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Gonadotropin binding sites in eel ovary : Autoradiographic visualization and new data on specificity

C. SALMON, J. NUNEZ-RODRIGUEZ (*), J. MARCHELIDON, Françoise LE MENN (*), Y. A. FONTAINE

Laboratoire de Physiologie Générale et Comparée,
Muséum National d'Histoire Naturelle, et Laboratoire d'Endocrinologie Comparée,
U.A. 90, C.N.R.S., 7, rue Cuvier, 75231 Paris Cedex 05.

(*) Laboratoire de Biologie Marine, Institut de Biologie Animale,
Université de Bordeaux I, Avenue des Facultés, 33405 Talence Cedex.

Summary. An autoradiographic study of cGTH binding sites in eel ovary indicates that they were mainly located in the follicular layers. Eel ovary membranes exhibited specific ^{125}I -hCG binding *in vitro*. In control fish we observed no cross-reaction between cGTH and hCG, whatever tracer hormone was used. On the contrary, carp pituitary extract (CPE) inhibited the binding of ^{125}I -hCG as well as of ^{125}I -cGTH. These results show that a fish pituitary factor, different from the cGTH we used, had binding sites in the ovary. It is suggested that this factor might be an isoform of the gonadotropin. Lastly, we have shown in *in vivo* CPE-treated eels that cGTH and hCG cross-reacted, demonstrating that the receptor properties changed during CPE-induced gonadal development.

Introduction.

The existence of saturable specific binding sites for carp gonadotropin (cGTH) in the silver eel (*Anguilla anguilla*) ovary was previously shown (Salmon *et al.*, 1987). Binding was not significant in other eel organs. Scatchard analysis showed that the sites could be separated into two classes ; one showed a low capacity and a high affinity and was thus compatible with the expected receptor properties. We describe here an attempt to localize these sites with an autoradiographic method.

Human chorionic gonadotropin (hCG) binding was also observed consistently. Earlier data on the stimulation of *in vitro* cyclic AMP (cAMP) production by eel ovary led us to hypothesize that several types of GTH receptors might exist in eel ovary (Salmon *et al.*, 1985, 1986). In the present study, we further examined this hypothesis. The effects of an *in vivo* carp pituitary treatment on *in vitro* binding were also considered.

Material and methods.

Animals and in vivo treatments. — Silver eels weighing 240 to 650 g were captured in ponds (Etangs de la Somme, France). They were kept in large tanks in running tapwater and under natural photoperiod (February to March, 1987). Water temperature varied between 9 and 12 °C.

In a first experiment, we used 3 control fish having a mean weight and gonadosomatic index (GSI) of 432 ± 106 g and 1.8 ± 0.1 , respectively.

In a second experiment, two groups of eels ($n = 5$) were injected i.p. three times a week for 2 months; control and treated eels received, respectively, NaCl 0.9 % (0.5 ml/250 g of body weight) and carp pituitary extract (CPE : 0.25 mg of dry pituitary/0.5 ml NaCl 0.9 %/250 g of body weight). At the time of sacrifice, mean body weights and GSIs were 258 ± 8 g and 1.8 ± 0.2 for the control group and 286 ± 3 g and 4.8 ± 0.4 for the treated eels.

The fish were decapitated; the treated ones were killed 3 days after the last CPE injection. The ovaries were dissected, weighed for GSI determination and immediately frozen (-20°C) until membrane preparation.

Hormones. — cGTH was prepared in collaboration with Dr Burzawa-Gérard according to Burzawa-Gérard (1971). Carp pituitary was extracted in NaCl 0.9 % and diluted to 0.5 mg/ml; its activity was then about $12.5 \mu\text{g}$ cGTH 6-178/0.5 ml as assessed by stimulation of *in vitro* cAMP production by eel ovary (Fontaine-Bertrand *et al.*, 1978). hCG (CR 121, about 13 000 IU/mg) was the kind gift of Dr Canfield (NIH).

