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Original article

Effects of bovine follicular fluid and partially purified bovine inhibin on FSH and LH release by bovine pituitary cells in culture

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Summary — We have established a dispersed bovine pituitary cell culture system to study the effects of charcoal-extracted bovine follicular fluid (BFF) or bovine inhibin, partially purified by immunoaffinity chromatography (IPI), on the spontaneous release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Pituitary cells were plated at 0.25, 0.5 or 1 x 10^6 viable cells/well (c/w) and incubated for 48 h. The medium was replaced and BFF (0, 0.54, 2.7, 13.7, 68.7 or 343.5 μg protein) or IPI (0, 0.01, 0.06, 0.29, 1.45 or 7.25 μg protein) added to the cultures and the incubation was continued for 48 h. Concentrations of FSH and LH in spent medium were determined by RIA and data analyzed by ANOVA with means compared by Student–Neuman–Keuls (SNK) test. We have shown an increase in spontaneous FSH and LH release attributable to both number of bovine pituitary cells plated and to the length of incubation. The addition of BFF reduced spontaneous FSH release over 48 h incubation. The dose-dependent inhibition curves observed in culture in which different numbers of cells were plated, indicates that inhibition was greater when 1 x 10^6 c/w were plated compared to 0.25 or 0.5 x 10^6 c/w. Bovine follicular fluid at 0.45 μg of protein (equivalent to 0.01 μl of BFF) incubated with 1 x 10^6 c/w, suppressed FSH release by 10.6% compare to control. Maximal suppression of 34.1% was obtained with 50 μg (equivalent to 1.56 μl of BFF). Immunopurified bovine inhibin at 1.45 μg tended to suppress FSH release and at 7.25 μg significantly reduced FSH release. Neither BFF nor IPI had a measurable effect on LH release. We conclude that BFF and IPI suppress the spontaneous release of FSH from bovine pituitary cells in culture in a dose-dependent manner, without concomitant suppression of LH release.

FSH / LH / inhibin / follicular fluid / pituitary / bovine

Résumé — Effet du liquide folliculaire bovin et d'inhibine bovine partiellement purifiée sur la sécrétion de FSH et de LH par des cellules hypophysaires bovines en culture. *Nous avons établi un système de culture de cellules hypophysaires bovines dispersées en culture afin de démontrer les effets du liquide folliculaire traité au charbon (BFF) ou de l'inhibine bovine partiellement purifiée par chromatographie d'immuno-affinité (IPI) sur la relâche spontanée de l'hormone folliculo-stimulante (FSH) et lutéinisante (LH). Ces dernières ont été mises en culture à des concentrations*

de 0,25, 0,5 ou 1 x 10^6 cellules/puit (c/p) et incubées pour 48 h. Le milieu de culture fut remplacé et additionné de BFF (0, 0,54, 2,7, 13,7, 68,7 ou 343,5 μg de protéine) ou IPI (0, 0,01, 0,06, 0,29, 1,45 ou 7,25 μg de protéine) par puit et l'incubation poursuivit pour 48 h. Les concentrations en FSH et LH ont été analysées dans le milieu de culture par RIA, les données analysées par ANOVA et les moyennes comparées par SNK. Nous avons observé une augmentation de la relâche spontanée de FSH et de LH attribuable au nombre de cellules hypophysaires bovines par puit et le temps d'incubation. L'addition de BFF a réduit la relâche spontanée de FSH. Les courbes d'inhibition de relâche spontanée de FSH en relation au nombre de cellules par puit démontre qu'une plus forte inhibition a été obtenue lorsque l'ensemencement était de 1 x 10^6 c/p que lorsqu'il était de 0,25 ou 0,5 x 10^6 c/p. Le BFF à 0,45 μg de protéine (équivalent à 0,01 μl de BFF) incubé à 1 x 10^6 c/p a diminué la relâche de FSH de 10,6% comparativement au contrôle et une inhibition maximale de 34,1% a été obtenue avec 50 μg de protéine (équivalent à 1,56 μl de BFF). L'IPI à raison de 1,45 μg a légèrement diminué la relâche de FSH et une différence significative a été obtenue à 7,25 μg. Nous n'avons pas montré d'effet mesurable du BFF et de l'IPI sur la relâche spontanée de LH. Nous concluons que le BFF et l'IPI diminuent spécifiquement la relâche spontanée de FSH par les cellules hypophysaires bovines dispersées en culture sans diminution concomittante de LH.

