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Characterization of Interleukin-2 (IL-2) receptor expression and action of IL-2 and IL-6 on normal anterior pituitary cell growth

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The pituitary gland is known to express cytokines and their receptors. Interleukin-2 (IL-2) and IL-2 receptor (IL-2R) transcripts and protein products in corticotrophic cells have been previously described. IL-2R were also observed in PRL and GH-producing cells. The synthesis of IL-1 and IL-6 and their receptors by pituitary cells has also been reported. We recently demonstrated that the cytokines in addition to their regulatory effects on anterior pituitary hormone secretion are involved in the autocrine or paracrine regulation of pituitary growth. In the present study we show in normal rat anterior pituitary cells: (a) expression of IL-2Rα chain mRNA, (b) the co-localization of IL-2Rα chain with TSH, FSH and LH-producing cells, (c) the percentage of co-localization of IL-2R with all types of anterior pituitary hormone producing cells: PRL>>> ACTH>> GH>TSH=FSH=LH. (d) that [^{3}H]-thymidine is incorporated into the nucleus of all types of hormone-producing cells without incorporation into other cell types, following IL-2 and IL-6 stimulation. Our results suggest that IL-2 acts on all types of anterior pituitary hormone-producing cells and, through specific functional receptors on the same or other cells, constitutes, as well as IL-6, an inter or intra-cellular factor involved in the coordinate regulation not only of hormone secretion but also of the proliferation of anterior pituitary hormone-producing cells.

Keywords: interleukin-2; interleukin-2 receptor; interleukin-6; anterior pituitary growth

Introduction

It has been described that the endocrine tissues may not only be a target but also a site of origin of lymphokines. Thus, interleukin-1 β (IL-1β), IL-6 and IL-6 receptor (IL-6R) were found in the hypothalamus (Breder *et al.*, 1988; Spangelo *et al.*, 1990a; Gadient & Otten, 1993; Molenaar *et al.*, 1993). IL-1 is also produced in the pituitary, increasing after bacterial lipopolysaccharide treatment of the animals (Koenig *et al.*, 1990; Takao *et al.*, 1993). In addition, IL-1 receptors and mRNA were characterized in mouse pituitary cells and AtT-20 corticotrophs (DeSouza *et al.*, 1989; Bristulf *et al.*, 1991; Parnet *et al.*, 1993; Takao *et al.*, 1993). Furthermore, after LPS injection, pituitary IL-1β and IL-1R are reciprocally modulated (Takao *et al.*, 1993). IL-6 production by cells obtained from rat anterior pituitary gland has also been demonstrated (Vankelecom *et al.*, 1989; Spangelo *et al.*, 1990b), stimulated among others by IL-1 (Yamaguchi *et al.*, 1990; Spangelo *et al.*, 1991). The expression of IL-6 mRNA in rat anterior pituitary (Spangelo *et al.*, 1990a), corticotrophic adenoma cell cultures (Velkeniers *et al.*, 1991) as well as in different types of anterior pituitary adenomas (Jones *et al.*, 1994) and the release of IL-6 from human pituitary adenoma cultures (Jones *et al.*, 1991, 1994) have been reported. The presence of IL-6 in different types of pituitary adenomas was also found using immunocytochemistry (Tsagarakis *et al.*, 1992). Adrenalectomy further enhances IL-6 mRNA levels in the pituitary (Schöbitz *et al.*, 1993). Furthermore, the expression of IL-6R was observed in rat anterior pituitary cells (Ohmichi *et al.*, 1992). We have recently described the expression of IL-2 and IL-2R by pituitary corticotrophic cells of different species: both human corticotrophic adenoma and mouse AtT-20 cells showed detectable amounts of IL-2R mRNA and by immunofluorescence, IL-2R membrane expression (Arzt *et al.*, 1992). In the normal rat, IL-2R co-localization with ACTH, PRL and GH producing cells has also been described (Arzt *et al.*, 1993).

After the first description of the stimulatory action of IL-1 on hormone secretion by the hypothalamic-pituitary-adrenocortical (HPA) axis (Besedovsky *et al.*, 1986), this and other cytokine actions have been extensively studied. It is now well accepted that cytokines not only are a lymphocyte message but also constitute autocrine and paracrine factors in the regulation of hormone secretion by the HPA axis, especially at the pituitary level. IL-1 is the most potent cytokine stimulating the axis and seems to act at hypothalamic, pituitary and adrenal level to regulate CRH, ACTH and cortisol secretion (reviewed in Bateman *et al.*, 1989; Hermus *et al.*, 1990). IL-1 also regulates PRL, LH, FSH, GH and TSH release by anterior pituitary cells (reviewed in Bateman *et al.*, 1989; Hermus *et al.*, 1990). The other inflammatory cytokines, IL-6 (reviewed in Gorospe *et al.*, 1993) and tumor necrosis factor α, share these biological actions of IL-1 (reviewed in Bateman *et al.*, 1989; Hermus & Sweep, 1990).

