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Somnogenic Activities of Synthetic Lipid A

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Bacterial infections and various immune response modifiers, including endotoxin and its lipid A moiety, alter sleep duration. The purpose of this study is to amplify our understanding of lipid A structure–somnogenic-pyrogenic activity relationships. Four synthetic disaccharide analogs of lipid A (LA-15-PP, LA-15-PH, LA-16-PH, and LA-18-PP) and a biosynthetic monosaccharide analog of lipid A (lipid X) were tested in rabbits for their effects on slow-wave sleep, rapid-eye-movement sleep, electroencephalographic slow-wave (0.5- to 4.0-Hz) amplitudes, and brain-colonic temperatures. Substances were injected intravenously and intracerebroventricularly. Compound LA-15-PP was the most potent; it significantly increased slow-wave sleep, delta electroencephalographic amplitudes, and brain-colonic temperatures while reducing rapid-eye-movement sleep. Compound LA-15-PH (monophosphoral analog of LA-15-PP) induced effects similar to those of LA-15-PP, although the responses were weaker. Compound LA-18-PP induced increases in slow-wave sleep and delta amplitudes only after high doses, whereas compound LA-16-PH was devoid of these activities at the doses tested. Intracerebroventricular, but not intravenous, injections of lipid X induced small but significant increases in both slow-wave sleep and rapid-eye-movement sleep without affecting delta amplitudes or brain-colonic temperatures. These data suggest that the somnogenic actions of these lipid A analogs depend on the acylation or phosphorylation pattern and backbone structures of the molecules. Their soporific activities parallel their relative behaviors in other biological assays.

During the past few years, several lines of evidence have suggested that sleep is associated with infectious processes (16, 28). Muramyl peptides are sleep-promoting substances isolated from mammalian brain and urine (10, 13, 14); they are also the monomeric building blocks of bacterial cell wall peptidoglycans. Somnogenic muramyl peptides identical in structure to those obtained from brain and urine have been isolated from enzymatic digests of *Neisseria gonorrhoeae* (15). Although there are no known mammalian synthetic pathways for muramic acid and diaminopimelic acid, both of these substances are in hydrolyzable linkage in mammalian tissue (13, 22). Further, during the processing of gram-positive bacteria by mammalian macrophages, muramyl peptides are released (29), as are other somnogenic substances of low molecular weight (possibly muramyl peptides) (L. Johannsen, J. Wecke, and J. Krueger, Soc. Neurosci. Abstr. **13**:260, 1987). In addition, it was recently shown that enhanced slow-wave sleep is observed after intravenous inoculation of rabbits with viable *Staphylococcus aureus* (28). Preliminary results (L. Toth, Soc. Neurosci. Abstr. **14**:1308, 1988) indicate that gram-negative bacterial infections (i.e., *Escherichia coli*) are also associated with enhanced slow-wave sleep, although the response course induced by *E. coli* was distinct from that induced by *S. aureus*. Endotoxin (lipopolysaccharide) and its lipid A moiety are also somnogenic (12).

Muramyl peptides and lipopolysaccharides-lipid As share many biological activities. For example, certain substances from both classes stimulate the acute-phase response (18), potentiate nonspecific resistance to infection (20), and induce fever (27). Both classes of substances also stimulate the synthesis and release of interleukin-1 (reviewed in reference 1 and 24). It is postulated that these bacterial cell wall products exert some of their effects via the production and release of interleukin-1 (12) and other cytokines; interleukin-1β, alpha interferon subtype 2, and tumor necrosis factor are also somnogenic.

4′-Monophosphoryl and bisphosphoryl lipid A derived from *Salmonella typhimurium* were previously described as being somnogenic (12). These substances induced dose-dependent increases of slow-wave sleep duration, electroencephalographic slow-wave (0.5- to 4.0-Hz) voltages, and body temperature, as well as decreases in rapid-eye-movement sleep. The recent chemical identifications and syntheses of different lipid A molecules and several precursors have allowed a new approach to the characterization of these materials. The purposes of the present study were to enhance our understanding of lipid A structure-activity relationships relative to sleep effects and to determine whether the somnogenic activity of these substances correlates with their other known biological activities. We report here that some of these synthetic substances are somnogenic.

MATERIALS AND METHODS

Test materials. Synthetic *E. coli*-type lipid A (compound LA-15-PP), its 4′-monophosphoryl analog (compound LA-15-PH) (Fig. 1), and a bisphosphoryl tetraacyl compound, an analog of a biosynthetic precursor Ia (IVa) (LA-18-PP), were tested. In addition, a synthetic *Salmonella minnesota*-type 4′-monophosphoryl lipid A analog, compound LA-16-PH (Fig. 1), was used. These substances were synthesized and characterized for certain biological activities as previously described (2, 4–9, 17; J. Hildebrandt, A. Haslberger, and P. Mayer, J. Immunol. Immunopharmacol. **6**[Suppl.]:190, 1986). Lipid X, a diacyl monosaccharide partial structure of lipid A, was a gift from N. von Jeney of Sandoz Research Institute. All samples were dissolved or suspended as homogeneously as possible by vigorous shaking at 1 mg/ml in 0.1% (vol/vol) triethylamine aqueous solution. Samples were in