FSH / LH / inhibine / liquide folliculaire / hypophyse / bovin

INTRODUCTION

Charcoal-extracted bovine follicular fluid (BFF) specifically inhibits FSH release when added to dispersed rat pituitary cells in culture (Padmanabhan *et al,* 1984; Robertson *et al,* 1985, 1986, 1987; Fukuda *et al,* 1986). Inhibin has been identified as one of the proteins responsible for suppression of spontaneous FSH release (Ying, 1988; Ling *et al,* 1990; Knight, 1991). In cattle, it has been shown that injection of either BFF or purified inhibin reduces endogenous FSH concentration in the systemic circulation of intact (Miller *et al,* 1979; Johnson *et al,* 1985; Quirk and Fortune, 1986; Lussier and Carruthers, 1989) hemiovariectomized (Johnson *et al,* 1985; Lussier *et al,* 1988) and ovariectomized cows (Ireland *et al,* 1983; Beard *et al,* 1990). Dispersed pituitary cells collected from *post-partum* cows that had been previously treated with BFF for 10 d showed no effect on the *in vitro* release of FSH following stimulation with GnRH (Hinshelwood *et al,* 1991). To our knowledge, no reports are available describing the effects of either BFF or partially purified bovine inhibin on cultured bovine pituitary cells. Therefore, the objectives of the following experiments were to establish a dispersed bovine pituitary cell culture to study the effects of BFF or partially purified bovine inhibin on spontaneous FSH and LH release.

MATERIALS AND METHODS

Preparation of bovine follicular fluid and partially purified bovine inhibin

Bovine ovaries were collected at an abattoir and put on ice until follicular fluid was aspirated from all follicles < 20 mm in diameter. Cyst-like follicles > 20 mm in diameter were discarded. The pooled follicular fluid was centrifuged (2 060 *g,* 15 min, 4°C), and the supernatant stored at –20°C. When the follicular fluid was thawed, activated charcoal (Norit A; BDH Chemicals) was added (10 mg/ml) and the solution stirred for 3 h at 4°C. The charcoal was removed by centrifugation (2 060 *g,* 15 min, 4°C) followed by filtration first through a Whatman No 1 filter and subsequently by a 0.45-μm filter (Millipore, Bedford, MA). The charcoal treatment of BFF removed > 98% estradiol-17β (0.66 ng/ml; following ex-

traction), testosterone (0.08 ng/ml) and progesterone (1.28 ng/ml), as previously reported (Lussier and Carruthers, 1989). The initial protein concentration of BFF was determined to be 55 mg/ml with the Bradford protein assay (Bio-Rad) using bovine serum albumin as standard.

Monoclonal antibody immunoaffinity chromatography

A monoclonal antibody (H256) directed against the α-subunit of bovine inhibin (20 kDa) was obtained from Miyamoto *et al* (1986) and received as an ammonium sulfate precipitate pellet of ascites fluid. To prepare an immunoaffinity gel, the pellet was dissolved in 5 ml of 0.1 M $NaHCO_3$, pH 8.5 and dialyzed extensively against 0.1 M $NaHCO_3$, pH 8.5. The antibody solution was mixed with an equal volume of Affi Gel 10 (Bio-Rad) in an ice bath for 4 h and incubated overnight at room temperature (22 °C) with gentle agitation. To block any active ester sites that might remain on the gel, 1 M ethanolamine–HCl, pH 8.0, was added to the mixture at a ratio of 0.1 ml/ml of gel. The affinity gel column was equilibrated with 50 mM Tris–HCl, pH 7.5. Bovine follicular fluid, collected as described previously, without charcoal extraction was diluted with an equal volume of 50 mM Tris–HCl, pH 7.5 and filtered (Whatman No 1) before being applied to the affinity column. Ten ml of diluted BFF was applied on a 5-ml gel bed at a flow rate of ≈ 0.5 ml/min. The column was washed with 10 to 15 ml of 50 mM Tris–HCl, pH 7.5, 0.2 M NaCl and 0.005% phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor. The protein fractions bound to the affinity gel, referred as immunopurified bovine inhibin (IPI), were eluted with 0.1 M glycine–HCl, pH 2.0, 0.005% PMSF. The eluate was immediately neutralized with 2 M Tris–HCl, pH 8.5. The column was regenerated with 10 ml of 6 M guanidine–HCl than re-equilibrated with 50 mM Tris-HCl, pH 7.5 for other purification cycles (Miyamoto *et al*, 1987). The eluate was collected and frozen at –20 °C. The collected fractions were concentrated by ultrafiltration using a 10 kDa cut-off membrane (YM-10; Amicon) under positive nitrogen gas pressure. The protein concentration of IPI was evaluated by optical density reading at 280 nm, assuming that 1 unit of absorbance represents 1 mg/ml protein (Scopes, 1982).