The T cell cytokine IL-2 also acts on the HPA axis. IL-2 *in vitro* has been shown to enhance proopiomelanocortin (POMC) gene expression (Brown *et al.*, 1987; Low *et al.*, 1987) and induce ACTH release (Farrar, 1984; Smith *et al.*, 1989; Karanth *et al.*, 1991a) in the murine AtT-20 cell line and in rat anterior pituitary cell cultures. IL-2 at picomolar concentrations not only regulates ACTH, but also PRL, LH, FSH, GH and TSH secretion (Karanth, 1991; Karanth & McCann, 1991). Thus, the presence of IL-2R on the various cell types was considered to be the most likely explanation for the effects of IL-2 on the secretion of the various

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pituitary hormones (Karanth, 1991). However, the expression of the IL-2R on TSH, LH and FSH-producing cells remained unknown. The stimulation of PRL release induced by IL-2 is blocked by dopamine (Karanth & McCann, 1991). A direct stimulatory effect of IL-2 on CRH release by hypothalami *in vitro* has also been described (Cambronero *et al.*, 1992; Karanth *et al.*, 1993). *In vivo*, when administered to human cancer patients, IL-2 increases β-endorphin, ACTH and cortisol levels (Lotze *et al.*, 1985; Denicoff *et al.*, 1989). In addition it induces rises in β-endorphin and ACTH levels when administered in allografted (Zakarian *et al.*, 1989) or normal (Naito *et al.*, 1989) rats, respectively. After IL-2 injection in rats an increment in POMC mRNA was observed in the pituitary without changes in CRH mRNA levels in the hypothalamic paraventricular nucleus, suggesting a direct stimulatory effect of IL-2 on the pituitary (Harbuz *et al.*, 1992). Several reports failed to find effects of IL-2 in the above mentioned systems. However, a study demonstrated that in the rat, rat but not human IL-2 had an ACTH-secreting activity (Naito *et al.*, 1989). Moreover, rat IL-2, but not human IL-2, increased corticosterone levels in a dose-dependent manner during 24 h of rat adrenal cultures (Tominaga *et al.*, 1991). This species specificity may account, at least partly, for some previous controversial results.

We have recently shown that IL-2 and IL-6 regulate pituitary cell growth (Arzt *et al.*, 1993). In the pituitary tumor cell line GH_3 both IL-2 (1–100 U/ml) and IL-6 (10–500 U/ml) significantly stimulate [^{3}H]-thymidine incorporation and cell count. In contrast, both IL-2 and IL-6 at the same concentrations have inhibitory effects on normal rat anterior pituitary cell growth, the effects being clearly observed under conditions that restrict fibroblast growth (MEM D-valine culture medium) (Arzt *et al.*, 1993). There was no direct correlation between the effects of IL-2 and IL-6 on cell growth and hormone secretion.

In the present report, we: (1) studied the transcription and expression of the receptor for IL-2 in order to establish whether all types of normal anterior pituitary cells express the receptor, and (2) characterized the action of IL-2 as well as IL-6 on the growth of pituitary cells by [^{3}H]thymidine autoradiography combined with staining for hormone secretion, in order to further clarify the role of the different types of anterior pituitary cells in the growth regulatory effects of IL-2 and IL-6.

Results

IL-2 receptor mRNA expression in normal anterior pituitary cells

The expression of the mRNA of the IL-2R in normal pituitary cells was studied by Northern blot. Normal pituitary cells were cultured for 24 h under basal conditions or stimulated with PMA. PMA was chosen because it has been shown to stimulate the different types of pituitary cells and induce membrane expression of IL-2R in about 50% of the normal rat anterior pituitary cells (Arzt *et al.*, 1992).

When RNA was hybridized with the rat IL-2R p55 (α subunit) probe, a single hybridizing band of about 3.5 kb was always detected (Figure 1). The position of the band is identical and weaker to that obtained with ConA stimulated rat splenocytes (Figure 1). These results indicate that there is a constitutive expression of IL-2R mRNA in the anterior pituitary cells that is further upregulated by PMA (Figure 1), in accordance with the protein expression pattern previously reported (Arzt *et al.*, 1992). These results show that in normal anterior pituitary rat cells the IL-2R gene is effectively transcribed and translated.

