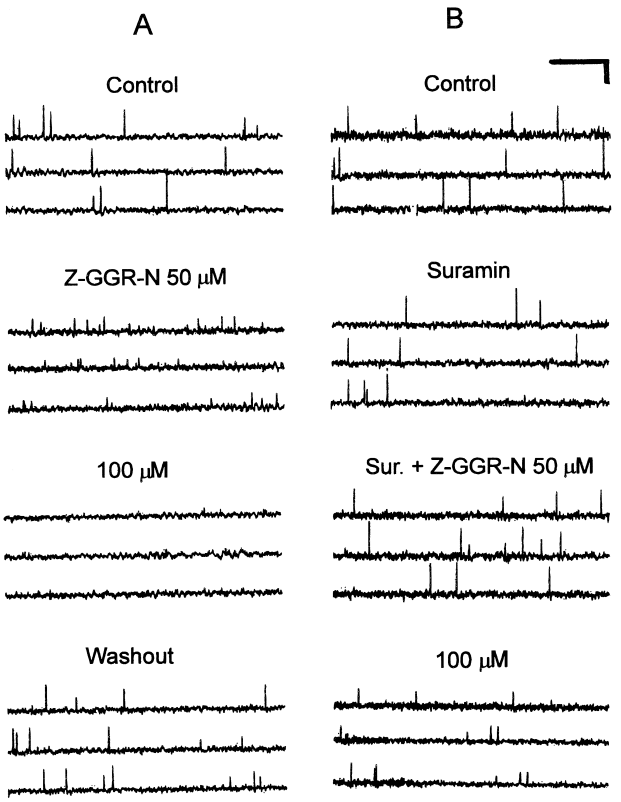

Fig. 4. Antagonism by suramin of the effect of Z-GGR-N on miniature end-plate potentials (mepps) of the mouse diaphragm. Z-GGR-N (0.05–0.1 mM) produced an inhibitory effect on the amplitude of mepps, while the frequency of mepps was increased by Z-GGR-N (0.05 mM) (A). Suramin (0.1 mM) pretreatment antagonized the decreased amplitude and increased the frequency of mepps induced by Z-GGR-N (B). Calibrations: 1 s and 1 mV.

3.2. Antagonism by suramin of the inhibitory effects of Z-GGR-N on end-plate potentials and nerve-evoked muscle contractions

3.2.1. Low-frequency stimulation (0.1 Hz)

Addition of Z-GGR-N (0.1 mM) produced a marked inhibition of end-plate potentials (epps) in response to 0.1 Hz stimulation (Fig. 2), the IC_{50} being 0.05 mM. Suramin, which has been shown to reverse the effect of non-depolarizing relaxants in rat diaphragm (Henning et al., 1992), could also significantly prevent the inhibition of the epps and muscle contractions induced by Z-GGR-N (Fig. 1B and Fig. 2; Tables 2 and 3).

3.2.2. High-frequency stimulation (20 Hz)

The tetanic fading phenomena could be produced by Z-GGR-N (0.05 mM) on both nerve-evoked muscle contractions and epps at 20 Hz nerve stimulation. These tetanic fading phenomena were similar to those induced by other nicotinic acetylcholine receptor antagonists, such as D-tubocurarine. Suramin (0.1 mM) was able to prevent the tetanic fading induced by Z-GGR-N of both nerve-evoked muscle contractions and epps (Fig. 3; Table 2).

3.3. Antagonism by suramin of the effects on miniature end-plate potentials induced by Z-GGR-N

Treatment with Z-GGR-N (0.05–0.1 mM) for 10–20 min significantly decreased the mean miniature end-plate potential (mepp) amplitudes, but increased the frequency of mepps to ~3.5-fold of control at 0.05 mM Z-GGR-N (Fig. 4). The depression of mepp amplitudes and the increase in mepp frequencies could be restored to normal levels by either washout or pretreatment with suramin (0.1 mM) (Fig. 4 and Table 3).