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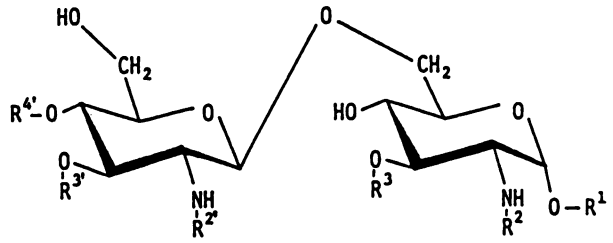

Compound	$R^{3'}$	$R^{2'}$	R^3	R^2	$R^{4'}$	R^1
LA-15-PP (506)	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-OH	P	P
LA-15-PH (504)	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-OH	P	H
LA-16-PH (514)	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{16})	P	H
LA-18-PP	C_{14}	C_{14}-OH	C_{14}	C_{14}-OH	P	P
Lipid X	---	---	C_{14}-OH	C_{14}-OH	-	P

FIG. 1. Structures of lipid A molecules and lipid X used in this study. Abbreviations: P, $PO(OH)_2$; C_{14}, tetradecanoyl; C_{14}-OH, (*R*)-3-hydroxytetradecanoyl; C_{14}-O-(C_{12}), (*R*)-3-dodecanoyloxytetradecanoyl; C_{14}-O-(C_{14}), (*R*)-3-tetradecanoyloxytetradecanoyl; C_{14}-O-(C_{16}), (*R*)-3-hexadecanoyloxytetradecanoyl.

polypropylene plastic test tubes; portions were then taken for further dilution and immediate use, and the remainder was stored frozen at −20°C until used. Compounds LA-15-PP, LA-18-PP, and lipid X completely dissolved, whereas compounds LA-16-PH and LA-15-PH were less soluble and produced a slightly turbid suspension. Appropriate samples were then further diluted in pyrogen-free saline (Abbott Laboratories, North Chicago, Ill.) before injection into animals; after these dilutions, all solutions were transparent. Diluted compounds were used within 30 min of preparation. All syringes, needles, containers, and solutions were sterile.

Preparation of assay rabbits. Adult male New Zealand White rabbits (3 to 4 kg) free from *Pasteurella* spp. were provided with six electroencephalograph electrodes, a brain thermistor, and a cerebral ventricular guide cannula as previously described (30). Briefly, the animals were anesthetized with ketamine-xylazine (35.0:5.0 mg/kg, subcutaneous), and stainless-steel screws were placed over the frontal, parietal, and occipital cerebral cortices. A 1-mm-diameter glass bead 50-kΩ thermistor (Fenwell Electronics) was inserted 3 mm into the parietal cortex. Wires fitted into a nine-pin Amphenol plug were fixed to the electrodes and thermistor leads. A ventricular guide tube was positioned 4 mm lateral to the bregma. After surgery, the incision was treated with a topical antibiotic (bacitracin; Eli Lilly & Co., Indianapolis, Ind.), and 150,000 U of Duracillin (Lilly) were given intramuscularly. After surgery, a minimum of 1 week passed before a rabbit was used for experimentation.

Experimental procedure. Rabbits were housed under a 12:12-h light-dark cycle (lights on, 0600 to 1800 h) at 21°C. The experimental chambers (Hotpack model 352600) were kept on the same light-temperature regimen as the animal facility. Food and water were available ad libitum. Each recording chamber was equipped with a multichannel electronic swivel (Air Precision) connecting the Amphenol head plug in the rabbit (via a flexible umbilical cable) to a polygraph (model 7D; Grass), thus allowing the animals free movement.

Rabbits were placed into the experimental chambers for overnight acclimation the day before an experiment. They were connected to the recording cables for a 1-h habituation period before injections; then they were removed from the chambers, and their colonic temperatures were recorded with a calibrated digital thermistor probe (Yellow Springs Instrument Co., Yellow Springs, Ohio) inserted 10 cm into the colon. The lipid A analog was then injected. Materials to be injected intracerebroventricularly were diluted such that the correct dose was in a volume of 50 μl, which was then injected slowly over 5 min. For intravenous injections, substances were diluted to 500 μl and then injected (ca. 30 s) into a marginal ear vein. After injections, rabbits were returned to their cages, and a 6-h recording period began, after which colonic temperature was again measured. Rabbits did not receive two intravenous injections of the same material, nor were they used again for at least 1 week after any assay. No signs of tolerance to lipid A were observed under these conditions. Control recordings were taken on different days by identical procedures, except that no materials were administered, providing a matched-pairs experimental design. It was previously shown that intracerebroventricular or intravenous injections of control substances do not alter subsequent sleep in rabbits (15). Doses of substances injected intracerebroventricularly are expressed in nanograms because adult rabbit brains weigh about 8 g, regardless of body weight.

