A new approach for assaying LH by using a target cell response

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Summary. Replicate cultures of 50000 mouse Leydig cells were used to obtain linear log dose–response curves to LH. This hormone augmented the activity of an endogenous alkaline phosphatase, which was subsequently allowed to act on *p*-nitrophenyl phosphate, the coloured end-point being measured with a spectrophotometer. The linear portion of the curve extended from 0.2 to 3.2 m.i.u. LH/ml.

Introduction

Two types of sensitive bioassays for luteinizing hormone (LH) and human chorionic gonadotrophin (hCG) are now available. The cytochemical method (Buckingham, Chayen, Hodges, Robertson & Weisz, 1979) utilizes an endogenous enzyme pathway in target cells to give a colorimetric end-point, but it is time-consuming and requires costly apparatus. Methods based on aliquots of cell suspensions permit simpler tissue replication (Shirley & Stephenson, 1973; Channing, 1974; Dufau, Pock, Neubauer & Catt, 1976; Romani, Robertson & Diczfalussy, 1976; Boggis, Bowman, Holder, Robinson & Ryle, 1978) but with one exception (Boggis *et al.*, 1978), involve estimation of a metabolite by radioimmunoassay (RIA), which increases the work required and the sources of error. The procedure outlined below combines aspects of both types of assay. Although circumstances have prevented the authors from characterizing it as a fully valid assay, the method is reported so that others can investigate its usefulness.

Materials and Methods

Culture medium. Medium 199 buffered with 20 mM-*N*-2-hydroxyethyl-piperazine-*N'*-ethane-sulphonic acid (HEPES), 4 mM-L-glutamine and 'Donor' calf serum were purchased from Flow Laboratories Ltd, Irvine, Ayrshire. Approximately 5 mM-isobutyl-1-methylxanthine (IBMX; Sigma (London) Chemical Co. Ltd, Poole, Dorset) was prepared by dissolving 5.6 mg in 1 ml highly purified ethanol (Fison's Scientific Apparatus Ltd, Loughborough, Leics) and diluting it with 4 ml glass-distilled deionized water. Immediately before use complete culture medium was prepared by adding 2 ml L-glutamine solution, 10 ml calf serum and 0.5 ml IBMX solution to 88 ml Medium 199 and adjusting the pH to 7.6.

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Leydig cell preparation. Outbred male mice derived from LACA stock (M.R.C. Laboratory Animals Centre, Carshalton, Surrey) were bred and reared in standardized conditions (Ryle, 1980). Groups of up to 10 mice were used when about 26 days old (14–16 g). The testes were removed aseptically, the tunicae slit and the contents extruded into a dish containing complete culture medium. The presence of serum throughout preparation proved essential for Leydig cell survival. After teasing the tubules apart, the contents of the dish were drawn gently in and out of a 10 ml syringe 3 times, to disperse the Leydig cell clumps, and transferred to a centrifuge tube. After 5 min, when the tubules and remaining cell clumps had settled, the supernatant was removed and centrifuged, the cells were resuspended in fresh medium, and an aliquot was stained with crystal violet (0.1% in 0.1 M-citric acid). The density of the Leydig cells was estimated with a haemocytometer and the suspension was diluted to give ~250 000 cells/ml. Each mouse yielded about 10^6 Leydig cells. Preliminary coverslip cultures showed that contaminating isolated Sertoli and germ cells rarely survived during culture and that the cells identified as Leydig cells (by their small round nuclei with distinct nucleoli) contained 3β -hydroxysteroid dehydrogenase activity which was augmented by LH.

Cell culture. The cells were cultured in 0.5 ml medium in soda-glass test-tubes, 7.5×1 cm (Anchor Glass Co., London NW2 1JS). Three replicate cultures were set up for each hormone dilution. Three additional tubes received no hormone and three which received neither hormone nor cells served as enzyme blanks. All components of the culture mixture were prepared in complete medium and dispensed from MLA automatic pipettes with disposable sterile tips (Frost Instruments Ltd, Wokingham, Berks). Medium (0.2 ml) was added first to coat the tube, followed by 0.1 ml of hormone dilution and 0.2 ml of the cell suspension. The rows of tubes were sealed with strips of autoclave tape and the racks shaken before incubating at 32°C for 44–48 h. After incubation the medium was removed by suction, using a filter-pump attached to a Pasteur pipette with a bent tip, which allowed the cell monolayer to be left undisturbed. Tris–sucrose buffer (0.3 M-sucrose–10 mM-Trizma base, pH 7.4; 0.1 ml/tube) was added and the racks were stored for up to 5 days at –20°C in order to disrupt the cells but protect the enzyme.

