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Fragments of galanin message-associated peptide (GMAP) modulate the spinal flexor reflex in rat

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Abstract

We have previously reported that galanin message-associated peptide (GMAP), a fragment of galanin precursor protein, is present in dorsal root ganglion cells and upon intrathecal (i.t.) administration influences the spinal nociceptive flexor reflex in a complex manner in the rat. GMAP elicited a moderate facilitation of the flexor reflex, but when administered prior to conditioning stimulation of C-afferents, it dose dependently blocked spinal cord hyperexcitability. The present study examined the effects of four fragments of GMAP-(1–60), GMAP-(1–12), GMAP-(10–24), GMAP-(25–44) and GMAP-(37–60), on the flexor reflex and compared them with the effects of the complete peptide sequence. All four GMAP fragments facilitated the flexor reflex. However, this effect was dose-dependent only for GMAP-(1–12) and the effect of GMAP-(1–12) was stronger than GMAP-(1–60). In contrast, only GMAP-(25–44) dose dependently blocked the facilitation of the flexor reflex induced by the C-fiber conditioning stimulation. The potency of the blocking effect of GMAP-(25–44) was one order of magnitude lower than that of GMAP-(1–60). The results indicated that two fragments of GMAP are pharmacologically active and produce effects which are similar to the full sequence. It is possible that the complex effect of GMAP may be mediated by different subtypes of GMAP receptors which recognize different portions of the GMAP sequence.

Keywords: Flexor reflex; Galanin; GMAP (galanin message-associated peptide), fragments; Nociception; Spinal cord

1. Introduction

Galanin message-associated peptide (GMAP) is a 59- or 60-amino-acid residue flanking peptide located in the sequence of preprogalanin, the precursor protein that gives rise to the neuropeptide galanin (Rökaeus and Brownstein, 1986; Rökaeus and Carlquist, 1988; Vrontakis et al., 1987; Kaplan et al., 1988). The sequence of GMAP shows striking homology in all species studied so far (Table 1, Rökaeus and Brownstein, 1986; Rökaeus and Carlquist, 1988; Vrontakis et al., 1987; Kaplan et al., 1988; Evans and Shine, 1991; Lundkvist et al., 1995), indicating a possible functional role. The distribution of GMAP-like immunoreactivity in the central and peripheral nervous system of the rat overlaps with the distribution of galanin-like immunoreactivity in most tissues (Hökfelt et al., 1992; Xu et al., 1994). However, in some areas, such as retinal cones, prolactin cells in the anterior lobe of the pituitary and in the insulin-secreting cells in the islets of Langerhans in the pancreas, GMAP-like immunoreactivity is stronger and detected in more cells than galanin-like immunoreactivity, indicating a possible tissue specific, post-translational differential processing of preprogalanin (Hökfelt et al., 1992). GMAP occurs in a small population of dorsal root ganglion cells (Hökfelt et al., 1992; Xu et al., 1995a) and a GMAP-LI positive network has been described in the superficial laminae of the dorsal horn (Xu et al., 1994).

We have recently studied the effects of intrathecal (i.t.) administration of GMAP on the flexor reflex and found that it caused a moderate facilitation of the flexor reflex, indicating a possible excitatory effect of this peptide (Xu et al., 1995b). However, when given as pretreatment, GMAP potently suppressed the strong facilitation of the

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flexor reflex induced by repetitive stimulation of C-fibers (Xu et al., 1995b). GMAP also reduced the reflex facilitatory effect of i.t. substance P. These data suggest that GMAP, like galanin, also exerted an inhibitory effect on spinal reflex excitability (Xu et al., 1995a,b).

Fragments of a peptide or of a protein can exert biological effects which are similar or different from their parent peptides (Martinez and Potier, 1986). In some cases different fragments of peptides may exert opposite effects, such as with substance P (Skilling et al., 1990; Lei et al., 1991; Hedlund et al., 1994). It is possible that some effects of some peptides are mediated by their fragments, rather than by the full peptide.

The sequence of the 60-amino-acid-long GMAP is separated by possible monobasic cleavage sites (Schwartz, 1987), raising the possibility that some of its fragments may be formed in vivo and are biologically active. The present study was undertaken to survey the effects of four possible proteolytic fragments of GMAP on flexor reflex excitability. We compared the effects of these four fragments with that of the full sequence of GMAP on the baseline flexor reflex and following spinal hyperexcitabiliy induced by repetitive C-afferent stimulation. The choice of the four fragments was based on the presence of monobasic cleavage sites and structural conservation among species (Table 1).

