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Direct Effects of Nerve Growth Factor on Thecal Cells from Antral Ovarian Follicles*

G. A. DISSEN, J. A. PARROTT, M. K. SKINNER, D. F. HILL, M. E. COSTA, AND S. R. OJEDA

Division of Neuroscience, Oregon Regional Primate Research Center, Oregon Health Sciences University (G.A.D., D.F.H., M.E.C., S.R.O.), Beaverton, Oregon 97006-3448; and Center for Reproductive Biology, School of Molecular Bioscience, Washington State University (J.A.P., M.K.S.), Pullman, Washington 99164-4231

ABSTRACT

TrkA, the nerve growth factor (NGF) tyrosine kinase receptor, is expressed not only in the nervous system, but also in nonneural cells, including discrete cellular subsets of the endocrine and immune system. In the rat ovary, trkA receptor abundance increases strikingly in thecal-interstitial cells during the hours preceding the first ovulation. Blockade of either trkA transducing capacity or NGF biological activity inhibited ovulation, suggesting a role for NGF in the ovulatory process of this species. To identify some of the processes that may be affected by trkA activation in the thecal compartment, we used purified thecal cells/thecal fibroblasts from bovine ovaries (heretofore referred to as thecal cells). Ribonuclease protection assays employing bovine-specific cRNA probes demonstrated the presence of the messenger RNAs (mRNAs) encoding NGF and its receptors, p75 NTR and trkA, in the thecal compartment of small, medium, and large antral follicles and showed that trkA mRNA is also expressed in granulosa cells. *In situ* hybridization and immunohistochemical examination of intact ovaries confirmed these cellular sites of NGF and trkA synthesis. TrkA mRNA, but not NGF mRNA, was lost within 48 h of placing thecal cells in culture. Thus, to study trkA-mediated actions of NGF on these cells we transiently expressed the receptor by transfection with a vector containing a full-length rat trkA complementary DNA under transcriptional control of the cytomegalovirus promoter. Because ovulation is preceded by an LH-dependent increase in androgen and progesterone production, the ability of NGF to modify the release of these steroids was determined in freshly plated cells still containing endogenous trkA receptors and in cells undergoing luteinization in culture that were transiently transfected with the trkA-encoding plasmid. NGF stimulated both androgen and progesterone release in freshly plated thecal cells, but not in luteinizing cells provided with trkA receptors. As ovulation in rodents requires an increased formation of PGE_2 and has been shown to be antedated by proliferation of thecal fibroblasts, we determined the ability of NGF to affect these parameters in trkA-transfected thecal cells. The neurotrophin rapidly stimulated PGE_2 release and amplified the early steroidal response to hCG in trkA-expressing cells, but not in cells lacking the receptor. Likewise, NGF stimulated [^{3}H]thymidine incorporation into trkA-containing cells, but not into cells that had lost the receptor in culture. Induction of ovulation in immature rats by gonadotropin treatment verified that an increased cell proliferation in the thecal compartment, determined by the incorporation of bromodeoxyuridine into cell nuclei, occurs 4–5 h before ovulation in this species. These results suggest that the contribution of NGF to the ovulatory process includes a stimulatory effect of the neurotrophin on steroidogenesis, PGE_2 formation, and proliferative activity of thecal compartment cells. (*Endocrinology* **141:** 4736–4750, 2000)

THE NEUROTROPHINS (NTs) are target-derived growth factors required for the survival and development of discrete neuronal populations in the central and peripheral nervous systems (1, 2). Although initial observations led to the conclusion that the biological actions of NTs are restricted to the nervous system (3, 4), it is now clear that they can also affect nonneuronal cells (5), including cells of the endocrine system. Several members of the NT family and their respective receptors have been detected in the mammalian ovary, including nerve growth factor (NGF), brain-derived neurotropic factor (BDNF), NT-3, and NT-4/5 (6, 7; for additional references, see Ref. 8).

