Biosynthesis of oxytocin in the corpus luteum

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In this report we demonstrate that ovine and bovine luteal cells synthesise oxytocin by way of a precursor protein similar to that found in the hypothalamus. Isolated ovine or bovine luteal cells were incubated for up to 12 h with [^{35}S]cysteine. Neurophysin–Sepharose column separation and HPLC of cell extracts demonstrated the presence of [^{35}S]oxytocin. Incorporation of [^{35}S]cysteine was confirmed by performic acid oxidation. Immunoprecipitation of cell extract with anti-rat oxytocin-neurophysin followed by SDS–PAGE yielded 2 radioactive bands of 14 kDa and 11–12 kDa. Immunoprecipitation with anti-oxytocin yielded 1 band at 14 kDa. On SDS–PAGE the 14 kDa band had a similar mobility to rat-hypothalamic oxytocin precursor.

Oxytocin biosynthesis *Corpus luteum* *Bovine luteal cell* *Ovine luteal cell* *Oxytocin precursor*

1. INTRODUCTION

Oxytocin, vasopressin and neurophysin have been shown, by radioimmunoassay, to be present in the corpus luteum of the sheep [1,2], cow [3–6] and human [7,8]. The presence of neurophysins together with the hormones suggests that they are synthesised in the corpus luteum since both molecules arise as fragments of a large common precursor protein in the hypothalamo-neurohypophysial system [9–11]. We now validate this suggestion by showing that dispersed ovine and bovine luteal cells incorporate [^{35}S]cysteine into oxytocin and into a putative oxytocin-precursor similar to that previously described in the hypothalamus [12–15].

2. MATERIALS AND METHODS

2.1. Tissue

Bovine corpora lutea, 5–10 days post-ovulation [16], were collected fresh from an abattoir. Sheep corpora lutea, 3 days post-ovulation (estimated by use of a vasectomised ram) were obtained from animals slaughtered on the premises by a captive-bolt.

2.2. Cell isolation and incubation

Luteal tissue was minced in minimum essential medium (Gibco Europe) with no L-cystine but containing 2.8 μg/ml L-cysteine (cyc-MEM). Minced luteal tissue was dissociated by two or three 45-min incubations in a shaking water bath at 37°C in cys-MEM containing 0.2% collagenase (Worthington, type CLS), 0.005% DNase and 0.5% bovine serum albumin with 100 units/ml penicillin and 100 μg/ml streptomycin. After each incubation, free cells were aspirated with the dissociation medium, collected by centrifugation at 150 × g for 4 min and washed twice in cys-MEM. Cells were resuspended in 1.0 ml cys-MEM containing 10% dialysed adult cow serum and two 0.4-ml aliquots (bovine, 1×10^7 cells/ml; ovine 3×10^6 cells/ml) were incubated with [^{35}S]cysteine (Amersham International, England, 1200 Ci/mmol) at 1 mCi/ml at 37°C in an atmosphere of 95% air: 5% CO_2.

2.3. Hormone and precursor extractions

Incubations of luteal cells with [^{35}S]cysteine were terminated by adding HCl to a final concentration of 0.1 M, and 500 μg L-cysteine. Cells were sonicated (2 × 2 s at 50 W) and left to extract overnight at 4°C. In some experiments, the medium

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was withdrawn prior to the addition of HCl and the cells and medium were extracted separately. Extracts were centrifuged (10000 × *g* for 10 min) and the pellet re-extracted in 1.0 ml of 0.1 M HCl. Unincorporated label and salts were removed by passing the combined supernatants through a Sep-Pak (Waters Associates, MA) which had been pre-wetted with acetonitrile (ACN) and then washed with 0.1% trifluoroacetic acid (TFA). After loading the sample the Sep-Pak was washed through with 200 ml of 0.1% TFA and the bound peptides and proteins were then eluted in 3 ml of 80% ACN in 0.1% TFA. Half the eluate was dried down under a stream of N_2 prior to analysis with reverse-phase high-performance liquid chromatography (RP-HPLC) and radioimmunoassay, while the other half was dried down in 5 aliquots (now referred to as 'crude extract') prior to immunoprecipitation and neurophysin–Sepharose affinity chromatography.