2. Materials and methods

2.1. Flexor reflex experiments

Female Sprague-Dawley rats weighing 200–250 g (ALAB, Sweden) were used and the magnitude of the polysynaptic hamstring flexor reflex in response to activation of high threshold afferents was examined. The animals were briefly anesthetized with methohexital (Brietal, Lilly, Indianapolis, IN, USA, 70 mg/kg, i.p.), ventilated and decerebrated by aspiration of the forebrain and midbrain. The spinal cord was exposed by a laminectomy at mid-thoracic level and sectioned at Th8-9. An i.t. catheter (PE 10) was implanted caudal to the transection with its tip on the lumbar spinal cord (L4–5). The flexor reflex was elicited by supramaximal electric shocks applied to the sural nerve innervation area in the left foot (0.5 ms, 10 mA, 1/min) that activated A- and C-fibers (11). In some experiments, a conditioning stimulus train (0.9 Hz, 20 stimuli) of the same strength as the test stimulus was administered to evoke a facilitation of the reflex. A stable reflex baseline (defined as less than 15% variation) was established for at least 20–30 min before administration of drugs or the C-fiber conditioning stimulation.

The flexor reflex was recorded as electromyographic (EMG) activity via stainless steel needle electrodes inserted into the ipsilateral posterior biceps femoris/semitendinosus muscles. The number of action potentials exceeding the level of spontaneous EMG activity was integrated over 2 s and recorded on a chart recorder (Gould 2400 S). During the experiments the heart rate and rectal temperature of the rat were monitored.

2.2. Peptides

GMAP and its fragments were synthesized in a stepwise manner on a solid support using a Peptide Synthesizer (Model 431A, Applied Biosystems, Foster City, CA, USA) using the standard dicyclohexylcarbodiimide (DCC)/hydroxybenzotriazole (HOBt) Solvent-Activation strategy on a 0.1 mmol scale. All cleaved peptides were purified on high performance liquid chromatography (LKB) using Polygosil 60-7 C_{18} reversed-phase column using a linear gradient in a system of 0.1% (v/v) trifluoroacetic acid/H_2O and 0.1% (v/v) trifluoroacetic acid/acetonitrile. Molecular masses of the peptides were determined using a Plasma Desorption Mass Spectrometer (Model Bioion, Applied Biosystems). The calculated values ± 1 were obtained in each case.

All peptides were dissolved in 0.9% normal saline and aliquoted. They were stored under −70°C and used directly after thawing. The i.t. injections were made in 10 μl volume followed by 10 μl saline to flush the catheter.

Table 1
Amino-acid sequences and homologous regions GMAP in different species

	1–9 (10)	10–19 (11–20)	20–29 (21–30)	30–39 (31–40)	40–49 (41–50)	50–59 (51–60)
Human [a] (59aa)	**EL**RP**E** DD**M**K	P**G**SFDRSI P**E**	NNIM**RTI**I**EF**	**LSFLHLKEAG**	**AL**DR**LL**DL**P**A	**AASSED**I EA**S**
Cow [b] (59aa)	**EL**EP**E** DEAR	P**G**SFDRPLA**E**	NNVV**RTI**EF	**L**TF**LHLKDAG**	**AL**ER**L**PSL**P**T	**AESAEDAERS**
Pig [c] (59aa)	**EL**EP**E** DEAR	P**G**GFDRLQS**E**	DKAI**RTIMEF**	**LAFLHLKEAG**	**AL**GR**L**PGL**P**S	**AASSED**AGQ**S**
Rat [d] (60aa)	**EL**PL**E**VEEGR	L**G**SVAVPLP**E**	SNIV**RTIMEF**	**LSFLHLKEAG**	**AL**DSLPGI **P**L	**ATSSED**L EQ**S**
Mouse [e] (60aa)	**EL**EL**E**VEERR	P**G**SVDVPLP**E**	SNIV**RTIMEF**	**LSFLHLKEAG**	**AL**DS**L**PGI **P**L	**ATSSED**L EK**S**
	1	11	21	31	41	51

This table shows the sequence of GMAP from several different species and indicates the conserved blocks of amino acids (in bold type) and the single basic cleavage sites (underlined). [a] Evans and Shine, 1991. [b] Rökaeus and Carlquist, 1988. [c] Rökaeus and Brownstein, 1986. [d] Vrontakis et al., 1987. [e] Lundkvist et al., 1995.

