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Immunoreactive prolactins of the neurohypophyseal system display actions characteristic of prolactin and 16K prolactin

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We have described the expression of the prolactin (PRL) gene and the occurrence of PRL-like immunoreactive proteins in the hypothalamic-neurohypophyseal system of the rat. Here, we investigated the nature of neurohypophyseal PRL-like antigens, by studying the biological activity of medium conditioned by incubated neurohypophyses in specific bioassays for PRL and for the 16 kDa N-terminal fragment of PRL (16K PRL). Neurohypophyseal conditioned medium (NHCM) obtained after incubating neurohypophyseal lobes (1 h at 37°C) was enriched with proteins of 14 kDa and 23 kDa, that crossreacted with PRL- and 16K PRL-directed antisera. The NHCM stimulated in a dose-dependent fashion the proliferation of Nb2-lymphoma PRL-dependent cells. This effect paralleled that of PRL and 16K PRL standards and was neutralized by different dilutions of both PRL- and 16K PRL-antisera. Also, the NHCM inhibited the proliferation of endothelial cells in culture, an antiangiogenic-effect exerted by 16K PRL. The antiangiogenic effect of the NHCM was parallel to that of 16K PRL standard and neutralized by 16K PRL antiserum in a dose-dependent fashion. These results indicate that NHCM contains proteins that share receptor activation properties as well as antigenic determinants with both PRL and 16K PRL.

Keywords: brain prolactin; prolactin isoforms; neurohypophysis; angiogenesis; Nb2 cells

Introduction

Prolactin (PRL) is a polypeptide hormone that exhibits a wide spectrum of molecular polymorphism. The major variant form of PRL, constituted by a single polypeptide chain with a molecular mass of 23 kDa (23K PRL), is known to be subjected to post-translational modifications that include glycosilation, phosphorylation and specific proteolytic processing (Sinha, 1992). Posttranslational events have been proposed as mechanisms for generating diversity in the biological actions of PRL (Sinha, 1992). Accordingly, it has been shown that the proteolytic cleavage and subsequent reduction of 23K PRL generates molecular variants with specific functions. Cleaved PRL, a variant with a nick at Tyr 145, specifically enhances proliferation of gonadotropes and thyrotropes, an effect not caused by 23K PRL (Andries *et al.*, 1992). Moreover the 16 kDa N-terminal fragment of PRL (16K PRL) inhibits the proliferation of endothelial cells as well as other events of angiogenesis, i.e., the formation of new capillary blood vessels (Ferrara *et al.*, 1991; Clapp *et al.*, 1993). 23K PRL does not affect such events of the angiogenic process (Ferrara *et al.*, 1991; Clapp *et al.*, 1993).

Although the anterior pituitary gland is known to be the major site for the synthesis and secretion of the PRL family of hormones, there are other known sources, including the brain (Dutt *et al.*, 1994). It has been recognized that the PRL gene is expressed in the central nervous system, particularly in the hypothalamus, although the PRL transcript is present in very low abundance (Wilson *et al.*, 1992; Dutt *et al.*, 1994). The molecular heterogeneity of brain PRL is substantiated by described differences in the molecule's epitopes (Harlan & Scammell, 1991), size (Emanuele *et al.*, 1986, 1987; DeVito, 1988) and bioactivity (Emanuele *et al.*, 1986) with respect to anterior pituitary PRL. Moreover, both a PRL mRNA with identical sequence to that of anterior pituitary PRL (Wilson *et al.*, 1992), as well as an alternatively spliced PRL mRNA have been identified in brain tissue (Emanuele *et al.*, 1992).

In a recent study, with the use of the reverse transcription-polymerase chain reaction we found the full size PRL mRNA in the hypothalamic-neurohypophyseal system of the rat (Clapp *et al.*, 1994). Consistent with this result, PRL-immunoreactive proteins with a predominant molecular mass of 14 and 23 kDa were detected in neurohypophyseal extracts and found to be secreted by neurohypophyses in culture (Clapp *et al.*, 1994; Torner *et al.*, 1994). Moreover the medium conditioned by incubated neurohypophyses inhibited the proliferation of endothelial cells in culture (Clapp *et al.*, 1994). Thus we have hypothesized that PRL gene is expressed in the hypothalamic-neurohypophyseal system and that the protein is modified, through tissue-specific mechanisms, to yield variant forms with specific functions.