2.4. Hormone and precursor purification

To purify the labelled hormone one aliquot of crude extract was dissolved in 600 μl of 0.1 M ammonium acetate (pH 5.8) and passed down a 1.5 ml neurophysin–Sepharose column (13.4 mg porcine neurophysin coupled to 3 g Sepharose; Pharmacia, Uppsala) [17,18] which was pre-equilibrated and washed with the same buffer. Bound material was eluted with 1.0 M acetic acid, freeze-dried and re-chromatographed on the same column. The bound label from the second affinity column was then chromatographed by RP-HPLC [15]. A high-pressure mixing HPLC system (Gilson, France SA) was used to pump the solvents, monitor the effluent for UV absorbance and collect the fractions. Samples were injected onto a 5 mm × 10 cm column packed with Hypersil ODS, 5 μ (Shandon Southern Products, Cheshire) and washed in with 0.05 M phosphate (adjusted to pH 2.5 with orthophosphoric acid) at a flow rate of 1.0 ml/min. Proteins and peptides were eluted from the column with an increasing gradient of ACN and 1.0 ml fractions collected for β-counting in a liquid-scintillation counter (1217 Rackbeta, LKB, Bromma, Finland).

Oxytocin-related components were isolated by immunoprecipitation with anti-oxytocin (anti-oxy) or IgG purified from an antiserum raised against rat oxytocin-neurophysin (anti-Np). Immunoprecipitates were obtained from either crude extracts, or following RP-HPLC of crude extracts, and separated by SDS–PAGE as in [15]. In one experiment, the supraoptic nucleus of Wistar rats was labelled in vivo with [^{35}S]cysteine for 1 h and acid extracts

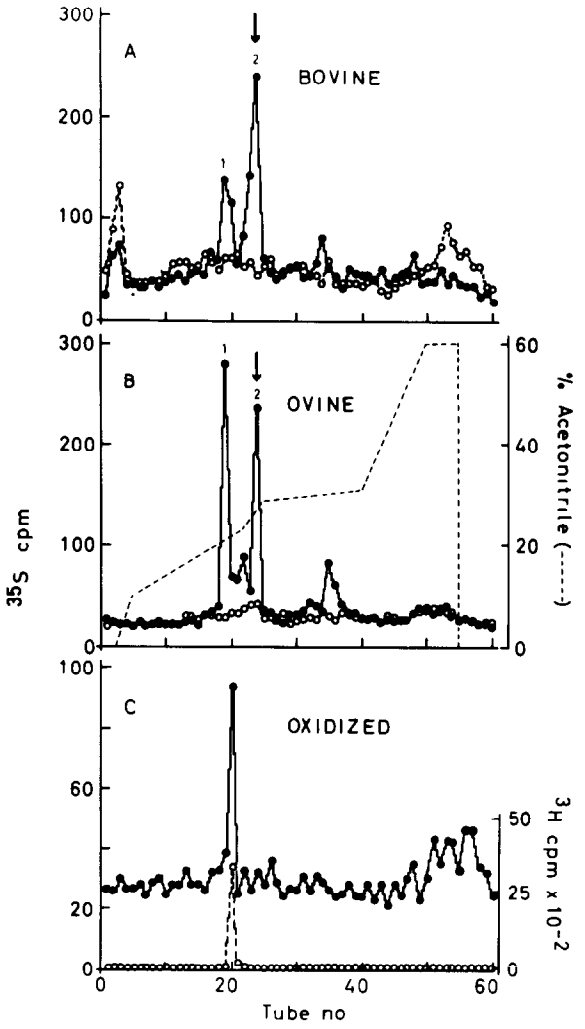

Fig.1. Reverse-phase HPLC of neurophysin-bound material present in extracts of luteal cells incubated with [^{35}S]cysteine. (A) Bovine cells following a 2 h (○---○) or 12 h (●——●) incubation (cpm/ml); (B) ovine cells following a 2 h (○---○) or 7 h (●——●) incubation (cpm/0.25 ml); (C) performic acid-oxidized tritiated oxytocin (Cambridge Research Biochemicals, England) (○---○) and performic acid oxidized ovine (B) peak 2 (●——●). Peaks numbered 1 elute in the position of vasopressin while those numbered 2 correspond to oxytocin. The elution position of authentic oxytocin is indicated by vertical arrows.

were either acetone precipitated or immunoprecipitated with anti-Np.