Hormone radioiodination. — cGTH and hCG were radioiodinated as described previously (Salmon *et al.*, 1987). Briefly, cGTH was labelled by the lactoperoxidase method according to Maghuin-Rogister *et al.* (1978). ^{125}I -cGTH was purified by elution in Tris-HCl 10 mM, pH 7.4, NaN_3 0.1 % on a column (18×1.1 cm) of Ultrogel AcA-54 (LKB); the most active fractions in the radioreceptor assay were pooled. Specific binding activity of the pool increased with membrane concentration and was about 10 % of the total radioactivity in the presence of an excess membrane preparation (Salmon *et al.*, 1987).

hCG was labelled with the Iodogen method according to Salacinski *et al.* (1981). Labelled hCG was purified by elution in Tris-HCl 10 mM, pH 7.4, NaN_3 0.1 % on a column of Sephadex G50 (20 cm in a disposable 5-ml pipette). The fraction containing the peak of radioactivity was used for hCG binding studies; its specific binding activity increased with membrane concentration and reached 20 to 30 % of total radioactivity in the presence of excess membrane preparation (data not shown).

Autoradiography (from Lara and Droz, 1970; Bienarz and Kime, 1986). — One eel weighing 268 g was killed and its ovaries dissected (GSI = 1.73). Pieces (about 50 mg) of ovaries were preincubated 20 min at 4°C in 2 ml of Krebs Ringer-Henseleit (KRB, half-concentration of Ca^{++}) then transferred to 2 ml of fresh medium containing either labelled cGTH alone (2×10^6 cpm), for total binding (TB) determination, or labelled GTH plus $20 \mu\text{g}$ of native GTH for non-specific binding (NSB) determination. Incubation lasted 3 h, at the end of which the ovarian pieces were washed five times in cold KRB. They were then frozen on dry ice before being cut into $10\text{-}\mu\text{m}$ sections with a cryostat at -18°C . Sections were put on gelatinized slides and dip-coated in Ilford K5 emulsion. After 2-month exposure, the autoradiographs were developed in D19 and counterstained with 2 % toluidine blue.

Membrane preparation. — Membranes were prepared as previously described (Salmon *et al.*, 1987). Frozen ovaries were thawed then crushed with a press in one volume (1 ml/g of fresh weight) of KRB containing aprotinin (50 KIU/ml). They were washed 6 times in the same buffer and transferred to one volume of Tris-HCl 10 mM, pH 7.4, aprotinin (50 KIU/ml) for homogenization on ice in an Ultra-Turrax (maximal speed: 2×10 sec). The homogenate was centrifuged ($1\,000 \times g$) for 1 min at 4 °C; the pellet and fat layer were discarded. The supernatant was filtered on nylon (200 μ m), then centrifuged ($23\,000 \times g$) for 90 min at 4 °C. The pellet was suspended in about one-tenth volume of Tris-HCl aprotinin and kept frozen at - 20 °C overnight. After thawing, it was suspended in one to two volumes of Tris-HCl aprotinin and centrifuged ($5\,000 \times g$) for 30 min at 4 °C. The pellet was washed 5 to 6 times in the same conditions until the supernatant was clear. It was then suspended in Tris-HCl 10 mM, pH 7.4, glycerol 5 % (1 ml/10 g of ovary for control eels and 1 ml/40 g of ovary for treated eels) in order to obtain similar protein concentrations as assessed according to Bradford (1976).

Binding. — As previously described, *in vitro* binding was determined in low adsorption tubes (Minisorp, Nunc); ^{125}I -cGTH binding to the tube was about 0.1 % of total radioactivity. To 50 μ l of Tris-HCl 10 mM, pH 7.4, CaCl_2 60 mM were added 100 μ l of Tris HCl 10 mM either without cold hormone (TB), with an excess of it (NSB) (1 μ g of cGTH or 10 μ g of hCG according to labelled hormone), or with an increasing dose of hormone (inhibition studies), 50 μ l of radioactive hormone and 100 μ l of membrane suspension (0.1-0.15 mg of proteins). Incubation lasted 72 h at 4 °C and was terminated by dilution in 2 ml of cold Tris-HCl 10 mM, pH 7.4, and centrifugation ($10\,000 \times g$) for 10 min at 4 °C; the supernatant was discarded and pellet radioactivity was measured in a multiwell gamma counter. Specific binding (SB) was the difference between TB and NSB and was expressed as a percentage of total radioactivity.