In this paper, we have tested the above hypothesis by investigating the nature of the neurohypophyseal PRL-immunoreactive proteins through the characterization of their biological activities in cell proliferation assays specific for 16K PRL (endothelial cells in culture) and 23K PRL (Nb2 lymphoma cells).

Results

Western blot detection of PRL-like antigens in medium conditioned by incubated neurohypophyses

Crossreactivity of proteins in medium conditioned by incubated neurohypophyses with PRL antisera was examined by Western blot analysis. As previously described (Clapp *et al.*, 1994), Western blots probed with the 16K PRL antiserum showed that the predominant immunoreactive protein present in the neurohypophyseal conditioned medium (NHCM) has an apparent molecular mass of 14 kDa (Figure 1). A 23 kDa immunoreactive protein was also consistently observed. These two immunoreactive proteins present in NHCM were also detected by an antiserum raised against 23K PRL (Figure 1A). Both the 14 kDa and the 23 kDa proteins were detected after reducing or not the samples with 5% β-mercaptoethanol (Figure 1B). In Western blots probed with pre-immune serum, no bands of 14 kDa or 23 kDa were detected (Figure 1A).

Effect of medium conditioned by incubated neurohypophyses on proliferation of Nb2 cells

The biological activity of NHCM was tested on the proliferation of Nb2 cells in culture and compared to the activities of 23K PRL and 16K PRL standards (Figure 2). Both PRL standards stimulated the proliferation of Nb2 cells in a dose-

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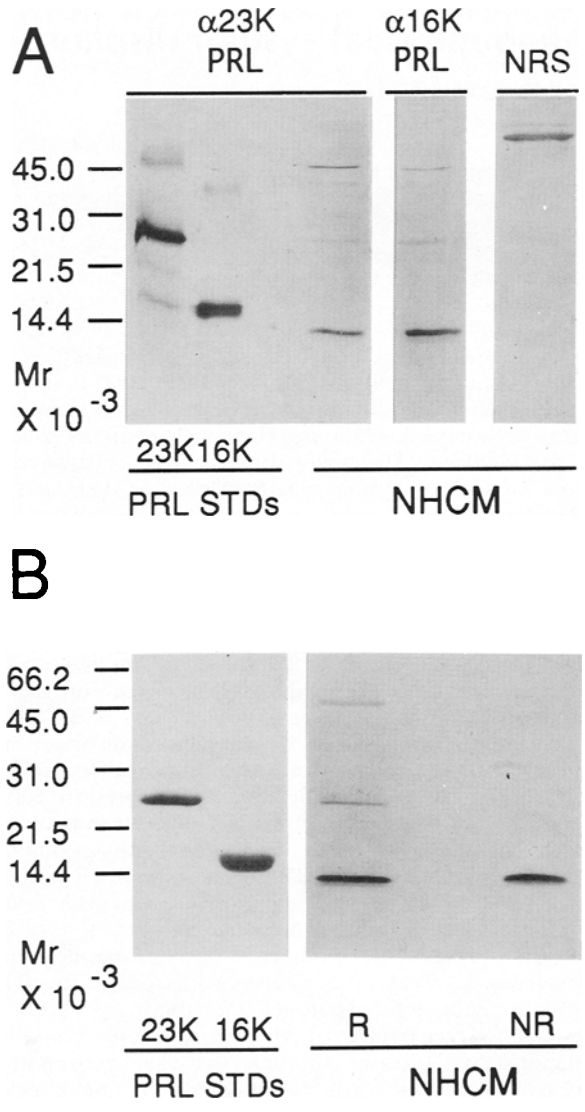

Figure 1 Western blot identification of PRL-like antigens in the medium conditioned by incubated neurohypophyses. (**A**) Blots from neurohypophyseal conditioned medium (NHCM, neurohypophyses were incubated 1 h in Krebs Ringer at 37°C), were probed with a 1:500 dilution of the 23K PRL antiserum (α23K PRL), 16K PRL antiserum (α16K PRL) or normal rabbit serum (NRS). (**B**) Blots from NHCM were probed with the antiserum to 16K PRL (1:500) under non-reducing (NR) or reducing (R) conditions. The migration rate (M_r) of molecular weight markers are indicated at left.