3. RESULTS AND DISCUSSION

No labelled hormone was recovered from either ovine or bovine luteal cells incubated with [^{35}S]cysteine for 2 h. After longer incubation times, however, extracts from both species showed peaks of radioactivity in the region of oxytocin and vasopressin (fig.1A,B). To confirm that the label had indeed been incorporated into the hormone and not merely adsorbed (especially important for [^{35}S]cysteine) the material in the radioactive peak corresponding to oxytocin (fig.1B, peak 2) in the sheep was oxidized with performic acid and this produced a new peak coincident with performic acid-oxidized [^{3}H]oxytocin (fig.1C). The peak of label eluting in the position of vasopressin (peak 1) does not correspond to vasopressin itself since it gave two new peaks after treatment with performic acid but neither of these co-eluted with oxidised vasopressin. Interestingly, if the medium was separated from the cells at the end of the incubation nearly all the radioactivity in peak 1 was found in the medium, while the radioactive oxytocin (peak 2) was present primarily in the cells. Neither of these peaks was observed when medium alone was incubated with label in the presence or absence of added oxytocin.

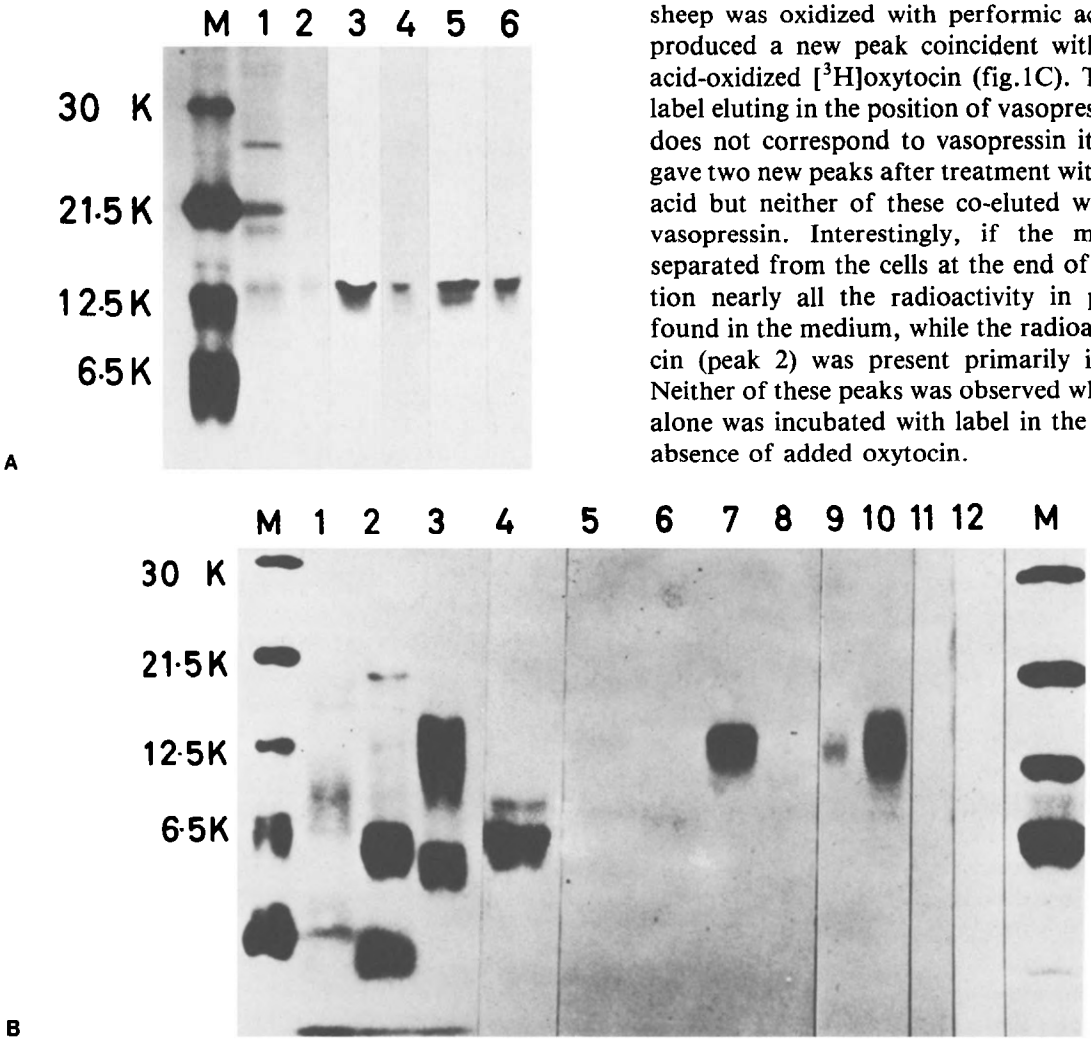

Fig.2. A composite picture of SDS–PAGE and fluorography of labelled proteins obtained from RP-HPLC or following immunoprecipitation with anti-oxy or anti-Np. (A) Acetone precipitate (lane 1) and immunoprecipitate with anti-Np (lane 2) of rat supraoptic nucleus extract following incubation with [^{35}S]cysteine in vivo. Ovine luteal cells; incubated for 2 h and immunoprecipitated with anti-Np (lane 3) or anti-oxy (lane 4); incubated for 7 h and precipitated with anti-Np (lane 5) or anti-oxy (lane 6). (M) Molecular mass markers. (B) Bovine luteal cell extract following a 6 h incubation with [^{35}S]cysteine. Lanes 1–4 correspond to peaks 1–4 of RP-HPLC of a crude extract. Immunoprecipitates of peaks 1–4 with anti-NP are shown in lanes 5–8, respectively. Only peak 3 contained labelled immunoprecipitable material and this peak was further analysed by immunoprecipitation with either antibody alone (anti-oxy, lane 9; anti-Np, lane 10) or with antibody pre-adsorbed with excess cold antigen (anti-oxy plus 10 μg oxytocin, lane 11; anti-Np plus 10 μg rat oxytocin-neurophysin, lane 12). (M) Molecular mass markers.

