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INHIBITION BY PENTOBARBITONE AND URETHANE OF THE *in vitro* RESPONSE OF THE ADENOHYPOPHYSIS TO LUTEINISING HORMONE-RELEASING HORMONE IN MALE RATS

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1 The effect of urethane (ethyl carbamate) and sodium pentobarbitone on the luteinising hormone-releasing hormone (LH–RH)-stimulated secretion of luteinising hormone (LH) was investigated with hemipituitaries obtained from male rats and incubated *in vitro*.

2 Urethane and pentobarbitone were added to the incubation medium to provide final concentrations of 2.2 mg or 4 μg/ml and 100 μg or 0.1 μg/ml respectively. The high doses of these anaesthetics blocked the LH–RH stimulated secretion of LH. The low doses significantly reduced the amounts of LH released in response to LH–RH but did not block the response completely.

3 Both concentrations of urethane reduced the basal release of LH.

4 The inhibitory action of the anaesthetics was reversible.

5 The results indicate that the two anaesthetics most commonly used in neuroendocrine experiments have a significant inhibitory action on the release of LH from the adenohypophysis.

Introduction

Many investigations into hypothalamic regulation of hypophyseal secretions have been performed on anaesthetized animals. The effect of the anaesthetics, commonly urethane (ethyl carbamate) or barbiturate (sodium pentobarbitone), upon the normal functioning of parts of the neuroendocrine system has been recognised and even exploited for many years. For example, the observation by Everett & Sawyer (1950) that two hours of barbiturate anaesthesia in the pro-oestrous rat delays ovulation for 24 h, has for nearly 30 years provided the standard model for investigations into the neural control of ovulation (e.g. Everett, 1964; Dyer & Mayes, 1978). However the effects of the anaesthetics on neuroendocrine responses provide an undesirable complication in the design of many experiments. A number of studies have sought to assess the interaction of urethane and pentobarbitone with the neuroendocrine mechanisms. There is some evidence that pentobarbitone is a more potent drug than urethane (e.g. Cross & Dyer, 1971; Dyball & McPhail, 1974; Dyer, ter Haar & Mayes, 1978) and it has been questioned whether urethane significantly interferes with the neural processes controlling ovulation (Haller & Barraclough, 1968; Lincoln & Kelly, 1972). Pentobarbitone has been reported to have a direct inhibitory action on the adenohypophysis (Wuttke, Gelato & Meites, 1972) but there are no data about the influence on the anterior pituitary of concentrations of urethane and pentobarbitone likely to be encountered in neuroendocrine experiments. The present work was undertaken to investigate the effects of urethane and pentobarbitone upon the anterior pituitary; and in particular to discover any interaction of these drugs with the luteinising hormone-releasing hormone (LH–RH) stimulated secretion of luteinising hormone (LH).

Methods

Animals

Male rats of between 120 and 160 g were used for the experiments. These animals were obtained from our own inbred Wistar strain and had been housed under controlled lighting (14 h light:10 h dark) and temperature (20°C).

The rats were killed by decapitation and the brains rapidly removed. The pituitary glands were carefully lifted out of the sella turcica and, after removal of the neurohypophysis, were bisected in a sagittal plane.

Incubation procedure

Individual hemipituitaries were incubated in 1 ml of a solution which consisted of 10% Medium 199 (Gibco, Bio-Cult Ltd.) and 10% castrated rat serum. The latter had previously been heated to 57°C and

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then stored at −10°C. The incubation medium was gassed with a mixture of 95% O_2 and 5% CO_2 which was warmed and hydrated before introduction into the incubation chamber by bubbling through water at 37°C. When gassed the pH of the medium was 7.3 to 7.4. The incubation chamber was partly submerged in a shaking water bath set to maintain the temperature of the medium at 37°C. In general, the procedures were similar to those developed by Saffran & Schally (1955) with the major difference that in our experiments each hemipituitary was incubated singly. Luteinising hormone released from these hemipituitaries was assayed in samples of incubation medium (50 µl) taken at appropriate times (see results) during the 6 h experimental period.