FPLC Superose-12 size exclusion chromatography

The concentrated IPI was applied directly on a size exclusion column (Superose-12; FPLC Pharmacia) and eluted in denaturing conditions. Solutions for chromatography were made in distilled deionized water, filtered through a 0.22-μm filter and degassed. A solution of 8 M urea was deionised by a single passage through an AG 501-X8 resin (Bio-Rad) and 0.001% PMSF added. The Superose-12 column was developed with a denaturing urea buffer solution composed of 8 M urea, 25 mM Tris–HCl, pH 7.5, 0.2 M NaCl, 0.05% of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and 0.001% PMSF. A IPI concentration of 1.28 mg/ml was applied through a 400-μl loading loop and the column developed at a flow rate of 1.0 ml/min.

FPLC PRO-RPC-5/10 reverse-phase chromatography

Immunopurified bovine inhibin was applied on a reverse phase column (PRO-RPC-5/10; FPLC Pharmacia) and developed using a technique adapted from Miyamoto *et al* (1985). The column was loaded with a 400-μl loading loop containing 514 μg IPI. Two solvents were employed to develop the reverse-phase column. Solvent A was 100% H_2O, 0.1% tifluoroacetic acid (TFA), and solvent B was 100% acetonitrile (BDH Chemicals), 0.1% TFA. The IPI was injected in 10% solvent B during the first 10 min. The column was developed with a linear gradient from 10% to 24% solvent B over 5 min, then to 60% over 25 min, and 5 min at 60% of the same solvent. The flow rate of the eluting phase was set at 1.0 ml/min. Eluates from the column and molecular weight markers (phosphorylase B: 97.4 kDa; bovine serum albumin: 66.2 kDa; ovalbumin: 45 kDa; carbonic anhydrase: 31 kDa; soybean trypsin inhibitor: 21.5 kDa; lysozyme: 14.4 kDa; Bio-Rad) were mixed with non-reducing sample buffers prior to unidimensional vertical slab sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE; Protein II, Bio-Rad). Gels were made according to Laemmli (1970) with stacking and separating gels, and were fixed and silver-stained (Bio-Rad).

Dispersed bovine pituitary cells in culture

The dispersed bovine pituitary cell culture system was based on modifications of previously described techniques (Padmanabhan and Convey, 1978; Bicknell and Chapman, 1983; Tsonis *et al*, 1986). The Dulbecco's phosphate-buffered saline (DPBS), Ca^{2+} and Mg^{2+} free DPBS ($DPBS^-$), Dubelcco's modified Eagle's medium (DMEM), fetal calf serum (FCS), glutamine, penicillin and streptomycin were obtained from Gibco-BRL. The glucose, bovine serum albumin (BSA: fraction V), collagenase (Type I; 300 UI/mg), $NaHCO_3$, HEPES sodium salt, EDTA and trypan blue stain were acquired from Sigma, and 24-well culture plates (#3524) from Costar. DMEM was supplemented with the following filter sterilized (0.22-μm filters; Millipore) additives at final concentrations of 10 mM $NaHCO_3$, 2 mM glutamine, 20 mM HEPES, 100 IU penicillin and 100 μg streptomycin per ml of medium.