3.4. Effects of Z-GGR-N and hexamethonium on train-epps and mepps

Both Z-GGR-N (0.05 mM) and hexamethonium (0.8 mM) produced tetanic fading of the epp amplitude at 20 Hz nerve stimulation. Z-GGR-N (0.05 mM) and hexamethonium (0.8 mM) reduced the amplitude of the train-epps

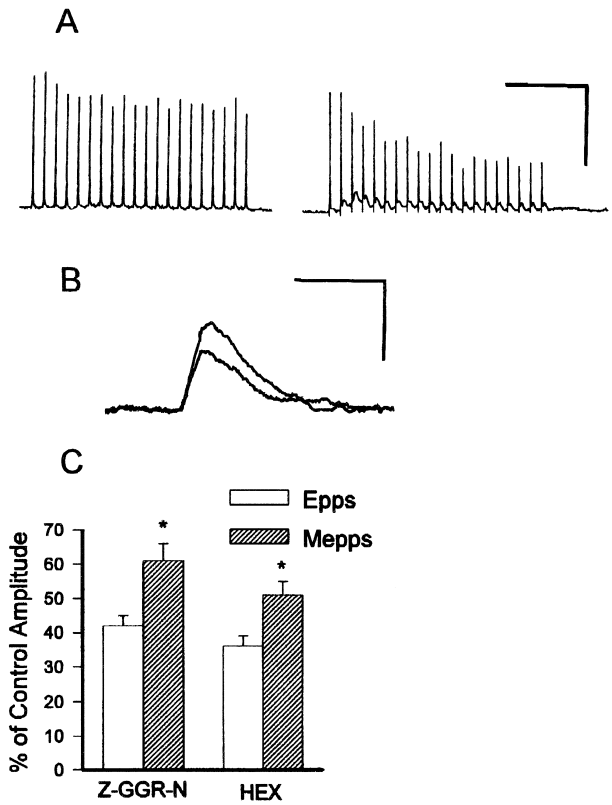

Fig. 5. Comparison on the inhibitory effects of Z-GGR-N and hexamethonium on the amplitude of train-epps and miniature end-plate potentials (mepps). Z-GGR-N (0.05 mM) produced a greater inhibitory effect on train-epps than on mepps. The percent inhibition produced by Z-GGR-N (A) on train-epps (20 Hz) was 42 ± 3% of control (amplitude before/amplitude after Z-GGR-N, in both cases the amplitude of the 20th epp was measured) as compared to the inhibitory effect (61 ± 5% of control) on mepp amplitudes (amplitude before/amplitude after Z-GGR-N) (B). This effect of Z-GGR-N was similar to that of hexamethonium (HEX, C). Calibrations: 0.4 s and 6 mV (A); 5 ms and 1 mV (B). $^{*}P < 0.05$ as compared with the respective inhibition percentage (mean ± S.E.) of epp (blank column).

to $42 \pm 3\%$ ($n = 4$) and $36 \pm 3\%$ ($n = 3$) of the control amplitude, respectively. The percent inhibition is presented as the amplitude before drug relative to the amplitude of the epp after drug; in both instances the amplitude of the twentieth epp was used. Z-GGR-N reduced the amplitude of the mepps to $61 \pm 5\%$ ($n = 4$) of the control amplitude and hexamethonium reduced it to $51 \pm 4\%$ ($n = 4$) of control (Fig. 5).