Recording and analyses. Movement (Grass tremor transducer model SPAI), electroencephalogram, and brain temperature were recorded throughout the 6-h assay period. The electroencephalographic signals were rectified and processed through bandpass filters (Buxco Electronics) to obtain

TABLE 1. Effects of lipid A compounds and lipid X on rabbit slow-wave sleep (SWS), rapid-eye-movement sleep (REMS), electroencephalographic delta voltages, and 6-h colonic temperatures[a]

Substance	Injection route[b]	Dose	n	Percent SWS		Percent REMS		Delta voltage		Colonic temp (°C)	
				Control	Expt	Control	Expt	Control	Expt	Control	Expt
LA-15-PP	i.v.	0.03 μg/kg	4	48 ± 4	62 ± 4[c]	4.6 ± 0.8	2.5 ± 1.5	67 ± 3	86 ± 15[c]	38.9	39.3
	i.v.	0.3 μg/kg	4	56 ± 3	83 ± 3[c]	4.6 ± 0.8	0.1 ± 0.1[c]	75 ± 8	120 ± 16[c]	39.0	39.6[d]
	i.c.v.	1 ng	4	36 ± 2	42 ± 4[c]	4.8 ± 0.5	5.7 ± 0.6	75 ± 7	88 ± 6[c]	39.3	39.7
	i.c.v.	10 ng	4	44 ± 2	46 ± 3	5.8 ± 0.8	3.3 ± 0.7	113 ± 8	126 ± 11[c]	39.2	40.4[d]
LA-15-PH	i.v.	0.3 μg/kg	4	50 ± 3	50 ± 1	6.9 ± 0.4	2.1 ± 0.9[c]	73 ± 5	72 ± 5	39.2	39.3
	i.v.	3.3 μg/kg	8	53 ± 1	65 ± 2[c]	3.8 ± 0.8	1.1 ± 0.4[c]	104 ± 9	121 ± 12[c]	39.3	39.9[d]
	i.c.v.	1 ng	4	55 ± 4	54 ± 2	3.4 ± 0.9	2.0 ± 0.4	107 ± 2	116 ± 6	39.5	39.6
	i.c.v.	10 ng	8	50 ± 3	53 ± 2	4.2 ± 1.5	2.6 ± 0.6	NA[e]	NA	39.5	40.3[d]
LA-18-PP	i.v.	0.3 μg/kg	4	38 ± 2	41 ± 3	4.9 ± 0.8	4.7 ± 0.9	95 ± 6	102 ± 11	39.1	39.3
	i.v.	3.3 μg/kg	4	36 ± 2	53 ± 2[c]	4.8 ± 0.5	3.6 ± 0.5	75 ± 7	99 ± 8[c]	39.3	39.3
	i.c.v.	1 ng	4	44 ± 2	41 ± 2	5.8 ± 0.8	5.5 ± 0.3	113 ± 8	111 ± 6	39.1	39.4
	i.c.v.	10 ng	8	39 ± 2	42 ± 3	4.9 ± 0.3	4.8 ± 0.6	81 ± 4	90 ± 4[c]	39.3	40.1[d]
LA-16-PH	i.v.	0.3 μg/kg	4	50 ± 3	52 ± 4	6.3 ± 1.2	3.1 ± 0.7[c]	97 ± 5	101 ± 7	39.3	39.4
	i.v.	3.3 μg/kg	4	55 ± 4	49 ± 2	3.6 ± 0.8	3.0 ± 0.9	107 ± 2	115 ± 4[c]	39.5	39.5
	i.v.	33.0 μg/kg	4	48 ± 4	45 ± 2	3.0 ± 0.9	2.3 ± 0.3	79 ± 11	76 ± 8	39.6	39.5
	i.c.v.	1 ng	3	50 ± 4	52 ± 4	4.9 ± 0.3	2.5 ± 0.9	82 ± 1	84 ± 10	39.1	39.4
	i.c.v.	10 ng	4	54 ± 2	52 ± 2	4.7 ± 1.0	2.4 ± 0.5	105 ± 16	105 ± 11	39.2	39.5
	i.c.v.	100 ng	4	52 ± 1	50 ± 2	1.8 ± 0.7	2.4 ± 0.7	78 ± 5	84 ± 7	39.2	39.1
Lipid X	i.v.	3.3 μg/kg	4	36 ± 2	35 ± 3	4.7 ± 0.8	6.1 ± 0.6	85 ± 5	89 ± 5	39.5	39.2
	i.v.	33.0 μg/kg	4	41 ± 2	44 ± 3	6.1 ± 0.8	4.2 ± 0.4	131 ± 5	132 ± 9	39.2	39.2
	i.c.v.	10 ng	4	43 ± 2	38 ± 3	5.0 ± 0.3	4.6 ± 0.9	88 ± 2	91 ± 2	39.2	39.5
	i.c.v.	100 ng	4	38 ± 2	43 ± 2[c]	4.9 ± 0.8	6.6 ± 1.2[c]	95 ± 6	92 ± 4	39.1	39.3

[a] Data shown are means ± standard errors; all temperatures, standard error ≤ 0.1°C.
[b] i.v., Intravenous; i.c.v., intracerebroventricular.
[c] Wilcoxon matched-pairs test, $P \leq 0.05$.
[d] Temperature increased by ≥0.5°C.
[e] NA, Not available.