All procedures were performed under sterile conditions. Two bovine pituitary glands per bioassay were collected at the slaughterhouse in ice-cold supplemented DMEM within 20 min of stunning. Pituitary glands were dissected ≈ 40–45 min *post-mortem* in Petri dishes containing DPBS with 7.5 mM glucose. The neurohypophysis and remaining meninges were gently dissected from the adenohypophyses. The glands were cut into 1- to 2-mm^3 blocks which were washed 6 times or until the washing solution remained clear with 5 to 10 ml of DPBS, 7.5 mM glucose and 0.1% BSA. Tissues pieces were transferred to a 25-ml spinner flask with 20 ml prewarmed 0.1% collagenase solution (DPBS, 0.1% collagenase, 7.5 mM glucose, 0.1% BSA) and stirred very gently for 30 min at 37 °C. The supernatant, which contained mostly red blood cells and debris, was discarded. Fresh collagenase solution (20 ml) was added and incubation continued for 90 to 105 min at 37°C with very gentle stirring. The solution was decanted and centrifuged (300 *g*, 4°C, 10 min), and the pellet resuspended and washed with 20 ml supplemented DMEM. Twenty ml $DPBS^-$ with 2 mM EDTA and 0.1% BSA was added to the spinner flask and incubated for 10 min at 37°C. After the incubation, the pieces of adenohypophysis were vigorously pipetted with a large bore pipette. The solution was decanted and centrifuged (300 *g*, 4°C, 10 min). After centrifugation, the cells were washed with 20 ml supplemented DMEM. This cycle was repeated with decreasing bore pipettes to allow dissociation of smaller cell clumps until only connective-like tissue remained. The resuspended cells were pooled and washed twice with 20 ml supplemented DMEM and the final resuspension made in a known amount of supplemented DMEM with 10% serum. Viability was estimated by trypan blue exclusion. Approximately 1.4 x 10^8 cells were obtained per pituitary gland with a mean viability of 80 ± 3%. Cells were diluted in supplemented DMEM and 10% FCS to concentrations of 0.25, 0.5 or 1 x 10^6 cells/ml which were plated at 1 ml/well in a random pattern for the BFF or IPI cell treatment. Incubation at 37°C in a 95% air:5% CO_2 for 48 h ensued. Medium was removed by gentle aspiration and immediately replaced with 950 μl of prewarmed supplemented DMEM with 10% FCS and 50 μl of previously prepared and diluted test materials (see below). Following the second incubation, the medium was removed and stored at –20°C until assayed for FSH and LH. Bioassays were conducted in quadruplicate for each dose of sample expressed as μg of protein added per well. Using this culture system we have examined: 1) the accumulation of FSH and LH in the culture medium as influenced by the number of cells plated per well (0.5 or 1 x 10^6 c/w) and the time of the second incubation (48, 72 or 94 h); 2) the effects of charcoal-extracted BFF on FSH and LH release in relation to the number of cells plated per well (0.25, 0.5 or 1 x 10^6 c/w); 3) the effects of different doses of BFF or IPI on FSH and LH release when 1 x 10^6 c/w and incubated for 48 h.

Follicular fluid protein was diluted in supplemented DMEM with 10% FCS to obtain final BFF concentrations of 0.54, 2.7, 13.7, 68.7 and 343.5 μg protein/50 μl, which corresponded to 0.01, 0.06, 0.31, 1.56 and 6.25 μl BFF. IPI at a concentation of 392 μg/ml, was diluted in supplemented DMEM with 10% FCS to obtained final concentrations of 0.01, 0.06, 0.29, 1.45, 7.25 μg of protein in 50 μl volume. The control (or 0 μg protein) for BFF was 50 μl culture medium, whereas for IPI, the control was the elution buffer used during the immunoaffinity chromatography equivalent to the lowest dilution of IPI used.

Endocrine and statistical analysis

The FSH concentrations for each bioassay were determined in a single radioimmunoassay as

previously described (Lussier and Carruthers, 1989; first antibody: NIAMDD-anti-oFSH; tracer: NIAMDD-oFSH-I-1) except that bovine FSH (USDA-bFSH-BP-1) was used as standard instead of ovine FSH (NIAMDD-oFSH-RP-1). Ovine and bovine FSH standard curves were analyzed by regression analysis and parallelism was assessed by comparison of slopes (*T*-test: Kleinbaum and Kupper, 1978; SAS, 1985). Slopes of the bovine and ovine FSH standards did not differ significantly. At a logit value of zero (50% bound), a dose ratio of 43.4 was obtained when comparing bovine to ovine standards. The sensitivity of the assay using USDA-bFSH-BP-1 was 1.0 ng/tube and the intra-assay coefficient of variation was 4.47%. The LH concentrations were determined in a single assay as previously described (Lussier and Carruthers, 1989; first antibody: anti-ovine-LH, GDN#15 obtained from GD Niswender, Colorado State University; tracer: bovine LH, LER-1716-2 obtained from LE Reichert, Union University; standard: NIH-bLH-B10). The sensitivity of the assay was 0.063 ng/tube and the intrassay coefficient of variation was 12.05%. Concentrations of FSH or LH in the spent culture medium were analyzed by ANOVA (Kleinbaum and Kupper, 1978; SAS, 1985) with concentrations of BFF or IPI as the main effect and the means compared by Student–Newman–Keuls (SNK) test ($P < 0.05$).