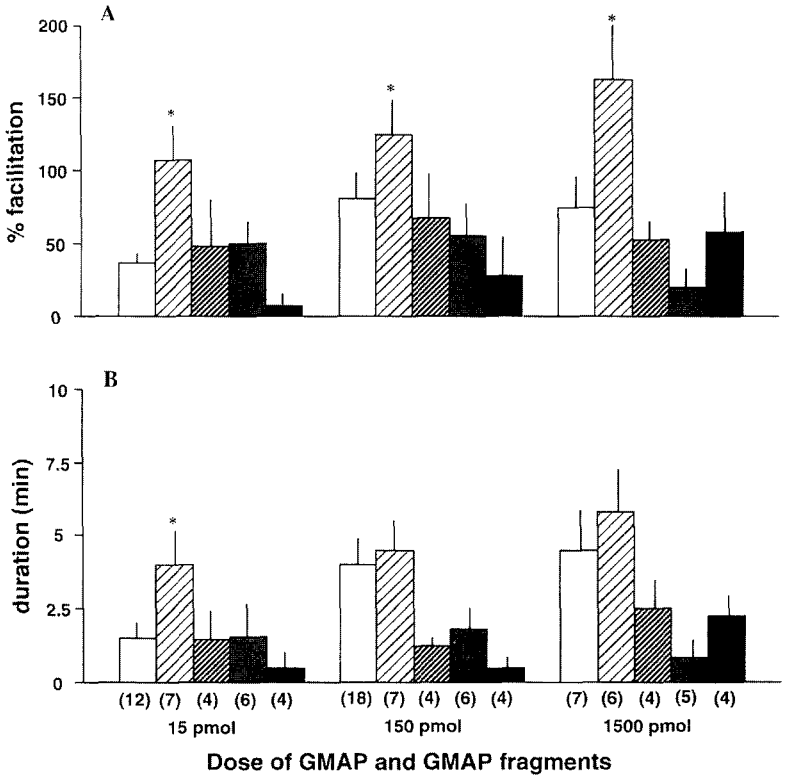

Fig. 1. Summary of the facilitatory effect of i.t. GMAP-(1–60, 1st column), -(1–12, 2nd column), -(10–24, 3rd column), -(25–44, 4th column) and -(37–60, 5th column) on the flexor reflex. The data are expressed as means ± S.E.M. and the number of experiments and the doses of peptides are indicated under the columns. ANOVA revealed significant overall difference of the magnitude, but not duration, of reflex facilitation at all three doses of GMAP and its fragments. Individual comparisons were made with Fisher PLSD test, * = $P < 0.05$ compared to GMAP-(1–60).

2.3. Data collection and statistics

A stable baseline reflex magnitude was established for at least 20 min before each i.t. injection or conditioning nerve stimulation. The effect of i.t. peptides and the nerve CS on the flexor reflex were expressed as percentage change in reflex magnitude compared to baseline, which is defined as 100%. Data were analyzed by one-way analysis of variance (ANOVA) followed by Fisher PLSD (protected least significant difference) test. Variability is expressed as S.E.M.

3. Results

3.1. Facilitatory effect of i.t. GMAP and its fragments

I.t. administration of GMAP-(1–60) caused a moderate and brief facilitation of the flexor reflex which was dose-dependent between 15 and 150 pmol (Fig. 1). Further increasing the dose of GMAP to 1500 pmol did not produce greater and longer facilitation of the reflex. I.t. GMAP-(1–12) caused strong and dose-dependent facilita-

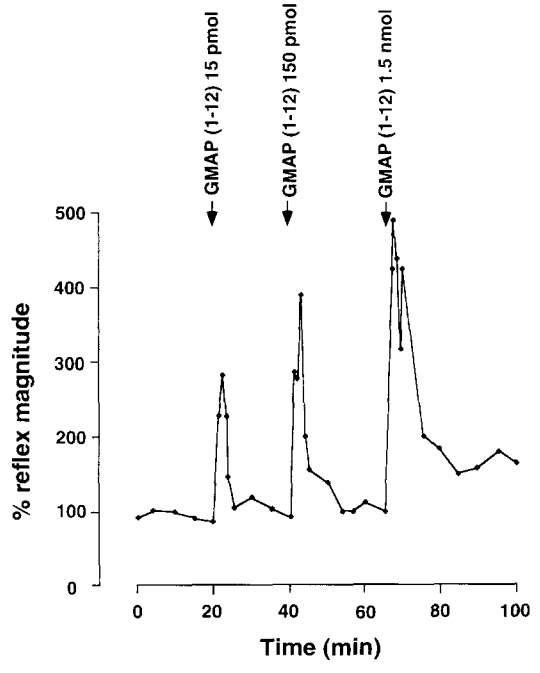

Fig. 2. Illustration of the potent and dose-related facilitatory effect of i.t. GMAP-(1–12) on the flexor reflex in one experiment. Baseline reflex magnitude is defined as 100%.

