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Isolation and partial characterization of a pair of prolactins released *in vitro* by the pituitary of a cichlid fish, *Oreochromis mossambicus*

(hormone variants/sequence homology/bioassay)

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ABSTRACT **The pituitary of the cichlid fish tilapia secretes two prolactins (PRLs) of molecular masses 20 kDa and 24 kDa. The 20-kDa PRL has an isoelectric point in the range of those of mammalian PRLs (pI 6.7), but the 24-kDa PRL is unusually basic (pI 8.7). Partial sequence information indicates that the PRLs are homologous but distinct proteins, differing by five amino acids within the first 29 NH_2-terminal residues. Homology in the known region is higher with chum salmon PRL than with known mammalian PRLs. Reversed-phase HPLC permits isolation of these two PRLs and a single tilapia growth hormone from culture medium or from the pituitary in a single step. HPLC and radio-HPLC analysis of [^{3}H]leucine pulse–chase experiments reveal that each PRL is secreted *in vitro* at remarkably high rates (21 pmol per gland per hr) and that the two PRLs are released in approximately equimolar amounts, suggesting the coordinate regulation of the secretion. Both PRLs exert characteristic PRL activity in that they prevent the loss of Na^+ from the plasma of hypophysectomized tilapia in fresh water.**

In teleost fishes, pituitary prolactin (PRL) production is confined to the rostral pars distalis (RPD). The RPD of the cichlid fish tilapia (*Oreochromis mossambicus*) consists almost entirely of PRL-secreting cells and accounts for almost one-third of the entire pituitary (1, 2). This regionalization of PRL cells differs from the scattered distribution of PRL cells in the pituitaries of other vertebrates and has made the teleost pituitary a useful model for the study of PRL cell function.

The aim of the investigations reported here was to make use of this organizational simplicity in order to isolate and characterize both stored and secreted PRL from the tilapia pituitary. This research direction was taken following reports that mammalian PRL might be modified during release (3–6) and because we thought that such modification might explain why tilapia PRL isolated previously exhibited anomalous electrophoretic behavior (7–9), even though its immunological and biological characteristics were similar to those of other vertebrate PRLs (10, 11).

Our approach has been to culture the PRL-producing lobes and to isolate secreted proteins from the culture medium using reversed-phase HPLC. To exclude the possibility that the isolated PRL was growth hormone (GH), we also isolated GH released into the medium by the organ-cultured proximal pars distalis (PPD) of the tilapia. We now report that a pair of PRLs is stored by and released at remarkably high rates from the tilapia pituitary in equimolar amounts. Only a single GH appears to be stored and released. We developed a bioassay using hypophysectomized tilapia and demonstrated characteristic "Na^+-retaining activity" for each protein of this novel PRL pair.

MATERIALS AND METHODS

Organ Culture of Pituitary Lobes. Pituitaries were removed from freshwater tilapia of both sexes weighing from 10 to 60 g. The RPD was dissected under sterile conditions and cultured in Krebs–Ringer bicarbonate supplemented with Eagle's minimal essential medium (KRb/MEM) at 27°C as described (12). The KRb/MEM for the RPDs was made hyposmotic (290 mosM) to stimulate PRL release (13). The PPD was cultured in isosmotic (320 mosM) KRb/MEM with cortisol added (200 ng/ml) to stimulate GH release (14). Ten RPDs or PPDs were cultured per well in 2 ml of KRb/MEM. The medium (KRb/MEM containing released hormones) was collected daily and replaced with fresh KRb/MEM.

Isolation by Reversed-Phase HPLC. Following 1-, 7-, or 21-day incubations, the media were centrifuged to remove cellular debris. For preparation of milligram quantities of secreted hormones, fresh media were pressure-filtered with Amicon YM10 membranes (M_r cutoff, 10,000) and rinsed twice with water in order to concentrate the samples and to remove the majority of the KRb/MEM constituents. The media were clarified by centrifugation (1000 × *g*, 15 min) and pumped directly onto a 10-μm octylsilyl silica column (25 × 0.46 cm, 300-Å pore diameter, Brownlee RP-300, Santa Clara, CA) with a Milton-Roy miniPump in lieu of an injector. Absorbed proteins were eluted with a 60-min linear gradient of 40–60% acetonitrile in water (both containing 0.1% trifluoroacetic acid) (15) at a flow rate of 1 ml/min; UV absorption was monitored at 214 or 280 nm (Waters and Altex-Beckman chromatographs and detectors). In smaller-scale analytical runs, clarified medium aliquots were injected directly onto the column.

