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Effect of Osmotic Pressure on Prolactin Release in Rainbow Trout: *in Vitro* Studies

F. GONNET, P. PRUNET,¹ M. C. TONON,* P. DUBOURG,† O. KAH† AND H. VAUDRY*

*Laboratoire de Physiologie des Poissons, INRA, Campus de Beaulieu, 35042 Rennes Cédex; *Groupe de Recherche en Endocrinologie Moléculaire, UA CNRS 650, Unité alliée à l'INSERM, Faculté des Sciences, Université de Rouen, 76160 Mont-St-Aignan; and †Laboratoire de Physiologie des Interactions Cellulaires, Université de Bordeaux I, Av des Facultés, 33405 Talence Cédex, France*

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To investigate a possible effect of osmotic pressure on prolactin (PRL) release in rainbow trout, we developed a technique for *in vitro* perfusion of trout pituitaries. Changes in osmotic pressure similar to those observed in fish plasma during transfer experiments did not induce significant modifications of PRL release. In contrast, high-amplitude variation of osmotic pressure resulted in clear modifications of PRL secretion: hyperosmotic medium caused a reduction in PRL release, while infusion of hyposmotic medium induced a transitory increase in PRL release. By using different concentrations of mannitol, we found that the modifications of prolactin secretion could not be ascribed to alterations of the ionic composition of the medium but actually resulted from variations in the osmotic pressure of the incubation medium. In further experiments osmotic pressure was decreased from 300 to 220 mOsm/kg or from 400 to 300 mOsm/kg; a similar transitory increase in PRL release was observed. Measurement of gonadotropin (GtH) in the perfusion effluent medium showed that PRL and GtH secretion followed similar patterns. Thus, our results suggest a possible mechanical effect of wide changes in osmotic pressure on pituitary cell membranes. These data indicate that the rainbow trout differs notably from nonsalmonid teleost species thus far studied in the lack of sensitivity of its PRL cells to osmotic pressure. © 1988 Academic Press, Inc.

In some teleost fish, prolactin (PRL) secretion appears to be controlled through two different mechanisms: neuroendocrine pathways involving hypothalamic aminergic and peptidergic neurons and a systemic pathway (changes in plasma osmotic pressure) often associated with changes in ambient salinity (see reviews by Clarke and Bern, 1980; Ball, 1981; Bern, 1983; Specker *et al.*, 1984). Numerous histological studies have shown that transfer of teleosts to environments of altered salinity is associated with morphological changes in the PRL cells which appear more active in fresh water (FW) than in seawater (SW) (cf., e.g., Dharmamba and Nishioka, 1968; Abra-

ham, 1971; Nagahama *et al.*, 1973, 1975, 1977; Dubourg *et al.*, 1980; Wendelaar Bonga and Van der Meij, 1980; Olivereau *et al.*, 1981). Experiments with fish bearing autografted pituitaries suggest that in goldfish, goby, and tilapia, PRL cells may be controlled directly by plasma osmotic pressure, with higher osmolarity being inhibitory (Leatherland and Ensor, 1973; Nagahama *et al.*, 1974, 1975). These results have been confirmed using incubated or perfused pituitary fragments *in vitro* (Ingleton *et al.*, 1973; Nagahama *et al.*, 1975; Wigham *et al.*, 1977; Grau *et al.*, 1981, 1986, 1987; Batten *et al.*, 1983); however, further experiments with *Oreochromis mossambicus* transferred to environments of high osmolarity or with high Ca^{2+} concentrations indicate that plasma osmolarity

¹ To whom requests for reprints should be addressed.

may not be an important regulatory factor for PRL cells (Wendelaar Bonga *et al.*, 1985). In salmonids, several studies reported changes in prolactin cell activity in salmon acclimated to hyper- or hyposmotic medium (Leatherland and McKeown, 1974; Leatherland and Lin, 1975; Nagahama *et al.*, 1977), while others could not detect any effect of salinity on prolactin cells (Cook and Van Overbeeke, 1969; McKeown and Leatherland, 1973). Moreover, no clear change in PRL release occurred in low- or high-sodium medium *in vitro*, although sodium has been reported to affect the production of newly synthesized PRL (Baker and Ingleton, 1975).

