MURAMYL PEPTIDES

Variation of Somnogenic Activity with Structure

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Slow-wave sleep-promoting "factor S" was described in the past fifteen years by Fencl et al. (1) and Pappenheimer and colleagues (2, 3) as a constituent of cerebrospinal fluid (CSF)[1] derived from sleep-deprived animals. More recently, factor S obtained from urine and brain was provisionally identified as a low molecular mass muramyl peptide (~1,000 D) (4). In rabbits, 10 pmol of this material was sufficient to induce excess slow-wave sleep (SWS) for six or more hours (4). In cats, similar increases in SWS (5) were induced by amounts of factor S calculated to be ~100 pmol. The similarity of the chemical composition of factor S to subunits of some bacterial peptidoglycans led to investigations of the effects on sleep of synthetic muramyl peptides such as *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (*N*Ac-mur-L-ala-D-isogln) (MDP). MDP or its L-lysine derivative were found to induce excess SWS in rabbits (6), cats (6), and monkeys (7) at molar levels 10-fold that of factor S. The dose required in the rabbit was dependent on the route of administration, with the highest dose required in oral administration, followed by intraperitoneal, intravenous, and intraventricular.

MDP is an immunostimulant that was first described as the minimal adjuvant-active structure that can substitute for mycobacteria in Freund's complete adjuvant (8). It is also a pyrogen (9, 10) and many of its derivatives have been examined for these two activities (11). Furthermore, it may act as a bactericidal agent in at least two ways. It may act on macrophages so that they produce augmented amounts of oxygen radicals (12, 13) and it also enhances production of interleukin 1 by macrophages (14). More recently supernates from activated macrophages have been shown to induce excess SWS.[2]

In the present paper, the list of MDP derivatives that induce SWS has been extended. Further, some analogs that do possess immunostimulatory and pyro-

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[1] *Abbreviations used in this paper:* CSF, cerebrospinal fluid; dap, diaminopimelic acid; E_a, mean rectified slow-wave voltage during waking periods; E_s, mean rectified slow-wave voltage during slow-wave sleep periods; EEG, electroencephalograph; MDP, *N*Ac-Mur-L-ala-D-isogln; SWS, slow-wave sleep.

[2] Krueger, J. M., J. Walter, C. A. Dinarello, S. M. Wolff, and L. Chedid. Sleep-promoting effects of endogenous pyrogen (interleukin 1). Manuscript submitted for publication.

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genic activities have been shown to lack somnogenic activity. We also present some tentative conclusions as to the structural requirements for somnogenic activity. Of these the most notable is that amidation of the free γ-carboxyl of MDP and several of its analogs results in a loss of somnogenic activity.

Materials

The MDP used was from Institut Pasteur Productions, Paris, France. Derivatives of MDP were obtained from the following sources (compound numbers are those shown in Table I): Numbers 2–11 (inclusive) and 15 were obtained from Institut Choay, Paris; numbers 13 and 14 were gifts from Dr. T. Y. Shen, Merck and Co., Inc., Rahway, NJ; number 12 was isolated from *Bacillus cereus* grown in the presence of vancomycin and was a gift from Dr. J. F. Petit, Institut de Biochimie, Universite de Paris-Sud, Orsay, France (15); number 16 was prepared as previously described (6). All other chemicals were reagent grade. Needles, syringes, glassware, and solutions were sterile and pyrogen free.

Methods

Male New Zealand rabbits (3–5 kg) were provided with chronically implanted electroencephalographic (EEG) electrodes and cerebral ventricular guide tubes under Nembutal anesthesia (15–30 mg/kg), as previously described (16). At least 1 wk was allowed for recovery from the operation. Before each recording period animals were brought to the experimental cages for an overnight acclimation period. Each substance was dissolved in artificial CSF (155 mM NaCl, 3 mM KCl, 1.15 mM $CaCl_2$, and 0.96 mM $MgCl_2$) (16); 0.3 ml of each solution was infused into a lateral cerebral ventricle at a rate of 7 μl/min. After the infusion, the EEG, its rectified slow-wave component (0.5–4 Hz), and bodily movements were recorded for the next 6 h as previously described (16). The 0.5–4 Hz EEG component was also electronically integrated and integrals were printed on tape every minute. Rectal temperatures were taken using a calibrated thermistor probe (Yellow Springs Instrument Co., Yellow Springs, OH) inserted 10 cm into the rectum immediately after the infusion and 3 and 6 h later. Both experimental and housing rooms were on a 12-h light-dark cycle (0600–1800 h, light). Infusions took place between 0800 and 1000 h.