Structural Characterization. Molecular masses were estimated by $NaDodSO_4$/polyacrylamide gel electrophoresis (PAGE) (16). The proteins were boiled for 2 min in solubilization buffer (0.062 M Tris·HCl, pH 6.8/2% $NaDodSO_4$/10% glycerol/5% 2-mercaptoethanol). Further details appear in the legend of Fig. 2*A*.

Isoelectric points were initially estimated in horizontal capillary thin 5% polyacrylamide gels by using Bio-Rad ampholytes with a broad pH range (Bio-Lyte 3/10). Each protein was then electrofocused by using ampholytes with a narrower pH range. Details are given in the legend of Fig. 2*B*.

Partial sequences of the 24-kDa and 20-kDa PRLs were obtained by using a gas-phase sequencer (Applied Biosystems model 470A) and the phenylthiohydantoin amino acid

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Abbreviations: PRL, prolactin; RPD, rostral pars distalis; PPD, proximal pars distalis; GH, growth hormone.

residues were identified by reversed-phase HPLC (Spectra Physics, Santa Clara, CA).

Determination of Release Rates of the PRLs: [^{3}H]Leucine Pulse–Chase Analysis. Rostral lobes were obtained from adult male tilapia and incubated at 27°C in KRb without MEM (pH 7.4, 10–16 RPD in 1.2 ml of medium) containing 2.4 nmol of [4,5-^{3}H]leucine (52.5 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) for 1 hr, rinsed three times, and then chased for 0.5, 1, 4, 8, and 24 hr with hyposmotic KRb/MEM containing 400 μmol of unlabeled leucine. Aliquots of medium from each interval were analyzed directly by reversed-phase HPLC as before. The column effluent was monitored first with an UV detector (at 214 or 280 nm) and then admixed with scintillation fluid and monitored for ^{3}H with a flow-through scintillation counter (Nuclear Enterprises Isoflo with ISO-2 data system). The UV/^{3}H peak lag was 2.0 min.

Bioassay for PRL Activity. A bioassay for PRL using hypophysectomized tilapia was developed based on the observation that PRL prevents the decrease in plasma [Na^+] that occurs in many species of hypophysectomized teleosts in fresh water (17) and was derived more specifically from previous assays using hypophysectomized tilapia (18) and *Fundulus* (19). Juvenile tilapia (8–12 g) were hypophysectomized via an orbital approach (20) and then held for 4 or 5 days in one-third strength (11 parts per thousand) seawater. Each fish was injected intraperitoneally with 5 μl of 0.7% saline without or with 10^{-14}, 10^{-13}, 10^{-12}, or 5×10^{-12} mol of hormone per g of body weight (approximately 21 ng to 12 μg per fish) while lightly anesthetized (0.03% 2-phenoxyethanol) at 20 hr and again at 20 min before being transferred to fresh water. Blood was collected 18 hr later from the severed caudal vasculature and the plasma was analyzed immediately to ascertain [Na^+] by atomic absorption spectrophotometry (Perkin-Elmer). The coefficient of variation of this method was 1.9% (n = 10). The slopes and elevations of the dose–response lines were compared by using analysis of covariance (SAS General linear model program).

RESULTS

Isolation by Reversed-Phase HPLC. The isolation of two major hydrophobic proteins from medium in which RPDs were cultured for 24 hr, using a single chromatographic step, is shown in Fig. 1*A*. The possibility that either protein was GH was excluded by the concurrent culture of the PPD and purification of a single and chemically distinct GH by the same methods, as shown in Fig. 1*B*. The purity of each preparation is demonstrated in the second elution profiles in Fig. 1*C*. Purity was judged to be >95% on the basis of rechromatography by using a shallower gradient with the same chromatographic system, by eluting the proteins with two different solvent systems, and by the appearance of the band when 10 μg rather than 2 μg was loaded on polyacrylamide gels (lanes 6–8, Fig. 2*A*). Recovery from the column was judged to be >95% for all three proteins on the basis of repeated chromatography (not shown).