These contradictory results in salmonids led us to reinvestigate more precisely, in rainbow trout, the possible effect of osmotic pressure on PRL release by pituitary fragments. For this purpose we developed a technique for *in vitro* perfusion associated with a specific radioimmunoassay (RIA) for salmon PRL, to study the kinetics of PRL secretion by the trout pituitary. Our results indicate that osmotic pressure does not seem to be an important factor controlling PRL release in rainbow trout.

MATERIALS AND METHODS

Animals. Immature rainbow trout, *Salmo gairdneri* (50–100 g), were kept in recirculating tap water at $13 \pm 1^\circ$ under natural photoperiod. All fish used in our experiments were of similar age (1 year old). Pituitaries were removed after decapitation, between 8 and 9 AM. For PRL experiments the rostral pars distalis (RPD) was dissected under a dissecting microscope; however, the RPD was partially contaminated with the proximal pars distalis (PPD) and the neurohypophysis (NH). For experiments in which gonadotropin (GtH) was measured all the pars distalis was used and this material was partially contaminated with the NH. Pituitary fragments were preincubated for 10 min in Ringer solution (NaCl 140 mM, KCl 2 mM, CaCl_2 2 mM, Hepes 15 mM) gassed with 95% O_2 –5% CO_2 . Immediately before use, the Ringer solution was supplemented with 2.5 g/liter glucose and 0.3 g/liter bovine serum albumin (fraction V, Sigma) and the pH adjusted to 7.4. The osmotic pressure was 300 mOsm/kg.

Perfusion. The perfusion system, previously described by Tonon *et al.* (1980), was composed of silicized glass columns (0.9×12 cm) delimited by Teflon plungers. The volume of the chamber was 1.5 ml. The rostral lobes (usually 3–5 per column) were suspended in a Bio-Gel P2 support matrix and perfused with the medium described above at constant flow rate (0.2 ml/min) and temperature ($15 \pm 1^\circ$). Samples (1.5 ml) were collected at 7.5-min intervals; hormone levels were measured on the day of perfusion, or samples were frozen at -20° until assay. Unless specified, osmotic pressure changes were produced by addition of mannitol (Sigma) to a Ringer solution containing 112 mM NaCl until the desired osmotic pressure was attained. Osmolarity was determined with a Roebling osmometer.

Radioimmunoassay. PRL secreted from rainbow trout pituitaries was determined using a PRL salmon RIA according to the technique described by Hirano *et al.* (1985) and modified by Prunet *et al.* (1985). This RIA has been demonstrated to be specific for measurement of PRL in rainbow trout as the absence of cross-reactivity with plasma from hypophysectomized rainbow trout and parallelism between the standard curve (chinook PRL) and dilution curves of rainbow trout plasma (Hirano *et al.*, 1985; Prunet *et al.*, 1985). Moreover, serial dilution of perfusate gave inhibition curves parallel to those of chinook PRL standard. Routinely the volume of perfusate assayed was 150 μ l. The sensitivity of the assay was estimated to be 0.4 ng/ml, and the intraassay coefficient of variation was 4.7% for 11 ng/ml ($n = 10$). GtH levels in the perfusate were determined in 20- μ l aliquots as described by Breton *et al.* (1978).

Calculation. Unless specified, each perfusion pattern was calculated as the mean amount of PRL or GtH released in three independent experiments. Baseline secretion was achieved within an hour; accordingly, all tubes collected during the first hour were discarded. As basal level could change from one column to the other, hormone secretion was expressed as a percentage of the basal level. The basal level (indicated as 100%) was calculated as the mean three samples just preceding the first change in osmotic pressure in each experiment.

Electron microscope studies. Two groups of pituitaries were examined to determine the possible ultrastructural changes occurring during perfusion. Control pituitaries were dissected out and immediately fixed by immersion in 6.25% glutaraldehyde (Soerensen buffer, 0.015 M, pH 7.4) according to Dubourg *et al.* (1983). After 15 min, the pituitaries were rinsed in Soerensen buffer, postfixed in 1% osmium tetroxide (Michaelis buffer, pH 7.4), dehydrated, and embedded in Epon. The experimental pituitaries were perfused for 7 hr under the conditions described above. Then the fixative solution was introduced into the perfusion

chamber and the pituitaries were removed from the chamber and postfixed as in the control group.

