Summary

We have studied the anaesthetic potencies of 5α-pregnanolone albumin solution (PAS) and 5α-pregnanolone Intralipid emulsion (PLE) at equivalent concentrations in male rats using an EEG threshold method. The criterion of anaesthesia was burst suppression of the EEG of 1 s or more (the "silent second" (SS)) as a sign of deep anaesthesia. The potency of the two formulations was assessed by comparing the threshold doses of 5α-pregnanolone at three dose rates (1.0, 2.0 and 3.0 mg kg⁻¹ min⁻¹). We found that SS was initiated in all rats after infusions of PAS, while no SS could be induced in rats after infusion of PLE at a larger dose. A higher concentration of 5α-pregnanolone was found in all brain and peripheral tissues of PAS-treated rats than in those treated with PLE. In rats with PAS-induced anaesthesia (3.0 mg kg⁻¹ min⁻¹), the highest concentrations were detected in striatum (mean 19.40 (SD 1.21) ng mg⁻¹). Although there was a small insignificant reduction in threshold doses with dose rates at 2.0–3.0 mg kg⁻¹ min⁻¹, the tissue concentrations in striatum, frontal cortex and occipital cortex were found to be significantly increased. We conclude that PAS was more potent than PLE in inducing anaesthesia. Brain distribution of 5α-pregnanolone varied regionally in a manner similar to the variation in GABA_A receptor sensitivity to this neuroactive steroid. (Br. J. Anaesth. 1995; 74: 553–557)

Key words
Anaesthetics i.v., pregnanolone. Brain, anaesthesia, molecular effects. Rat.

The hypnotic and anaesthetic properties of steroid hormones have been investigated extensively since the initial studies of Seyle [1]. Some 3α-hydroxy ring A-reduced C-21 and C-19 steroids have been shown to bind to the GABA_A receptor-gated chloride ionophore complex (GRC) with high affinity and potentiate the inhibitory effects of GABA [2]. In particular, the ring A-reduced metabolites of progesterone, 5α-pregnanolone (3α-hydroxy-5α-pregnane-20-one) and etanolone (3α-hydroxy-5β-pregnane-20-one), have been found to modulate chloride influx via a putative steroid site on or near GRC [3]. Further investigation revealed that ring A-reduced metabolites were responsible for the anaesthetic activity when progesterone was used to induce anaesthesia [4]. Both 5α-pregnanolone and etanolone were anaesthetically active when administered to animals and humans [5, 6]. 5α-Pregnanolone was more potent than etanolone in anaesthetic activity [5].

Althesin, a mixture of the two 5α-reduced progesterone derivatives (alphaxalone and alphadolone) in saline by addition of a polyoxyethylated castor oil derivative (Cremophor EL), appeared to be a good i.v. anaesthetic [7]. Unfortunately, serious allergic reactions to Cremophor EL caused this preparation to be withdrawn [8]. A new formulation of steroids in 10–20% Intralipid has been developed recently and proved to be suitable for clinical use. The steroids are solubilized in the soya bean oil phase of Intralipid and emulsified in a way similar to diazepam found in Diazemuls [6]. However, infusions of Intralipid have been shown recently to exert a negative inotropic effect on the cardiovascular system [9]. It is well known that circulating ovarian steroids are to a great extent bound to either sex hormone binding globulin, transcortin or albumin. Only the free and albumin bound fractions were found to be taken up by the brain [10]. As progesterone can be dissolved in an albumin solution [11], the 5α-pregnanolone albumin solution could be another i.v. anaesthetic.

The aim of this study was to compare the anaesthetic properties of 5α-pregnanolone albumin solution (PAS) with those of 5α-pregnanolone Intralipid emulsion (PLE). In addition, the regional brain distribution of 5α-pregnanolone was investigated.

Materials and methods

Thirty-nine male, adult Sprague-Dawley rats (MOL:SPDR Mollegaard, L. Skensved, Denmark), weighing 290 (15) g, approximately 60 days old, were kept at a constant temperature of 24 °C and artificial light with access to food and water ad libitum. The study was approved by the Regional Ethics Committee in Umeå.
5α-Pregnanolone (3α-hydroxy-5α-pregnan-20-one) (CoCensys, Inc., Irvine, CA, USA) was dissolved in human albumin 20 mg ml⁻¹ (Novo Nordisk, Bagsværd, Denmark) in 15-ml aliquots and solubilized by sonification. 5α-Pregnanolone was dissolved in Intralipid, an emulsion commercially available from Pharmacia (Stockholm, Sweden). Both preparations contained 5α-pregnanolone 0.3 mg ml⁻¹. Sonification has been shown not to impair the effect of the steroid [12].