Results.

Autoradiography. — The autoradiographic visualization of ^{125}I -cGTH binding in control eels is presented in figures 1 (TB) and 2 (NSB); in each figure, part (a) (dark field) shows the distribution of silver grains while part (b) (normal light) shows the ovarian structures. In TB, the silver grains were concentrated around the oocytes; in NSB, the sections were uniformly labelled with a low-grain density. This suggests that the particular localization of the grains in TB was related to specific cGTH binding. A comparison with the structures indicates that the silver grains in TB were associated with follicular layers.

In vitro binding of ^{125}I -cGTH and ^{125}I -hCG to untreated eel ovary membranes (experiment I). — Untreated eel ovary membrane bound labelled cGTH and hCG (fig. 3). SB was 4 and 2 %, respectively, of total radioactivity. ^{125}I -cGTH SB was inhibited by increasing doses of cGTH but not by hCG, even at high concentrations (fig. 3, upper part); bound ^{125}I -hCG was displaced by hCG, but

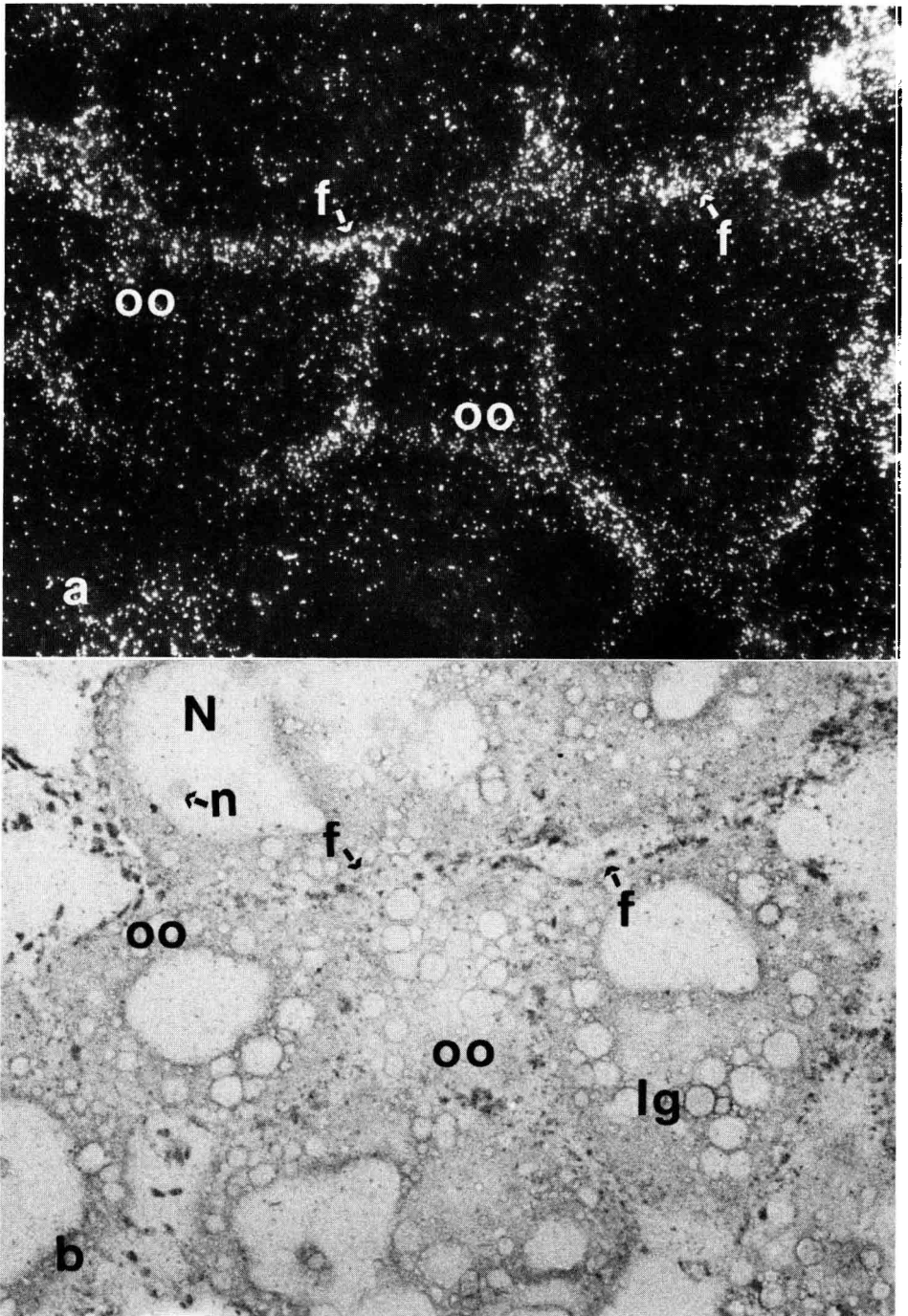


FIG. 1. — Autoradiograph of control eel ovary section. a : Total binding of ^{125}I -cGTH observed in dark field conditions. b : Same section observed in normal light conditions. f : follicle, lg : lipidic globule, N : nucleus, n : nucleolus, oo : oocyte. ($\times 250$).

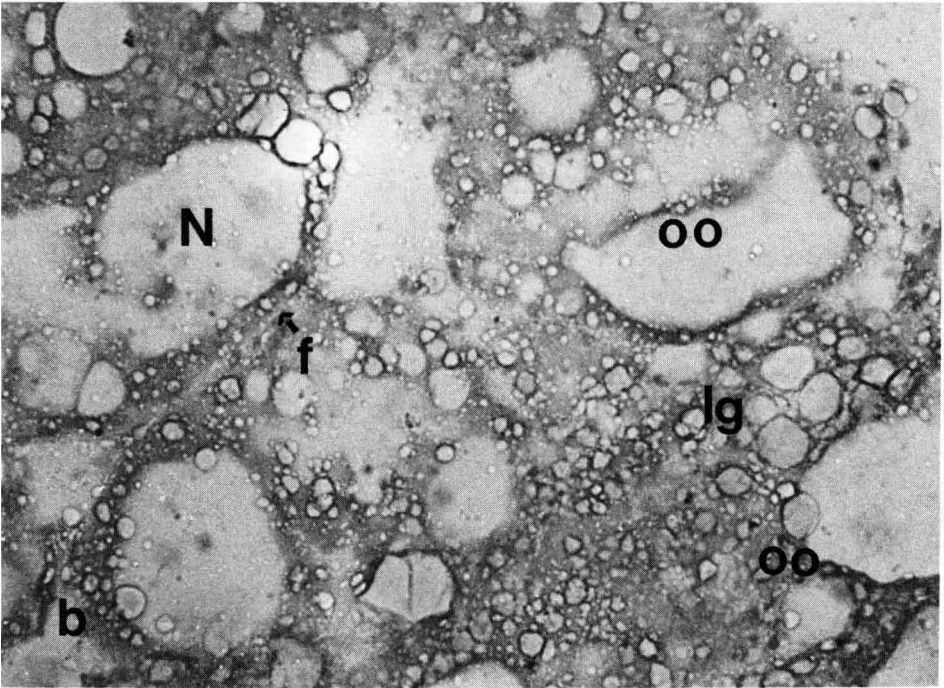
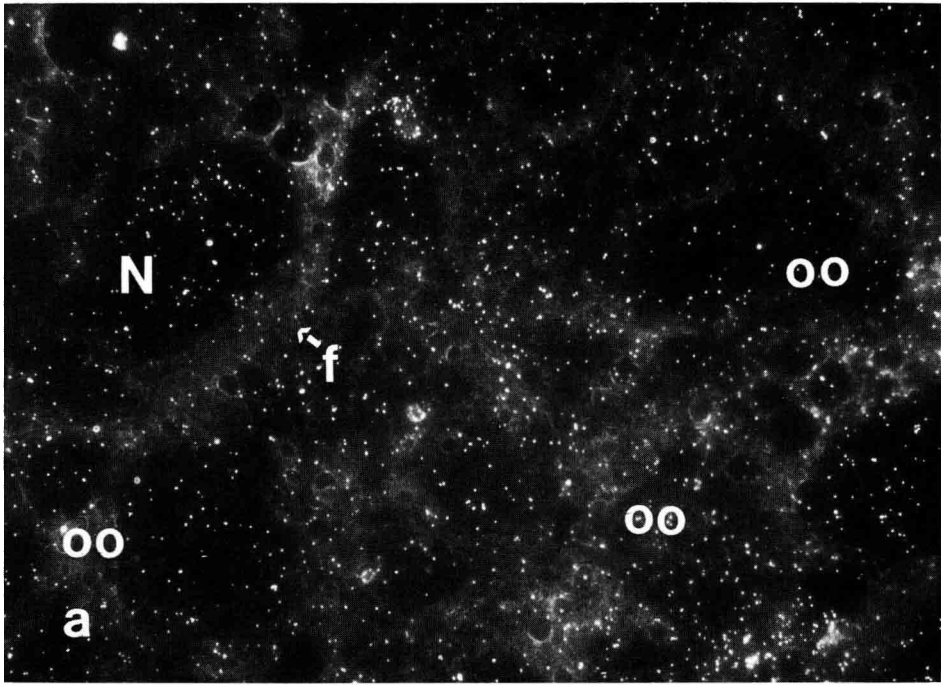