Structural Characterization. NaDodSO$_4$/PAGE analysis of the proteins isolated by reversed-phase HPLC indicated that the more polar PRL ran as a protein of ≈24 kDa and the less polar PRL ran as a protein of ≈20 kDa. GH appeared as a protein of ≈21 kDa. These three proteins ran as single bands (lanes 1–3), even with as much as 10 μg of each on a single lane (lanes 6–8). It was possible to resolve the proteins by using NaDodSO$_4$/PAGE (lane 4, Fig. 2*A*).

The isoelectric point (pI) of the larger PRL (8.7) was found to be uncharacteristically basic (Fig. 2*B*). The pI of the smaller PRL was 6.7, and the pI of GH was 5.9. Previous isoelectric focusing using Bio-Lyte 3/10 indicated pI values of 8.3, 7.5, and 6.2, respectively (21).

Microsequencing permitted sequence determination of residues 1–29 and 1–28 of the 20-kDa and 24-kDa PRLs,

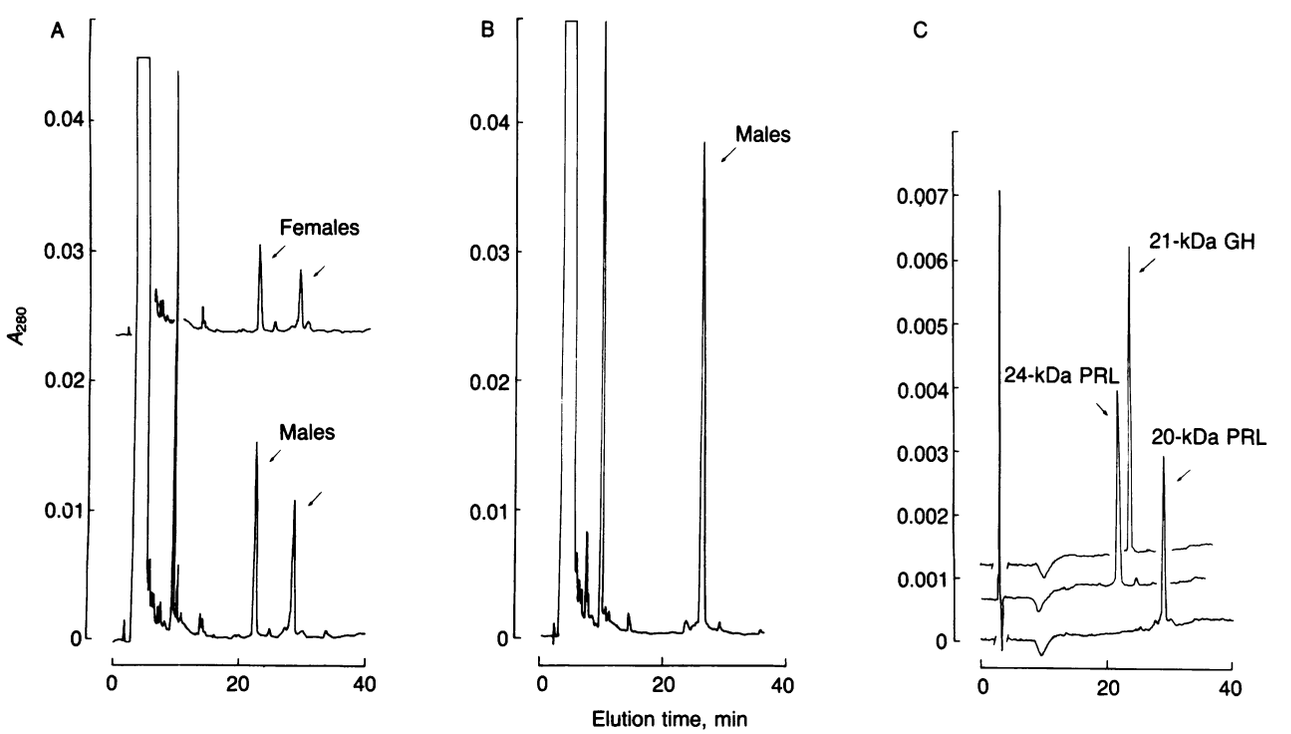

FIG. 1. (*A*) HPLC separation of a pair of PRLs (arrows) from culture medium following a 24-hr incubation of RPDs from three female tilapia (upper tracing) or from three male tilapia (lower tracing). Both sexes of adult tilapia release the PRL pair in approximately the same proportion. (*B*) Separation of GH from medium following a 24-hr culture of PPDs from three adult male tilapia, using the same chromatographic conditions as described for the PRLs. (*C*) Demonstration of purity of preparations by rechromatography of 1 μg of each tilapia hormone isolated (as in *A* and *B*), lyophilized, and weighed.