1-min average voltages of the delta (0.5- to 4.0-Hz), theta (4.0- to 8.0-Hz), alpha (8.0- to 12.0-Hz), and beta (12.0- to 25.0-Hz) frequency bands; these values were printed. The theta/delta ratio was continuously computed and simultaneously displayed with the above-mentioned recordings on the polygraph tracings.

Recordings were analyzed visually to determine the durations of wakefulness, slow-wave sleep, and rapid-eye-movement sleep. In rabbits, episodes of slow-wave sleep and rapid-eye-movement sleep are of relatively short duration (<3 min average) and alternate with wakefulness throughout the day (30). Slow-wave sleep is characterized by increased electroencephalographic slow-wave voltages, little body movement, low theta/delta ratios, and decreasing brain temperature. Wakefulness was identified by a low-voltage electroencephalogram, increased body movements, midlevel theta/delta ratios, and decreasing brain temperature after rapid-eye-movement sleep or increasing brain temperature after slow-wave sleep. Rapid-eye-movement sleep was characterized by a low-voltage electroencephalogram, phasic body movements, high theta/delta ratios, and a relatively rapid increase in brain temperature. The duration of any vigilance state was measured by entering episode length (distance along the recording paper) into a computer with a calibrated digitizer board (Jandel Scientific). These were then analyzed by a computer program to produce cumulative statistics based on the measured distances (durations) of the different sleep states.

The percentage of time spent in each vigilance state (slow-wave sleep, rapid-eye-movement sleep, wakefulness) was calculated for each hour for each rabbit (Table 1), and average electroencephalographic voltages were calculated similarly. The changes in brain temperature were calibrated to changes in colonic temperature as previously described (14). Treatment groups were compared with their own controls by a Friedman test (nonparametric two-way analysis of variance) to determine possible treatment effects. If significant differences were found, the Wilcoxon matched-paris test was used to determine when the differences occurred. In some cases, additional analyses were performed with data collected only during postinjection hours 2 to 4 (for intravenous injections) or 3 to 5 (for intracerebroventricular infusions). This was done because previous work with lipid A molecules showed that somnogenic effects were maximal during these periods. The number of animals tested for each compound is shown in the Table and figure legends. Alpha levels of 5% were used.

RESULTS

Compound LA-15-PP. Compound LA-15-PP, a hexaacyl bisphosphoryl lipid A (Fig. 1), was the most active compound tested in this study. After either 0.03 or 0.3 μg/kg was administrated intravenously, significant increases in slow-wave sleep for the 6-h recording period were observed (Table 1). The greatest increases in slow-wave sleep were observed 2 to 4 h after intravenous injection, whereas after intracerebroventricular administration, the largest effects on slow-wave sleep were observed 3 to 5 h after injections, confirming previous results with other lipid A substances