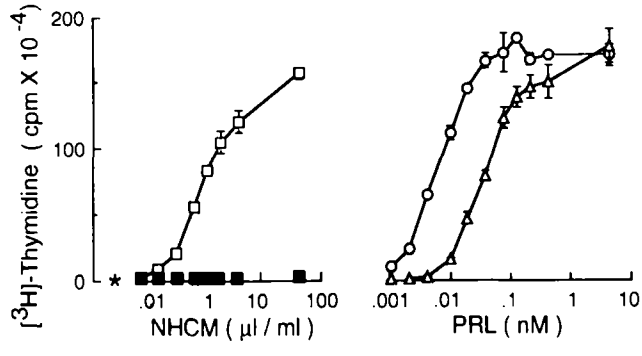

Figure 2 Stimulation by neurohypophyseal conditioned medium of Nb2 cell proliferation. [^{3}H] thymidine incorporation into Nb2 cells was measured in response to increasing concentrations of neurohypophyseal conditioned medium (NHCM) (□), and compared to the effects of increasing concentrations of 23K PRL (○), 16K PRL (△), or preconditioned medium (■). The basal incorporation of [^{3}H] thymidine is shown (*). Starting at the 0.02 µl/ml concentration, the NHCM values were significantly higher ($P<0.05$) than basal values.

dependent fashion. The proliferative effect induced by 16K PRL showed a lower potency than the one induced by 23K PRL, i.e., 16K PRL had a one-half maximal response concentration (EC_{50}) seven times greater than that of 23K PRL or 45 pM *vs* 6 pM, respectively. Increasing concentrations of the NHCM stimulated the proliferation of Nb2 cells in a manner parallel to that of both PRL standards, while preconditioned medium had no effect. The concentration of the NHCM to give a half-maximal proliferative effect was 1 µl/ml. Western blotting and densitometric analysis of the NHCM, calibrated against different concentrations of 23K PRL standard, were used to estimate the concentration of the 23 kDa and the 14 kDa immunoreactive proteins in the medium as 1.2 and 7.2 ng/µl, respectively. Thus, the activity of the NHCM to stimulate the proliferation of Nb2 cells, expressed as a function of the estimated concentrations of either the 23 kDa or the 14 kDa immunoreactive proteins gave EC_{50} values of 0.05 and 0.45 nM, respectively.

Inhibition of the Nb2 cell proliferative effect of neurohypophyseal conditioned medium by 23K PRL and 16K PRL antisera

The ability of antisera raised against 16K PRL and 23K PRL to inhibit the mitogenic activity of NHCM was tested (Figure 3). Nb2 cells were incubated with NHCM (1 µl/ml) to obtain a half-maximal proliferative effect, in the presence or absence of either PRL antisera. Serial concentrations of 23K PRL or of 16K PRL antisera inhibited in a dose-dependent fashion the stimulation of Nb2 cell proliferation induced by NHCM or by 23K PRL standard. Pre-immune serum had no effect.

Effect of neurohypophyseal conditioned medium on bFGF-stimulated proliferation of bovine brain capillary endothelial (BBCE) cells

Increasing concentrations of NHCM were tested on the proliferation of BBCE cells stimulated by bFGF and their effects

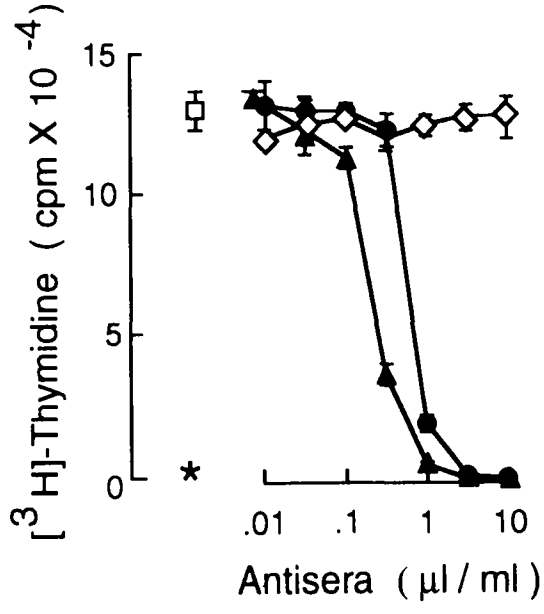

Figure 3 Blockage by PRL antisera of the stimulation of Nb2 cell proliferation induced by neurohypophyseal conditioned medium. [^{3}H] thymidine incorporation into Nb2 cells was measured in response to neurohypophyseal conditioned medium (NHCM, at the EC_{50} value of 1 µl/ml) in combination with increasing concentrations of 23K PRL antiserum (●), 16K PRL antiserum (▲) or normal rabbit serum (◇). The basal incorporation of [^{3}H] thymidine (*), and that induced by NHCM alone (□) are shown. The values for the anti-16K PRL and the anti-23K PRL antisera co-culture, starting at the 0.5 and 1.0 µl/ml antiserum concentrations, respectively, were significantly ($P<0.05$) lower than that of NHCM alone.