Immunoassay procedure

The samples of incubation medium were stored at −20°C. The amount of luteinising hormone in these samples was measured in duplicate by a homologous double antibody radioimmunoassay system with kits provided by NIAMDD. The standard curve consisted of eight dilutions (3.8 to 480 ng LH-RP1/ml) of the reference preparation assayed in triplicate. Only the part of the curve between the 10% and 90% intercepts of total counts bound was used. To ensure that the samples fell on the most linear section of this curve they were generally diluted 1:100 in phosphate buffered saline; although further dilution was sometimes required. Pooled plasma containing three different hormone concentrations, and plasma from hypophysectomized rats, were included as duplicate samples in each assay. Any duplicates differing by more than ±7.5% from the mean were reassayed. Further details of this rat LH radioimmunoassay system may be found in Brown-Grant & ter Haar (1977). For the results presented below, the mean intra-assay coefficient of variation was 9% and the mean inter-assay coefficient of variation was 18%.

Chemicals

Both urethane (ethyl carbamate, Fisons Ltd.), and sodium pentobarbitone (Sagatal, May & Baker Ltd.) were diluted from stock solutions with 0.9% w/v NaCl solution (saline) and added to the incubation medium to give final concentrations for urethane of either 2.2 mg or 4 µg/ml and for pentobarbitone of either 100 µg or 0.1 µg/ml. The higher concentrations were the same as those used by Dyball (1975) and the lower concentrations were calculated by assuming an equal rate of dispersal of the anaesthetics amongst the various body compartments. The osmolarity of the incubation medium, estimated by a depression of freezing point method with a Halbmikron Osmometer, was not significantly altered by the addition of these anaesthetics.

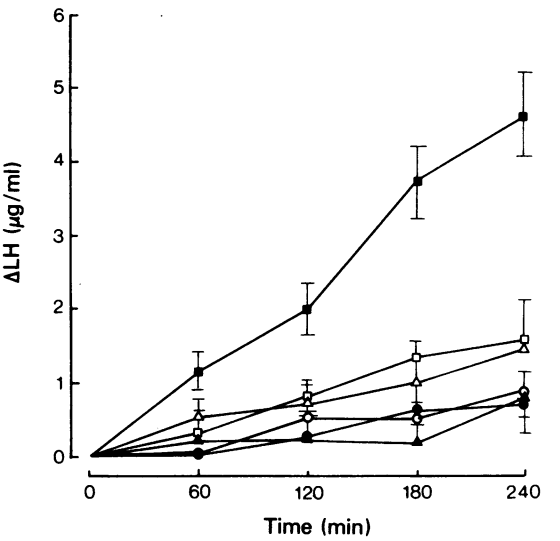

Figure 1 Graphs showing concentration of luteinising hormone (LH) in the medium after incubation of hemipituitaries in anaesthetic-free medium (■ or □), with 2.2 mg/ml urethane (● or ○) and with 100 µg/ml pentobarbitone (▲ or △). Mean values are shown; vertical lines indicate s.e. mean. The closed symbols represent data obtained when luteinising hormone-releasing hormone (LH-RH, 12 ng/ml) was added to the incubation medium. Note that both anaesthetics depressed the LH-RH stimulated release of LH and urethane depressed the basal release of LH ($n \geqslant 13$ for all observations).

Luteinising hormone-releasing hormone (LH-RH) was stored at −20°C in 50 µl aliquots of a 100 µg/ml solution. The aliquots were diluted with saline to produce a final concentration of 240 ng/ml and then 50 µl of this solution was added to the incubation medium.

Statistics

All significance levels were calculated by the use of Student's *t* test for non-paired observations.

Results

Effect of urethane and pentobarbitone on the LH-RH stimulated release of LH

The unstimulated hemipituitaries released LH into the incubation medium at a steady rate of approximately 0.3 µg LH/h. By contrast, when LH-RH (12 ng/ml) was added to the incubation medium the rate