RESULTS

Validation of the Perfusion Technique

Figure 1 shows typical patterns of PRL release during a 6-hr perfusion at three different osmotic pressures. An initial decrease was observed when the pituitaries were perfused with hyposmotic (220 mOsm/kg), isosmotic (300 mOsm/kg), and hyperosmotic (400 mOsm/kg) Ringer solution; however, within 1 hr, the secretion rate reached its basal level and remained stable throughout the experiments. Since the basal release varied from one column to the other, values in all subsequent experiments were expressed as percentages of the spontaneous secretion rate. The total amounts of PRL secreted during 6-hr perfusions have been measured in three independent experiments. The total PRL released during the perfusion was 1.8 ± 0.29 μ g/gland, while the tissue PRL content at the end of the experiment was 4.19 ± 0.66 μ g/gland. The ultrastructural characteristics of perfused pituitaries are shown in Fig. 2. The PRL cells of rainbow trout are easily recognized owing to their follicular arrangement. In perfused glands, blood cells still present in the capillaries were ob-

served, and their ultrastructure did not appear to be affected by the experimental procedure. In addition, mitosis was occasionally observed in perfused glands as in controls. In both control and perfused tissues, cells exhibited a marked polarity with the nucleus located at the basal pole. As compared with controls (Fig. 2a) the fine structure of perfused PRL cells (Fig. 2b) showed a reduction in cytoplasmic vacuoles; however, these differences were minor and after a 6-hr perfusion, PRL cells were still loaded with secretory granules as were control cells. It thus appears that the viability of PRL cells was not altered during perfusion.

In Vitro Effects of Osmotic Pressure on PRL Release

The effects of changes in osmotic pressure on PRL release *in vitro* were initially studied via alterations in NaCl concentrations. Stepwise increases in Na⁺ concentration from 112 mM (220 mOsm/kg) to 130 mM (260 mOsm/kg) and then to 170 mM (344 mOsm/kg) did not induce significant changes in basal PRL release (Fig. 3). In another experiment (Fig. 4) perfused pituitary fragments were successively exposed to 160 mM Na⁺ (310 mOsm/kg) and 200 mM Na⁺ (380 mOsm/kg) without major modifications of PRL release; the tendency toward a decrease with high-osmotic-pressure medium was not significant.

These first results led us to investigate the effects of high-amplitude variation of osmotic pressure. After exposure to isosmotic medium for half an hour (140 mM Na⁺, 290 mOsm/kg), the medium was switched to hyperosmotic medium (400 mOsm/kg) which caused an immediate decrease in PRL release ($30.9 \pm 4.6\%$). This decrease was maintained during the whole period (1 hr) of hyperosmolar perfusion (Fig. 5). Subsequent exposure to hyposmotic medium (220 mOsm/kg) for 1 hr resulted in a transient stimulation of PRL re-

FIG. 1. Comparison between the PRL secretion patterns of trout rostral pars distalis perfused for 6 hr at three different osmotic pressures. $\circ$, Isosmotic Ringer solution (300 mOsm/kg); $\bullet$, hyperosmotic Ringer solution (400 mOsm/kg); $\square$, hyposmotic Ringer solution (220 mOsm/kg).

FIG. 2. Electron micrographs of prolactin cells before (a) and 7 hr after (b) perfusion. No major differences were observed between perfused and control groups. PRL, prolactin cell; CA, cellular apex; FL, follicular lumen.

FIG. 3. Effect of stepwise increases in Na^+ concentration from 112 mM (220 mOsm/kg) to 130 mM (260 mOsm/kg) and then to 170 mM (344 mOsm/kg) on PRL release by perfused trout rostral pars distalis. The basal level (100% value), 34.6 ± 6.6 ng/ml, was calculated for each experiment as the mean for three samples (○) just preceding the first change in osmotic pressure in each experiment.

lease ($90 \pm 13.8\%$) with a progressive return to basal levels (Fig. 5). During the last hour of perfusion with isotonic medium, a tendency toward a decrease in hormone release was observed. To determine whether the effect on PRL response was due to ionic composition or osmotic pressure, a similar experiment was designed in which osmotic pressure was altered by adding appropriate amounts of mannitol in the presence of a constant NaCl concentration (112 mM). In this case, PRL release varied in the same

FIG. 5. Effect of sequential changes in sodium concentration on PRL release by perfused trout rostral pars distalis. The basal level (100% value) was 4.9 ± 0.4 ng/ml. See legend to Fig. 3 for other details.

manner as in the previous experiment (Fig. 6).