were compared to those of different concentrations of carbamidomethylated 16K PRL standard (CAM-16K PRL) (Figure 4). CAM-16K PRL was used for this purpose, since blockage of disulfide bond formation by carbamidomethylation enhances the potency of 16K PRL to inhibit endothelial cell proliferation (Ferrara *et al.*, 1991). Administration of NHCM inhibited the bFGF-stimulated proliferation of BBCE cells in a dose-dependent fashion that paralleled the inhibitory effect of CAM-16K PRL. The activity of NHCM to inhibit BBCE cells proliferation expressed as a function of the estimated concentration of the 14 kDa immunoreactive protein (7.2 ng/μl) gave an EC_{50} value of 5.4 nM, close to the EC_{50} value of 13 nM observed for CAM-16K PRL.

Blockage of the effect of neurohypophyseal conditioned medium on endothelial cell proliferation by 16K PRL antiserum

We next examined whether increasing concentrations of PRL-directed antisera were capable of neutralizing the inhibitory effect of NHCM on the bFGF stimulated-BBCE cell proliferation. BBCE cells were incubated with NHCM (12 μl/ml) to give a half maximal inhibition of cell proliferation, in the presence or absence of either PRL antisera. Increasing concentrations of 16K PRL antiserum blocked, in a dose-dependent manner, the inhibition by NHCM of bFGF-stimulated BBCE cell proliferation (Figure 5). Conversely, 23K PRL antiserum or pre-immune serum at dilutions of up to 1:100 had no significant effects on the inhibitory activity of NHCM.

Discussion

Our results demonstrate that PRL-like immunoreactive proteins found in the medium conditioned by incubated neurohypophyses share bioactivities with both PRL and 16K PRL. Proliferation of lymphoma Nb2 PRL-dependent cells was stimulated by NHCM, and this effect was neutralized by a 23K PRL-directed antiserum. Proliferation of endothelial cells was also inhibited by NHCM, and this effect was neutralized by a 16K PRL antiserum. These results indicate that biologically active PRLs are secreted by the hypothalamic-neurohypophyseal system, including a predominant 14 kDa PRL-like fragment with antiangiogenic effects.

In a recent study we described the presence of immunoreactive PRLs in the hypothalamic-neurohypophyseal system. With the use of an antiserum directed against the N-terminal 16 kDa fragment of PRL, we found that the hypothalamic-neurohypophyseal system contained two PRL-like antigens, i.e., a predominant 14 kDa protein and small amounts of a 23 kDa protein (Clapp *et al.*, 1994). The PRL nature of both antigens and their local production was substantiated by the amplification, via the polymerase chain reaction, of the full size PRL mRNA in isolated hypothalamic paraventricular nuclei (Clapp *et al.*, 1994). These results were consistent with the expression of PRL in the hypothalamic-neurohypophyseal system and with its posttranslational modification into a lower molecular weight fragment. The precise nature of the neurohypophyseal immunoreactive proteins requires further analysis.

In the present study we have confirmed that the neurohypophyses secrete 14 kDa and 23 kDa proteins that react with the 16K PRL antiserum, and showed that these two proteins also crossreact with an antiserum directed against 23K PRL. Thus it is concluded that both immunoreactive proteins share antigenic determinants with the 16K and the 23K PRL molecules.

We further investigated the identity of the neurohypophyseal PRL-like immunoreactive proteins, by analysing biological activities of the NHCM. In support to the PRL-like nature of the neurohypophyseal immunoreactive proteins, the NHCM stimulated in a dose-dependent fashion the proliferation of Nb2 lymphoma cells. The effect was parallel