Co-localization of IL-2R with hormone-producing cells in the anterior pituitary

By dual immunofluorescence studies we observed co-localization of the anti-rat p55 (α subunit) IL-2R monoclonal antibody with TSH (Figure 2A and B), LH (Figure 2C and D) and FSH (Figure 2E and F) cells. Thus, we now provide evidence that all types of anterior pituitary cells express the IL-2R. Since immunofluorescence studies are generally considered to provide semi-quantitative results, an estimation of the co-localization of IL-2R with hormone positive cells was made for all experiments, over the whole slide area. The highest percentage of cells displaying the IL-2R was found among the PRL-producing cells (Table 1).

Effect of IL-2 on anterior pituitary cell growth

Simultaneous immunocytochemistry and autoradiographic studies show that in cells cultured in MEM D-Valine medium without serum the [^{3}H]thymidine is incorporated only into the nucleus of hormone-secreting pituitary cells without any incorporation into other cells (Figure 3). Co-localization studies of [^{3}H]thymi-

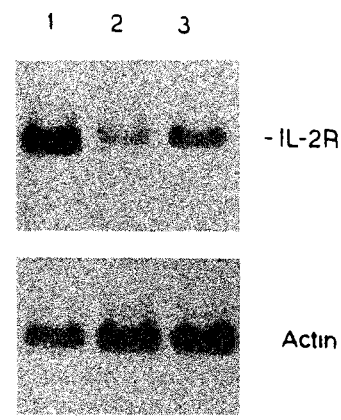

Figure 1 IL-2 receptor mRNA expression in normal rat anterior pituitary cells Northern blot of RNA (10 μg total RNA per lane) extracted from rat spleen cells stimulated for 18 h with Con A (2.5 μg/ml) (lane 1) and anterior pituitary cells cultured for 24 h under basal conditions (lane 2) or stimulated for 24 h with 10 nM PMA (lane 3). After hybridizing with a 1.1 kb ^{32}P-labeled rat IL-2R cDNA at 60°C and washing at high stringency, the autoradiograms were obtained after 2 days of exposure. One band of 3.5 kb, identical to that present in the spleen cells (lane 1), is observed in the pituitary cells (lanes 2–3). Similar results were obtained in four different blots. At the bottom: the same Northern blot but completely eluted of radioactivity and reprobed with a 1 kb ^{32}P-labeled actin cDNA fragment.

Table 1 Relative co-localization of IL-2R with anterior pituitary hormone producing cells

Hormone	*Co-localization*[a]
PRL	50–60%
ACTH	30–40%
GH	10–20%
TSH	5–10%
FSH	5–10%
LH	5–10%

[a]co-localization was determined as shown in Figure 2 and is expressed according to the number of cells positive for a specific hormone that also stain for the IL-2R.

dine and immunocytochemistry with antibodies for all types of hormones secreted by anterior pituitary cells demonstrate that under incubation with IL-2 and IL-6 the [^{3}H]thymidine incorporation is observed in all types of anterior pituitary hormone producing cells and not only in a specific hormone producing population (Figure 3). The examples shown in Figure 3, for each hormone with either IL-2 or IL-6 stimulation, are representative of all the effects of IL-2 and IL-6 on all types of hormone-secreting cells.

For all experiments, over the whole slide area, less [^{3}H]thymidine incorporation in hormone-producing cells (20–40% of controls) was observed for IL-2 and IL-6 stimulated anterior pituitary cultures. The inhibitory effects were not due to cell death, as the viability of cells was over 95% in all cases.

Discussion

In the present report we show that the IL-2R expression in the membrane of normal anterior pituitary cultures is co-localized with all types of anterior pituitary hormone-secreting cells. This finding provides evidence for a direct site of action for previous functional observations that IL-2 influences secretion of all different anterior pituitary hormones (Karanth, 1991). The expression of IL-2R on all different types of hormone-producing cells in the anterior pituitary that we show in the present report, in concert with the direct regulatory effects of IL-2 *in vitro* (Karanth, 1991), strongly suggests a peptide-direct-mediated physiological regulation of pituitary function.