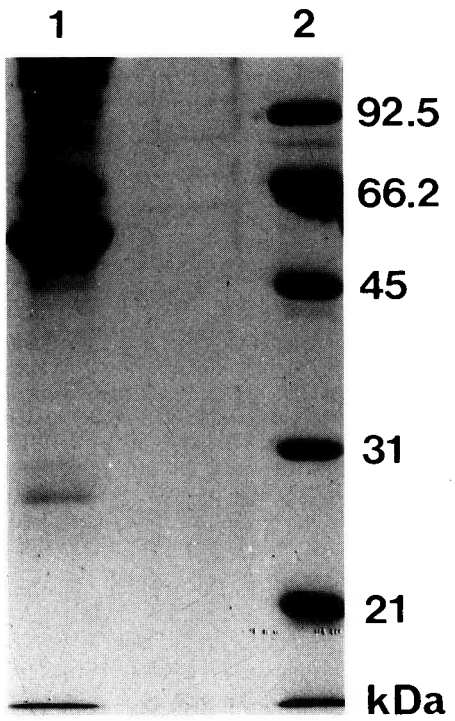

Fig 1. Electrophoresis of non-reduced immunopurified bovine inhibin in 12% SDS–PAGE gel stained with Coomassie blue. Lane 1 = 30 μg of non-reduced immunopurified bovine inhibin; lane 2 = reduced molecular weight standards (97.4-kDa phosphorylase B; 66.2-kDa albumin; 45-kDa ovalbumin; 31-kDa carbonic anhydrase; 21.5-kDa soybean trypsin inhibitor).

RESULTS

Characterization of immunoaffinity purified bovine inhibin

Different protein bands were obtained following electrophoresis of IPI on 12% SDS–PAGE gels stained with Coomassie blue (fig 1). The band of interest is in the 25–35 kDa region which is believed to represent bovine inhibin and corresponds to the published MW of 32 kDa. It should be underlined that the MW standards were subjected to chemical reduction by the suppliers whereas the IPI was not. Since reduced standards were applied, a reduced and non-reduced purified BSA protein fraction (Sigma: fraction V) was also applied to correct for protein band migration in the gel. The results showed the presence of different protein bands of the following approximate molecular weights: 31.4, 58.4, 68.9, 75, 80.4 and higher MW proteins. The elution profile of IPI obtained on FPLC Superose-12 provided a good resolution of the lower MW protein bands (fig 2). The absorption at 280 nm shows a large peak corresponding to the protein bands of high

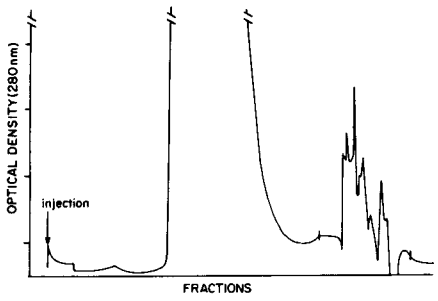

Fig 2. Protein elution profile of 514 μg of immunopurified bovine inhibin applied on a size exclusion chromatography, FPLC Superose-12, and eluted with 46 ml of 8 M urea buffer at a flow rate of 1 ml/min. Eluted proteins were detected by optical density at 280 nm.

MW, higher than 55 kDa obtained previously (fig 1) and a complex peak for the lower MW proteins ≈ 25–35 kDa. At least 3 to 4 protein peaks were detected in this region and suggested that the lower MW proteins is not composed solely of 32-kDa bovine inhibin. The fractions collected following IPI injection in a FPLC reverse phase (PRO-RPC-5/10), were separated on a 10% SDS–PAGE under non-reducing conditions, and identified by silver-staining (fig 3), show the presence of low MW components that were eluted at different concentrations of acetonitrile, indicative of proteins of different hydrophobic properties. Figure 3 shows in the 25–35-kDa region, the presence of 2 protein bands eluted at 35–36% acetonitrile (fraction 14), 1 or 2 protein bands at 38–39% acetonitrile (fractions 17–18) and one protein band at 43–44% acetonitrile (fraction 21). This protein profile in the 25–35-kDa region is in agreement with the protein elution profile obtained on the FPLC Superose-12 (fig 2).