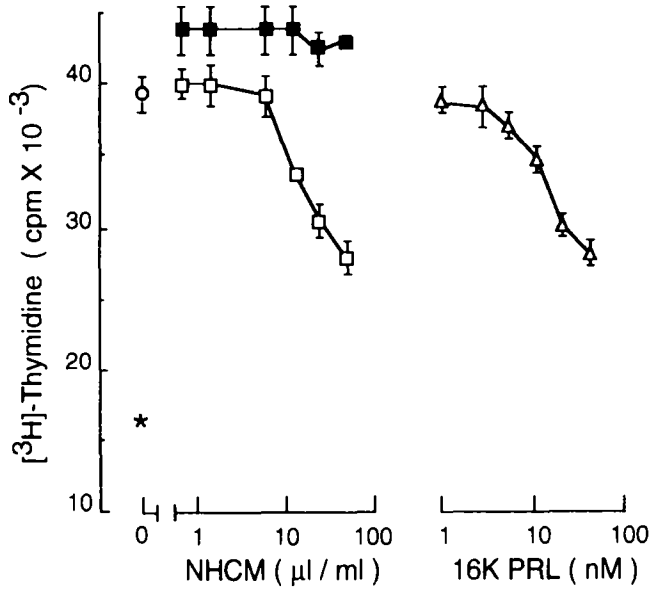

Figure 4 Inhibition by neurohypophyseal conditioned medium of bFGF-stimulated proliferation of endothelial cells. [^{3}H] thymidine incorporation into BBCE cells was measured in response to bFGF (50 pM) alone (○) or in combination with increasing concentrations of neurohypophyseal conditioned medium (NHCM) (□), preconditioned medium (■), or 16K PRL (△). The basal incorporation of [^{3}H] thymidine (*) is shown. Starting at the 12.5 μl/ml concentration, both the NHCM and the 16K PRL values were significantly lower ($P<0.05$) than that of bFGF alone.

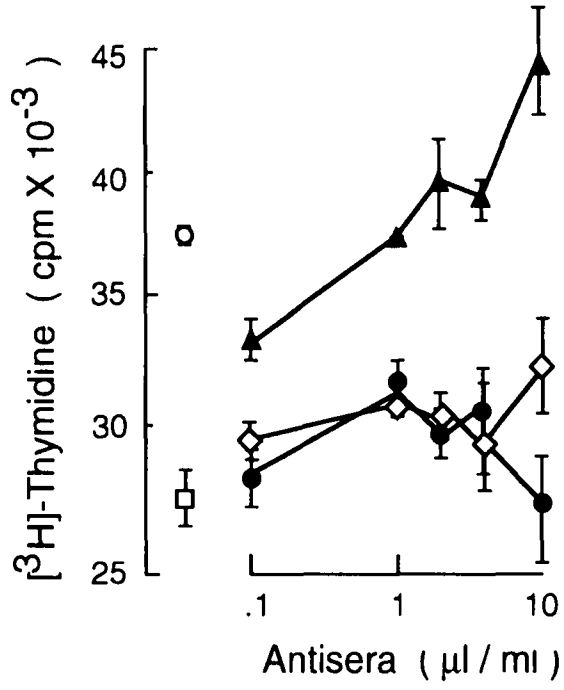

Figure 5 Blockage by 16K PRL antiserum of the inhibition by neurohypophyseal conditioned medium of bFGF-stimulated proliferation of endothelial cells. [^{3}H] thymidine incorporation into BBCE cells was measured in response to bFGF (50 pM) alone (○) or in combination with neurohypophyseal conditioned medium (NHCM, at the EC_{50} value of 10 μl/ml) in the absence (□) or presence of increasing concentrations of 16K PRL antiserum (▲), 23K PRL antiserum (●) or normal rabbit serum (◇). All values for the anti-16K PRL co-culture were significantly ($P<0.05$) higher than the NHCM (□) control. Values of 23K PRL antiserum- or normal rabbit serum- co-cultures were not significant from the NHCM control value.

to that elicited by 23K and 16K PRL standards, and was blocked by both 23K and 16K PRL-directed antisera. Accordingly, in addition to antigenic determinants, the proteins of the NHCM share PRL-receptor activation properties with both 23K and 16K PRL.

The Nb2 cell proliferation activity of the NHCM could be due to the combined action of both the 14 kDa and the 23 kDa PRL antigens, or to the independent effect of either one of them. The bioactivity of the NHCM expressed in terms of the sum of the estimated concentrations of both antigens gave an EC_{50} value 10- to 80-fold higher than the values obtained with 16K PRL and 23K PRL standards respectively. The apparently low biological potency of both PRL antigens could be explained in one of several ways. It is possible that the concentration of both antigens was overestimated, perhaps due to a high reactivity of these proteins with the PRL antisera. Alternatively, both neurohypophyseal PRLs could have a low biological activity. This possibility is consistent with previous determinations of PRL-like bioactivity from hypothalamic extracts in which a low PRL bioassay to immunoassay ratio was demonstrated (Emanuele *et al.*, 1986, 1987). A low biological potency can be expected for PRL fragments. The EC_{50} value observed for 16K PRL (45 pM) was 7.5 times higher than that of 23K PRL (6 pM). An additional possibility would be that the 14 kDa antigen is acting as an antagonist of the PRL receptor. According to this possibility the 23 kDa antigen would be expected to have a reduced effect in the presence of the 14 kDa immunoreactive protein. Accordingly, the bioactivity of the conditioned medium expressed solely in terms of the 23 kDa antigen showed an EC_{50} value (0.05 nM) 8-fold higher than the respective value of 23K PRL standard (0.006 nM).