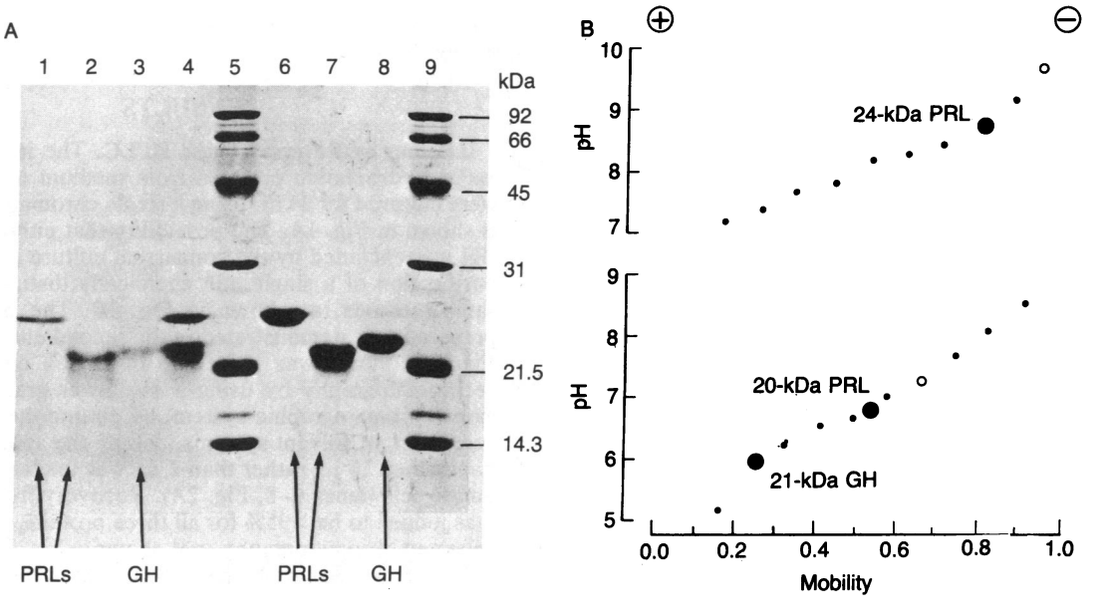

FIG. 2. (*A*) NaDodSO$_4$/PAGE analysis of molecular mass and purity of tilapia hormones isolated by reversed-phase HPLC. The proteins were stacked at constant voltage (50 V) in a 4% acrylamide gel (30 mm, pH 6.8) and run at constant current (20 mA) in a 15% acrylamide gel (90 mm × 1.5 mm, pH 8.8), stained with Coomassie brilliant blue in 45% methanol/10% acetic acid, and destained in 10% methanol/7% acetic acid. Apparent molecular mass was estimated by migration distance relative to standards in lanes 5 and 9 (phosphorylase b, 92 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21 kDa; lysozyme, 14 kDa). Lanes 1 and 6, 1 μg and 10 μg of the first PRL to elute from the column; lanes 2 and 7, 1 μg and 10 μg of the second PRL; lanes 3 and 8, 1 μg and 10 μg of GH; lane 4, 2 μg of each tilapia hormone. (*B*) Isoelectric focusing in horizontal capillary thin 5% polyacrylamide gels. The pH of every 10-mm gel slice was measured in distilled water. Top: position of 24-kDa PRL focused in 1:1 Bio-Lytes 8/10 and 6/8 (Bio-Rad). Open circle is standard, RNase, pI 9.5. Bottom: positions of 20-kDa PRL and 21-kDa GH focused in Bio-Lyte 6/8. Open circle is standard, horse myoglobin, pI 7.4. The proteins were stained with 0.05% crocein scarlet/0.04% Coomassie brilliant blue R250 in 27% ethanol/10% acetic acid/0.5% CuSO$_4$ and destained in 0.05% CuSO$_4$/12% ethanol/7% acetic acid.

respectively (Fig. 3). Comparative aspects of these partial structures are discussed below.