Polygraph recordings were analyzed visually to determine duration of SWS; values obtained by this method are shown in Table I and Fig. 1. Printed integrals were used to calculate the mean, rectified slow-wave voltages associated with SWS periods (E_s) and waking periods (E_a); such values are applied in Fig. 1 and in the text. Details of the infusion, recording, and analysis methods have been given elsewhere (3, 16).

Results

Dose-Response Relationship for MDP. The effects of five different doses of MDP are shown in Fig. 1 and Table I. Infusion of 10 pmol of MDP was insufficient to induce excess SWS. Doses of 50 pmol or greater induced significant increases in duration of SWS. The time course of sleep responses observed after doses of 50 and 100 pmol was similar to that previously reported (6). During the first hour after infusion, SWS values were similar to control values. Excess SWS became manifest during the second hour postinfusion and continued throughout the routine 6-h assay period. When doses were increased to 1 and 10 nmol, excess SWS was evident during the 1st h postinfusion. At all doses, SWS appeared normal in the sense that it remained episodic; animals could be aroused and they awoke spontaneously from time to time to eat, drink, and groom. However, after infusion of 10 nmol of MDP, other autonomic responses were observed; these included excess nasal and lacrimal secretions. Similar responses were observed

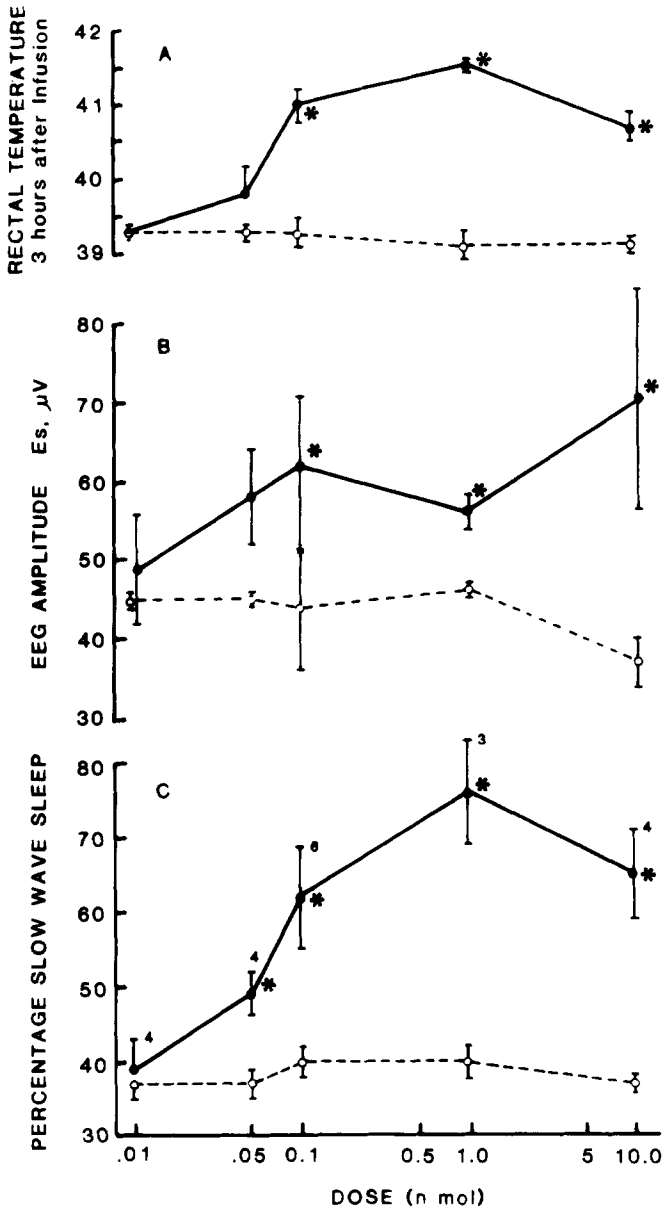

FIGURE 1. Effects of various doses of MDP on rectal temperatures, EEG slow-wave amplitude, and duration of SWS. (——) Effects of MDP after intraventricular infusion of MDP in 0.3 ml of artificial CSF; (– – –) control values from same rabbits. (*A*) Rectal temperatures were taken 3 h after the end of the infusion. At this time febrile responses were greatest. (*B*) Amplitude of EEG slow waves; slow-wave (0.5–4 Hz) voltages during periods of SWS (E_s) are shown. Voltages during waking periods (E_a) (not shown) were unaffected by MDP. (*C*) Duration of SWS; ordinate values are the percent of time occupied by SWS during a routine 6-h assay period after the infusion. The number above each point is the number