There are well known species differences in the immune system, particularly between mouse/human and rat, in which IL-2 constitutes one of the molecules that shows a high degree of species-specific activity (McKnight & Classon, 1992). For this reason we used rat IL-2 and species specific anti-IL-2R antibodies and cDNA to establish the presence of the IL-2R and its mRNA as well as IL-2 action on pituitary cells. The present demonstration that normal pituitary cells express IL-2R mRNA provides evidence that these cells effectively transcribe the IL-2R gene. The gene is constitutively expressed and can be up-regulated, for example with PMA. PMA has previously been shown to stimulate the expression of the receptor in the membrane of anterior pituitary cells (Arzt *et al.*, 1992). Thus, the up-regulation of IL-2R mRNA by PMA indicates that the stimulation occurs at the transcription level. Five distinct transcripts of IL-2R mRNA (in which the longest transcript predominates) and two transcripts of 3.5 and 1.5 kb are observed in mouse and human lymphoid IL-2R positive cells, respectively (Leonard *et al.*, 1984; Schimuzu *et al.*, 1985). Only one 3.5 kb transcript is detected in rat splenocytes (Dallman *et al.*, 1991; Page & Dallman, 1991). Accordingly, we found expression of only one similar transcript in normal rat pituitary cells. Interestingly, only this 3.5 kb transcript was detected in human pituitary adenoma cells (Arzt *et al.*,1992). Rat splenocytes have at least four IL-2 binding proteins (Chopra *et al.*, 1992). Species-specificity seems to be particularly important for IL-2 effects on the endocrine system: rat IL-2 induces corticosterone production by rat adrenal cells and ACTH release by normal rats *in vivo*, while human IL-2 has no effect (Naito *et al.*, 1989; Tominaga *et al.*, 1991). Specific binding characteristics or the particular environment of the IL-2R in endocrine cells could also account for its specificity. It has recently been hypothesized that since IL-2 receptor chains are expressed even in nonlymphoid cells, the IL-2 receptor component chains may associate with other noncytokine receptor molecules to create new signaling complexes (Taniguchi *et al.*, 1993). Further studies will be necessary to determine the binding properties and putative associations of the anterior pituitary IL-2R. Moreover the putative expression of the β and the recently cloned γ chain (Takeshita *et al.*, 1992) of the IL-2R in pituitary cells should be studied to further understand the mechanism of action of IL-2 on the pituitary.

The direct regulation by IL-2 and IL-6 of anterior pituitary hormone secretion has been previously described by several groups (reviewed in Bateman *et al.*, 1989; Hermus & Sweep, 1990; Gorospe & Spangelo, 1993). The actions of many peptide growth factors include both stimulation and inhibition of cell proliferation, as well as effects unrelated to control of cell growth (Sporn & Roberts, 1988). IL-2 and IL-6 seem to behave in this way, since they exert diverse effects on pituitary cell growth independently of their effects on hormone secretion (Arzt *et al.*, 1993). In the present report we show that the action of IL-2 and IL-6 on anterior pituitary cell proliferation is directly exerted on all different types of hormone-secreting cells as [^{3}H]thymidine is incorporated into the nucleus of all types of hormone-producing cells without incorporation into other cell types, following stimulation with IL-2 or IL-6.

Frequently associated with high levels of hormone production, the anterior pituitary can develop different sized benign tumors classified as micro and macroadenomas (Landolt *et al.*, 1988). Invasive tumor growth is associated with higher proliferation activity (Buchfelder *et al.*, 1991). The particular factors involved in the development of different types of pituitary tumors (micro-macroadenomas, invasive) as well as those responsible for the high incidence of nonmalignant pituitary tumors are not clear at present. Recent research has demonstrated that in experimental tumor models immunotherapy with low-dose IL-2 can be highly effective against metastatic cancer when applied locally at a site of tumor growth (Maas *et al.*, 1991). Further, insertion of a functional IL-2 gene into plasmocytoma (Bubenik *et al.*, 1992) or L1210 lymphoma (Chakravarty *et al.*, 1992) cells led to a loss of tumorigenicity of the IL-2 secreting cells. When injected into normal mice, murine fibrosarcoma cells that after transfection constitutively secrete IL-6 exhibit

reduced tumorigenicity (Mullen *et al.*, 1992). A paracrine role for IL-6 produced by breast fibroblasts in the inhibition of breast cancer cell growth has also been postulated (Speirs *et al.*, 1993). IL-6 production by folliculo-stellate cells in the pituitary (Vankelecom *et al.*, 1989; Spangelo *et al.*, 1990b), or IL-2 synthesis by human pituitary adenomas (Arzt *et al.*, 1992), could play a similar autocrine or paracrine role within the anterior pituitary gland by exerting a direct regulation of the growth of all different types of hormone-secreting pituitary cells, which, as shown in this report, are the targets of their action.

