Delayed luteal regression in ewes immunized against oxytocin

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Summary. Active immunization of sheep against oxytocin prolonged the luteal phase of the oestrous cycle, as judged by oestrous behaviour and circulating progesterone concentrations. Mean cycle length was extended by 3·7 days. The treatment resulted in a 10-fold increase in circulating oxytocin concentrations. Antisera produced were specific for oxytocin; cross-reactions with vasotocin, arginine vasopressin, and hypothalamic releasing factors were low. Cerebrospinal fluid contained low levels of antibodies directed against oxytocin. This finding supports the hypothesis that the luteolytic action of oestradiol-17 β in sheep may be mediated through a stimulatory effect on the endometrial oxytocin receptor concentration.

Introduction

Although there is a good deal of circumstantial evidence that prostaglandin (PG) F-2 α is the uterine luteolysin in sheep (Flint & Hillier, 1975; Goldberg & Ramwell, 1975; Horton & Poyser, 1976), little is known of the factors controlling its increased production by the uterus towards the end of the oestrous cycle. Oestradiol-17 β causes luteal regression in sheep when given after Day 8 of the cycle (Stormshak, Kelly & Hawk, 1969), and oxytocin administration induces histological changes characteristic of luteolysis (Milne, 1963). Both oestradiol and oxytocin potentiate PGF-2 α release by the uterus (Caldwell, Tillson, Brock & Speroff, 1972; Sharma & Fitzpatrick, 1964; Mitchell, Flint & Turnbull, 1975; Roberts, Barcikowski, Wilson, Skarnes & McCracken, 1975), and it has been suggested that both may be involved in causing luteal regression through an effect on uterine PGF-2 α production (Roberts, McCracken, Gavagan & Soloff, 1976). In this paper we present evidence in support of a function for oxytocin in the luteolytic process, based on results with ewes actively immunized against oxytocin.

Materials and Methods

Grazing Clun Forest ewes kept with a crayon-bearing vasectomized ram were brought into a yard for examination and blood sampling twice every week during the 1978/1979 breeding season. The ewes were inspected for crayon marks in the field each day. Blood samples, collected by jugular venepuncture into heparinized syringes, were centrifuged within 30 min and plasma was stored at -20°C before assay. Serum samples were stored in the same way after clotting at 4°C overnight. Cerebrospinal fluid was obtained by lumbar puncture, after inducing local anaesthesia with 2% lignocaine hydrochloride.

Oxytocin was conjugated to bovine serum albumin with glutaraldehyde (Kagan & Glick, 1974) and the conjugate was separated from unconjugated oxytocin by dialysis. Conjugation ratios (determined by incorporation of ^{125}I -labelled oxytocin) were in the range 3.3–5.6 mol oxytocin/mol bovine serum albumin. Ewes were immunized against either the conjugate, or bovine serum albumin. For injection the material (1 mg/animal on each occasion, in solution in 0.9% (w/v) NaCl) was emulsified in Freund's complete adjuvant and administered intradermally at several sites along the dorsal midline; injections (4 ml on each occasion) were made 4 times at weekly intervals.

Oxytocin was iodinated using the lactoperoxidase technique, with purification after iodination by means of ion-exchange resin and gel filtration (Dawood, Raghavan & Pociask, 1978); Dowex-1 (chloride) was used in place of Hycler resin. Antibody titres were evaluated at dilutions of 1:200 and 1:2000 by incubating samples of diluted serum (0.1 ml) with ^{125}I -labelled oxytocin (0.1 ml) overnight at 4°C in 0.05 M-phosphate-buffered 0.9% (w/v) saline containing 0.1% sodium azide and 0.2% bovine γ -globulin (pH 7.5). After incubation, γ -globulins were precipitated by the addition of polyethylene glycol 6000 (0.2 ml of 30% (w/v) solution in water). Bound ^{125}I -labelled oxytocin was measured in the pellet after centrifugation and aspiration; non-specific binding (in the absence of antibody) was <10%, and this was subtracted in calculating the percentage of label bound. The presence of oxytocin antibodies in cerebrospinal fluid was also evaluated in this way (at a final dilution of 1:200 only). For determination of the specificity of the antisera the same technique was used, authentic oxytocin (0–400 pg) or other substances (0–100 ng) being added.

