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Sleep-promoting effects of muramyl peptides

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ABSTRACT **A muramyl peptide that induces excess slow-wave sleep in rats, rabbits, and cats has recently been isolated from human urine. We now report that synthetic acetylmuramyl-L-alanyl-D-isoglutamine ("muramyl dipeptide") and its lysyl derivative (acetylmuramyl-L-alanyl-D-isoglutaminyllysine) can mimic the somnogenic effects of the natural peptide. Both compounds are also pyrogenic and may cause other disturbances of autonomic function. The pyrogenic effects of intravenously administered muramyl dipeptide can be suppressed by previous treatment with acetaminophen without blocking the sleep-promoting effects.**

A sleep-promoting factor derived from human urine has recently been described by Krueger *et al.* (1, 2). Correlation of amino acid and amino sugar analyses with sleep-promoting activities of purified fractions indicated that the sleep factor contained muramic acid, alanine, glutamic acid, and diaminopimelic acid. Chemical and physiological properties of the urinary factor resemble those of the sleep factor found in sterile cerebrospinal fluid and in acid/acetone extracts of brains from sleep-deprived animals (2, 3). In rabbits, intraventricular infusion of 10 pmol of the pure factor induces excess slow-wave sleep (SWS) for 5–10 hr after the infusion. The effects are primarily on the duration of SWS and on the amplitude of cortical slow waves; there is relatively little effect on rapid eye movement (REM) sleep. The excess SWS appears to be normal from a behavioral point of view; the animals can easily be aroused by noise or, if left to themselves, awaken spontaneously from time to time to eat, drink, and groom. Microinjection studies indicate that the site of action of the sleep factor is localized to a region between the basal forebrain and the mesodiencephalic junction (4).

Muramic acid and diaminopimelic acid are constituents of polymeric peptidoglycans in the cell walls of bacteria and the subunits are immunostimulants and pyrogens (5). The simplest synthetic analog that has both immunostimulatory and pyrogenic effects is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (AcMur-Ala-iGln, "muramyl dipeptide"; ref. 6) and many derivatives of this basic structure have been examined for their immunological and pyrogenic properties (5, 7, 8). Our discovery that the composition of sleep factor purified from brain or from urine is closely related to bacterial peptidoglycans led us to investigate the effects of some synthetic muramyl peptides on sleep. In the present paper, we show that AcMur-Ala-iGln and the tripeptide AcMur-Ala-iGln-Lys can mimic the sleep-promoting effects of sleep factor, although the response may be modified by pyrogenic effects and by other disturbances of autonomic function.

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MATERIALS AND METHODS

AcMur-Ala-iGln and its derivatives were gifts from E. Lederer (Université de Paris-Sud 91405 Orsay Cedex) and from P. Lefrancier (Institut Choay). The purity of these materials was checked by amino acid and amino sugar analyses of unhydrolyzed and hydrolyzed (6 M HCl, 100°C, 6 hr) samples using a Beckman 121 MB analyzer.

Surgical procedures for implantation of ventricular guide tubes and electroencephalogram (EEG)/EOG electrodes have been described (9, 10). Calibrated glass bead thermistors (Fenwall; 1.2-mm outside diameter, 50 Ω/°C) were implanted through burr holes in frontal bone: the thermistor leads were buried in a mound of dental cement that also insulated the screw electrodes and supported a 9-pin miniature electrical connector. In some experiments, the rectal temperatures were measured at intervals by using a calibrated thermistor probe; temperatures estimated from the implanted brain thermistors were generally within 0.3°C of simultaneously recorded rectal temperature.