Bovine pituitary cell culture

To verify the accumulation of FSH and LH in the culture medium, 0.5 or 1 x 10^6 bovine pituitary cells were plated and following a first 48-h incubation with medium change, the cells were incubated for an additional 48, 72 or 96 h without further medium changes. Accumulated medium concentrations of FSH and LH (table I) relative to the number of pituitary cells plated and the time of incubation, demonstrate the viability of the bovine pituitary cells obtained using our dissociation and culture conditions. At 1 x 10^6 c/w for 96 h culture without medium changes the culture medium had begun to change color, indicating exhaustion of its buffering capacity. When

Table I. Effects of incubation time (48, 72 or 94 h) and the number of bovine pituitary cells per well (0.5 or 1 x 10^6 c/w) on spontaneous release of FSH and LH (mean ± SEM; $n = 8$).

Incubation time (h)	*FSH (ng/ml)*		*LH (ng/ml)*	
	0.5 x 10^6 c/w	*1 x 10^6 c/w*	*0.5 x 10^6 c/w*	*1 x 10^6 c/w*
48	269.0 ± 14.25^a	524.3 ± 14.23^c	314.1 ± 15.50^a	557.6 ± 19.0^c
72	365.3 ± 22.58^{a,b}	584.8 ± 16.89^d	349.1 ± 16.20^{a,b}	745.2 ± 44.25^d
94	392.3 ± 24.12^b	655.2 ± 18.37^e	420.2 ± 17.55^b	

Superscripts not in common within columns and rows are significantly different ($P < 0.05$).

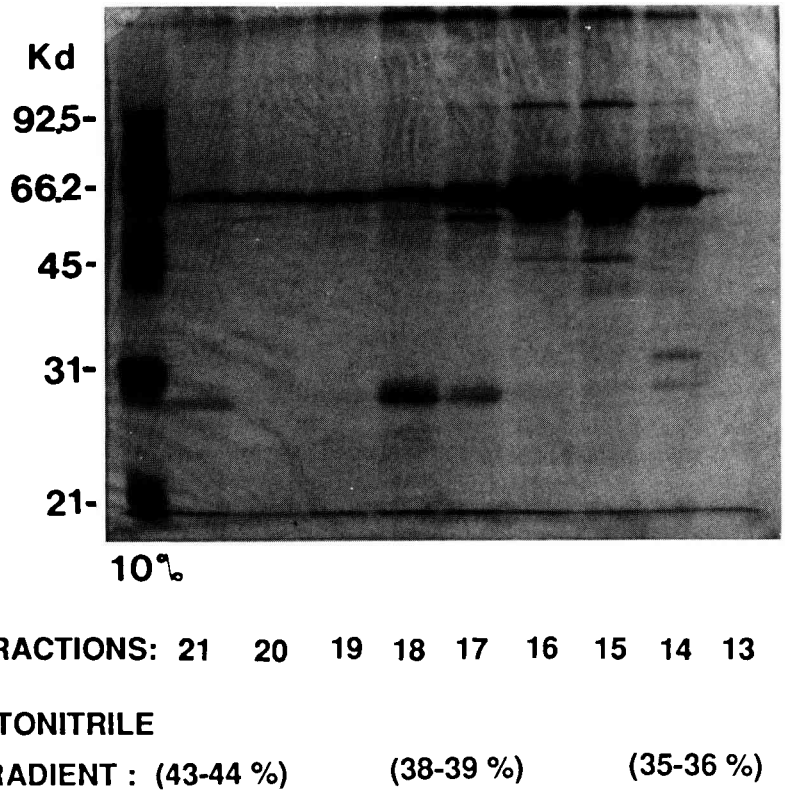

Fig 3. Fractions of immunopurified bovine inhibin collected from FPLC reverse-phase PRO-RPC 5/10 column that was developed with a 24% to 60% linear gradient of acetonitrile with 0.1% TFA at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected, lyophilized, and applied on a 10% SDS–PAGE under non-reducing conditions and proteins visualized by silver staining.

1 x 10^6 c/w were plated, the mean concentrations of FSH and LH were significantly different ($P < 0.05$) between each incubation time. For the following investigations we chose 48 h incubation time.