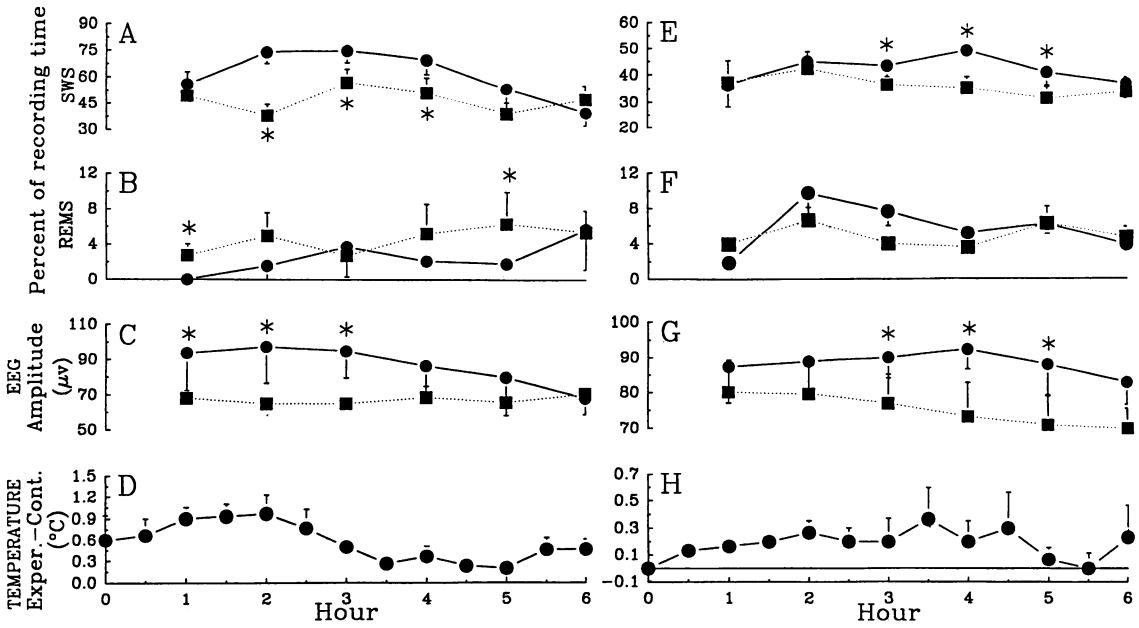

FIG. 2. Time courses of slow-wave sleep (SWS) (A and E); rapid-eye-movement sleep (REMs) (B and F), electroencephalographic slow-wave (0.5- to 4.0-Hz) amplitudes (C and G), and brain temperatures (D and H) after intravenous injection (A to D, 0.03 μg/kg) and intracerebroventricular injection (E to H, 1.0 ng) of lipid A analog LA-15-PP (●) or control values (■) from the same rabbits. $n = 4$ for all data points. Brain temperature values are expressed as difference from control values. *, $P < 0.05$, Wilcoxon matched-pairs test; error bars indicate standard error of mean.

(12). Thus, if we restrict the analysis to hours 3 to 5 after intracerebroventricular administration, both doses tested intracerebroventricularly (1 and 10 ng) significantly enhanced slow-wave sleep; values (percent time) were (± standard error of mean) 34 ± 4 control and 45 ± 4 experimental after 1 ng, and 44 ± 3 control and 53 ± 4 experimental after 10 ng.

Quantification of average delta-wave electroencephalographic voltages (slow wave; 0.5 to 4.0 Hz) is a sensitive electronic method that may be used as an objective measure to amplify and verify visual scoring of electroencephalographic records (23). Significant increases of electroencephalographic delta amplitudes were found for all the doses tested after compound LA-15-PP was given either intravenously or intracerebroventricularly (Table 1). The time course of effects on electroencephalographic slow-wave voltage was similar to that observed for slow-wave sleep, although both intravenous and intracerebroventricular injections of compound LA-15-PP enhanced amplitudes of electroencephalographic slow waves during the first hour postinjection (Fig. 2).

Intravenous administration of compound LA-15-PP induced decreases in rapid-eye-movement sleep. After the lower intravenous dose (0.03 μg/kg), three of the four animals tested had less rapid-eye-movement sleep than that observed under control conditions, although this effect was not significant (Table 1). After the higher intravenous dose (0.3 μg/kg), a significant decrease in rapid-eye-movement sleep was observed. In contrast, rapid-eye-movement sleep was not significantly reduced for the 6-h recording period after either intracerebroventricular dose. However, if we restrict the analysis to postinjection hours 3 to 5, the period during which maximum effects on slow-wave sleep were observed, a significant decrease in rapid-eye-movement sleep was evident after intracerebroventricular injection of 10 ng of compound LA-15-PP (6.2% ± 0.4% control versus 2.9% ± 0.7% experimental).

Although rabbit behavior was not systematically quantified in these studies, compound LA-15-PP at the doses tested did not induce gross abnormal behavior. Thus, the animals continued to cycle through the three major states of vigilance in a relatively normal fashion, although durations of individual states were altered. Animals could easily be aroused and showed normal responses when colonic temperatures were measured at the end of the 6-h recording period.

Colonic temperatures taken at the end of the 6-h recording period were also elevated after administration of compound LA-15-PP, although only the higher doses induced significant increases (Table 1). The courses of brain temperature responses after intravenous injections were different from those observed after intracerebroventricular injections. Thus, after intravenous injections, brain temperature increased within the first hour, and by 3.5 h postinjection, values returned to near control values (Fig. 2). In contrast, intracerebroventricular injection of compound LA-15-PP produced a relatively slow onset of elevated brain temperature, and maximum increases were observed at the end of 6 h. Another aspect of temperature regulation remained undisturbed after injection of compound LA-15-PP, that is, brain temperature changes that are coupled to states of vigilance (30) continued to fluctuate in a normal fashion (data not shown). However, these vigilance state-coupled changes in brain temperature were small in magnitude (0.1 to 0.3°C) compared with the febrile responses induced by compound LA-15-PP.

Compound LA-15-PH. Compound LA-15-PH is the 4′-monophosphoryl analog of compound LA-15-PP (Fig. 1) and, in general, had effects on sleep and colonic temperature that were similar to, although weaker than, those observed with compound LA-15-PP. Only the higher intravenous dose tested (3.3 μg/kg) significantly enhanced the duration of slow-wave sleep and amplitudes of electroencephalographic slow-wave voltages (Table 1). Neither intracerebroventricular dose of compound LA-15-PH enhanced the duration of