New Zealand rabbits (male, 3–5 kg) or domestic cats were adapted to the infusion cages in a temperature-controlled room (21 ± 2°C) on a 12:12 light/dark cycle. Intraventricular infusions or systemic injections were carried out between 0800 and 1000 hr and were followed by 6 or more hr of recording without disturbing the animals. The vehicle for intraventricular infusion was 0.3 ml of pyrogen-free artificial cerebrospinal fluid (155 mM NaCl/3 mM KCl/1.15 mM $CaCl_2$/0.96 mM $MgCl_2$) and the rate of infusion was 3–18 μl/min in rabbits and up to 32 μl/min in cats. Indomethacin for intravenous injection was dissolved in a small amount of EtOH and diluted with 150 mM $NaHCO_3$ to a concentration of 5 mg/ml. Acetaminophen (Sigma) was dissolved in sterile pyrogen-free saline.

EEG, electro-oculogram, and bodily movements were recorded on Grass polygraphs. The amplitude of EEG slow waves was recorded as the rms-rectified output from a 0.5- to 4-Hz bandpass filter (Buxco Electronics, Sharon, CT); the rms component was integrated and the integral was printed on tape every 2 min, thus providing an objective measure of the product of amplitude and duration of EEG slow waves. Each record was also analyzed visually for duration of SWS in rabbits and for both SWS and REM sleep in cats. Details of the infusion and recording systems and examples of analysis of records have been given elsewhere (3, 4, 11).

RESULTS

Effects of Intraventricular Infusions of AcMur-Ala-iGln or AcMur-Ala-iGln-Lys. *Rabbits.* Intraventricular infusion of 75–150 pmol of AcMur-Ala-iGln or AcMur-Ala-iGln-Lys increased the hourly percentage of SWS in rabbits from control

Abbreviations: SWS, slow-wave sleep; REM, rapid eye movement; Mur, muramic acid; iGln, isoglutamine; EEG, electroencephalogram.

values of about 40% to about 60% for 6 hr or more after the infusion. The amplitude of slow waves during episodes of SWS was increased and there was an increase of 1 to 2°C in body temperature. The results obtained in a single experiment using AcMur-Ala-iGln-Lys are shown in Fig. 1, and similar results obtained from 12 rabbits given either AcMur-Ala-iGln or AcMur-Ala-iGln-Lys are summarized in Table 1. The somnogenic effects of AcMur-Ala-iGln resemble those elicited by factor S but the amount of synthetic peptide required to induce a comparable increase of SWS for 6 hr is roughly 10 times that of the natural product.

The excess sleep induced by intraventricular administration of AcMur-Ala-iGln or of AcMur-Ala-iGln-Lys in rabbits appears to be normal in spite of the development of fever. The episodic nature of sleep is retained and the induced excess sleep results primarily from an increase in the number of sleep episodes lasting for more than 8 min (Fig. 2). The animals can be aroused easily and, even during maximal responses involving 80% SWS, they awaken spontaneously from time to time to eat, drink, or groom. The amplitude of slow waves during each episode was also increased (Fig. 1 and Table 1) as it is following administration of sleep factor or during the deep sleep that occurs during recovery from sleep deprivation (3).

Cats. Each of four cats was infused intraventricularly one or more times with AcMur-Ala-iGln or AcMur-Ala-iGln-Lys (200–500 pmol). The responses were more complex than those in rabbits. The febrile responses to synthetic muramyl peptides were associated with lethargy and seeming inability to enter deep (stage II) SWS for 2–4 hr after the infusion; REM sleep was completely suppressed during this period. If the investigator entered the experimental chamber during this period, the cats remained curled up in their litter boxes and did not come forward to be petted as usual. Only after the febrile response had reached its peak did the cats start to sleep for abnormally long periods in deep stage II SWS alternating with periods of REM sleep. The SWS and temperature responses of one cat to 250 pmol of AcMur-Ala-iGln-Lys delivered intraventricularly during 15 min prior to start of recording is shown in Fig. 3. Similar results were obtained from each of the other three cats although the time courses of the febrile response and the periods of sleep suppression were variable. However, all cats receiving the synthetic muramyl peptides underwent an abnor-