of animals tested at each dose; the same animals were used to determine control values. In *A*, *B*, and *C*, values shown are mean ± SEM; (*) denotes significant increase above corresponding control value; $P < 0.05$ (paired *t* tests). After 10 pmol of MDP, experimental values were similar to corresponding control values. When the dose was increased to 50 pmol, the increases in rectal temperatures (*A*) and slow-wave amplitudes (*B*) were not significant but significant increases in duration of SWS (*C*) were observed. Larger doses induced significant increases in all three response parameters.

TABLE I

Biological Effects of Cerebral Intraventricular Administration of MDP and Related Compounds.

Compound	Dose	n‡	Percent SWS 6 h postinfusion		Effect		
			Control	Expt.	Somnogenic	Pyrogenic*	Immunostimulatory§
	nmol						
Group I							
1. MDP	0.01	4	37 ± 2	39 ± 4	−	−	+ (8, 21)
	0.05	4	37 ± 2	49 ± 3‖	+	+	
	0.1	6	40 ± 2	62 ± 7‖	+	+	
	1.0	3	40 ± 2	76 ± 7‖	+	+	
	10.0	4	37 ± 1	65 ± 6‖	+	+	
2. NAc-Mur-L-ala-D-glu	0.2	4	38 ± 1	43 ± 3	−	−	+ (11, 21)
	2.0	4	36 ± 3	51 ± 3‖	+	+	
3. NAc-Mur-L-ala-D-gln	0.2	2	28–44	32–32	−	−	+ (11)
	2.0	4	46 ± 3	45 ± 1	−	+	
4. NAc-Mur-L-ala-D-glu-(NH_2)-NH_2	2.0	3	42 ± 3	43 ± 1	−	−	+ (21, 25)
5. NAc-Mur-L-ala-D-glu-(OMe)-OMe	2.0	4	43 ± 4	66 ± 4‖	+	+	+ (21)
6. NAc-Mur-L-ala-D-glu-α-OMe	0.2	2	38–40	47–61	+	+	+ (22)
	2.0	4	42 ± 4	72 ± 4‖	+	+	
7. NAc-Mur-L-ala-D-isogln-γ-OMe	2.0	6	39 ± 3	56 ± 7‖	+	+	+ (21)
8. NAc-Mur-L-ala-D-gln-α-OMe¶	0.5	2	34–42	31–45	−	−	+ (23)
9. NAc-Mur-L-ala-D-gln-n-Bu ester	0.25	2	37–42	41–47	−	−	+ (11, 22)
Group II							
10. NAc-Mur-L-ala-D-isogln-L-lys	0.13	5	35 ± 2	60 ± 3‖	+	+	+ (23)
11. NAc-Mur-L-ala-D-isogln-L-tyr-OMe	0.15	2	36–44	68–68	+	+	ND
12. NAc-Mur-L-ala-γ-D-glu-meso-dap-D-ala-D-ala	0.02	3	41 ± 2	46 ± 3	−	−	+ (15)
	0.2	3	41 ± 2	65 ± 5‖	+	+	
Group III							
13. 3-O-(D-2-propionyl-L-ala-D-isogln)-D-glucose	0.2	2	33–44	35–43	−	−	ND
	2.0	2	42–43	40–45	−	−	
14. NAc-glucosaminyl (1–4) β-MDP	0.2	4	45 ± 3	60 ± 5‖	+	+	+ (24)
15. NAc-Mur(N-Me)-L-ala-D-isogln	0.2	2	37–42	32–47	−	−	+ (11)
16. Periodate-cleaved NAc-Mur-L-ala-D-isogln	0.2	2	40–44	38–42	−	ND	ND

* (+) >0.5 °C change; (−) no change. ND, not determined.