EEG THRESHOLD METHOD

The anaesthetic potencies of PAS and PLE were assessed using the EEG threshold method [13]. PAS or PLE was infused into the tail vein at a constant dose rate (see table 1), and the EEG was monitored continuously via s.c. stainless steel electrodes. The infusion was stopped immediately when the first burst of EEG suppression for 1 s or more was noted (the “silent second” (SS)). The appearance of SS occurs at a much deeper level of anaesthesia than loss of righting reflex. The time to reach SS was recorded and the amount of steroid needed to induce the effect was calculated. This dose was considered the threshold dose. Data for a dose-rate curve were generated by repeated determinations with different dose rates. The dose rate which gave the lowest threshold dose when drug administration was terminated at SS was defined as the optimal dose rate [14].

EXPERIMENTAL PROCEDURE

A previous report from our laboratories using PLE at a concentration of 4.0 mg ml⁻¹ revealed an optimal dose rate of 2.0 mg kg⁻¹ min⁻¹ [5]. In the present study the animals in both PAS and PLE groups were allocated randomly to three subgroups. In these subgroups, PAS was infused at dose rates of 1.0, 2.0 and 3.0 mg kg⁻¹ min⁻¹, with eight rats in each subgroup. In the PLE group, PLE was infused at dose rates of 1.0, 2.0 and 3.0 mg kg⁻¹ min⁻¹, with five rats in each subgroup (table 1). The infusion was stopped after the first SS was recorded. If no SS was obtained, the infusion was stopped when a dose of 5α-pregnanolone 10.0 mg kg⁻¹ had been injected. A maximum permissible dose limit of 10.0 mg kg⁻¹ was set up before the tests in order to limit the infusion volume. The dose limit was based on our previous study using PLE treatment at a concentration of 4.0 mg ml⁻¹, revealing a threshold dose of 6.7 mg kg⁻¹ for SS in young rats (age 44-46 days) and 5.1 mg kg⁻¹ for adult rats (age 109-110 days) [5]. All rats that reached the dose limit had lost the righting reflex.

TISSUE SAMPLE PREPARATION

The rats were killed by decapitation after the first SS was initiated or when a dose of 10.0 mg kg⁻¹ had been infused. The brain was dissected immediately into the following areas: cerebral cortex, hypothalamus, midbrain, hippocampus, striatum, medulla oblongata, cerebellum and amygdala [15]. The cerebral cortex was separated further into frontal and occipital parts. Fat tissue from retroperitoneal and s.c. abdominal areas, together with psoas muscle tissue were removed from each rat. After weighing, the tissue was frozen at -70 °C until analysis. The samples were extracted later with 95 % ethanol 10 ml for 7 days at +4 °C. The recovery of steroid in this process has been shown previously to be 100 % [16].

CELITE CHROMATOGRAPHY

5α-Pregnanolone was separated from other cross-reacting steroids by celite column chromatography, as described by Bäckström and co-workers [17] and verified by Corpechot and colleagues [18]. Glass columns (inner diameter 5 mm) were packed tightly with a mixture of celite (Mansville, Denver, CO, USA), heated to +600 °C overnight, and propylene glycol (Merck, pro analysi) (weight:volume = 1:1), brought to a height of 50 mm. Iso-octane 10 ml was percolated through the columns before sample applications. The sample (1 % of the total) was dissolved in 1.0 ml of iso-octane saturated with ethylene glycol and then applied to the column. The elution pattern was iso-octane 1.5 ml to obtain 5α-DHP followed by iso-octane 1.0-ml wash, iso-octane 4.0 ml to obtain progesterone and then iso-octane:toluene (60:40) 4.0 ml to obtain 5α-pregnanolone. Recovery of 5α-pregnanolone following chromatography was about 75 %. The 5α-pregnanolone-containing fractions were evaporated under nitrogen and then dissolved in ethanol. The final solutions were assayed for 5α-pregnanolone by means of radioimmunoassay (RIA).

HORMONE ASSAY

The concentrations of 5α-pregnanolone in brain and peripheral tissue extracts were measured by RIA. The antiserum was raised against 3α-hydroxy-20-oxo-5α-pregnan-11α-yl carboxymethyl ether coupled to bovine serum albumin (gift from R. H. Purdy). The specificity of this antiserum was tested earlier [19]. The sensitivity of this assay was 25 pg, with an intra-assay coefficient of variation of 6.5 % and an inter-assay coefficient of variation of 8.5 %.

STATISTICAL METHODS

The data were assessed using two way analysis of variance (ANOVA) followed ad hoc by the method of “least significant difference test (LSDT)” at a significance level of 0.05. The differences between the PAS and PLE treatments and the difference between subgroups within the PAS treatment were tested by the Mann–Whitney U test. Results are expressed as mean (SD).