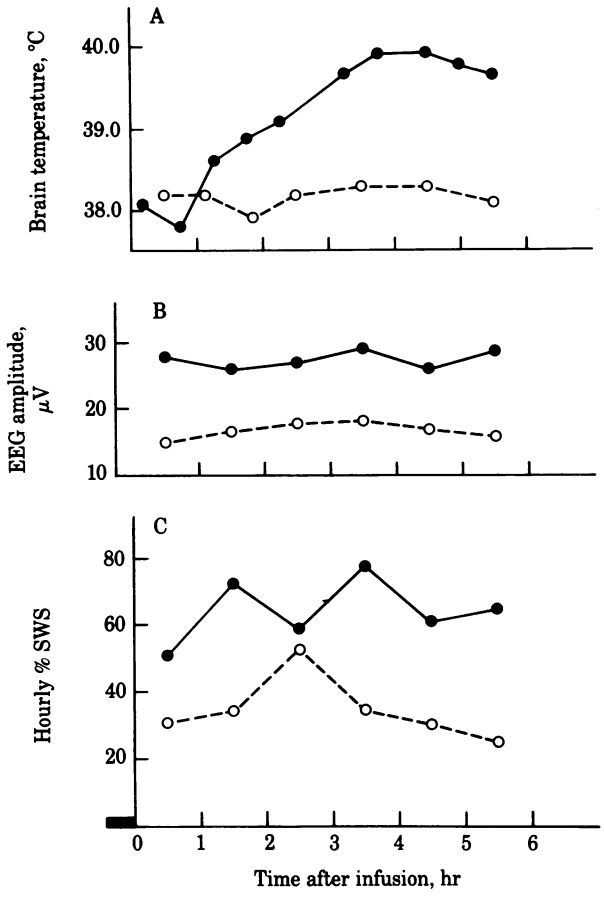

FIG. 1. Effects of AcMur-Ala-iGln-Lys on rabbit 9T illustrate in detail the 12 similar experiments summarized in Table 1. ○, Control, no infusion; ●, after intraventricular infusion of 150 pmol of AcMur-Ala-iGln-Lys (in 0.3 ml of artificial cerebrospinal fluid) for 20 min prior to zero time. The excess hourly % SWS (*C*) [64.5 ± 4.0 (mean ± SEM) vs. 34.5 ± 3.9 (control)] and the increased amplitude of EEG slow waves (*B*) began prior to onset of the febrile response (*A*). Effects of AcMur-Ala-iGln and AcMur-Ala-iGln-Lys may persist for 6–12 hr but recordings were ordinarily terminated after 6 hr.

Table 1. Effects of AcMur-Ala-iGln and of AcMur-Ala-iGln-Lys on sleep and brain temperature of rabbits

Rabbit	Infusate	Dose, pmol	% SWS		Amplitude* of slow wave, μV		ΔT_{max}, °C
			Control	Expt.	Control	Expt.	
62	AcMur-Ala-iGln	120	48	94	29	56	ND
65		120	43	69	28	42	ND
66		75	39	55	30	38	2.8
74		75	33	43	65	120	1.6
67		125	32	38	70	85	1.1
70		125	39	60	23	29	1.5
67	AcMur-Ala-iGln-Lys	100	32	62	70	145	1.7
69		150	32	51	38	54	ND
72		100	34	57	40	110	ND
76		100	35	62	52	77	ND
6T		150	46	57	24	25	2.0
8T		100	42	61	22	29	2.1
9T		150	41	67	21	30	2.2
Mean ± SEM			38 ± 2	59 ± 5	39 ± 5	65 ± 11	1.9 ± 0.2

% SWS was measured during hr 2–6 after infusion. Expt., experimental; ΔT_{max}, maximum increase in temperature; ND, not done.

* rms-Rectified voltage of 0.5- to 4-Hz band.