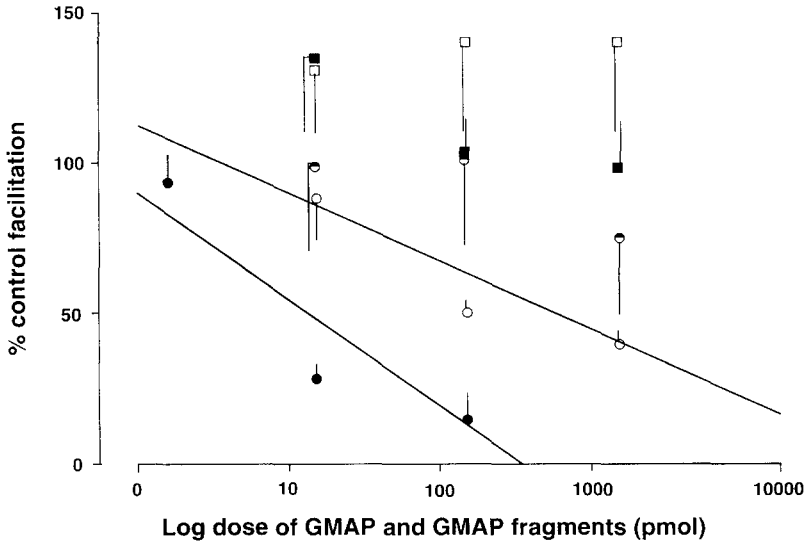

Fig. 3. The effect of i.t. GMAP-(1–60, filled circles), -(1–12, filled squares), -(10–24, open squares), -(25–44, open circles) and (37–60, shaded circles) on the facilitaiton of the flexor reflex induced by C-fiber conditioning stimulation (expressed as percentage of control facilitation). The data are expressed as means ± S.E.M. and 4–8 experiments are included for each fragment at each dose. ANOVA indicated that GMAP-(1–60) and GMAP-(25–44) dose dependently antagonised facilitation of the reflex ($F(1,16) = 23.8$ and 12.1 respectively, $P < 0.001$). The regression lines for GMAP-(1–60) and GMAP-(25–44) are drawn according to the formulae $y = -36.2x + 89.2$ and $y = -24.0x + 112.2$, respectively. ANOVA indicated that the regressions for the other three fragments are not significant.

tion of the flexor reflex (Fig. 1 and Fig. 2). The magnitude of reflex facilitation elicited by GMAP-(1–12) was significantly greater than after GMAP-(1–60) at the same dose (Fig. 1A). The other three GMAP fragments studied, 10–24, 25–44 and 37–60, moderately facilitated the reflex in some experiments. This effect was, however, not consistently observed and was not dose-related (Fig. 1).

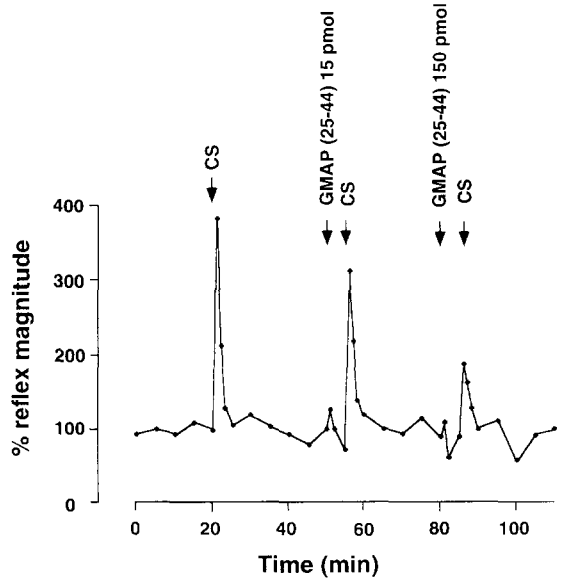

Fig. 4. Illustration of the effect of i.t. GMAP-(25–44) on the facilitation of the flexor reflex induced by C-fiber conditioning stimulation to the sural nerve in one experiment. The baseline reflex magnitude is defined as 100%.