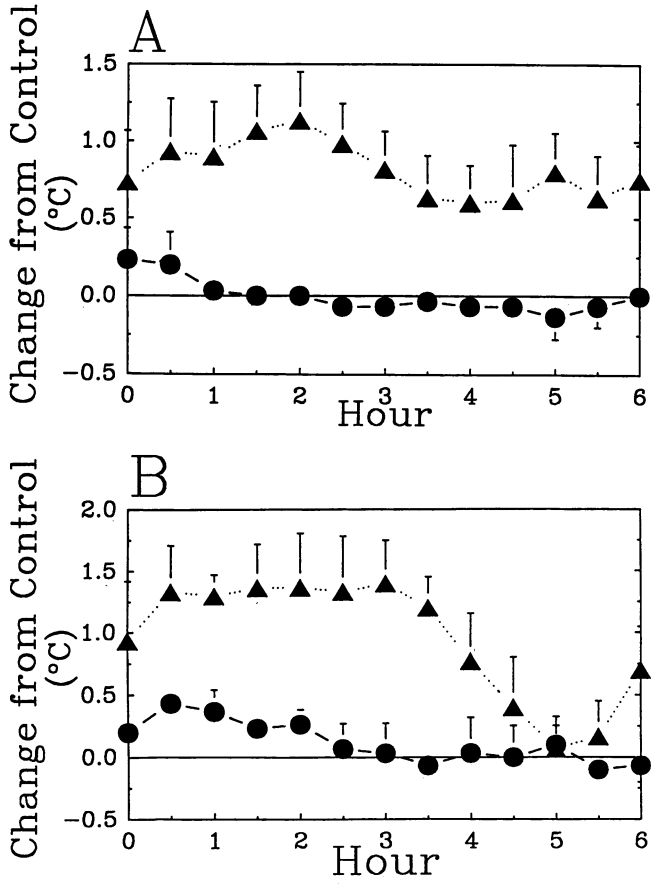

FIG. 3. Course of brain temperatures after intravenous injection of (A) lipid A analog LA-15-PH (0.3 μg/kg, $n = 3$ [●]; and 3.3 μg/kg, $n = 6$ [▲]) and (B) lipid A analog LA-18-PP (0.3 μg/kg, $n = 4$ [●], and 3.3 μg/kg; $n = 4$ [▲]). The higher dose of both these substances enhanced brain temperature, whereas the lower doses were ineffective. Error bars indicate standard error of mean.

slow-wave sleep. Both intravenous doses of compound LA-15-PH induced a significant decrease in rapid-eye-movement sleep. Rapid-eye-movement sleep was reduced after both intracerebroventricular doses, although neither effect was significant (Table 1). No abnormal behavior was observed after any dose of compound LA-15-PH.

The highest intravenous and intracerebroventricular doses of compound LA-15-PH also induced increases in colonic temperature measured after the 6-h recording period (Table 1). The time course of brain temperature changes induced after intravenous injection (3.3 μg/kg) of compound LA-15-PH was biphasic (Fig. 3). Changes in brain temperature after intravenous injection of the lower dose (0.3 μg/kg) were minimal. It is important that, as mentioned above, this dose significantly decreased rapid-eye-movement sleep. Brain temperature changes coupled to states of vigilance were not altered after administration of compound LA-15-PH.

Compound LA-18-PP. Compound LA-18-PP is an artificial tetraacyl bisphosphorylated disaccharide analog of lipid A molecules (similar to biosynthetic precursor Ia) (Fig. 1). This substance when given intravenously (3.3 μg/kg) significantly enhanced slow-wave sleep and electroencephalographic slow-wave voltages for the 6-h recording period (Table 1). Neither the lower intravenous dose (0.3 μg/kg) nor the two intracerebroventricular doses significantly altered the duration of slow-wave sleep. However, the higher intracerebroventricular dose (10 ng) of compound LA-18-PP significantly enhanced electroencephalographic slow-wave voltage, although this effect was small (Table 1). The higher intravenous (Fig. 3) and intracerebroventricular (Table 1) doses also enhanced brain and colonic temperature, respectively. As with the other compounds tested in this study, this substance did not induce abnormal behavior at the doses tested.

Compound LA-16-PH. Compound LA-16-PH, a heptaacyl monophosphoryl lipid A (Fig. 1), was almost devoid of any of the biological activities assayed in this study (Table 1; Fig. 4), even when tested with doses 10 times greater than those of the other three lipid A compounds. In addition, if we confined our analyses to hours 2 to 4 after intravenous injection or to hours 3 to 5 after intracerebroventricular injection (the periods during which maximum responses were observed with the other lipid As tested), no significant effects were observed (data not shown).

Lipid X. Lipid X is essentially the right-side half of a tetraacyl bisphosphorylated lipid A molecule (Fig. 1). It caused no significant changes in slow-wave sleep when given intravenously, but it did significantly increase slow-wave sleep after intracerebroventricular administration of 100 ng (Table 1). The delta electroencephalographic voltages did not change across all intravenous or intracerebroventricular doses of lipid X (Table 1). Thus, the slight increase of slow-wave sleep observed after the highest intracerebroventricular dose was not accompained by a concurrent increase of delta electroencephalographic voltages. No significant changes in rapid-eye-movement sleep were observed after intravenous administration of lipid X, but a significant, although small, increase of rapid-eye-movement sleep was induced by the 100-ng intracerebroventricular dose (Table 1).