Although NTs, and in particular NGF (9), are critical for development of the ovarian innervation, they also appear to exert direct actions on nonneuronal cells of the ovary as evidenced by the presence of trk tyrosine kinase receptors in these cells (6, 7). NGF is recognized by two different membrane-spanning receptor molecules, one displaying rapid dissociation kinetics, known as the low affinity NT receptor or $p75^{NTR}$, and another with a slow dissociation rate, known as trkA (10, 11). Although $p75^{NTR}$ binds all other NTs, including BDNF, NT-3, and NT-4/5, with similar low affinity (4, 12), the trkA receptor binds NGF preferentially and with high affinity (13, 14).

The $p75^{NTR}$ potentiates the effects of trkA activation (12, 15), but also has an independent role in apoptosis, initiated by activation of a ceramide-dependent signaling pathway (16). TrkA receptors, on the other hand, are endowed with a tyrosine kinase domain similar to that of other receptor tyrosine kinases (17, 18) and mediate the biological effects of NGF via activation of signaling pathways similar to those activated by mitogenic receptor tyrosine kinases (19). Expression of trkA in the rat ovary increases dramatically in cells of the follicular wall during the preovulatory surge of gonadotropins (6). This increase is LH dependent and is

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Address all correspondence and requests for reprints to: Dr. Gregory A. Dissen, Division Neuroscience, Oregon Regional Primate Research Center, 505 NW 185th Avenue, Beaverton, Oregon 97006-3448. E-mail: disseng@ohsu.edu.
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accompanied by an increase in NGF messenger RNA (mRNA) levels. Immunological or pharmacological blockade of NGF action reduces the rate of ovulation (6), suggesting that activation of trkA receptors plays a role in this process. In the present study we considered the possibility that activation of trkA receptors in thecal compartment cells contributes to events known to occur during the hours antedating the first ovulation. Although trkA receptor gene expression can be induced by preovulatory levels of LH or by the cytokine interleukin-1β (6), we chose not to use these agents to avoid potentially confounding effects unrelated to trkA activation. Experiments were, therefore, conducted in primary cultures of freshly plated purified thecal cells that contain endogenous trkA receptors or in thecal cells undergoing luteinization, transiently provided with the receptors via cationic lipid-mediated gene transfer.

Bovine thecal cells were chosen for these studies because they can be easily isolated in large quantities (20). In addition, the bovine ovary is monoovulatory and potentially similar to the human ovary in the mechanisms controlling follicular growth and differentiation. Because ovulation requires the intrafollicular action of progesterone (21) and the enhanced formation of PGE_2 (22, 23), we examined the ability of NGF to stimulate the production of both progesterone and PGE_2 from purified thecal cells. Because NGF binding to trkA receptors expressed in endocrine cells results in cell proliferation (24–26), and in view of the observations of thecal cell proliferation around the time of ovulation in the rat (27–29), studies were performed to determine whether NGF could affect the proliferative activity of cells cultured from the thecal compartment cells in culture. A partial report of these findings has appeared (30).

Materials and Methods

Animals

Ovaries from young nonpregnant cycling heifers were obtained less than 10 min after death and delivered to the laboratory, on ice, by Golden Genes (Fresno, CA).

Sprague Dawley rats (B&K Universal, Fremont, CA) were housed under controlled conditions of temperature (23−25 C) and light (14 h of light, 10 h of darkness; lights on from 0500–1900 h). They were provided *ad libitum* access to food (Purina laboratory chow, Ralston Purina Co., St. Louis, MO) and water. At 27 days of age (0900 h) the rats received a sc injection of either 8 IU PMSG in 0.2 ml saline or saline alone. Two days later hCG was administered sc to those rats that had been primed with PMSG, and the ovaries were collected 0, 4, 8, and 12 h later. Two hours before the scheduled time of tissue collection the rats were injected ip with 5-bromo-2′-deoxyuridine (BrdU; Roche, Indianapolis, IN; 50 μg/g BW) dissolved in saline solution-0.007 M NaOH.