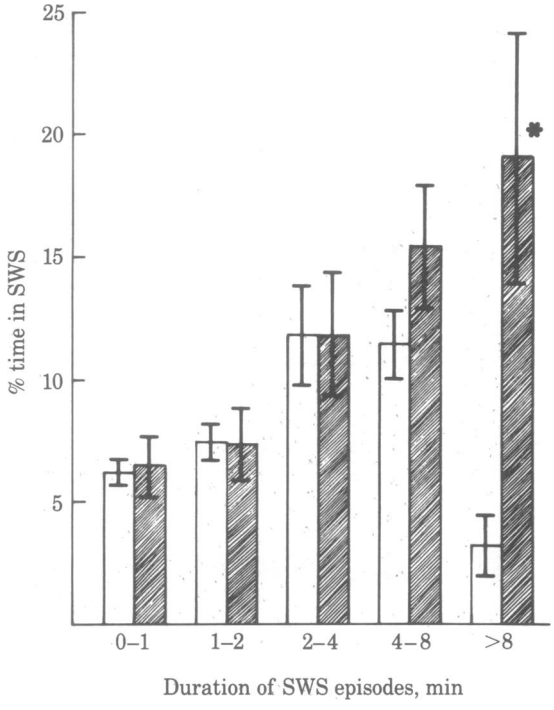

FIG. 2. Effect of AcMur-Ala-iGln on duration of sleep episodes. The duration of each episode of SWS was defined as that period of time during which high-amplitude EEG slow waves were not interrupted for more than 15 sec by the low-amplitude EEG characteristic of wakefulness or REM. The ordinate is the percentage of time spent in SWS in each duration class in the period from hr 2 to hr 6 after administration of AcMur-Ala-iGln. Each value is mean ± SEM of eight assays in eight rabbits. Striped bars show data after infusion of AcMur-Ala-iGln; open bars are data from same rabbits without infusion. The asterisk denotes a significant difference, $P < 0.0125$. AcMur-Ala-iGln increased the number of episodes of SWS having durations greater than 8 min but had no significant effect on the number of episodes of SWS having durations of 1–8 min.

mally long period of deep SWS after the initial period of sleep suppression. A similar delay in the onset of excess SWS was noted by Garcia-Arraras (10) after infusion of natural sleep factor in cats.

Effects of Intravenous, Intraperitoneal, and Enteric AcMur-Ala-iGln. Systemic administration of AcMur-Ala-iGln induces a febrile response in rabbits (8, 12), and we now report that somnogenic effects can also be elicited by intravenous and intraperitoneal injection or by administration via stomach tube. The average magnitude and time course of the somnogenic effects of AcMur-Ala-iGln administered intravenously to rabbits in doses of 150–200 nmol/kg (75–100 μg) is shown in Fig. 4. The sleep response reaches a maximum about 3 hr after injection and returns to near control values within 5 hr. During the first 3 hr, including the period of maximum sleep, the animals appear normal in the sense that they can be aroused easily and respond to handling. After 3 to 4 hr, however, the animals become unresponsive and may fail to open their eyes even when they are awake. In some animals, we noted abnormal lacrimation, nasal secretion, and inflammation of the conjunctiva. Evidently, the relatively large doses of AcMur-Ala-iGln required to induce pyrogenic or somnogenic effects via the intravenous route also cause disturbances of other autonomic functions: other deleterious effects of AcMur-Ala-iGln have been reviewed by Lederer (5). Masek *et al.* (13) reported that intravenous injection of bacterial peptidoglycans or of AcMur-Ala-iGln can cause suppression of REM sleep in rats for several hours; it may be that, in this species, as in the cat (Fig. 3), the