***In Vitro* Release Rates of the Pair of PRLs.** The pulse–chase experiments consistently demonstrated that the two PRLs were synthesized and released at similar rates (Fig. 4). In addition, radiochromatography detected no other proteins containing leucine in the culture medium. The specific activity of the PRLs released into the medium during each interval of a 24-hr culture period (Fig. 4*C*) suggests a short delay (<1.5 hr) between synthesis and release *in vitro*.

Biological Activity. Both proteins isolated from the RPD medium, but not the protein from the PPD medium, exerted Na^+-retaining activity in hypophysectomized tilapia in a dose-dependent manner (Fig. 5). The slopes and elevations of the dose–response curves for the 24-kDa and 20-kDa PRLs were not different in analysis of covariance.

DISCUSSION

Unlike mammals, which produce a single PRL with only trace amounts of PRL variants (29, 30), the tilapia produces two PRLs. The newly discovered tilapia PRL described here

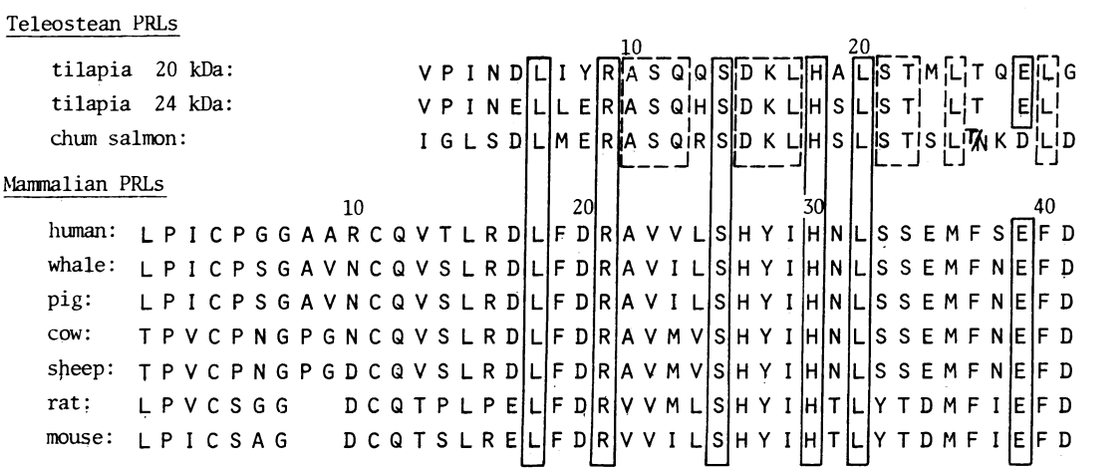

FIG. 3. Alignment of the partial amino acid sequences (represented by standard single-letter abbreviations) of the 20-kDa and the 24-kDa PRLs from tilapia with the corresponding NH_2 termini of PRLs from chum salmon (22), human (23), whale (24), pig (25), cow (26), sheep (25), rat (27), and mouse (28). Identical residues among the teleost PRLs are boxed with a broken line. Identical residues in teleost and mammal PRLs are boxed with a heavy line.