Progesterone was measured by radioimmunoassay using an antibody raised in sheep against progesterone 11 α -hemisuccinyl-bovine serum albumin (Flint, Anderson, Patten & Turnbull, 1974). The principal cross-reactants tested (with cross-reactions given in parentheses) were 11-deoxycorticosterone (4.0%), 5 α -pregnanedione (2.0%), pregnenolone (1.5%), 17 α -hydroxypregn-4-en-3-one (0.4%), testosterone and 20 α -hydroxypregn-4-en-3-one (0.35%). Other compounds tested (including 20 β -hydroxypregn-4-en-3-one, oestrone, corticosterone, cholesterol, androstenedione, 20 α -hydroxypregn-5-en-3-one, 5 β -pregnanolones, 5 β -pregnane-diols, cortisol, oestradiol-17 β and medroxyprogesterone acetate) cross-reacted <0.3%. Plasma samples were extracted with petroleum ether before assay; values were corrected for extraction efficiency determined in separate samples (mean $\pm$ s.e.m., 81.2 $\pm$ 1.6%). Assay blank (determined with plasma from an ovariectomized animal) was 24 pg/ml; sensitivity (determined from 2 $\times$ s.d. about the zero-binding point) was 9.6 pg. Intra-assay coefficient of variation was 8.2% ($n = 22$); inter-assay coefficient of variation was 5.2% ($n = 16$). Oxytocin was measured by radioimmunoassay as described by Mitchell, Mountford, Natale & Robinson (1980). Oxytocin was extracted from plasma onto powdered glass, and subsequently extracted from the glass with aqueous acetone. Extraction efficiencies were in the range 74–98%, and values were corrected for extraction losses. The antibody used was raised in rabbits against oxytocin conjugated to bovine serum albumin; ^{125}I -labelled oxytocin was prepared using chloramine T as oxidizing agent. The following cross-reactions were obtained with this antiserum (relative to oxytocin = 100%): arginine vasopressin, 0.13%; arginine vasotocin, 0.006%; lysine vasopressin, β -endorphin and melanocyte hormone inhibitory factor, <0.005%. The sensitivity of the assay (determined from 2 $\times$ s.d. about the zero-binding point) was 0.8 pg; assay blank was undetectable. The accuracy of the assay was determined by extracting and assaying plasma samples to which various amounts of oxytocin had been added; regression analysis of the resulting data gave y (mass recovered) = 0.90 x – 1.22 ($r = 0.98$). The intra-assay coefficient of variation was 9.4%; inter-assay coefficient of variation was between 12.9% (at 16 pg) and 17.6% (at 64 pg).

Oxytocin was obtained from Sandoz Inc., Basel, Switzerland, and Bachem Fine Chemicals Inc., Torrance, California 90505; synthetic 1–24 adrenocorticotrophin (Synacthen) was from Ciba Laboratories, Horsham, Sussex, and bovine γ -globulin was from Sigma Chemical Co., Kingston-upon-Thames, Surrey. Other substances were gifts.

Results

Two ewes immunized against oxytocin during the spring of 1976 were bled during the breeding season 1976/77 and these animals, which developed high titres of antibodies directed against oxytocin, had some unusually long cycles (up to 28 days). These animals were not kept with a ram, and this conclusion was reached on the basis of plasma progesterone levels only. An attempt to investigate this response in the season 1977/78 failed because we were unable to prepare an immunologically active oxytocin conjugate; 6 ewes which were treated with inert conjugate had normal ovarian function, while the 2 animals that had been immunized in the previous year again had lengthened cycles (up to 26 days). These preliminary observations indicated that cycle length might be extended after immunization against oxytocin, and the experiment was successfully repeated during the 1978/79 breeding season; only the results from that season will be reported here.

Before starting immunization 9 ewes were studied for 2 control cycles; during this period they all had oestrous cycles in the range 15–19 days (mean $\pm$ s.e.m., 16.7 ± 0.21 days, $n = 18$). Thereafter, 5 ewes were injected with oxytocin–bovine serum albumin conjugate (3.8 mol oxytocin/mol protein), and 4 received bovine serum albumin in an identical manner. After the start of immunization, control ewes (which had undetectable circulating oxytocin antibody concentrations throughout the experiment) had 15–18-day cycles, while cycles were significantly prolonged in the ewes immunized against oxytocin (Table 1). Analysis of the data by weighted analysis of variance showed that immunization against oxytocin caused prolongation of the cycle by 3.7 ± 1.5 days (mean $\pm$ s.e.m.; $P < 0.05$).