4. Discussion

In order to investigate the possible role of protease activity in transmitter release from mouse phrenic nerve-diaphragm preparations, we tested several protease inhibitors and substrates on nerve-evoked muscle contractions. The results obtained were unexpected in that *N*-carbobenzoxy-Gly-Gly-Arg-β-naphthylamide (Z-GGR-N) was found to be an antagonist at the nicotinic acetylcholine receptor and this activity was considered to be unrelated to its protease activity. Evidence for its curare-like action at the neuromuscular junctions is that: (1) it protected the diaphragm from the irreversible blocking action of α-bungarotoxin, which is a specific nicotinic acetylcholine receptor antagonist and (2) it had an inhibitory action on the amplitude of miniature end-plate potential (mepp) and end-plate potential (epp), producing tetanic fading of the response to high-frequency stimulation comparable to the effect of D-tubocurarine. An intriguing finding is that all of these effects of Z-GGR-N could be completely abolished by treatment with suramin, which has been found to be a competitive inhibitor of non-depolarizing muscle relaxants (e.g. D-tubocurarine and pancuronium) but not of a depolarizing agent (succinylcholine) (Henning et al., 1992). Since Z-GGR-N was unable to change the resting membrane potential of mouse diaphragm, it is considered to be a non-depolarizing relaxant. These effects of suramin were not due to the blockade of ATP-sensitive receptors, as has been reported in the rat diaphragm (Henning et al., 1992).

The presence of nicotinic acetylcholine receptor at presynaptic sites, the so-called presynaptic autoreceptors, has been claimed to be important in the regulation of the release of transmitters (Bowman et al., 1989). Whether Z-GGR-N acts on the presynaptic autoreceptor was tested. Wilson et al. (1995) found that hexamethonium as well as D-tubocurarine depressed the amplitude of epps significantly greater than that of mepps, indicating these drugs act on presynaptic autoreceptors, Z-GGR-N mimicked these effects of hexamethonium and D-tubocurarine. Therefore, Z-GGR-N could act on presynaptic sites. The increase in mepp frequency caused by Z-GGR-N (0.05 mM) could also be abolished by suramin, which suggests that their antagonistic interaction occurred not only at postsynaptic sites but also at presynaptic sites.

Concerning the other protease substrates, Z-GF-N and Z-PLG-N and inhibitors aprotinin and SCH 39370 (a neutral metalloprotease inhibitor, Sybertz et al., 1989), tested in this study, we found that none of them induced a comparable inhibitory effect on the nerve-evoked muscle contractions. At a higher concentration of 0.8 mM, Baxter et al. (1983) have reported that Z-GF-N depresses the epp amplitude to 60% of control. Similarly, we also found that 0.8 mM Z-GF-N inhibited the mepp amplitude to $52 \pm 3\%$ of control. Although the metalloprotease substrate, Z-GF-N, inhibited epps probably by interfering with protease activity, we considered that a postsynaptic inhibitory effect on nicotinic acetylcholine receptor had not been excluded. Nevertheless, among the polypeptides tested, Z-GGR-N stands out as being a relatively potent antagonist of nicotinic acetylcholine receptor.

There are many polyonium, tertiary and secondary amines with curare-like activity (Kitz et al., 1969; Kharkevich and Skoldinov, 1980). The typical nicotinic acetylcholine receptor antagonists, D-tubocurarine and succinylcholine, possess one or two quaternary ammonium groups. In our previous study, we have shown a curare-like action of tripyridine (Lin-Shiau et al., 1992; Hsu et al., 1993a) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Hsu et al., 1993b; Hsu et al., 1994) in the mouse neuromuscular junction. Tripyridine and MPTP contain three tertiary nitrogens and a mono-quaternary ammonium, respectively, and were less potent than d-tubocurarine, which contains one quaternary and one tertiary nitrogen. In the present studies, the chemical structure of Z-GGR-N was different from that previously reported: it contained three secondary nitrogens and one guanidinium group. The approximate IC_{50} values for the neuromuscular-blocking action of D-tubocurarine, Z-GGR-N, tripyridine and MPTP were 0.7, 50, 48 and 53 μM, respectively.

In conclusion, we demonstrated in this study that Z-GGR-N has a curare-like action on the mouse phrenic nerve-diaphragm. Suramin showed itself to be an antagonist of Z-GGR-N not only at postsynaptic but also at presynaptic sites. It is conceivable that these two agents react at presynaptic autoreceptors and this merits further investigation, as does the role of nicotinic acetylcholine receptor in transmitter release.

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