Possibilities like the ones raised above have been considered for the understanding of PRL signaling mechanisms. PRL fragments are currently being conceived as partial agonists and/or antagonists of the PRL receptor (Fuh *et al.*, 1993). It is now well accepted that PRL activates the PRL receptor by a sequential dimerization process that yields an active PRL-$(receptor)_2$ complex (Cunningham *et al.*, 1991; Fuh *et al.*, 1993; Hooper *et al.*, 1993; Rui *et al.*, 1994). This mode of activation implies that there are two receptor binding sites on the PRL molecule and that PRL binds through the first site (Site 1) to a receptor and then through the second site (Site 2) to a second receptor. PRL analogs with mutations at Site 1 or Site 2 act as partial agonists or antagonists of the receptor, respectively. For instance mutants of hGH which bind tightly with Site 1 but not with Site 2 are potent antagonist of the PRL receptor and virtually inactive on Nb2 lymphoma cells (Fuh *et al.*, 1993). On the other hand a hGH mutant with Site 2 intact, but with a compromised Site 1, acted as a partial agonist on Nb2 cells, with an EC_{50} value 12 times higher than that of native hGH (Cunningham & Wells, 1991; Fuh *et al.*, 1993). In a similar way, 16K PRL could be acting as a partial agonist for the PRL receptor because the C-terminal end of the molecule is at Tyr 145 (Baldocchi *et al.*, 1993) and thus a group of amino acid residues (167–191), presumed to be part of Site 1 (Cunningham & Wells, 1989), have been lost. Along the same line a role for the neurohypophyseal 14 kDa PRL as a partial agonist and/or antagonist at the PRL receptor level are open possibilities that need to be addressed following the purification and sequencing of the protein.

The functional significance of the PRL variants found in the hypothalamic-neurohypophyseal system may lie on their ability to subserve different functions. For instance, 16K PRL while being a weak agonist of the PRL receptor, is a potent inhibitor of angiogenesis (Clapp *et al.*, 1988, 1993; Ferrara *et al.*, 1991), an effect not shared with 23K PRL. We previously showed that the PRL-like molecules present in the hypothalamic-neurohypophyseal system exert an antiangiogenic-effect similar to that attributed to 16K PRL (Clapp *et al.*, 1994). Here we show that inhibition of endothelial cell proliferation by the NHCM occurs in a dose-dependent fashion that parallels the dose-related antiangiogenic effect of 16K PRL standard. In addition, serial dilutions of the 16K PRL antiserum neutralize, in a dose dependent manner, the antiangiogenic-effect of the NHCM. In support to the 14 kDa protein as the likely antiangiogenic-molecule, we found that the activity of the NHCM expressed in terms of the estimated concentration of the 14 kDa PRL antigen, showed an EC_{50} value close to that obtained with 16K PRL standard.

The 23K PRL antiserum, even at a 1:100 dilution, did not neutralize the antiangiogenic effect of the NHCM. The selectivity of the 23K PRL antiserum to neutralize the NHCM effect on Nb2 cells, but not on endothelial cells, could be explained in one of several ways. It is possible that this antiserum recognizes epitopes in neurohypophyseal PRLs that are part of the binding region to the PRL receptor but not to the 16K PRL receptor, and thus block the activation of the former but not of the latter. High affinity, specific binding sites for 16K PRL are present on endothelial cell membranes and have been proposed as the receptors mediating the antiangiogenic effect of 16K PRL (Clapp & Weiner, 1992). These 16K PRL receptors differ structurally from the PRL receptor and do not bind 23K PRL (Clapp & Weiner, 1992). Thus it is likely that epitopes associated to the 16K PRL receptor-binding region are not present in 23K PRL and thus not recognized by the 23K PRL antiserum. Alternatively, the 23K PRL antiserum may bind to epitopes distinct to either receptor-binding region. Once bound, the antibodies could alter the molecular conformation required for binding to the PRL receptor but not the one required for binding to the 16K PRL receptor. Similar mechanisms have been proposed to explain the selective neutralization activity of PRL monoclonal antibodies of the action of PRLs from different species. (Scammell *et al.*, 1990).