‡ If $n = 2$, range is given instead of SEM.

§ This term includes situations in which antiinfectious activity rather than true immunoadjuvanticity was measured. Relevant references appear in parentheses.

‖ Significantly different from control ($P < 0.05$).

¶ Four rabbits were given this derivative intravenously (0.16–0.33 mg/kg); none exhibited excess SWS.

after intravenous injection of relatively large amounts (75–100 μg) of MDP (6).

Dose-dependent increases in body temperature and amplitudes of EEG slow waves during SWS (E_s) also occurred. These increases were significant after the infusion of 0.1–10 nmol of MDP. Slow-wave voltages during periods of wakefulness (E_a) were not affected by any of the doses used. Previously it was reported that sleep-promoting factor S (3), MDP (6), and interleukin 1,[2] as well as sleep deprivation (3) cause similar increases in slow-wave amplitudes during SWS and that these increases were independent of the increases in body temperature.[2]

Comparison of Various Analogs of MDP. The effects of intraventricular infusion of MDP and several of its analogs on the duration of SWS are shown in Table I. Pyrogenic activities and information from the literature regarding immunostimulatory activities of these analogs are also summarized in Table I. In assessing the effectiveness of the various compounds, the following general criterion was used: Compounds were considered active if a dose of 2 nmol or less induced significant increases in SWS. For completeness, five compounds reported on earlier (Nos. 8, 9, 10, 15, and 16) are included, although data on these do not completely fit the criterion mentioned.

The first set of substances discussed (Nos. 1–9) (group I) is concerned with derivatives in which various changes in the D-isogln moiety of MDP are made. Replacement of isogln by glu (No. 2) results in reduced somnogenic potency; when isogln was replaced by gln (No. 3), the substance was not somnogenic at 2 nmol infused. However, at this dose, No. 3 did induce significant increases in rectal temperatures. The diamide analog of MDP (No. 4) failed to induce increases in either SWS or body temperature when 2 nmol were tested. On the other hand, the dimethyl ester analog (No. 5) induced significant increases in SWS and rectal temperatures. Similarly, the glu-α-methyl ester analog (No. 6) induced significant increases in SWS and temperature, and the γ-methyl, α-amide analog (No. 7) was also an effective sleep promoter. However, as previously reported (6), the α-methyl ester, γ-amide (No. 8) and the α-n-butyl ester, γ-amide (No. 9) were inactive, as might be expected from observations with substance No. 3 above. These data suggest that conversion of the free γ-carboxyl of the glu moiety of MDP to an unsubstituted amide results in the loss of somnogenic activity. In addition, derivatives with an unsubstituted amide on the α-carboxyl are more potent somnogenic agents than those with either a free carboxyl or an α-methyl ester.

Results obtained using derivatives in which the free carboxyl of the glu moiety of MDP was linked to a substituted amide (peptide link) (group II) were in marked contrast to those having an unsubstituted amide in this position. The coupling of either L-lys (No. 10) or L-tyr methyl ester (No. 11) to MDP resulted in active compounds. Moreover, the presence of a tripeptide (meso-dap-D-ala-D-ala) (dap, diaminopimelic acid) at the same position actually increased potency (compare Nos. 12 and 2).

We also tested several substances with modifications of the muramyl moiety of MDP (group III). If D-glucose is substituted for the *N*-acetyl-glucosamine moiety of MDP (No. 13), sleep-promoting and pyrogenic activities were lost. However, sleep-promoting activity was retained in the disaccharide dipeptide in which *N*-acetyl-glucosamine is in glycosidic linkage to carbon No. 4 of muramic acid (No.

14). Previously we reported that the *N*-methyl-L-ala derivative of MDP (No. 15) as well as periodate-cleaved MDP (No. 16) were inactive and these results are reproduced here.

The sleep responses elicited in rabbits by somnogenic analogs of MDP were similar to those elicited by MDP. Sleep remained episodic and appeared normal despite simultaneous febrile responses. In addition, each somnogenic analog also induced increases in EEG slow-wave amplitudes during SWS (E_s). Those analogs that failed to induce increases in SWS also failed to affect EEG slow-wave amplitudes. None of the inactive analogs appeared to decrease normal sleep at the levels tested.