We examined the effects of BFF on FSH release by bovine pituitary cells using the above culture conditions and with varying cell numbers of 0.25, 0.5 or 1 x 10^6 c/w. The results (fig 4) demonstrate that BFF reduced spontaneous FSH release by pituitary cells incubated for 48 h of culture. The dose-dependent inhibition curves obtained when different concentration of cells are plated, indicates a steeper inhibition curve was obtained when 1 x 10^6 c/w were plated compared with 0.25 or 0.5 x 10^6 c/w. At 1 x 10^6 c/w, the lowest concentration at which BFF significantly reduced FSH (10.6%; $P < 0.05$) was 0.01 μl or 0.45 μg of protein (table II) and maximal suppression (34.1%) occurred at 1.56 μl or 68.7 μl of protein for 1 x 10^6 c/w. No significant suppression of LH release was observed. IPI

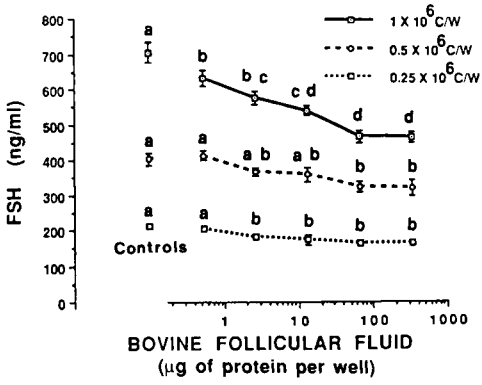

Fig 4. Dose-dependent suppression of FSH accumulation (mean ± SEM; $n = 4$) by charcoal-extracted BFF in culture medium of bovine pituitary cells; effects of pituitary cell number (0.25, 0.5 or 1 x 10^6 cells/well). Superscripts not in common on the same line are significantly different ($P < 0.05$).

Table II. Effects of charcoal-extracted bovine follicular fluid on spontaneous FSH and LH release (percent of control values; mean ± SEM; $n = 4$) in culture medium of bovine pituitary cells (1 x 10^6 cells/well) incubated for 48 h.

Protein content BFF (µg/ml)	*FSH*	*LH*
0	100 ± 3.97[a]	100 ± 3.17[a]
0.54	89.4 ± 3.08[a]	109.6 ± 4.19[a]
2.7	81.5 ± 2.52[b]	98.1 ± 8.47[a]
13.7	76.3 ± 1.79[b,c]	94 ± 3.09[a]
68.7	65.9 ± 2.45[c,d]	102.7 ± 4.76[a]
343.5	65.3 ± 2.22[d]	106.2 ± 5.94[a]

Superscripts not in common within columns are significantly different ($P < 0.05$).

at 1.45 µg incubated with 1 x 10^6 c/w for 48 h (table III) demonstrated a tendency to suppress FSH release and suppressed FSH release significantly at 7.25 µg by

Table III. Effects of immunopurified bovine inhibin on spontaneous FSH and LH release (percent of control values; mean ± SEM; $n = 4$) in culture medium of bovine pituitary cells (1 x 10^6 cells/well) incubated for 48 h.

Protein content IPI (µg/ml)	*FSH*	*LH*
0	100 ± 2.36[a]	100 ± 2.32[a]
0.058	92.6 ± 5.87[a]	98.3 ± 3.79[a]
0.29	95.7 ± 4.37[a]	105.3 ± 2.97[a]
1.45	89.7 ± 3.06[a]	101.0 ± 4.41[a]
7.25	78.0 ± 3.83[b]	101.8 ± 3.56[a]

Superscripts not in common within columns are significantly different ($P < 0.05$).

22% ($P < 0.05$). IPI had no effect on LH release. When the inhibitory activity was expressed per µg of protein, the effects of IPI and BFF were similar. This suggests that purification of biologically active bovine inhibin by immunoaffinity chromatography as determined by our dispersed bovine pituitary cell culture bioassay was not significant.

DISCUSSION

A dispersed bovine pituitary cell culture has been established to provide the first demonstration of the effects of charcoal-extracted BFF or IPI on the spontaneous release of FSH and LH in the bovine species. During work preliminary to these experiments, we found that the dissociation techniques described for rat (Scott *et al*, 1980) and sheep (Tsonis *et al*, 1989) pituitaries were not applicable to the bovine pituitary. Bovine pituitary cells enzymatically dissociated with trypsin as described by Tsonis *et al* (1986) for the ovine pituitary cells had poor viability (20–25%) as deter-

mined by trypan blue exclusion. This may reflect a higher sensitivity of bovine pituitary cell to trypsin. Collagenase dispersion had a less severe effect on viability with survival rates of 80 ± 3.0%. Furthermore, the results suggested that the number of bovine pituitary cells required per well to detect a BFF dose-dependent inhibition of spontaneous FSH release during 48 h of culture was much higher than the number of rat (0.125 x 10^6 c/w; Scott *et al,* 1980) or sheep (0.18–0.20 x 10^6 c/w; Tsonis *et al,* 1986) pituitary cells. The large number of bovine pituitary cells required may reflect lower gonadotropin secretory rates relative to other species or increased proportions of interstitial cells and other types of pituitary cells relative to the number of FSH secreting gonadotrophs. It may further suggest a requirement for cell contact or may reflect the sensitivity of bovine gonadotrophs to inhibin.