Table 1. Oestrous cycle lengths in ewes before and after active immunization against oxytocin–bovine serum albumin or bovine serum albumin

	Cycle length (days)								
	15	16	17	18	19	20	21	26	≥ 30
Control ewes (N = 4)									
Before immunization	1	2	4		1				
After immunization	1	5	15	1					
Experimental ewes (N = 5)									
Before immunization	1	2	7						
After immunization	1	5	6	3	3	1	2	2	2

As responses to immunization differed between animals, both in rapidity and in degree of antibody production, an attempt was made to relate prolongation of the cycle to the circulating antibody concentration (Text-fig. 1). In Ewe D antibody concentration remained low ($<10\%$ binding at 1:200 dilution), and cycle lengths were normal (17.0 ± 0.63 days, $n = 6$). In Ewes B and E antibody concentration rose slowly and one ewe exhibited 3 prolonged cycles, while the other had 4 normal cycles followed by one cycle of 21 days. In Ewes A and C antibody concentration increased rapidly and cycles were prolonged to various degrees. One of the prolonged cycles of Ewe C was interrupted by removal of the single corpus luteum found at laparotomy; after lutectomy, the progesterone concentration fell and ovulation occurred, as indicated by rising progesterone values. Although normal cycles were observed in some animals with serum oxytocin binding, notably in Ewes B and E, prolongation of the luteal phase was generally related to antibody concentration; plotting cycle length (y) against oxytocin binding (x) gave $y = 0.09x + 16.55$, $r = 0.6034$, $P < 0.001$ ($n = 25$; data for experimental ewes only).

Circulating progesterone concentrations confirmed, with few exceptions, the data on cycle length obtained from observations of oestrous behaviour; there was no indication that any of the prolonged inter-oestrous periods were caused by a failure to ovulate after luteal regression. How-

Text-fig. 1. Antiserum binding of labelled oxytocin and jugular venous progesterone concentrations in 5 ewes immunized against oxytocin by weekly injections for 4 weeks starting on 27 November. Sera were tested for binding to ^{125}I -labelled oxytocin at final dilutions of 1:200 (●) and 1:2000 (○). Peripheral plasma progesterone concentrations are indicated by broken lines (—), and observed oestrus by ▼, with the lengths of cycles (days) at the top of each panel. Luteal regression without oestrous behaviour is denoted by ▽. Lutectomy is indicated by ◆ (Ewe C only). Observations were made until the last oestrus of the breeding season, which is shown for all animals except Ewe D, which returned to oestrus for the last time on 15 March.

ever, in Ewe C, which had the highest antibody titre, luteal regression (i.e. reduced progesterone concentrations) was not accompanied by behavioural oestrus on 2 occasions, suggesting that a high oxytocin antibody concentration might interfere with oestrous behaviour.

The specificity of the antisera produced by the 5 ewes immunized against oxytocin was evaluated by testing with synthetic LH-RH and TRH, somatostatin, synthetic 1–24 ACTH (Synacthen), ovine LH (NIH-S17), ovine FSH (NIH-S6), ovine prolactin (NIH-S9), arginine vasopressin and arginine vasotocin. Serum from Ewe D cross-reacted slightly with LH-RH (0.2%) and vasotocin (0.06%), but sera from the other animals showed no significant cross reaction with any of the compounds tested (<0.02%).

To eliminate the possibility that the antibody was acting at a site in the central nervous system cerebrospinal fluid from 4 of the ewes was assayed for oxytocin binding activity. Binding was not detectable (<2%) in Ewes D and E, and low binding occurred (<10%) in Ewes A and C. These determinations were made at a time when serum binding was near maximal, and indicate that very little of the antibody entered the cerebrospinal fluid.

Immunization raised circulating oxytocin concentrations. The mean ($\pm$ s.e.m.) concentration in control animals, in 6–8 samples per animal obtained after immunization against bovine serum albumin, was 11 ± 3 pg/ml plasma; in the ewes immunized against oxytocin the

corresponding value was 111 ± 28 pg/ml ($P < 0.02$). Oxytocin concentrations in the experimental ewes varied widely both between animals and with time; the variation between animals was related to circulating antibody concentration (plotting oxytocin concentration, y , against oxytocin binding at 1:200 dilution, x , gave $y = 0.24x + 41.4$, $r = 0.6757$, $P < 0.05$).