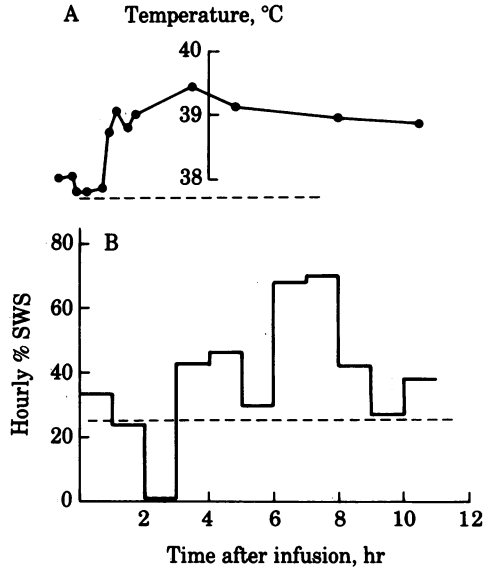

FIG. 3. Effects of AcMur-Ala-iGln-Lys on sleep (*B*) and brain temperature (*A*) in a cat; 240 pmol of AcMur-Ala-iGln-Lys was infused intraventricularly during 20 min prior to zero time. Normal sleep was partially inhibited during the chill phase of the pyrogenic response (0–3 hr). REM sleep (not shown) was completely suppressed in this period. Excess SWS induced by AcMur-Ala-iGln-Lys lasted for 3–8 hr after infusion. (*A*) ---, Control = 37.7 ± 0.2°C. (*B*) ---, Control = 27 ± 5%. Similar responses were observed following one or more infusions of AcMur-Ala-iGln or AcMur-Ala-iGln-Lys in each of four cats.

initial effect of AcMur-Ala-iGln is inhibitory.

In two rabbits, we recorded sleep responses after intraperitoneal injection of AcMur-Ala-iGln (160 μg/kg) and, in another two rabbits, sleep responses were recorded after administration of AcMur-Ala-iGln by stomach tube (5 mg/kg). Substantial sleep responses (*ca.* 50% increase of SWS for 6 hr) were noted in both cases.

Effects of Indomethacin and Acetaminophen. The time course of the pyrogenic response to AcMur-Ala-iGln was sometimes quite different from that of the somnogenic response and in some animals the natural muramyl peptide isolated from urine induced substantial excess SWS without significant alterations of temperature. This suggested the possibility that the somnogenic effect of AcMur-Ala-iGln might be dissociated from

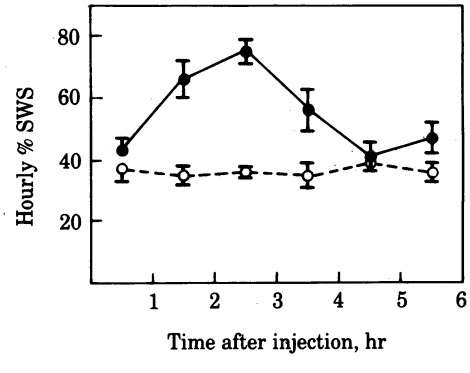

FIG. 4. Effect of intravenous AcMur-Ala-iGln on SWS in rabbits. ●, After single intravenous injection of 150–200 nmol/kg at zero time; ○, control values from same rabbits. Each value is mean ± SEM of 11 assays in eight rabbits. Intravenous AcMur-Ala-iGln, in doses sufficient to induce excess SWS and pyrexia also caused deleterious side effects after about 3 hr. In contrast, much smaller doses of AcMur-Ala-iGln delivered intraventricularly did not cause deleterious effects and the excess SWS continued for 6–10 hr (compare Figs. 4 and 1*C*).