The necessity of applying high-amplitude variations of osmotic pressure to obtain an effect on PRL release from perfused pituitary fragments led us to raise further questions about this effect. We therefore investigated the possibility that in the previous experiments, the changes in PRL release may have been due to osmotic shock rather than to an absolute value of osmotic pressure (hyper- vs hyposmotic medium). In a first series of experiments, we compared the effect of different abrupt decreases in osmotic pressure on PRL release; when os-

FIG. 4. Effect of 160 mM (310 mOsm/kg) and 200 mM (380 mOsm/kg) Na^+ on PRL release by perfused trout rostral pars distalis. The basal level (100% value) was 3.9 ± 0.3 ng/ml. See legend to Fig. 3 for other details.

FIG. 6. Effect of hyperosmotic (400 mOsm/kg) and hyposmotic (220 mOsm/kg) media with constant sodium concentration on PRL release. Osmotic pressure was modified by adding different mannitol concentrations. The basal level (100% value) was 12.6 ± 2.6 ng/ml. See legend to Fig. 3 for other details.

otic pressure was decreased from 300 to 220 mOsm/kg (Fig. 7) or from 400 to 300 mOsm/kg (Fig. 8), we observed the same pattern of PRL release, i.e., an immediate surge followed by a progressive decrease to basal level.

In a second series of experiments, we examined the response of a second pituitary hormone whose secretion could have been

FIG. 7. Effect of hyposmotic (220 mOsm/kg) and isosmotic (300 mOsm/kg) media with constant sodium concentration on PRL release. Osmotic pressure was modified by adding different mannitol concentrations. The basal level (100% value) was 17.1 ± 1.1 ng/ml. See legend to Fig. 3 for other details.

FIG. 8. Effect of hyperosmotic (400 mOsm/kg) and isosmotic (300 mOsm/kg) media with constant sodium concentration on PRL release. Osmotic pressure was modified by adding different mannitol concentrations. The basal level (100% value) was 12.3 ± 4.2 ng/ml. See legend to Fig. 3 for other details.

modified by these osmotic abrupt changes; as shown in Fig. 9, exposure to hyperosmotic medium followed by hyposmotic medium resulted in changes in GtH release similar to that observed with PRL using the same protocol (Fig. 6). In the same way, an abrupt decrease in osmotic pressure from 400 to 300 mOsm/kg induced a surge in GtH release (Fig. 10) closely resembling that obtained with PRL (Fig. 8).

FIG. 9. Effect of sequential changes in sodium concentration on GtH release by perfused trout proximal pars distalis. The basal level (100% value) was 2.8 ± 0.3 ng/ml. See legend to Fig. 3 for other details.

FIG. 10. Effect of hyperosmotic (400 mOsm/kg) and isosmotic (300 mOsm/kg) media with constant sodium concentration on GtH release. Osmotic pressure was modified by adding different mannitol concentrations. The basal level (100% value) was 2.4 ± 0.8 ng/ml. See legend to Fig. 3 for other details.

DISCUSSION

The *in vitro* perfusion system described in this paper appears to behave in most respects as would be expected for pituitary hormone secretion. During the first hour of perfusion, a sharp decrease in PRL release was observed, reflecting elimination of PRL that accumulated in the medium before application of pituitary fragments to the column and/or originated from some cell damage during dissection of the pituitary tissue. In all experiments, PRL secretion reached its basal level within 1 hr and remained stable for 6 hr (Fig. 1). This basal level may differ from one perfusion to another but in all cases it was high enough (>2 ng/ml) to be detected by our radioimmunoassay technique (sensitivity threshold: 0.4 ng/ml). That the perfused pituitary fragments continued to secrete PRL at a constant rate during perfusion and that the tissue still contained consistent amounts of PRL at the end of the perfusion argue in favor of the continued functioning and viability of pituitaries in our perfusion model. This was confirmed by ultrastructural analysis of perfused tissue which did not reveal any major differences from control pituitaries; after a 6-hr perfusion the appearance

of PRL cells and the follicular arrangement were maintained (Fig. 2). Thus, this perfusion technique, associated with a specific RIA for measurement of salmonid PRL, appears to be a useful tool for studying, in rainbow trout, the dynamics of pituitary PRL secretion and its possible control by osmotic pressure.