No significant changes of colonic temperatures (≥0.5°C) were observed after administration of lipid X at any dose tested, intravenously or intracerebroventricularly (Table 1), nor did lipid X alter brain temperature changes that are coupled to states of vigilance.

DISCUSSION

The somnogenic actions of *E. coli* endotoxin and lipid A derived from *S. typhimurium* have been previously described (12). The present study expanded these results by showing that different structural analogs of lipid A differentially affect sleep, electroencephalographic slow-wave activity, and colonic-brain temperatures. In general, the results presented here concerning the structure of lipid A molecules and their sleep-temperature effects parallel previous studies in which other biological activities of these substances were examined.

Compound LA-15-PP, a synthetic counterpart of *E. coli*-type lipid A, was the most active somnogenic and pyrogenic analog, and it produced these effects at lower doses than did any of the other compounds tested. Indeed, compound LA-15-PP was previously shown to possess full endotoxic activities and to have strong influences on pyrogenicity, leukopenia, chicken embryo toxicity, Shwartzman reactions, immunoadjuvancy, and macrophage stimulation (2, 9; Hildebrandt et al., J. Immunol. Immunopharmacol. 6[Suppl.]:190, 1986). Compound LA-15-PP is the substance corresponding to *E. coli* Re mutant F515 lipid A. This hexaacyl bisphosphoryl lipid A configuration has been described as the minimum structure which expresses the full range of typical endotoxic effects (21, 25; H. Loppnow, L. Brade, H. Brade, E. Rietschel, S. Kusumoto, T. Shiba, and

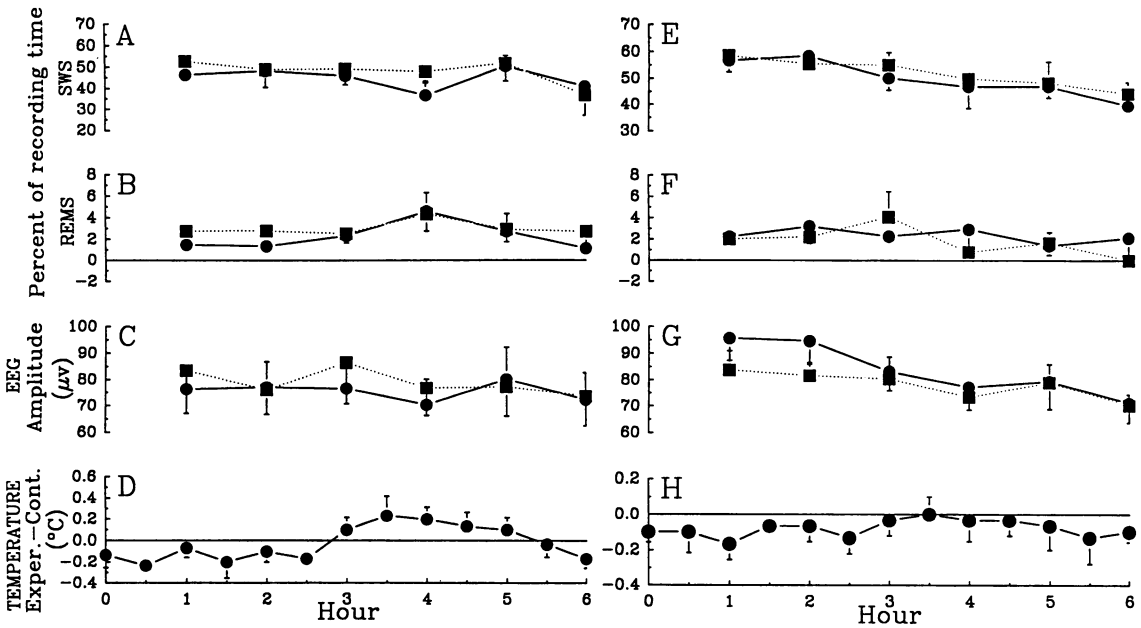

FIG. 4. Time courses of slow-wave sleep (SWS) (A and E), rapid-eye-movement sleep (REMS) (B and F), electroencephalographic slow-wave (0.5- to 4.0-Hz) amplitudes (C and G), and brain temperatures (D and H) after intravenous injection (A to D, 33.0 μg/kg) and intracerebroventricular injection (E to H, 100 ng) of lipid A analog LA-16-PH (●) or control values (■) from the same rabbits. $n = 4$ for all data points. Brain temperature values are expressed as difference from control values. *, $P < 0.05$, Wilcoxon matched-pairs test; error bars indicate standard error of mean.

H.-D. Fiad, J. Immunol. Immunopharmacol. 6[Suppl.]:203, 1986), and it is the most potent lipid A structure tested thus far for inducing sleep.