Some pituitary tumors appear to develop as a result of somatic mutation (Herman *et al.*, 1990). Such mutations could enhance growth by causing altered expression of growth factors or their receptors, as those for IL-2 or IL-6. The high prevalence of IL-2R on PRL, ACTH and GH producing cells, that we show in the present report, could be associated with the development of specific PRL, ACTH or GH secreting tumors.

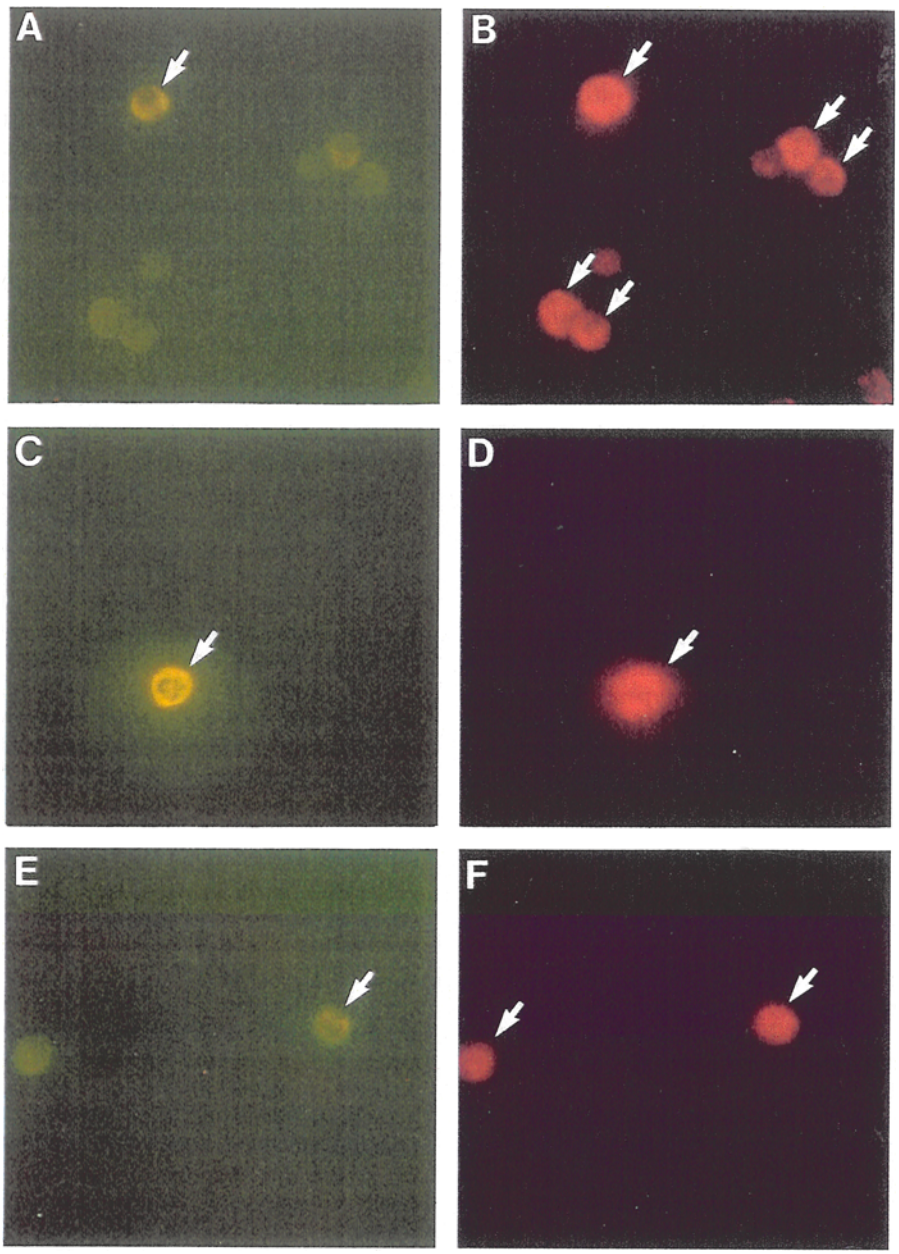

Figure 2 Co-localization of IL-2 receptor with TSH, LH and FSH-secreting rat pituitary cells. Dual immunofluorescence staining was performed in cells cultured for 24 h at basal conditions. After fixing, cells were pre-absorbed for 1 h with 10% normal goat serum and incubated overnight with the following first antibodies: (**B**) rabbit anti-TSH (1:2000), (**D**) rabbit anti-LH (1:3000), (**F**) rabbit anti-FSH (1:2000). After washing, a rhodamine conjugated goat anti-rabbit IgG second antibody was added. Cells were washed and then treated with a specific anti-rat IL-2R (p55 subunit) FITC-conjugated monoclonal antibody diluted 1:100. Co-localization of TSH, LH and FSH positive cells (arrows in **B**, **D** and **F**, respectively) with positive IL-2R staining (arrows in **A**, **C** and **E**) can be observed (400 ×). (**A**/**B**) in areas of the field selected with TSH positive cells, many TSH positive cells that are not positive for IL-2R are observed (co-localization results for all hormones are summarized in Table 1). Fibroblasts present in the cultures were always negative. Using an antibody of the same isotype as the corresponding anti-IL-2R antibody, 100% negative cells were observed for all the conditions, confirming the specificity of immunostaining. In addition, negative immunostaining was observed when second antibodies were added without previous addition of the primary antibody. Similar results were obtained in six different cell preparations for each hormone.

The study of paracrine and autocrine interactions within the anterior pituitary has been the focus of expanding research efforts in the last 5 years. Our data provide new insights into interleukin involvement in pituitary (patho)-physiology: as intrinsic factors of the gland, through specific functional receptors on the same or other cells, they constitute inter or intracellular factors involved in the coordinate regulation not only of hormone secretion but also of cell growth.

Materials and methods

Cell cultures

Unless stated, materials and reagents were from Flow (Meckenheim), Seromed (Berlin), Gibco (Karlsruhe), Falcon (Heidelberg) and Nunc (Wiesbaden) (Germany). Pituitary cell culture was performed as previously described (Stalla *et al.*, 1988, 1989; Arzt *et al.*, 1993). In brief, glands were obtained from male Sprague-Dawley rats (200–300 g) within minutes of death by decapitation and were treated as follows. The tissue was washed several times with preparation buffer