The present investigation, using this perfusion technique, clearly shows that a gradual increase in osmotic pressure does not induce significant modification of PRL release (Figs. 3 and 4). For these experiments, the osmotic pressures were selected to parallel physiological values measured in the plasma of SW- and FW-adapted rainbow trout (Conte and Wagner, 1965; Bath and Eddy, 1979; Eddy, 1982; Boeuf and Harache, 1984; Prunet and Boeuf, 1985). These results are in agreement with other *in vitro* studies. For instance, Baker and Ingleton (1975), using incubated rainbow trout pituitaries, did not observe any changes in PRL release; however, the technique (polyacrylamide gel electrophoresis at pH 9.5) used by these authors for measuring PRL content may not have been an adequate tool for analyzing trout PRL if this hormone is a basic protein as is salmon PRL (Kawauchi *et al.*, 1983; Prunet and Houdebine, 1984). Recently, Suzuki *et al.* (1987) did not observe a significant stimulatory effect of hypotonic medium on PRL release from incubated chum salmon pituitaries. The present findings with rainbow trout differ notably from other studies on the effect of osmotic pressure on PRL release; similar *in vitro* studies with other teleosts testing a range of osmotic pressures led to marked modification of PRL secretion (e.g., Nagahama *et al.*, 1975; Wigham *et al.*, 1977; Grau *et al.*, 1981). Moreover, recent studies using perfused tilapia rostral pars distalis confirmed that reduced osmotic pressure (280 mOsm/kg) stimulates release of PRL, while hyperosmotic medium (355 mOsm/kg) reduces it. This observed effect is rapid and lasts for at least 2 to 3 hr (Grau *et al.*, 1986, 1987). The reason for

such a discrepancy is not clear but may be related to species differences. In any case, it appears that the rainbow trout differs notably in the lack of sensitivity of its PRL cells to osmotic pressure from nonsalmonid teleost species studied so far.

Application of higher variations of osmotic pressure resulted in clear modification of PRL secretion; while an increase from 300 to 400 mOsm/kg decreased PRL release, a subsequent decrease of osmotic pressure from 400 to 220 mOsm/kg increased it (Fig. 5). This effect was due to osmotic pressure itself rather than changes in NaCl concentrations, as experiments with mannitol concentrations gave similar patterns of PRL release (Fig. 6). The levels of osmotic pressure used in these experiments appeared to be at the limit of the physiological range observed in rainbow trout, although values as high as 400 mOsm/kg have been recorded in fish 3 days following direct transfer from FW to SW (Prunet *et al.*, 1985). Thus, the need for large osmotic pressure variations to modify PRL release could be due to a low sensitivity of PRL cells to osmotic pressure in rainbow trout. It is also possible that these large variations induced osmotic shock, resulting in changes in hormonal release. Experiments designed to answer this last problem clearly showed that the PRL secretion surge obtained after exposure to 300 to 220 mOsm/kg was also observed when osmotic pressure was decreased from 400 to 300 mOsm/kg. It appears therefore that the induced change in PRL secretion is likely due to the magnitude of the change in osmotic pressure and not to the hyposmotic medium. In mammals, release of pituitary hormones from incubated bovine glands can be modified by alteration of the osmolarity of the medium to 10% above the control (Labella *et al.*, 1975). In particular, LH release from adenohypophyseal cells is inversely related to extracellular osmolarity (Greer *et al.*, 1985). The latter authors suggested that the effects observed were attributable to a mechanical effect of the osmotic pressure,

which may alter the cell volume and induce an expansion or a contraction of the outer cell membrane. We thus assume that a similar phenomenon may explain, in our experiment, the *in vitro* effects of a large variation in osmotic pressure on PRL release. Such a hypothesis would imply that these effects should not be specific to one particular hormone. By measuring GTH release in two series of perfusion experiments, we found parallel changes in GTH release and in PRL release.

In conclusion, we found that rainbow trout differ notably from other teleost fish in the control of PRL release and in its sensitivity to osmotic pressure. During the first 3 days following transfer from FW to SW or the converse, changes in plasma osmotic pressure may be sufficient to induce physiological modifications of PRL secretion (Prunet and Boeuf, 1985; Prunet *et al.*, 1985; Hirano *et al.*, 1985; Suzuki *et al.*, 1987). The origin of such regulation should be at the level of the central nervous system; the effects of external medium changes on hypothalamic neurohormone secretion require investigations.

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