At any event, it is likely that the 23K PRL antiserum does not recognize all the epitopes of the neurohypophyseal PRLs that are recognized by the 16K PRL antiserum. This is plausible since differences in three-dimensional structure of 16K PRL, attributed to the lost C-terminal end of the 23K PRL molecule, might have created new epitopes that are now recognized by 16K PRL-directed antibodies. Accordingly in assays where the three-dimensional structure of the molecules is preserved, the specificity of both the 23K PRL- and the 16K PRL-antisera increases. For instance, there is a low (1–2%) crossreactivity of 16K PRL and 23K PRL in the corresponding radioimmunoassays (Clapp *et al.*, 1988, 1994). Moreover, with immunocytochemistry, the 16K PRL antiserum clearly recognizes PRL-like antigens in the hypothalamic-neurohypophyseal system (Clapp *et al.*, 1994), while the 23K PRL antiserum fails to detect the neurohypophyseal PRL-like immunoreactivity (Siaud *et al.*, 1980; Harlan *et al.*, 1989; Paut-Pagano *et al.*, 1993).

In support to the biosynthesis of the PRL variants by the hypothalamic-neurohypophyseal system we have detected the PRL mRNA in isolated paraventricular nuclei (Clapp *et al.*, 1994). Moreover, these prolactin variants appear to enter the neurohypophyseal secretory pathway, as indicated by the observed *in vitro* release of PRL-like immunoreactive proteins following potassium-induced depolarization of hypothalamic-neurohypophyseal explants (Torner *et al.*, 1994). On the other hand, we have not detected the 14 kDa PRL-like immunoreactive protein in extracts of anterior pituitary glands, isolated lactotropes, or in the medium of cultured lactotropes (Torner *et al.*, 1994, unpublished observations). Moreover, we have not been able to detect immunoreactive PRLs in the hypophyseal intermediate lobe (Clapp *et al.*, 1994). These data allow excluding a possible contamination of neurohypophyseal PRLs with PRLs from adenohypophyseal lactotropes or hypophyseal-intermediate lobe cells. Furthermore PRL-like antigens were detected throughout the hypothalamic-neurohypophyseal system, i.e. somas, axonal

projections and terminals of magnocellular neurons from the paraventricular and supraoptic hypothalamic nuclei (Clapp *et al.*, 1994).

It is not clear what are the functional implications of neurohypophyseal PRLs and, in particular, of an anti-angiogenic-PRL molecule at this site. An effect on local vascularization is plausible. For instance, magnocellular neurons within hypothalamic paraventricular and supraoptic nuclei are known to express high levels of vascular endothelial growth factor (VEGF) mRNA (Ferrara *et al.*, 1992). On the other hand, the presence of this PRL variant in a neuroendocrine system raises its potential hormonal action upon distant target tissues. Accordingly, we have observed Ca^{2+}-dependent release of the 14 kDa antigen by incubated neurohypophyses and detected a 14 kDa PRL immunoreactive molecule in the circulation of the rat (unpublished observations).

The involvement of PRL-like molecules in the control of angiogenesis is being increasingly supported. Proliferin and proliferin-related protein, two proteins with extensive amino-acid sequence homology to PRL, were shown to stimulate and inhibit angiogenesis, respectively (Jackson *et al.*, 1994). Similarly our results suggest that the neurohypophyseal 14 kDa PRL, may constitute a new member of the PRL family likely to be involved in the regulation of angiogenesis, as well.

Materials and methods

PRLs

Rat PRL [B-6; National Hormone and Pituitary Program (NHPP)] was used as the 23K PRL standard and as the source for the production of 16K PRL. 16K PRL was isolated after the enzymatic proteolysis of 23K PRL by a particulate fraction from rat mammary gland homogenates, disulfide-bond reduction and gel filtration as reported (Clapp, 1987). A portion of the 16K PRL preparation was carbamidomethylated (CAM-16K PRL) by its reduction with dithiothreitol under denaturing conditions and subsequent alkylation with iodoacetamide (Ferrara *et al.*, 1991).