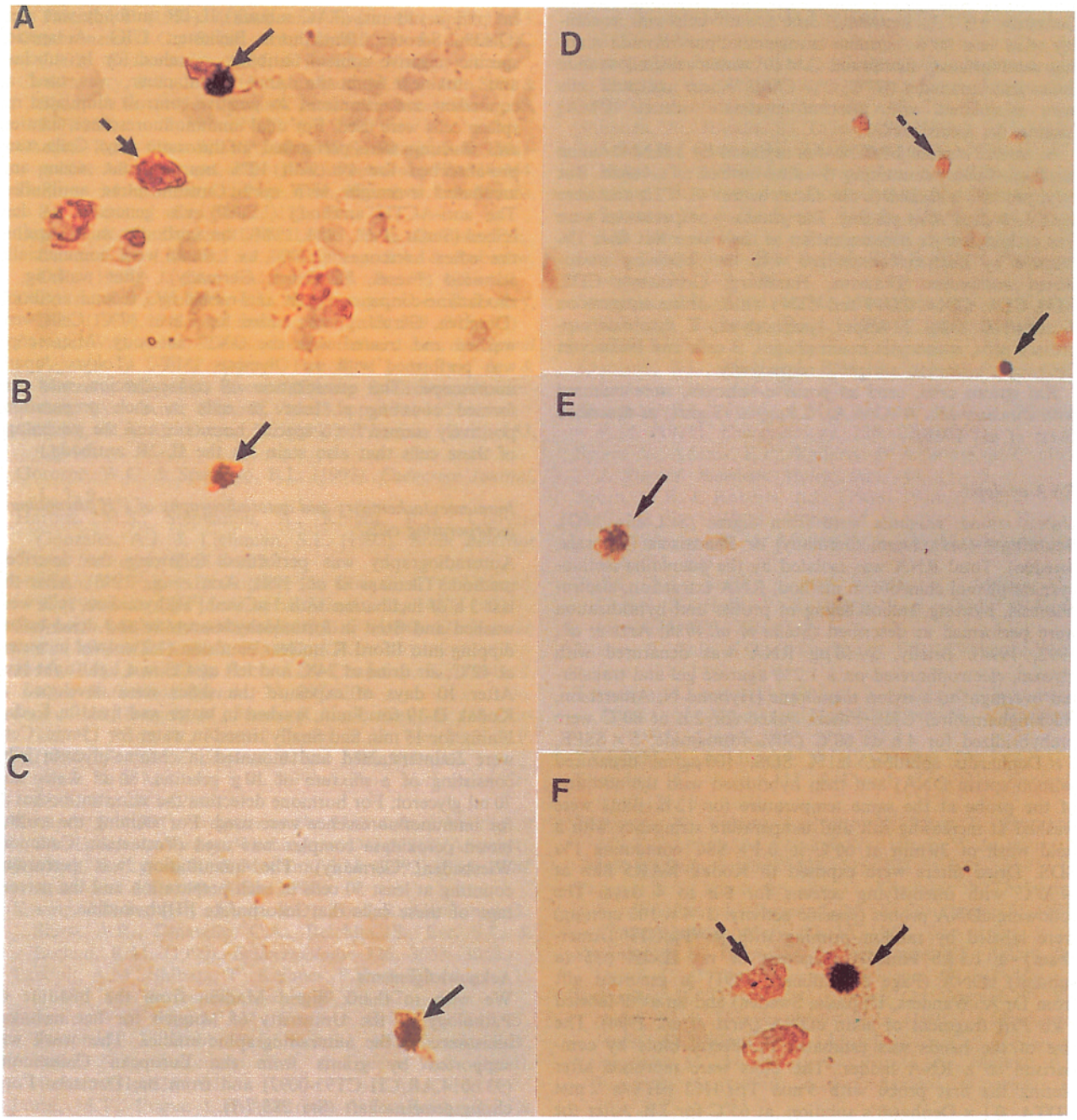

Figure 3 Immunocytochemistry and autoradiography of [^{3}H]thymidine incorporation on normal rat anterior pituitary cells. Cells were cultured in MEM D-valine with 10% FCS for 4 days when cells were washed and cultured for 24 h in MEM D-valine without FCS. Cells were then stimulated (**A** and **D**: IL-2 10 U/ml, **B**: basal, **C**, **E** and **F**: IL-6 100 U/ml) and cultured for 3 h with [^{3}H]thymidine. Autoradiography, immunocytochemistry (orange-red color for hormone positive cells) and microscopy (400 ×, except **D**: 100 ×) were performed as indicated in Materials and methods. [^{3}H]thymidine incorporation (arrows) in hormone positive cells can be observed (**A**: GH, **B**: PRL, **C**: ACTH, **D**: TSH, **E**: FSH, **F**: LH). No [^{3}H]thymidine incorporation is observed in cells negative for hormone staining. Some hormone positive cells (examples as broken arrows) which do not incorporate [^{3}H]thymidine are observed. Similar results were obtained in four different independent experiments.

(137 mM, NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 10 mM glucose, 15 mM HEPES pH 7.3, antibiotics). Sliced fragments were dispersed mechanically and enzymatically in preparation buffer containing 1000 units/ml collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA), 4 g/liter BSA, 10 mg/liter DNAase II, 1 g/liter soybean trypsin inhibitor and 2 g/liter hyaluronidase. Cells