Primary cell culture: freshly plated cells

The thecal compartment of bovine antral follicles was isolated by microdissection. The theca externa was isolated from the theca interna as previously described (20, 31). The theca externa layer is generally isolated with the initial removal of theca from the opened follicle. Adhering interstitial cells were scraped away, and the theca interna was microdissected by pulling the intact layer/shell of interna theca from the externa. This was done with two small tweezers, which allowed the theca interna shell to stay generally intact. The theca externa peeled away in pieces and was collected separately. The cells were dispersed with 2 mg/ml collagenase (Sigma, St. Louis, MO) in Ca^{2+}/Mg^{2+}-free buffer as previously described (31). The antral follicles were classified as small (5 mm), medium (5–10 mm), or large (>10 mm) before microdissection. Unless the size range of the follicles is noted, the thecal cells were derived from a mixed-sized population of antral follicles. Cell preparations obtained by this procedure have negligible contamination from granulosa and stromal-interstitial cells and show less than 3% contamination with endothelial cells (31). Greater than 97% of the cells stain for steroidogenic enzymes (Skinner, M. K., *et al.*, unpublished observation). The thecal cells were immediately plated in serum-free Ham's F-12 medium (Sigma) containing 0.1% BSA (Sigma) and were maintained at 37 C in a 5% CO_2 atmosphere. The cells were treated for 72 h with either NGF (25 ng/ml; Life Technologies, Inc., Grand Island, NY) or hCG (100 ng/ml; Sigma), and androstenedione and progesterone levels were measured as previously described (32). The sensitivities of the assays employed are 0.01 ng/ml for both steroids. Data were normalized to the DNA content per well as reported previously (32).

Established cultures

NGF release. Bovine thecal cells collected from a mixed-sized population of antral follicles were initially plated in T-75 flasks. Three days later the cells were subcultured into 12-well plates at 4,000, 10,000, and 20,000 cells/well in Ham's F-12 medium containing 0.1% calf serum (HyClone Laboratories, Inc., Logan, UT). The cells were incubated for 24 h, after which the medium was collected and assayed for NGF using the NGF Emax Immunoassay System (Promega Corp., Madison, WI).

PGE_2 and steroid release. Three-day-old primary cultures of bovine thecal cells were subcultured into 6-well plates at a density of 500,000 cells/well in Ham's F-12 medium containing 10% calf serum. The cells were allowed to recover for 72 h before transfecting them with an expression vector containing the coding region of rat trkA (pJM5) under the control of the cytomegalovirus promoter (33) or the plasmid alone (pCMV) as a control. The transfection employed 2 μg/ml of each plasmid and 5 μg/ml Lipofectamine (Life Technologies, Inc.) as the transfecting reagent in 1.0 ml OPTIMEM (Life Technologies, Inc.) (34). After 5 h, the transfection mixture was removed, and Ham's F-12 medium containing 1% calf serum was added to the wells for 24 h to allow expression of the trkA receptor. The medium was then changed to serum-free Ham's F-12 supplemented with 5 μg/ml insulin (Sigma), and the cells were treated with NGF (100 ng/ml), hCG (1 μg/ml), or hCG plus NGF for 1, 2, or 8 h. Each well received 2.5 ml medium to allow repeated sampling (0.5 ml) at the time points indicated. PGE_2, progesterone, and androstenedione released into the medium were measured as previously described (35–37).

Cell proliferation. Three-day-old cultures of bovine thecal cells were subcultured into 24-well tissue culture plates at a density of 10,000 cells/well. Transfections were performed 3 days later with either pJM5 or pCMV, as outlined above, then the medium was changed to Ham's F-12 containing 0.1% calf serum. The following day, growth factors [NGF, BDNF, NT-3,NT-4 (gift from G. Yancopoulos, Regeneron Pharmaceuticals, Inc., Tarrytown, NY), EGF, bFGF (Collaborative Research, Bedford, MA)] were added (100 ng/ml) in serum-free DMEM (Sigma). After 22 h, [^{3}H]thymidine (Amersham Pharmacia Biotech, Arlington Heights, IL) was added (2.5 μCi/well, in 0.5 ml medium), and the plates were incubated for 4 additional h at 37 C. At this time the cells were dissociated in a 0.5-ml solution containing 10 mM Tris (pH 7.5), 5 mM EDTA, 20 mM NaCl, and 0.25% trypsin at 37 C for 15 min. The samples were then frozen at −85 C until mechanical homogenization. The incorporated [^{3}H]thymidine was bound to DE-81 filters, and the filters washed extensively and then counted in 10 ml scintillation fluid as previously reported (38).