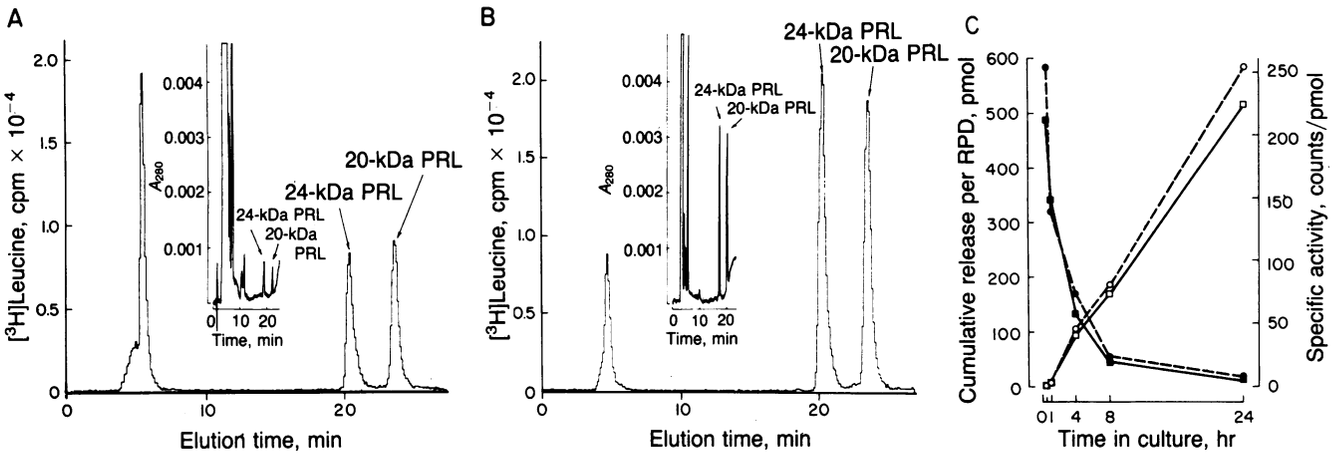

FIG. 4. Radiochromatographic analysis of secretory rates for the PRL pair in a [^{3}H]leucine pulse–chase experiment. (*A*) Reversed-phase HPLC of the medium collected after incubation of 13 PRDs after the 0.5- to 1-hr chase interval, indicating the elution of the proteins (*Inset*: absorbance at 280 nm) and elution of [^{3}H]leucine (5 min), the [^{3}H]leucine-labeled 24-kDa PRL (20 min), and the [^{3}H]leucine-labeled 20-kDa PRL (23 min). (*B*) Reversed-phase HPLC of the medium collected after incubation of 3 RPDs after the 4- to 8-hr chase interval. (*C*) Equal and constant release of both PRLs (○, 24-kDa PRL; □, 20-kDa PRL) during the 24-hr chase. Steady decline in specific activity (●, 24-kDa PRL; ■, 20-kDa PRL) of both PRLs in the medium indicates an interval of <1.5 hr between synthesis and release in these culture conditions.

(24 kDa) is 4 kDa larger than the previously described tilapia PRL (7, 8), and is atypically basic (pI 8.7) compared to other PRLs. Application of reversed-phase HPLC permits the isolation in a single step of both PRLs or GH from media in which pituitary regions containing PRL- or GH-secreting cells have been incubated. By this method, large quantities (milligram) of proteins in this family of hormones can be isolated from culture medium to >95% purity and with >95% recovery in <30 min. The elution profiles (not shown) of PRLs isolated directly from the RPD and of GH isolated directly from the PPD were identical with the profiles of the secreted hormones, suggesting that these proteins are not altered during release. The somewhat harsh conditions of the isolation procedure do not destroy the biological activity of the hormones as measured *in vivo* (see below).

We have no evidence for the independent regulation of secretion of the two PRLs. Instead, two peptides (somatostatin and urotensin II) appear to inhibit the *in vitro* release of both PRLs similarly (31). Pulse–chase experiments designed to detect a prohormone–hormone relation between the 24-kDa and 20-kDa PRLs revealed instead remarkably high (21 pmol per gland per hr) and essentially identical synthesis and release rates for the two PRLs. Furthermore, no other secretory proteins of the rostral lobe were detected by radiochromatography.