were centrifuged and resuspended in culture medium (Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum (FCS), 2.2 g/liter $NaHCO_3$, 10 mM HEPES, 2 mM glutamine, 10 ml/liter non-essential amino acids, 10 ml/liter MEM vitamins, antibiotics, 5 mg/liter insulin, 20 μg/liter selenium, 5 mg/liter transferrin, and 30 pM triiodothyronine (Henning, Berlin, Germany, pH 7.3). Between 3 and 6×10^6 cells with a viability of at least 80% (acridine orange/ethidium bromide staining) were isolated, distributed (2×10^5 viable cells/ml) to slide flasks and incubated (37°C, 5% CO_2). Where indicated cells were stimulated with phorbol myristate acetate (PMA) (Sigma, St. Louis, MO).

In several studies DMEM was replaced by MEM D-valine medium (Gibco) containing D-valine instead of L-valine. Rat pituitary cells attached to the dishes within 48–72 h and were used 4–6 days after plating. The pituitary preparations were free of lymphocyte contamination as they were less than 1% reactive by immunofluorescence with the following monoclonal antibodies (Dianova, Hamburg, Germany): CD2, CD4, CD8, CD14, CD19 and CD45 which define antigens on T cells/NK cells, T helper lymphocytes, T cytotoxic/suppressor cells, monocytes/macrophages, B cells and leukocytes (leukocyte common antigen), respectively.

Rat spleen cells, used as positive controls, were cultured with concanavalin A (Con A), 2.5 μg/ml (Sigma) as described (Arzt *et al.*, 1988).