Results

SS was obtained in all rats with infusions of PAS at the dose rates 1.0, 2.0 and 3.0 mg kg⁻¹ min⁻¹. In this group, there was a dose rate-related significant decrease in the threshold dose between 1.0 mg kg⁻¹
There was no significant difference in threshold dose between the subgroups of 2.0 and 3.0 mg kg$^{-1}$ min$^{-1}$ in the PAS group. In the PLE group, no SS was obtained in any subgroup during the time observed after infusion of PLE to a fixed dose of 10 mg kg$^{-1}$. All rats had lost righting reflex at the end of the PLE infusion. The PAS-treated rats were killed directly after the first SS appeared, while the PLE-treated rats were killed after a dose of 10 mg kg$^{-1}$, a dose slightly higher than that inducing SS in the PAS group. As we found that the concentrations of 5a-pregnanolone in striatum, frontal cortex and occipital cortex were significantly higher than those in the subgroup 2.0 mg kg$^{-1}$ min$^{-1}$ (P < 0.05, Mann-Whitney U test).

Brain concentrations of 5a-pregnanolone were also measured in PLE-treated rats. The difference in regional distribution between the PAS- and PLE-treated rats was compared at a dose rate of 3.0 mg kg$^{-1}$ min$^{-1}$ (fig. 1). A significantly higher level of 5a-pregnanolone was found in all brain regions, fat and muscle areas in PAS-treated rats ($F_{1,168} = 40.68$, $P < 0.01$, ANOVA). There was a mean increase of 200% in all brain regions in PAS-treated rats and the concentrations in all brain regions were significantly higher than those in fat and muscle tissues ($P < 0.05$, LSDT). Thus despite the infusion of a larger dose in the PLE group, the brain concentration in the PAS group was much higher, pointing to a pharmacokinetic difference in potency between the two formulations.

**Discussion**

The clinical use of steroid anaesthetics is hindered by poor water solubility. A negative cardiovascular effect was observed after i.v. infusion of 5a-pregnanolone in lipid emulsion [20]. In addition, the lipid vehicle of Intralipid might encapsulate the steroid molecule and block uptake at the target site in vivo. On the other hand, it is known that most steroids can be prepared readily in albumin solution.
However, the main drawback of PAS is that the highest steroid concentration obtainable is 0.7–0.9 mg ml\(^{-1}\), whereas for PLE a concentration of 4.0 mg ml\(^{-1}\) can be reached [6]. If an adequate concentration of this steroid is possible in a protein formulation, it should be preferred in comparison with a lipid formulation because of a better pharmacokinetic distribution pattern found in the present study.

The anaesthetic properties of PAS and PLE were studied on the basis of equivalent steroid concentrations. We concluded that PAS 0.3 mg ml\(^{-1}\) was more potent than PLE for induction of anaesthesia at all of rates of infusion. PAS but not PLE induced SS in the EEG. After infusion of lower doses in the subgroup 3.0 mg kg\(^{-1}\) min\(^{-1}\) (table 2), the brain concentration of 5α-pregnanolone in the PAS-treated rats was almost tripled in all brain regions compared with the corresponding PLE-treated rats, possibly because the lipid emulsion at the concentration tested diminished uptake of the anaesthetic steroid. At a concentration of 0.3 mg ml\(^{-1}\), the binding affinity between pregnanolone and albumin may be lower than that between pregnanolone and lipid droplets. This indicates that i.v. lipid emulsion can potentially alter the clinical behaviour of concurrently administered drugs.

It is reasonable to suggest that SS can be initiated also when the steroid concentration in PLE is raised to a much higher level (4.0 instead of 0.3 mg ml\(^{-1}\) in the present study), as reported previously [5]. In such a saturated lipid solution the release of steroid from the lipid droplets may be easier and more rapid. The diluted solution and larger volume used in the present experiments may have reduced the local drug concentrations at the uptake sites.

As an optimal dose rate was found to be 2.0 mg kg\(^{-1}\) min\(^{-1}\) with PLE 4.0 mg ml\(^{-1}\) [5], we included three dose rates, 1.0, 2.0 and 3.0 mg kg\(^{-1}\) min\(^{-1}\), in this experiment. There was a dependence of threshold dose on dose rate (table 1). Theoretically it should be a concave relationship and there exists an optimal dose rate for every anaesthetic provided the pharmacokinetic and pharmacodynamic properties of the substance are stable. However, in the present study there was no significant difference between the threshold doses in the subgroups 2.0 and 3.0 mg kg\(^{-1}\) min\(^{-1}\). Thus the optimal dose rate cannot be defined without testing higher dose rates. We can only assume that a rate of 3.0 mg kg\(^{-1}\) min\(^{-1}\) is close to optimal. Previous experience with higher concentrations of PLE support such an assumption [5].