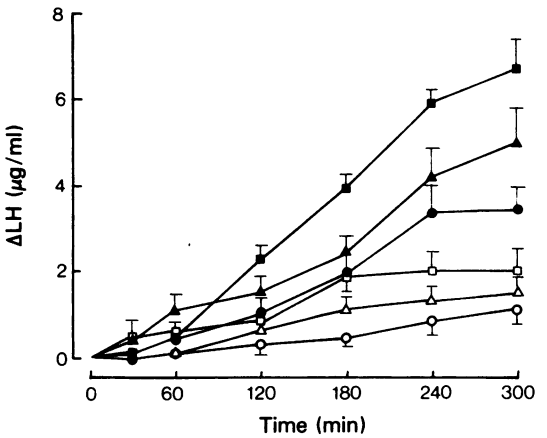

Figure 2 Graphs showing concentration of luteinising hormone (LH) in the medium after incubation of hemipituitaries in anaesthetic-free medium (■ or □), with 4 μg/ml pentobarbitone (▲ or △) and with 0.1 μg/ml urethane (● or ○). Mean values are shown; vertical lines indicate s.e. mean. The closed symbols represent data obtained when luteinising hormone-releasing hormone (LH–RH, 12 ng/ml) was added to the incubation medium. Note that, compared with responses shown in Figure 1, the LH–RH stimulated significant LH release in the presence of the anaesthetics although basal release was still suppressed by urethane ($n \geqslant 13$ for all observations).

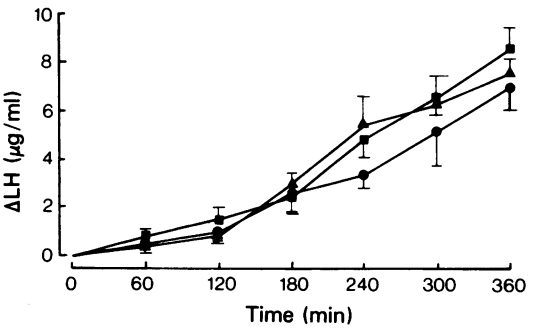

Figure 3 Graph showing that luteinising hormone-releasing hormone (LH–RH)-stimulated luteinising hormone (LH) secretion is unaffected by pre-incubation for 2 h in medium containing urethane (2.2 mg/ml; ●) and pentobarbitone (100 μg/ml; ▲), when compared with hemipituitaries pre-incubated in anaesthetic-free medium (■). Note that there was no LH–RH present during the pre-incubation period. ($n = 7$ for all observations).

of release increased to 1.3 μg LH/h. After 3 h of incubation there was significantly more LH released from the hemipituitaries incubated in medium containing 12 ng/ml LH–RH ($P < 0.01$). However, when urethane and pentobarbitone were added to the incubation medium, to give concentrations of 2.2 mg/ml and 100 μg/ml respectively, this stimulatory action of LH–RH was totally blocked (Figure 1). Indeed there was a tendency for all hemipituitaries incubated in these high concentrations of anaesthetic, regardless of whether LH–RH was present or not, to release less LH than the unstimulated pituitaries incubated in the absence of anaesthetics. This depression of the basal release of LH was also observed when the pituitaries were incubated in the presence of urethane and pentobarbitone at the lower concentrations of 4 μg/ml and 0.1 μg/ml respectively. For urethane, the depression was statistically significant ($P < 0.02$ after 4 h) at both concentrations. However, in the presence of low concentrations of urethane and pentobarbitone there was significant LH–RH-stimulated release of LH ($P < 0.02$ at 4 h for both anaesthetics when compared with basal release in media containing the same low concentration of the anaesthetics; Figure 2).

Evidence for the reversible nature of the inhibitory effect of urethane and pentobarbitone on LH release

In a second series of experiments hemipituitaries were pre-incubated for two hours in medium containing either urethane (2.2 mg/ml) or pentobarbitone (100

Table 1 Influence of urethane, pentobarbitone and the incubation media on the estimation by radioimmunoassay of three concentrations of luteinising hormone (LH) ($n = 8$ for all estimations)

	Original concentration of LH (ng LH–RP1/ml)		
	15	60	240
Additions to assay	*Concentration of LH calculated from immunoassay (ng/ml ± s.d.)*		
50 μl phosphate buffered saline	19.7 ± 1.5	70.1 ± 6.2	250.1 ± 13.8
50 μl incubation media	19.8 ± 1.2	65.3 ± 4.0	239.7 ± 28.2
50 μl urethane (2.2 mg/ml)	18.7 ± 3.4	66.4 ± 2.3	251.1 ± 16.6
50 μl pentobarbitone (100 μg/ml)	19.3 ± 3.5	66.4 ± 4.2	232.6 ± 18.5

μg/ml). The hemipituitaries were then transferred to anaesthetic-free medium which contained LH–RH (12 ng/ml). Figure 3 shows that the inhibitory effects of the anaesthetics did not persist and that release of LH from the pre-treated hemipituitaries was indistinguishable from LH release from control pituitaries pre-incubated in medium alone.