FIG. 2. — *Autoradiograph of control eel ovary section.* a: Binding of ^{125}I -cGTH in presence of $20\ \mu\text{g}$ cGTH observed in dark field conditions. b: Same section in normal light conditions. For abbreviations, see figure 1. ($\times 250$).

not by cGTH, even at high concentrations (fig. 3, lower part). A 50 % inhibition of labelled hormone SB was obtained between 9 and 27 ng of cGTH/tube for ^{125}I -cGTH and between 243 and 729 ng of hCG/tube for ^{125}I -hCG.

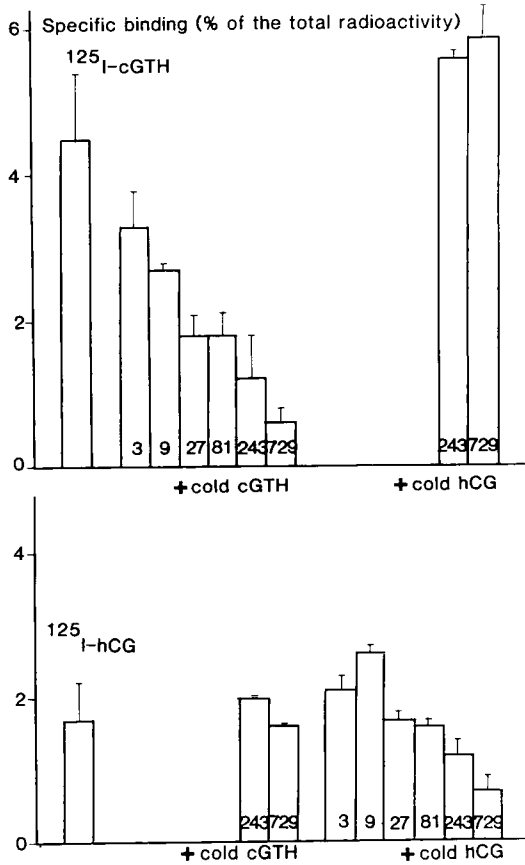


FIG. 3. — Specific binding of ^{125}I -cGTH and ^{125}I -hCG to ovarian membranes of untreated silver eels (experiment I). Total radioactivity: 27 000 cpm/tube. Unlabelled hormone concentrations: ng/tube. Values are the means of two incubations (vertical lines: range between the two determinations).