Measurement of enzyme activity. Preliminary tests showed that Leydig cells, like the steroidogenic cells of the immature mouse ovary (Bramley, 1974; Bramley & Kent, 1976), contained an alkaline phosphatase, the activity of which was increased when they were cultured in the presence of LH. The enzyme activity measured was the hydrolysis of *p*-nitrophenyl phosphate, with estimation of the quantity of *p*-nitrophenol liberated in a given time. The reaction was carried out in the following buffer, prepared immediately before use: 5 parts of 0.3 M-piperazine–HCl, 1 part each of 5 mM-EDTA and 50 mM-magnesium acetate and 1 part of water. The pH was adjusted to 10.7 and 0.8 ml was dispensed to each of the thawed culture tubes. The tubes were ‘vortexed’ briefly and, after warming to 32°C, 0.1 ml 50 mM-*p*-nitrophenyl phosphate was added to each. The racks were shaken vigorously and incubated at 32°C for 2 h. The reaction was stopped by dispensing 2 ml 0.5 N-NaOH–0.5% EDTA to every tube. The absorption was read at 420 nm with a CE 373 spectrophotometer (Cecil Instruments, Cambridge), the tubes being handled in a random sequence. Since *p*-nitrophenol is stable, delays before reading the tubes were not critical. The readings ($\times 1000$) were transformed to logarithms before applying standard statistical procedures (Finney, 1952).

Hormone preparations. One ampoule of the First International Reference Preparation of Human Pituitary LH for Radioimmunoassay (68/40; Storrington, Bangham, Cotes, Gaines & Jeffcoate, 1978) was dissolved in Medium 199, pH 7.6, supplemented with 0.25% (w/v) bovine serum albumin (Sigma), to give a concentration of 10 i.u./ml. The same buffer was used to prepare a working stock solution of 1 i.u. LH/ml. Aliquots of this solution were diluted to 32 mi.u./ml with Medium 199 supplemented with 10% calf serum (pH 7.6). All concentrations were stored as 0.5 ml aliquots at –20°C. For each standard curve doubling dilutions (0.5 ml of each) were prepared, in complete culture medium, from an aliquot of 32 mi.u./ml. Each dilution was

further diluted ($\times 5$) in the culture tubes. This LH preparation did not require filtering before use with cell cultures.

The following human pituitary hormones were used in preliminary checks for cross-reactions and interactions. They were prepared and purified in this laboratory (by Dr M. Bluck) and their potencies were estimated by RIA, using the M.R.C. standard preparations indicated: follicle-stimulating hormone (FSH; 1350 units/mg; 69/104); thyroid-stimulating hormone (TSH; 8.5 i.u./mg; 68/38); prolactin (9.0 units/mg; 71/222). The TSH preparation was not highly purified and contained 233 units LH/mg (69/104). Human chorionic gonadotrophin (hCG; 2nd International Standard Preparation) was also tested.

Results

Log dose-response curves

The linear portion of the log dose-response curve regularly extended from about 0.2 to 3.2 mi.u. LH/ml. In preliminary experiments, carried out before the culture conditions had been optimized, the response to concentrations above 3.2 mi.u./ml sometimes diminished again. However, since substantial dilution of test samples is desirable, most attention was subsequently given to the lower part of the dose range. Table 1 summarizes the properties of 7 standard curves in which the responses to 0.2, 0.4 and 0.8 mi.u. LH/ml were measured, using 3 replicate tubes at each concentration in each curve. The mean log responses, with the exception of Exp. A, were all of the same order, and in no experiment was there significant curvature. The indices of precision were high but each was derived from only 9 observations. The sensitivity was assessed by comparing the mean response to 0.2 mi.u./ml with that in the absence of LH and calculating Student's *t*. A significant difference ($P < 0.05$) occurred in 5 of the experiments and in the remaining 2 the value of *P* was less than 0.1. Experiments D and E illustrate the variation between experiments set up in parallel using the same cell suspension. Experiment C is of interest because the medium was supplemented with serum taken from a lactating cow shortly after milking and likely to contain much prolactin.