Discussion

These results show that immunization of ewes against oxytocin causes a significant prolongation of the cycle, and that the degree of prolongation is in general related to the concentration of the circulating antibodies. Measurements of circulating progesterone levels indicate that the prolongation reflects a lengthening of the functional life-span of the corpus luteum. The findings are consistent with the mechanism proposed by Roberts *et al.* (1976) to account for the increase in uterine PGF-2 α production at luteal regression in sheep, i.e. that a rise in follicular oestrogen production causes an increased endometrial oxytocin receptor concentration, thereby facilitating the stimulatory effect of oxytocin on PGF-2 α production. Evidence in support of this mechanism includes an increase in uterine oxytocin receptor concentration at the end of the cycle in sheep (Roberts *et al.*, 1976), and the observation that uterine oxytocin receptor concentration is stimulated in the rat (Soloff, 1975) and rabbit (Nissenson, Flouret & Hechter, 1978) by oestradiol-17 β .

Active immunization against potentially important hormones has been widely used as a method of investigating their effects, and has been applied in the study of proteins, steroids and prostaglandins as well as small peptides (Scaramuzzi, Baird, Wheeler & Land, 1973; see also Edwards & Johnson, 1976). The use of active immunization against PGF-2 α to investigate its luteolytic function in sheep (Scaramuzzi *et al.*, 1973) is of particular relevance since the end-point (prolongation of the oestrous cycle) was the same as that employed here. One characteristic of the treatment is that it raises the circulating levels of the compound against which the animal is immunized, presumably by decreasing its metabolic clearance rate; such an increase in concentration was noted in the present study.

An important consideration in evaluating effects of active immunization is the specificity of the antisera produced, because of the possible existence of unknown cross-reactants. The antisera produced in the present study showed low cross-reactions with all the compounds tested, including those most closely related to oxytocin. None cross-reacted more than 0.06% with vasotocin, which has been shown to interfere with LH release in male rats when administered into the cerebral ventricles (Pavel, Luca, Calb & Goldstein, 1979), and it appears unlikely therefore that prolongation of the cycle was due to a central effect. This is supported by the low levels of antibody found in cerebrospinal fluid. The antisera also failed to cross-react with a variety of other compounds involved in the maintenance of cyclic ovarian function. However, it cannot be assumed that active immunization against oxytocin interferes with luteal regression solely through an effect on uterine PGF-2 α production.

It has been suggested (Robinson, Challis, Furr, Louis & Thorburn, 1976) that the sheep corpus luteum is subject to tonic inhibition during the luteal phase of the oestrous cycle by a continuous low level of uterine PGF-2 α release. If this mid-cycle release is influenced by oxytocin, then circulating progesterone concentrations might be expected to rise after immunization against oxytocin. In fact, no clear evidence was obtained in support of such a rise, possibly because samples were taken for progesterone analysis too infrequently.

Roca *et al.* (1978) found that passive immunization against oxytocin blocks ovulation in rabbits in response to hCG. In the present study no effect of circulating oxytocin antibodies was observed on luteinization, and it was not determined whether the treatment had any effect on ovulation. Luteinization of unruptured follicles cannot be ruled out. Therefore we are unable to tell whether the suggestions made by Roca *et al.* (1978) also apply in the sheep.

The data presented here suggest that oxytocin plays a facilitatory role in the control, through

uterine prostaglandin production, of luteal function. The results of experiments with cows are consistent with this finding: oxytocin is luteolytic in intact cows (Armstrong & Hansel, 1959), and this effect is prevented by hysterectomy (Hansel & Wagner, 1960). The involvement of oxytocin may therefore represent a mechanism of general importance in ruminants.

We thank Sandoz Inc., Basel, Switzerland, for gifts of oxytocin; Hoechst U.K. Ltd, Milton Keynes, Bucks, for LH-RH and TRH; Professor B. T. Pickering, Department of Anatomy, University of Bristol, for vasotocin; Dr R. G. Dyer, A.R.C. Institute, Babraham, for somatostatin and arginine vasopressin; and the NIAMDD, NIH, Bethesda, U.S.A., for gifts of anterior pituitary hormones. We are indebted to Mr D. E. Walters, A.R.C. Statistics Group, for carrying out the statistical analysis and to the animal house staff at Babraham and Oxford, for help with the animals.

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Received 16 August 1979