The two 4′-monophosphoryl analogs tested here (compounds LA-15-PH and LA-16-PH) exhibited effects similar to, but weaker than, those of compound LA-15-PP in terms of slow-wave sleep enhancement, rapid-eye-movement sleep reduction, and pyrogenicity. 4′-Monophosphoryl lipid A analogs exert less of an effect on other biological and immunological activities (e.g., chicken embryo lethality, pyrogenicity, immunoadjuvant activity, and induction of tumor necrosis factor, interferon, and interleukin-1) than do the corresponding bisphosphoryl compounds (3, 5, 7, 9). Thus, it could be predicted that bisphosphoryl analogs would exert stronger sleep-temperature effects than would monophosphoryl compounds, as was previously described for lipid A compounds derived from *S. typhimurium* (12). Current results support this hypothesis in that compounds LA-18-PP and LA-15-PP, both bisphosphoryl lipid As, were somnogenic and compound LA-16-PH, a monophosphoryl analog of salmonella-type lipid A, had little activity. In contrast, compound LA-15-PH, the monophosphoryl analog of compound LA-15-PP, was somnogenic, although it was less effective than compound LA-15-PP. This finding seems to be consistent with the fact that *E. coli* lipid A (compound LA-15-PP) is more biologically active than compound LA-16-PP, a main component of salmonella-type lipid As.

Additional structural differences among the four lipid A compounds tested here (besides the number of phosphate groups) that may contribute to variations of their biological activities are the number, structure, and position of acyl residues on the glucosamine backbone of the lipid A molecule. Both analogs LA-15-PP and LA-15-PH have the optimal acyl group configuration for endotoxic activity (two acyloxyacyl groups on C-2′ and C-3′), while LA-18-PP is tetraacyl with no acyloxyacyl residues (Fig. 1), partially explaining its low activity. Analog LA-16-PH is a 4′-monophosphoryl derivative of the parent heptaacyl bisphosphoryl molecule (compound LA-16-PP, not tested here). Acyl chain 7 on LA-16-PH comes from an acyloxyacyl group at C-2 rather than a hydroxyacyl as is found on compounds LA-15-PP and LA-15-PH. The additional C-2 acyloxyacyl group lowers bioactivity (5, 7, 25), and compound LA-16-PH was also found here to be less active in terms of sleep-temperature effects. Additional evidence indicating that acylation pattern or the number of acyl residues is important for biological activity stems from experiments in which lipopolysaccharide was treated with naturally occurring enzymes that cleave nonhydroxylated acyl groups from the hydroxyl groups of 3-hydroxymyristoyl residues (i.e., acyloxyacyl hydrolysis), but leave the rest of the molecule intact. After such cleavage treatment, a loss of bioactivity directly related to the degree of deacylation (acyloxyacyl removal) and reduced tissue toxicity was observed (19). The somnogenic activities of the compounds tested here further support the hypothesis that hexaacyl lipid A molecules with acyloxyacyl chains on C-2′ and C-3′ but not on C-2 have generally more biological activity. Thus, the number, structure, and position of acyl residues, as well as the presence of phosphate groups, influence the bioactivity of different lipid A molecules.

Naturally occurring lipid A compounds enhance the synthesis and release of interleukin-1 (1; Loppnow et al., J. Immunol. Immunopharmacol. 6[Suppl.]:203, 1986) as well as that of tumor necrosis factor and interferons (3, 6, 7). These lymphokines are also somnogenic (11, 23) and probably participate in a cascade of events leading, by one or more pathways, to the induction of sleep (23). Hexaacyl lipid A molecules are more potent stimulators of interleukin-1 (7, 25; Loppnow et al., J. Immunol. Immunopharmacol. 6[Suppl.]: 203, 1986), interferon α/β (3, 6, 7, 9, 25), and tumor necrosis factor (3, 5–7, 9, 25). The corresponding 4′-monophosphoryl

analogs (i.e., LA-15-PH, LA-16-PH) were poorer inducers of these lymphokines. The less toxic lipid A analog, LA-18-PP, has also displayed the ability to induce tumor necrosis factor and interferon, but to a lesser degree than the hexaacyl analogs (5, 6). Analog LA-16-PH, synthesized according to the structure proposed for *S. minnesota* lipid A, is a weak tumor necrosis factor inducer and is unable to stimulate interferon (7, 25). Thus, it is possible that lipid A and other similar compounds exert their different effects on sleep because of their differential abilities to influence cytokine production.

Lipid X is reported to be essentially nontoxic as compared with other disaccharide lipid A synthetic analogs and natural lipid A (8, 25, 26) and 10^5 times less pyrogenic (A. Haslberger, E. Schütze, H. Obenaus, and I. Macher, J. Immunol. Immunopharmacol. 6[Suppl.]:189, 1986). However, 100 ng of lipid X injected intracerebroventricularly increased slow-wave sleep and rapid-eye-movement sleep slightly, but did not significantly alter body temperature. This effect may be related to the weak but detectable ability of lipid X to increase serum levels of tumor necrosis factor (25; Hildebrandt et al., J. Immunol. Immunopharmacol. 6[Suppl.]:190, 1986). Tumor necrosis factor itself is pyrogenic (23) and has the capacity to induce interleukin-1 production. Thus, the possibility exists that some other yet undefined mechanisms is involved in the somnogenic actions of lipid X and the other substances tested here.

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