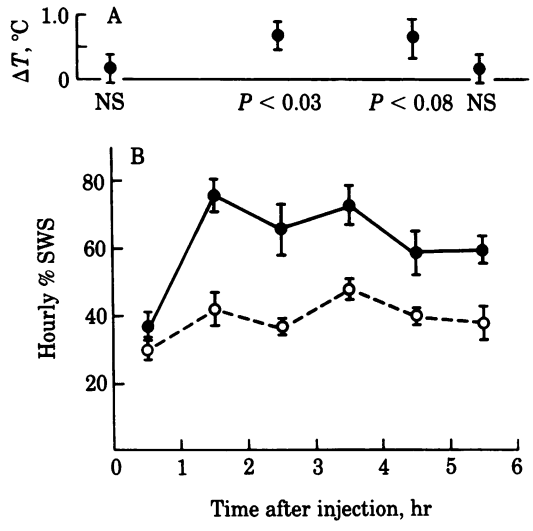

FIG. 5. Effects of acetaminophen (AAP) on somnogenic (*B*) and pyrogenic (*A*) actions of AcMur-Ala-iGln. AAP at 10 mg/kg was injected intravenously prior to time zero. ○, AAP alone; ●, AAP followed by 120 μg of AcMur-Ala-iGln intravenously at time zero. Each value is mean ± SEM of six assays in six rabbits. In *A*, the average of the individual increases (ΔT values) in rectal temperature induced by AcMur-Ala-iGln together with AAP is compared with that induced by AAP alone in each of six rabbits. AAP alone had no significant effects on either sleep or rectal temperature nor did it block the sleep-inducing effects of AcMur-Ala-iGln [hourly % SWS: AAP alone, 41 ± 3 (mean ± SEM); AAP together with AcMur-Ala-iGln, 67 ± 3]. However, the pyrogenic effects of AcMur-Ala-iGln were completely or partially suppressed by AAP. The average ΔT after AcMur-Ala-iGln together with AAP was <0.7°C as compared with 1.9 ± 0.2°C after AcMur-Ala-iGln alone (Table 1). NS, not significant.

its pyrogenic effect by previous treatment with antipyretics. In preliminary experiments, we used indomethacin as an antipyretic because Parant *et al.* (14) had shown that this drug (in doses of 40 mg/kg) was effective in blocking the pyrogenic response to intravenously administered AcMur-Ala-iGln. However, we found that indomethacin alone caused toxic reactions in rabbits and cats, even in doses as low as 1 mg/kg; toxic reactions included diarrhea, lethargy, and inhibition of normal sleep. For this reason, we used the less toxic acetaminophen to suppress the pyrogenic effects of AcMur-Ala-iGln. The results for six rabbits injected intravenously with AcMur-Ala-iGln at 120 μg/kg 30 min after intravenous injection of acetaminophen at 10 mg/kg are summarized in Fig. 5. It is clear that, under these conditions, a substantial somnogenic effect was obtained for 3 to 4 hr with only a weak associated febrile response. In some animals, there was no significant increase in temperature during the sleep response. After about 4 hr, the animals developed toxic reactions similar to those that follow the intravenous administration of AcMur-Ala-iGln alone.

Indomethacin failed to block either the pyrogenic or the somnogenic effects of intraventricularly administered AcMur-Ala-iGln in 10 out of 10 trials in six rabbits. Similar results were obtained with acetaminophen in two rabbits. At present, we have no explanation for the fact that these drugs suppress the pyrogenic effects of AcMur-Ala-iGln when it is delivered intravenously but not when it is delivered intraventricularly in rabbits. For purposes of the present paper, however, it is sufficient to note that the somnogenic effect of intravenously administered AcMur-Ala-iGln can be dissociated from its pyrogenic effect by prior treatment with acetaminophen.

Effects of Some AcMur-Ala-iGln Derivatives. Several AcMur-Ala-iGln derivatives were infused intraventricularly into rabbits with a view to determining (*i*) relationships between structure of muramyl peptides and somnogenic activity and (*ii*) whether some derivatives might be found that would induce sleep without fever. The results are summarized in Table 2 and compared with the effects of AcMur-Ala-iGln and AcMur-Ala-iGln-Lys. The stereoisomers of AcMur-Ala-iGln (DD and LL) are nonpyrogenic and they failed to induced sleep; they are also ineffective as immunostimulants (5, 15). The methyl and *n*-butyl esters and the *N*-Me derivative of AcMur-Ala-iGln are immunostimulants but they have no pyrogenic or somnogenic effects. Similar results were obtained after intravenous injection of 500 μg of the methylated derivatives. The muramyl moiety of AcMur-Ala-iGln was cleaved by gentle periodate oxidation to remove carbons 5 and 6 from the glucosamine ring. This treatment abolished the somnogenic effect, indicating that the muramyl component plays an essential role in the biological activity. However, *N*-acetylmuramic acid by itself had no effect on sleep.