Several laboratories have reported the presence of 5α-pregnanolone in plasma and brain of male Sprague-Dawley rats. Using the same antisera as ours after separation of 5α-pregnanolone by HPLC, Purdy and co-workers [21] reported that the basal concentration of this steroid was very low or undetectable in plasma, but detectable in both cerebral cortex (2.4 (0.33) ng g\(^{-1}\)) and hypothalamus (1.7 (1.0) ng g\(^{-1}\)) in non-stressed male rats. After exposure to swim stress, increases in 5α-pregnanolone concentrations were observed in both the brain and plasma. In the present study, 5α-pregnanolone concentrations in cerebral cortex and hypothalamus were about 4000 and 6000 times higher during PAS-induced anaesthesia than the basal values mentioned above. Similarly, a 5000–6000 times increase in progesterone concentration was observed in striatum and hypothalamus during progesterone-induced anaesthesia compared with the value in the follicular phase of female rats [22, 23].

The brain concentrations of 5α-pregnanolone measured at SS were approximately 30 nmol g\(^{-1}\) (amygdala) to 60 nmol g\(^{-1}\) (striatum), which are well within the range of concentrations previously shown to augment GABA-activated chloride currents in electrophysiological experiments and to stimulate the binding of \(^{3}H\)-muscimol in GABA\(_{A}\) receptor ligand binding assays [24, 25]. The steroid concentrations showed significant regional variation. The concentration of 5α-pregnanolone in striatum was highest among all brain regions studied. Its distribution in the medulla oblongata, midbrain and hippocampus was also high but these did not differ from each other. The regional brain distribution of progesterone and its intermediate metabolite 5α-pregnanedione (5α-DHP) has been investigated by Bixo and Bäckström [23]. In progesterone-induced anaesthesia, a variation in regional distribution was revealed in rat brain and high concentrations of these two steroids were detected also in the striatum and medulla oblongata. In addition, the transformation ratios of progesterone to pregnanedione were also found to be the highest in the same areas. Regional differences in neuroactive steroid modulation of the GABA\(_{A}\) receptor complex, as measured by \(^{35}S\)-TBPS binding and \(^{36}Cl\)\(^{-}\) uptake, have been demonstrated in rat brain [26, 27]. It is interesting to note that inhibition of \(^{35}S\)-TBPS binding to GABA\(_{A}\) receptor by 5α-pregnanolone is most potent in striatum compared with other brain regions [28]. These findings imply that the GABA\(_{A}\) receptor sensitivity to 5α-pregnanolone is highest in striatum and there might exist heterogeneous populations of GABA\(_{A}\) receptor complexes containing neurosteroid modulation sites [28]. Our data also revealed that 5α-pregnanolone concentrations in striatum correlated significantly with the threshold doses in PAS-treated rats at a dose rate of 3.0 mg kg\(^{-1}\) min\(^{-1}\) (r = 0.76, b = 0.48, P < 0.05, df = 6). This distinct distribution pattern of 5α-pregnanolone in striatum and its correlation with the threshold doses indicate that this area might play a role in steroid-induced anaesthesia.

The efficacy and potency of GABA\(_{A}\) receptor active steroid in a specific brain region depend on the difference in its bioavailability among brain regions; the differences in affinity with the steroid binding sites on the receptor and the GABA\(_{A}\) receptor density. As discussed above, the steroid bioavailability and receptor sensitivity to 5α-pregnanolone varied among brain regions. However, Jussufie [29] compared receptor density (nmol/kg protein) before and after acute administration of 5α-pregnanolone in rats and found no change in five brain areas, namely the frontal cortex, hippocampus hypothalamus, cerebellum and medulla oblongata. They also studied the allosteric effects of this steroid on \(^{3}H\)-muscimol binding in different brain regions and revealed that the binding affinity was not
changed in cerebellum, frontal cortex and hippocampus after acute administration of 5α-pregnanolone. The alteration of 3H-muscimol binding affinity in striatum was not measured. These data are not in agreement with those obtained using 35S-TBPS binding assay, which shows variation in the potency of 5α-pregnanolone modulation in brain tissue of the cortex, cerebellum, hippocampus and striatum [28]. It is known that the relative potency of 5α-pregnanolone as modulator of CI− uptake parallels its potency as inhibitor of 35S-TBPS binding [26], while 3H-muscimol binding closely reflects the affinity of GABA to its binding site. As far as the mechanism of action of neuroactive steroids on GABA_A receptor is concerned, it is uncertain if alteration in the affinity of 3H-muscimol binding reflects steroid effects on GABA-gated chloride channel function, whereas the relationship between the anaesthetic effect of steroids and 35S-TBPS binding is well documented. The present study has shown clear regional differences in 5α-pregnanolone distribution, similar to variation in GABA_A receptor sensitivity. In addition, there was a clear difference in brain concentration which correlates well with depth of anaesthesia and type of vehicle used.

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References