Discussion

Sleep deprivation, which results in the accumulation of endogenous sleep promoters (1), is followed by excess SWS, which in rabbits (3) and rats (17) remains episodic, alternating with bouts of rapid eye movement (REM) sleep and wakefulness. In rats, the amount of excess SWS observed after sleep deprivation is dependent upon the time of day. During daylight hours, there is little increase in sleep above normal daytime values (17, 18), but large increases are observed during dark hours (17). Normally, rats sleep 70–80% of the time during daylight hours, during which time levels of endogenous sleep promoters are probably high, whereas during dark hours rats are normally active. Interestingly, intraperitoneal injection of MDP into rats at the beginning of daylight hours does not induce an increase in the duration of SWS (19), whereas administration of MDP to rats at the onset of dark hours results in excess sleep (20). These observations suggest that MDP may elicit its effects on sleep through the same mechanisms as do endogenous substances. It is also noted that in rabbits, the duration of SWS after 24 h of sleep deprivation (3) was similar to the values reported here (Fig. 1) after high doses of MDP.

Many analogs of MDP have been examined for their immunostimulatory and pyrogenic properties (11); some of these were found to be immunostimulatory but not pyrogenic (11). However, all those that were pyrogens were also found to be immunostimulants. Similarly, in the present paper we showed that some immunostimulants (Nos. 3, 8, 9, 15) and one pyrogenic derivative (No. 3) were not somnogenic. However, each agent that was somnogenic was also an immunostimulant and a pyrogen. It was previously shown (6) that pyrogenic actions of MDP could be suppressed with antipyretics without affecting sleep responses. Thus, through the use of various derivatives or through pharmacologic manipulation, these major actions of muramyl peptides can be separated, in part, from each other.

In the present study, we have amplified our understanding of structural requirements for somnogenic activity by muramyl peptides. Previously we reported (6) that the adjuvant-inactive stereoisomers of MDP (*N*Ac-mur-L-ala-L-isogln and *N*Ac-mur-D-ala-D-isogln) were inactive and that periodate oxidation of the muramyl moiety of MDP also led to the loss of somnogenic activity. With regard to this latter observation, we have now shown that replacement of the *N*-acetyl-glucosamine moiety of MDP by glucose also led to inactivity. Addition of *N*-acetyl-glucosamine in β 1-4 linkage to muramic acid in MDP (i.e., the disac-

charide monomer form found in bacterial cell wall peptidoglycans) does not block sleep-promoting activity. We also present evidence indicating that the presence of an unsubstituted amide on the free carboxyl of MDP leads to inactivity. However, if this position is in peptide linkage, somnogenic activity was retained or even enhanced. Our findings lead to the interesting hypothesis that perhaps specific mammalian amide-synthesizing or -hydrolyzing enzymes exist in the body that have the capacity to control the somnogenic activity of muramyl peptides.

Currently it is thought that the immunological and pyrogenic effects of muramyl peptides are mediated through the monokine, interleukin 1. Furthermore, we have recently shown that cerebral intraventricular administration of interleukin 1 can also induce dose-dependent increases in SWS.[2] Thus, it is possible that the functional role(s) of exogenous muramyl peptides in mammalian physiology could be analogous to those of vitamins and/or essential amino acids in that they may be incorporated into larger entities in order to exert their normal physiological action(s).

Summary

Sleep-promoting activities of muramyl dipeptide (MDP) (NAc-Mur-L-ala-D-isogln) and the naturally occurring muramyl peptide(s), factor S, have recently been demonstrated. We now have amplified our understanding of structural requirements for somnogenic activity. The effects of several analogs of MDP on rabbit slow-wave sleep are presented and these results are compared to the dose-response relationship for MDP. Some tentative conclusions as to structural requirements for somnogenic activity are presented; most notably, amidation of the free γ-carboxyl of MDP and several of its analogs resulted in the loss of somnogenic activity. MDP also can induce febrile and immunostimulatory responses. In the present paper, we show that some analogs possess immunostimulatory and pyrogenic activity but not somnogenic activity, thus suggesting that these biological activities of muramyl peptides may, in part, be mediated by separate mechanisms.

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