Effects of in vivo carp pituitary treatment on in vitro cGTH and hCG binding (experiment II). — Figure 4 shows that labelled hormones exhibited the same binding behaviour in the control group of experiment II as in normal eels studied in experiment I. Again, no cross-reaction was observed between cGTH and hCG. Surprisingly, SB of both labelled hormones was inhibited by carp pituitary extract; this inhibition was dose-dependent with ^{125}I -cGTH as tracer, while it was maximal with the lowest dose of CPE in the case of ^{125}I -hCG.

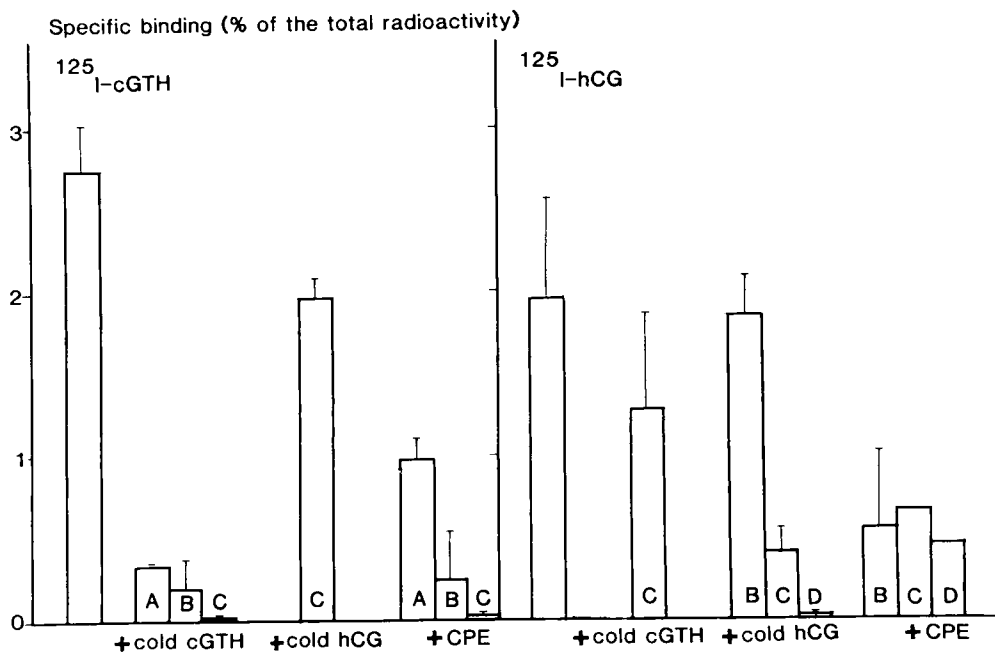


FIG. 4. — Specific binding of $^{125}\text{I-cGTH}$ and $^{125}\text{I-hCG}$ to ovarian membranes of control eels (experiment II). Total radioactivity: 37 000 cpm. Unlabelled hormone concentration (ng/tube): A = 9, B = 81, C = 729, D = 6 560. CPE concentration is expressed as cGTH equivalent. Values are the means of two incubations (vertical lines: range between the two determinations).

In vivo treatment with CPE induced an important change in binding properties *in vitro* (fig. 5). hCG became able to completely inhibit $^{125}\text{I-cGTH}$ SB, and cGTH could displace at least part of the bound $^{125}\text{I-hCG}$. CPE kept the ability to inhibit the binding of mammalian and fish hormones; this inhibition needed higher doses for $^{125}\text{I-hCG}$ than for $^{125}\text{I-cGTH}$.

Discussion.