Under our experimental conditions, ovine cells appeared to synthesise more hormone than did those from the cow. Owing to the availability of material we routinely used about 3-times more bovine cells than ovine luteal cells yet the sheep cultures incorporated 4-times more isotope into oxytocin. Whether this reflects differences in species, freshness of tissue or the age of the corpus luteum is currently under investigation.

Crude extracts of ovine luteal cells, immunoprecipitated with anti-NP showed radioactive bands on SDS gels which ran with the apparent molecular masses of approx. 14 and 11–12 kDa (fig.2A). The 14-kDa molecule, which has a similar molecular mass to the rat oxytocin-precursor (fig.2A, lane 2), could also be precipitated with anti-oxy while the smaller molecule could not. Only the 14-kDa band was seen in the 2-h incubates while both bands were visible after 7 h. Extracts of bovine luteal cells, incubated for 6 h with [^{35}S]cysteine, were chromatographed on RP-HPLC and 4 peaks of radioactivity were found. These 4 peaks were run on SDS–PAGE either directly (fig.2B, lanes 1–4) or following immunoprecipitation with anti-Np (fig.2B, lanes 5–8). As can be seen, peak 3 from HPLC contained an approx. 14-kDa molecule which was immunoprecipitable with both anti-oxy and anti-Np. The specificity of the immunoprecipitations was tested by addition of excess oxytocin or rat oxytocin-neurophysin and in these experiments no radioactive bands were seen.

Thus, as occurs in the hypothalamus, the biosynthesis of luteal oxytocin involves the formation of an approx. 14-kDa precursor protein which is subsequently cleaved to form neurophysin and hormone although at present we cannot say if the luteal precursor is identical with the hypothalamic one. While there can now be no doubt that oxytocin is an ovarian hormone, and recent immunocytochemical evidence [19,20] points to the 'large' luteal cells as its source, we still do not understand the role of this hormone in the regulation of reproductive processes; although it has been suggested that oxytocin may be involved in luteolysis [21–23]. Even less is known about vasopressin which is also present but in very much smaller amounts [4–7]. The demonstration [24] that these peptides are also to be found in the testis tempts the suggestion that they are related to steroidogenesis [25,26]. We also have findings which are in accord with a testicular function related to the motility of the seminiferous tubules [27].

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