DISCUSSION

Muramyl dipeptide was first synthesized with a view to determining the simplest chemical structure capable of mimicking the immunostimulatory properties of the bacterial peptidoglycans (6). Subsequent work showed that AcMur-Ala-iGln and several of its derivatives are pyrogenic, although some derivatives that are immunostimulants are not pyrogens (7). The pyrogenic effects of systemically administered AcMur-Ala-iGln may be mediated by formation of leukocytic pyrogens (14) but

Table 2. Somnogenic and pyrogenic effects of AcMur-Ala-iGln and related compounds

Compound	Dose, pmol	*n*	% SWS		Pyrogenic effect	Immunostimulatory effect
			Control	Expt.		
AcMur-Ala-iGln	75–150	6	40 ± 2	62 ± 7	+	+ (6)
AcMur-Ala-iGln-Lys	100–150	5	35 ± 2	60 ± 3	+	+ (15)
AcMur-D-Ala-D-iGln	100–1,000	3	33 ± 1	35 ± 2	−	− (5)
AcMur-L-Ala-L-iGln	200	4	38 ± 2	38 ± 4	−	− (15)
AcMur-L-Ala-D-iGlnOMe	500	2	34–42	31–45	−	+ (15)
AcMur-L-Ala-D-iGln *n*-Bu ester	250	2	37–42	41–47	−	+ (5)
AcMur(*N*-Me)-L-Ala-D-iGln	200	2	37–42	32–47	−	+ (5)
Periodate-cleaved AcMur-Ala-iGln*	200	2	40–44	38–42	ND	ND
AcMur	500	2	35–36	29–35	ND	ND

% SWS was measured during hr 2–6 after infusion. Results are mean ± SEM or ranges for *n* rabbits. ND, not done.

* AcMur-Ala-iGln was treated with periodate to remove two carbon atoms from the muramic acid ring. One hundred nmol of AcMur-Ala-iGln was incubated with 0.18 M Na metaperiodate in a volume of 40 μl overnight at 3°C. The next day, 4 μl of ethylene glycol was added at room temperature. This solution was kept in the dark for 30 min and then applied to a 19-ml G-10 Sephadex column equilibrated and developed in 50 nM acetic acid. The void volume of 6 ml was discarded; the next 5.5 ml was saved. Aliquots from this fraction were subjected to amino acid analyses before and after acid hydrolysis. The amount of glucose released by hydrolysis was used to calculate the dose.

it is also true, as shown by Riveau *et al.* (12) and confirmed in the present paper, that pmol doses of AcMur-Ala-iGln administered intraventricularly elicit long-lasting febrile responses. In this case, AcMur-Ala-iGln may act directly on temperature-regulating neurons or indirectly through centrally generated endogenous pyrogens.

In the present paper, we have reported that AcMur-Ala-iGln and AcMur-Ala-iGln-Lys induce excess SWS in rabbits and cats. It is not surprising that this somnogenic effect was overlooked by previous investigators interested in the pyrogenic or immunostimulatory properties of the muramyl peptides. The excess sleep induced by AcMur-Ala-iGln is easily disturbed by procedures such as the insertion of rectal probes for measurement of temperature or even by the presence of human activity in an open laboratory. It is unlikely that the somnogenic effects of AcMur-Ala-iGln would be noted without recording EEGs from animals maintained in an environment free from disturbances. We were led to investigate the somnogenic effects of AcMur-Ala-iGln only because the sleep-promoting factor we had isolated from human urine turned out to be a complex muramyl peptide (2), and positive somnogenic effects obtained with relatively simple synthetic analogs would provide circumstantial evidence that the material isolated from urine was indeed responsible for the observed sleep-promoting effects. Results reported here show that AcMur-Ala-iGln and AcMur-Ala-iGln-Lys, administered intraventricularly to rabbits in doses of 75–150 pmol per rabbit, can mimic the somnogenic effects of the urinary muramyl peptide. The extremely low doses involved suggest the presence in brain of receptors that have a high affinity for muramyl peptides and it would not be surprising if such compounds, or some endogenous equivalent, play an important role in the physiology of sleep and temperature regulation. The muramyl peptide isolated from urine may well be such an endogenous neuromodulator.

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