3.2. Suppression of C-fiber CS-induced reflex facilitation by GMAP and its fragments

The facilitation of the flexor reflex by the C-fiber conditioning stimulation applied to the sural nerve innervation area was potently and dose dependently suppressed by pretreatment with GMAP-(1–60) (Fig. 3) with an ED_{50} of 12.1 pmol. GMAP-(25–44) also dose dependently antagonised the C-fiber stimulation-induced reflex facilitation with an ED_{50} of 397.6 pmol (Fig. 3 and Fig. 4). Thus, the dose-response curve for GMAP-(25–44) was shifted to the right compared to GMAP-(1–60), with the former peptide being about one order of magnitude less potent (Fig. 3). None of the other three fragments antagonised the C-fiber conditioning stimulation-induced reflex facilitation at the doses examined (Fig. 3). In fact, the C-fiber conditioning stimulation-induced facilitation was frequently enhanced by GMAP-(1–12) or GMAP-(10–24) (not illustrated).

4. Discussion

I.t. administration of two fragments of GMAP elicited clear and dose-dependent effects on the flexor reflex, namely, a facilitation of the baseline reflex by GMAP-(1–12) and a suppression of the C-fiber conditioning stimulation-induced reflex facilitation by GMAP-(25–44). Both effects have been observed after administration of GMAP-(1–60) (Xu et al., 1995a,b). It appears from the present study that the excitatory and inhibitory effects of GMAP

may be mediated by different receptors recognizing the N-terminal fragments and a middle (25–44) portion of the GMAP-(1–60) sequence, respectively. This is similar to the effect of substance P where the C-terminal fragment acting on the NK_1 receptor exerts an excitatory effect on spinal mechanisms whereas the N-terminal fragment produces inhibition (Skilling et al., 1990; Lei et al., 1991; Goettl et al., 1994). Thus, this effect of GMAP is different from that of galanin where the N-terminal fragments seems to mediate both spinal excitation and inhibition, depending on the dose (Xu et al., 1990).

The amino-acid sequence of all GMAP fragments studied are well preserved among species and are separated in the GMAP sequence by cleavage sites. It is therefore possible that the effects observed after i.t. GMAP-(1–60) are mediated by its fragments after proteolytic degradation of GMAP in vivo. This may be particularly the case with the inhibitory effect of GMAP since it occurs after a long latency following i.t. administration (Xu et al., 1995b). It can be postulated that the structure of GMAP is globular and that it prevents the interaction between the middle portion of the sequence and the inhibitory receptors. However, as the efficacy of GMAP-(25–44) in producing inhibition is lower than that of the full-length peptide, it is also possible that the inhibitory effect is normally produced by GMAP-(1–60). The initial excitatory effect of GMAP has a rapid onset, which may indicate a direct interaction between the full-length GMAP-(1–60) peptide and the receptor that recognizes its N-terminal.

GMAP-(1–12) is more efficacious in facilitating the flexor reflex than GMAP-(1–60) whereas GMAP-(25–44) is about 10-fold less potent than GMAP-(1–60) in producing inhibition. It is however difficult to assert that this truly reflects differences in the affinity of GMAP and its fragments in interacting with their receptors. The potency and efficacy of peptides in vivo are limited by factors such as their size, rate of penetration and sensitivity to degradation. While short fragments may penetrate better, they may be more sensitive to enzymatic degradation, as is the case for GAL-(1–29) and GAL-(1–16), respectively (Land et al., 1991; Bedecs et al., 1995). Furthermore, the synthetic GMAP fragments studied here may not be the optimal sequences as they need not represent natural products of GMAP degradation.

The mechanism by which GMAP and its fragments exert effects on spinal reflex function is unclear. We have previously presented evidence indicating that the effect of GMAP may be independent of an action on galanin receptors (Xu et al., 1995a,b). A study examining whether GMAP, like galanin, inhibits glucose-stimulated insulin secretion from isolated rat and mouse islets showed no effect of GMAP in that system (Gregersen et al., 1994). Thus, the β cells may not respond to holo-GMAP. There have been no other functional studies on the biological effects of GMAP, due to limited access to this long peptide which is not commercially available. Studies on in vivo degradation of GMAP have also been hampered by the lack of access to large quantities of GMAP, which is difficult to produce by solid phase peptide synthesis. We have now expressed GMAP in bacteria and hope to be able to study its degradation by CSF or its processing by vesicle enzymes, since galanin and GMAP are stored in the same large core dense vesicles where GMAP may be further processed.

In summary, the present results showed that at least two fragments of GMAP exert biologically measurable activity, indicating that it is possible to use short fragments of GMAP to study the biological or pharmacological effects and binding profile of this long and potentially important neuroendocrine peptide.

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