We have shown that a small amount of BFF (0.45 μg of protein equivalent to 0.01 μl of BFF) significantly suppresses spontaneous FSH released from bovine pituitary cells following 48 h of culture, without a concomitant suppression of LH release. Maximum specific inhibition of FSH release (34.1%) was obtained when 68.7 μg of protein (equivalent to 1.56 μl of BFF was added). This corresponds to the 35% to 40% suppression of circulating FSH observed when BFF is injected in intact (Lussier and Carruthers, 1989) or hemiovariectomized heifers (Lussier *et al,* 1988) suggesting that our bovine pituitary cell culture to some extent reflects *in vivo* relationships.

Culture of bovine pituitary cells with IPI showed a specific dose-dependent inhibiton of spontaneous FSH release without a concomitant inhibition of LH release. These results agree with the results obtained when either highly purified bovine or porcine inhibin was tested in rat pituitary cell culture (Robertson *et al,* 1985; Ling *et al,* 1987; Miyamoto *et al,* 1987) or injected in ovariectomized heifers (Ireland *et al,* 1983; Beard *et al,* 1990). Comparison of dose-dependent FSH inhibition curves showed no increase inhibition capacity of IPI compared to BFF in our bovine pituitary cell culture bioassay system. Miyamoto *et al* (1987) using a rat pituitary cell assay reported that a several hundred-fold purification of inhibin could be achieved by the same simple immunoaffinity chromatography procedure. When we compared our size exclusion chromatography protein elution profile and our results obtained on the 10% SDS–PAGE under non-denaturing conditions following reverse-phase chromatography with the protein elution profile on Sephacryl-S200 of Miyamoto *et al* (1987), it appears clear that purification of bovine inhibin does not differ from the latter purification; as only a limited number of proteins were retained on the column, the resultant inhibin should have been purified. As suggested by Miyamoto *et al* (1987), these proteins may represent either precursor or intermediate products of the lowest bioactive molecular weight form of inhibin (*ie* 32 kDa). Our protein chromatography profiles showed the presence of 3–4 protein peaks in the low MW region. It was recently shown that follistatin could bind the β-subunit of activin and inhibin (Nakamura *et al,* 1990; Kogawa *et al,* 1991; Shimonaka *et al,* 1991). Follistatin was shown to decrease FSH release by rat pituitary cells, to bind and prevent the biological activity of activin only (Nakamura *et al,* 1990; Kogawa *et al,* 1991; Shimonaka *et al,* 1991). Therefore, the purification of bovine inhibin with H256 as ligand may also copurify follistatin and activin,, and these bound proteins appear as a multiplicity of bands in the 25–35-kDa region. These results underline the potential problems of purifying inhibin by immunoaffinity chromatography from biological fluid. Furthermore, it was shown that the pituitary cells synthe-

sise activin (Corrigan *et al,* 1991; Kogawa *et al,* 1991) which acts as an autocrine mechanism to stimulate FSH release. Follistatin was recently isolated from bovine pituitary glands (Kogawa *et al,* 1991). Thus, the presence of inhibin, activin and follistatin in the bovine follicular fluid and serum as well as the secretion of activin and follistatin by the pituitary cells renders difficult the comparison of BFF and IPI in terms of biological activity. The presence of both factors may have been responsible for the apparent lack of purification of IPI *versus* BFF as measured in our bovine pituitary cell culture bioassay. Since follicular fluid and serum contain, and pituitary cells secrete activin and follistatin, there are significant problems interpreting inhibin bioassay potencies, as the effects of activin could lead to an underestimation and the presence of follistatin would result in an overestimation.

In summary, we have demonstrated that charcoal-extracted bovine follicular fluid or bovine inhibin partially purified by immunoaffinity chromatography suppress the spontaneous release of FSH in our bovine pituitary cell culture in a dose-dependent manner, without concomitant suppression of LH release.

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