PRL antisera

The anti-rat 23K PRL serum (S-9) distributed by the NHPP was used. 16K PRL has a low crossreactivity (2% that of 23K PRL) with this antiserum in the 23K PRL RIA (Clapp *et al.*, 1988). The 16K PRL antiserum was raised in rabbits against the 16K PRL non-carbamidomethylated preparation described above. The specificity of this antiserum in the 16K PRL RIA has been reported previously (Clapp *et al.*, 1994). Briefly, 23K PRL has a reduced crossreactivity (1% that of 16K PRL) with the 16K PRL antiserum, while growth hormone, oxytocin, vasopressin, neurophysins I and II and other peptides known to be present in the hypothalamic-neurohypophyseal system, do not show crossreactivity with this antiserum (Clapp *et al.*, 1994).

Animals

Adult male Wistar rats (200–250 g) were kept in an environment of constant temperature and controlled day-night cycles and received food and water *ad libitum*. Their pituitary glands were removed following decapitation and the anterior lobes were dissected out under the microscope.

Incubation of neurohypophyses

The neurohypophyseal lobes were washed in Ca^{2+}-deficient Krebs-Ringer solution for 10 min at 37°C and incubated individually in 20 µl of Krebs-Ringer solution for 1 h at 37°C. The resulting conditioned media were pooled, concentrated (10 ×, Centricon 10, Amicon, Beverly, MA) and stored in aliquots at −70°C.

Western blot-densitometric analysis

Conditioned medium (10 µl) was subjected to SDS–PAGE as previously described (Clapp *et al.*, 1994). Medium proteins were blotted onto nitrocellulose membranes, probed with a 1:500 dilution of the 16K PRL or the 23K PRL-antisera, and developed by using the alkaline phosphatase second antibody kit (Bio-Rad Labs). To determine the concentration of the 16K PRL reference preparation and of the PRL immunoreactive proteins of the conditioned media, different dilutions of the respective samples were blotted and probed with the 16K PRL antiserum. The blots were then subjected to a densitometric analysis (Fotoanalyst-Fotodyne, Greensboro, NC, USA) calibrated with different concentrations of the 23K PRL standard processed within the same blot, as previously described (Clapp, 1987).

Cell proliferation assays

Nb2 cells The PRL activity of the conditioned media was measured using the lactogen-dependent rat T cell lymphoma Nb2 cell line as detailed previously (Tanaka *et al.*, 1980). Briefly, the cells (kindly provided by Dr. Peter Gout, Cancer Control Agency of British Columbia, Vancouver, B.C., Canada) were maintained as suspension cultures in high glucose-Dulbecco's Modified Eagle's medium supplemented with 10% horse serum, 10% fetal calf serum, 10^{-4} M β-mercaptoethanol and penicillin/streptomycin (50 U/ml). The effect of the conditioned or non-conditioned media was tested on Nb2 cell growth, in the presence or absence of the PRL antisera. Cell proliferation was determined in triplicate 12-mm tissue culture wells at a starting density of 2.5×10^4 cells in 0.25 ml of the above incubation medium, but without fetal calf serum. The cells were incubated for 72 h, after which [^{3}H]-thymidine incorporation into cellular DNA was measured as an index of cell proliferation (Ferrara *et al.*, 1991).

Bovine brain capillary endothelial cells Bovine brain capillary endothelial (BBCE) cells were isolated as previously described (Ferrara *et al.*, 1991: Clapp *et al.*, 1993) and grown in low glucose-Dulbecco's Modified Eagle's medium, supplemented with 10% fetal calf serum, glutamine (2 mM) and antibiotics (50 U/ml penicillin/streptomycin) as reported (Ferrara *et al.*, 1991; Clapp *et al.*, 1993). To assess cell proliferation, BBCE cells were plated at 2.5×10^3 cells per 12 mm culture wells in 0.25 ml of the above culture medium. bFGF (50 pM: GIBCO/BRL) alone or together with medium conditioned or not by incubated neurohypophyses were added to the wells every other day for 4 days. For immunoneutralization studies different dilutions of either PRL antisera were added together with the conditioned medium. On day 4, [^{3}H]-thymidine incorporation into cellular DNA was measured as reported (Ferrara *et al.*, 1991).

Data analysis

The data in Figures 2 to 5 are presented as means ± SEM of triplicate determinations of representative experiments. Comparisons of means were carried out by one-way analysis of variance (ANOVA) and the Newman-Keuls test for determining statistical differences between two treatment means in multitreatment experiments.

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