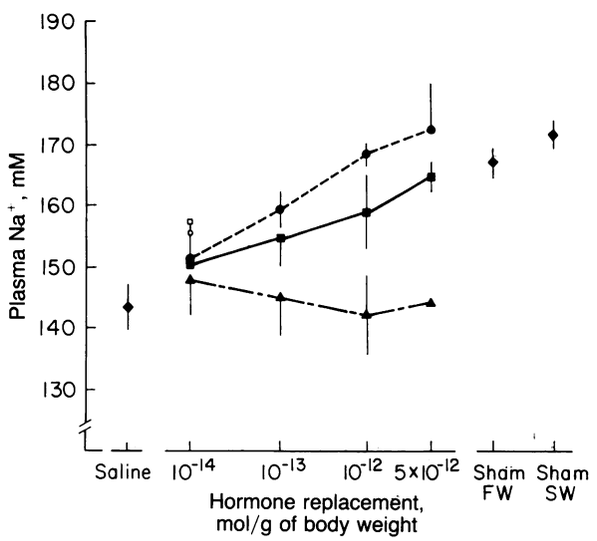

FIG. 5. Prevention of Na^+ loss from the plasma of hypophysectomized juvenile tilapia in fresh water by the 24-kDa PRL (●) and the 20-kDa PRL (■) but not by the 21-kDa GH (▲). Both PRLs fully restore plasma Na^+ concentration from that found in saline-injected, hypophysectomized tilapia (Saline) to that found in sham-hypophysectomized tilapia in fresh water (Sham FW) or in sham-hypophysectomized tilapia acclimated to one-third strength seawater (Sham SW). n = 10 for each point except 5 × 10^{-12} mol/g, where n = 3.

Partial sequence information confirms that the two PRLs are related but distinct molecular entities. The sequences of the first 28 or 29 amino acid residues of the two tilapia PRLs appear in Fig. 3 along with the comparable sequences of some other known PRLs. These partial sequence data indicate that the two tilapia proteins are highly homologous (81%, 21/26) in the NH_2-terminal region but are distinct proteins closely related (58%, 15/26) to the other partially sequenced fish PRL, chum salmon PRL (22). The site of N-glycosylation (corresponding to Asn^{31}-Leu-Ser, ref. 32) that is present in ovine, human, porcine, and whale PRLs was absent from both tilapia PRLs. All three known teleost PRLs lack the first 12 amino acid residues of mammalian PRLs. By aligning residue 1 of the tilapia PRLs with residue 13 of the mammalian PRLs, it becomes clear that the NH_2 termini of the tilapia PRLs are 23% homologous (6/26) to mammalian PRLs. If one considers homology less stringently by allowing single base substitutions in the presumed coding sequences, the homology within the sequenced regions of the two tilapia PRLs is 96% (25/26) and between the tilapia and mammalian PRLs is 62% (16/26). The proteins secreted by the tilapia RPD can thus be considered PRLs on structural grounds and belong to the protein family that includes proliferin (33), the GHs, and the placental lactogens.

Structural similarity throughout the remainder of the PRL molecule is indicated by immunological and biological criteria. Binding curves of polyclonal antiserum generated against the 20-kDa PRL, purified from frozen pituitaries by DEAE-cellulose chromatography (8), are the same for both tilapia PRLs purified by reversed-phase HPLC (21). In our bioassay, the 20-kDa PRL and 24-kDa PRL both prevent the decrease in plasma [Na^+] that occurred in hypophysectomized tilapia in fresh water. Both hormones exert an effect at a dose as low as 10^{-14} mol of PRL per g of body weight (21–24 ng of PRL per g of body weight) and are completely effective at 10^{-12} mol of PRL per g of body weight (210–240 ng of PRL per g of body weight). Thus, the tilapia PRLs are 200 times more potent in the tilapia than ovine PRL (18), underlining

the importance of species specificity among these protein hormones. Similarly, vertebrate class specificity may also exist in that salmon PRL has been shown to be more potent than mammalian PRL in a similar bioassay using another teleost, the killifish (19).

The significance of a pair of RPLs, with similar but not identical amino acid sequences, overlapping biological activity, similar rates of synthesis and release, and apparently similar regulation, remains to be elucidated. The predominance in the tilapia RPD of two classes of mRNA that translate into proteins of approximately 22 and 25 kDa (21) suggests that the two tilapia PRLs are distinct at the pretranslational, and possibly the genetic, level. Preliminary data indicate a teleost growth-promoting role for only the larger of the two tilapia PRLs (34). The similarity in release rates and response to release inhibitors argues against our earlier hypothesis that distinct cell populations are responsible for producing each of the PRL pair (34). Further biological testing is needed to determine whether each of these two PRLs serves specific functions in the tilapia and whether different physiological demands (e.g., reproduction) result in preferential production of one PRL over the other.

Note Added in Proof. H. Kawauchi and colleagues (personal communication) have isolated and sequenced two PRLs (as well as two growth hormones) from the pituitary of the chum salmon. The PRLs, present in approximately equal amounts, differ only slightly from each other, and the duplication of these pituitary hormones may reflect the tetraploid condition of the salmonid species.

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