The presence of gonadotropin binding sites in fish ovary is now increasingly documented (Van der Kraak, 1983; Salmon *et al.*, 1984; Breton *et al.*, 1986; Salmon *et al.*, 1987; Jamal Uddin and Bhattacharya, 1987; Kanamori *et al.*, 1987). Their specificity and biochemical properties are usually considered as evidence of their ability to be the GTH receptors. If so, they are supposed to stay mainly in the somatic cells of the follicle. In the present paper, we describe an attempt at autoradiographic visualization of cGTH specific binding. Radioactive hormone seems mainly associated with the follicular layers, although it is impossible to determine whether any of the layers are more concerned than

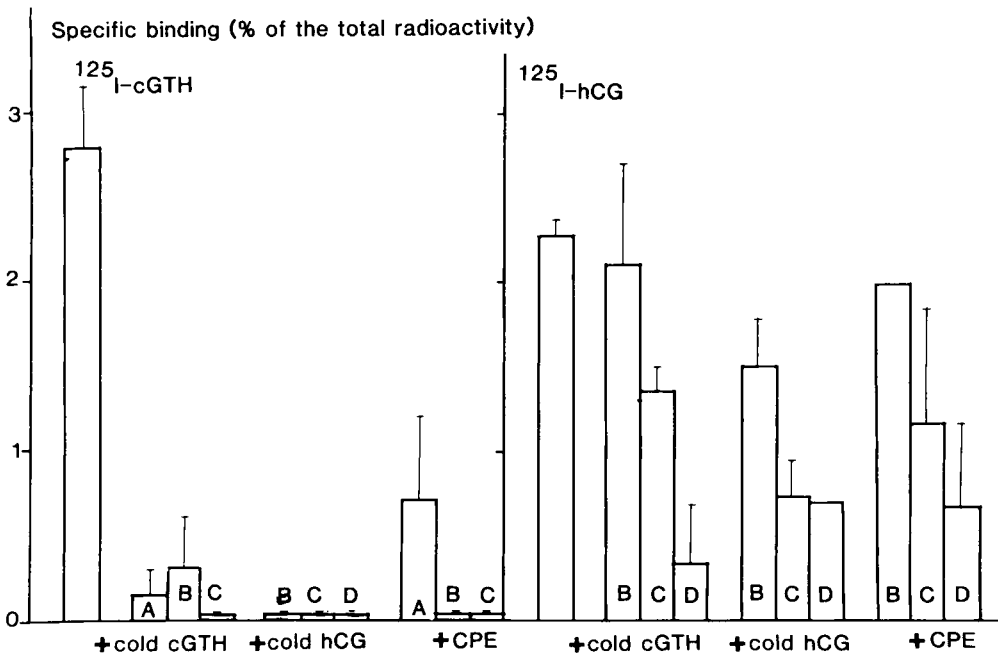


FIG. 5. — Specific binding of $^{125}\text{I-cGTH}$ and $^{125}\text{I-hCG}$ to ovarian membranes of CPE treated eels (experiment II). Total radioactivity : 37 000 cpm. Unlabelled hormone concentration (ng/tube) : A = 9, B = 81, C = 729, D = 6 560. CPE concentration is expressed as cGTH equivalent. Values are the means of two incubations (vertical lines : range between the two determinations).

others ; inner oocyte structures are more lightly labelled. Furthermore, only the peripheral silver grains disappeared when the tissue was incubated with excess cGTH, demonstrating cGTH specific binding in follicular layers. This result is in agreement with the work of Bienarz and Kime (1986) on common carp ovary.

We observed that a mammalian GTH, namely human chorionic gonadotropin, was able to bind to control eel ovary membranes and could not be displaced by cGTH. The inhibition of hCG SB by hCG needs about 30 times more cold hormone than does cGTH SB inhibition by cGTH. CPE inhibits both $^{125}\text{I-cGTH}$ and $^{125}\text{I-hCG}$, even though inhibition seems to be less complete and needs higher CPE doses in the case of $^{125}\text{I-hCG}$. This, and the high unlabelled hCG doses needed to inhibit hCG SB, may be related to the lower reversibility of hCG specific binding. As previously noted, hCG cannot displace bound labelled cGTH (Salmon *et al.*, 1987). Schulz *et al.* (1985) and Jamal Uddin and Bhattacharya (1987) also described hCG binding to ovarian membranes of previtellogenic or vitellogenic trout and mature murrel, respectively. In murrel, hCG (or ovine LH) could not displace more than 10 % of the bound fish GTH.