RNA analysis

Unless stated, reagents were from Sigma (St.Louis, MO), Boehringer (Mannheim, Germany) or Pharmacia (Uppsala, Sweden). Total RNA was isolated by the guanidine isothiocyanate-phenol-chloroform method. RNA extraction, electrophoresis, blotting, radiolabeling of probes and hybridization were performed as described (Stalla *et al.*,1988; Arzt *et al.*, 1992, 1994). Briefly, 5–10 μg RNA was denatured with glyoxal, electrophoresed on a 1.2% agarose gel and transferred overnight to a nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK). Filters baked for 2 h at 80°C were prehybridized for 4 h at 60°C (50% formamide, 5 × SSPE, 5 × Denhardts solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA) and then hybridized with the addition of the probe at the same temperature for 12 h. Blots were washed at increasing salt and temperature stringency with a final wash of 30 min at 60°C in 0.1 × SSC containing 1% SDS. Dried filters were exposed to Kodak XAR5 film at −70°C with intensifying screens for 6 h to 4 days. The following cDNA probes (specific activity, 2–4 × 108 cpm/μg) were labeled by random-priming with [α-^{32}P]dCTP (Amersham): a 1.1 kb HindIII fragment of rat IL-2R p55 (α subunit) cDNA (Page & Dallman, 1991) (a generous gift from Dr A. Wanders, Uppsala, Sweden) and an α-^{32}P-labeled 1 kb *PstI* fragment of actin cDNA (Arzt *et al.*, 1994). The size of the bands was estimated in several blots by comparison to a RNA ladder. The blots were reprobed after eluting the first probe with 5 mM Tris/HCl pH 8.0, 2 mM EDTA, 0.1 × Denhardt's solution, at 65°C for 2 h. After the previous signal was removed, confirmed by re-exposure of the filter, the blots were prehybridized and hybridized following methods described above. A control with the actin cDNA as probe was performed in each blot.

IL-2R immunofluorescence

IL-2R was determined by immunofluorescence, as described (Arzt *et al.*, 1988, 1992, 1993). After fixing the cells in formaldehyde-acetone, the antibody at serial dilutions was added for 30 min at 37°C. Cells were extensively washed with phosphate-buffered saline (PBS) alone and with PBS containing 1% Triton. A FITC-conjugated monoclonal mouse anti-rat p55 (α subunit) (TAC subunit) IL-2R antibody was used (OX39, Serotec, Blackthorn Bichester, UK). A species specific negative control antibody, matched for Ig subclass and obtained from the same manufactuer, was used at equivalent concentrations. As positive control, stimulated rat spleen cells were used. For dual immunofluorescence, fixation and washing were performed in the same way. Cells were pre-absorbed for 1 h with 10% normal goat serum and incubated overnight with rabbit anti-hormone antibodies. The anti-ACTH antibody (1:2000) was generated as described (Stalla *et al.*, 1988, 1989), the antibodies raised against the other hormones (1:2000 to 1:5000) were commercially acquired (Paesel, Frankfurt, Germany). After washing, a rhodamine-conjugated goat anti-rabbit IgG second antibody (Dianova, Germany) was added for 1 h at 37°C. Cells were washed and treated with the OX39 antibody. Microscopy was performed with an Olympus IMT-2 (Tokyo, Japan) microscope. The quantitation of co-localization was performed counting al least 30 cells in each preparation, positively stained for a specific hormone, and the percentage of these cells that also stain for the IL-2R antibody.

Immunocytochemistry and autoradiography of [^{3}H]thymidine incorporating cells

Autoradiography was performed following the described method (Tilemans *et al.*, 1991; Arzt *et al.*, 1993). After the last 3 h of incubation with 1 μCi/ml [^{3}H]thymidine, cells were washed and fixed in formaldehyde-acetone and dried before dipping into Ilford K nuclear emulsion (1:2 vol/vol in water) at 42°C, air dried at 24°C and left at 4°C in a light-tight box. After 10 days of exposure the slides were developed in Kodak D-19 for 3 min, washed in water and fixed in Kodak Unifix for 15 min and finally rinsed in water for 15 min. Cells were counterstained and mounted in chrome-glycerin jelly, consisting of a mixture of 10 g gelatine, 80 ml water and 70 ml glycerol. For hormone detection the same antibodies as for immunofluorescence were used. For staining the avidin-biotin peroxidase complex was used (Vectastain, Cammon, Wiesbaden, Germany). The quantitation was performed counting at least 50 cells in each preparation and the percentage of these cells that incorporate [^{3}H]thymidine.

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