Effect of the anaesthetics and incubation medium upon the immunoassay system used for estimation of LH release

For the observations presented above to be of pharmacological interest it was necessary to show that they were not the result of interference in the LH immunoassay system by the anaesthetics. This possibility was tested by adding high concentrations of urethane and pentobarbitone, as well as the solution used for incubating the hemipituitaries, to assay tubes containing 50 μl of the 15, 60 and 240 ng/ml standard preparations. No statistically significant differences in the values obtained for these LH concentrations were observed (Table 1). This experiment indicates that the inhibitory action of urethane and pentobarbitone on LH–RH-stimulated release is due to a direct action at the level of the pituitary.

Discussion

The results presented in this paper show that both urethane and pentobarbitone inhibit the *in vitro* secretion of LH from the pituitary. The importance of this observation depends on its relevance to the whole animal situation and in particular on any similarity between the drug concentrations found in the plasma of anaesthetized intact rats and the concentrations present in the media used for pituitary incubation. Rats given urethane (1 g/kg) or pentobarbitone (the sub-anaesthetic dose of 15 mg/kg) have plasma concentrations of these drugs 1 h after injection of 1.4 mg/ml and 12 μg/ml respectively (Boyland & Rhoden, 1949; Goldstein & Arrow, 1960). These values are about the same as or higher than the concentrations of urethane and pentobarbitone used for our *in vitro* experiments. Thus our results are likely also to be applicable to the *in vivo* situation.

For urethane, our findings are in direct contradiction to those of Blake & Sawyer (1972) who concluded from indirect evidence that the drug did not act on the adenohypophysis. The extent of the inhibitory effect we report here is such that in addition to blocking LH–RH-stimulated release of LH, urethane also reduced basal release from the unstimulated adenohypophysis. We used to think that the basal release of LH *in vitro* was a pathological consequence of the inevitable disruption of part of the anterior pituitary. However, the finding that this basal release can be reduced significantly suggests that some physiological mechanism is activated more strongly *in vitro* than *in vivo*, and that the gonadotrophs can be stimulated in the absence of releasing hormone.

Urethane and pentobarbitone are the anaesthetics of choice for many *in vivo* neuroendocrine experiments and thus absolute values of any LH measurements made in such experiments are likely to be lower than normal. Unfortunately it is not possible for us to state whether urethane or pentobarbitone exerts the greater inhibitory action at concentrations required for anaesthesia because we cannot be certain of the effective amount of these drugs to which the gonadotrophs are exposed. However, the magnitude of the inhibition is clearly dose-dependent (see Figure 1 and 2).

Finally our experiments have shown that the action of urethane on release of LH from the adenohypophysis is different from the action of this drug on secretion of oxytocin from the neurohypophysis. Whereas urethane inhibits LH release, the drug potentiates potassium-stimulated release of oxytocin (Dyball, 1975). The latter experiments also demonstrated that urethane increases the movement of calcium ions into neural lobe nerve endings. There is some evidence that release of both LH and oxytocin involves a depolarization-induced, calcium-mediated, exocytotic process (McCann, 1971; Douglas, 1973; Dreifuss, 1973; Taraskevich & Douglas, 1977). Supposedly, the difference between the presumed release processes in the two lobes of the pituitary is that depolarization is induced by an 'electrical' mechanism in the neurohypophysis but by a chemical messenger in the adenohypophysis. The very different actions of urethane on the two lobes of the pituitary might reflect these divergent mechanisms. For example, urethane may block the receptors for the releasing hormone and this action might predominate over the direct membrane effects observed with the neurohypophysis. However, if this hypothesis is correct how does urethane also block secretion of LH in the absence of releasing hormone?

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