Former data on *in vitro* stimulation of cAMP production by eel ovary suggest that ovine LH and hCG interact with part of the cGTH receptor pool in eel ovary (Salmon *et al.*, 1985, 1986). In these conditions there should be a cross-reaction

between cGTH and hCG. Its absence indicates that the binding sites for these hormones are likely to be different. This raises the question of the physiological meaning of hCG SB to a fish gonad. There may be some similarity between hCG and a fish hormone, such as a second gonadotropin (Idler *et al.*, 1975a, 1975b ; Kawachi *et al.*, 1986) or another factor controlling some gonadal function. There is strong evidence supporting the unicity of gonadotropin in fish (for review, see Fontaine, 1985) and it seems unlikely that an unrelated GTH receptor would bind hCG. Gonadotropin isoforms exist in mammals (*e.g.* Chappel *et al.*, 1983, 1985 ; Blum *et al.*, 1985 ; Matteri *et al.*, 1986) as well as in fish (Huang *et al.*, 1981 ; Goncharov *et al.*, 1983 ; Kuznetsov *et al.*, 1983 ; Swanson *et al.*, 1987). A general consensus is that isoforms are coded by the same gene but are differently processed (*e.g.* with regard to glycosylation). These isohormones may differ in their biological activities (Goncharov *et al.*, 1983) and may be dependent on the hormonal state of the organism (*e.g.* Chappel *et al.*, 1985) ; thus, they could play a role in the fine regulation of the gonadal functions. We suggest that isoform-specific GTH isoreceptors may exist in the gonad. From this viewpoint, the present demonstration of the dual activity of CPE in inhibiting both ^{125}I -cGTH and ^{125}I -hCG binding is of interest ; it shows that the specific hCG binding site of eel ovary interacts with a pituitary factor, likely to be an isoform of cGTH and absent from the preparations we used. Indeed, several isohormones with their own isoreceptors should be functionally equivalent to a plurihormonal system, at least during the physiological stages when receptors are highly specific ; gonadal regulation by pituitary hormones should then result from the differential processing of a single gene product instead of from the productions of two different genes.

Developmental influences on binding specificity have been assessed after a CPE treatment *in vivo* which induces vitellogenesis in eel. After 2 months, we observed an important shift in the binding site properties ; as in control eels, the inhibition of ^{125}I -hCG binding required higher cold hormone or CPE doses than did ^{125}I -cGTH, but hCG and cGTH could cross-react, whatever the tracer hormone was. This means that the binding sites and/or their membrane environment were modified according to the gonadal stage.

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Résumé. *Sites de liaison des gonadotropines dans l'ovaire d'anguille : visualisation autoradiographique et nouvelles données sur leur spécificité.*

Une étude autoradiographique des sites de liaison de la cGTH dans l'ovaire d'anguille argentée indique qu'ils sont situés essentiellement dans les couches folliculaires.

In vitro, des préparations membranaires d'ovaire d'anguille contiennent aussi des sites de liaison de la hCG, spécifiques de cette hormone. Nous n'observons pas, chez les témoins, de réaction croisée entre la cGTH et la hCG quelle que soit l'hormone marquée

utilisée. Par contre, un extrait d'hypophyse de carpe (CPE) inhibe la liaison non seulement de la ^{125}I -cGTH mais aussi de la ^{125}I -hCG. Ce résultat montre qu'un facteur présent dans l'hypophyse de poisson, différent de la cGTH utilisée dans cette expérience, possède des sites de liaison dans l'ovaire. Nous suggérons qu'il peut s'agir d'une isoforme de la gonadotropine.

Enfin, nous montrons que, pendant la vitellogenèse induite par un traitement avec CPE *in vivo*, cGTH et hCG acquièrent la capacité de faire des réactions croisées. Ceci montre que les propriétés des récepteurs changent pendant la maturation de la gonade.

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