Table 1. Properties of seven log dose-response curves*

	Experiment						
	A	B	C	D		E	
				i	ii	i	ii
Mean log response	1.77	1.94	1.95	1.91	1.93	1.93	1.87
Slope (b)	0.21	0.19	0.14	0.14	0.11	0.15	0.19
Index of precision (λ)	0.17	0.35	0.21	0.24	0.23	0.22	0.18
Significance of difference between responses to 0 and 0.2 mi.u./ml (<i>P</i>)	>0.05	>0.05	<0.05	<0.01	<0.001	<0.05	<0.02

* To 0.2, 0.4 and 0.8 mi.u. LH/ml.

Preliminary tests for interactions with other hormones

In a single experiment log dose-response curves to 0.2, 0.4 and 0.8 mi.u. LH/ml were obtained in the presence of 0, 0.54, 2.7 and 13.5 mi.u. FSH/ml. The slopes did not differ significantly and the mean log response was in each case 1.92. The effects of 0.1 and 10 μ units TSH/ml were examined similarly. The lower dose had no significant effect but with 10 μ units/ml

the slope was significantly flatter, probably due to the relatively greater effect, at the lower end of the dose-response curve, of the LH with which the TSH preparation was contaminated. hCG, like LH, stimulated alkaline phosphatase activity.

In an experiment carried out before the culture conditions had been optimized, responses to 0.1, 0.4 and 1.6 mi.u. LH/ml in the presence of 5 pmol testosterone/ml, 0.1 pmol oestradiol/ml and 100 pmol progesterone/ml were compared with the responses in the absence of added steroid. Analysis of variance indicated that none of these steroids significantly affected the results. In a separate experiment, however, 150 pmol cortisol/ml appeared to depress the response to LH although 50 pmol/ml was without effect. A single preliminary experiment with prolactin suggested that this hormone also may, over a limited concentration range in the region of 100 μ units/ml, depress the response to LH.

Discussion

The procedure described above measures only biologically effective hormone, and, when fully validated as an assay method, should be applicable to the serum LH of different species, as well as to hCG. It is less laborious than other cell-based bioassays for LH, for one person can set up 100–150 tubes in 4 h and carry out the enzyme incubation and readings in 3–4 h, but it is slower, requiring 3 days for completion. It needs little expensive apparatus, no specific antibodies and no radioactive isotopes. Its sensitivity is similar to that of the method of Romani *et al.* (1976), somewhat less than that of Dufau *et al.* (1976) (~ 100 and 25 μ units 2nd IRP hMG/ml respectively) and much less than that of Buckingham *et al.* (1979) which can detect as little as 10^{-4} mi.u./ml. Its present sensitivity is comparable to that of RIA and would permit detection of 2–4 mi.u. LH/ml in serum samples diluted at least 10-fold in the assay tubes. Such dilution is necessary to reduce interference by other serum components. Indeed, the preliminary results with cortisol and prolactin suggest that greater dilution would be advisable in view of possible interference by occasional high concentrations of these and other hormones. Further extensive checks for interactions between LH and a wide range of hormones are required because although the presence of 10% calf serum in the culture medium ensures a minimal supply of insulin and thyroxine, abnormally high levels of these and other active serum components might modify the cellular response to LH.

In experiments performed to optimize culture conditions IBMX doubled the sensitivity of the system, suggesting that cyclic AMP is involved in the activation of the alkaline phosphatase. The metabolic role of this enzyme is at present unknown but it has been suggested that the similar phosphatase in mouse ovaries may inactivate phosphorylated proteins involved in steroidogenesis (Bramley, Sage, Kent & Ryle, 1976). Other preliminary experiments showed that increased numbers of cells per tube, or more prolonged culture, reduced the slope of the log dose-response curve and increased variability without markedly increasing the mean response. This suggests that some feed-back inhibition limited the enhancement of the enzyme activity and underlines the importance of precise culture conditions. It also suggests that identification of the inhibitory factor(s) might indicate means of increasing the sensitivity of the system.

Our results suggest that it may be possible to develop bioassays for other hormones in serum by measuring increased activity of specific enzymes in small populations of appropriate target cells.

We thank Mrs V. Griffiths for assistance in this work, Dr H. G. Bray for supervision of the optimization of the enzyme step, Dr D. R. Bangham for the LH standard and Dr B. Setchell for the cow serum.

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Received 6 May 1980