Cell line culture

The NIH-3T3 cell line (39, 40) and NIH-3T3 cells stably transfected with a CMV-driven trkA construct (trkA-3T3), resulting in constitutive expression of the trkA receptor (41), were used in this study. The cells were maintained in DMEM containing 10% FCS (HyClone Laboratories, Inc.).

To determine the effect of NGF on PGE_2 release, the cells were plated at 100,000 cells/well in 24-well plates containing 0.5 ml DMEM lacking serum or supplemented with 0.1% FCS. As there was no difference in PGE_2 release between these two conditions the results obtained were

pooled. The PGE_2 concentration in the medium was determined by RIA as previously described (35).

The ability of NGF to transregulate the PG endoperoxide synthase 2 (cyclooxygenase-II; COX-2) gene promoter (COX-2-P) (42) was examined in both native NIH-3T3 cells and trkA-3T3 cells. The cells were transfected with either the promoterless luciferase reporter vector pGL2-Basic (Promega Corp., Madison, WI) or pGL2-Basic containing 2.7 kb of the rat COX-2 gene 5′-flanking region (pCOX-2) (42). The cells were plated into 6-well plates at 350,000 cells/well in DMEM containing 10% FCS. The following day, they were transfected with the reporter pCOX-2 plasmid (at 100 ng/ml) or the vector alone (at 500 ng/ml), using Lipofectamine at 2.5 μl/μg DNA in 1 ml OPTIMEM. In the wells receiving pCOX-2, the total amount of DNA was maintained at 500 ng/ml by adding 400 ng vector alone. All wells also received 20 ng/ml of pCMV·SPORT-βgal (Life Technologies, Inc.) to correct for transfection efficiency. After 5 h of transfection, the DNA mixture was replaced by DMEM containing 1% FCS. The following day growth factors (NGF, BDNF, and NT-3) were added at a concentration of 100 ng/ml. At different intervals thereafter (1, 2, and 24 h), the wells were rinsed with PBS, and the cells were removed into 160 μl cell lysis buffer for luciferase and β-galactosidase assays (43). The luciferase values were normalized using the corresponding β-galactosidase values.

Nucleic acid probes and complementary DNAs (cDNAs)

The antisense RNA probes used in these studies were complementary to the bovine mRNAs encoding NGF, trkA, $p75^{NTR}$, and cyclophilin. Cyclophilin mRNA, which is constitutively expressed in both the rat brain (44) and ovary (6, 7), was used as an internal marker to normalize the results of the ribonuclease (RNase) protection assay. Because no bovine cyclophilin nucleotide sequence has been reported, rat primers were used (see Table 1) to produce a 350-bp bovine cDNA, henceforth referred to as cyclophilin 350. The deduced amino acid sequence of this cDNA is identical to the first 93 amino acids of the known bovine cyclophilin protein (45). Bovine-specific internal primers were then designed based on this sequence (Table 1) and used in PCR to amplify a 107-bp cDNA template. An NGF cDNA fragment was PCR amplified from a bovine NGF cDNA template (provided by R. Heumann, Ruhr Universität, Bochum, Germany). To obtain bovine trkA, $p75^{NTR}$, and COX-2 cDNAs, the cDNAs of two different species were aligned (Table 1), and conserved sequences were selected to design amplifying primers (Table 1). Where only one nonbovine species is listed in Table 1, these primers had been synthesized for other purposes, but there was enough cross-species similarity for the amplification of the bovine sequence. The sizes of the bovine cDNAs generated by PCR are presented in Table 2, along with their accession numbers and the percent similarity to the human and rat sequences.

RT was carried out according to a previously published procedure (46) with the following exceptions: the reverse transcriptase used was Superscript II (200 U; Life Technologies, Inc.), the oligo(deoxythymidine) primer used was a 37-mer (5′-GGATCCAAGC-T_{27}-3′), and the protocol consisted of denaturing 1 μg total RNA at 65 C (5 min), followed by extension at 42 C for 1 h and at 50 C for 30 min. The RNA used to isolate trkA, cyclophilin 350, and COX-2 cDNAs derived from thecal cells collected from medium and large follicles. RNA from bovine basal forebrain (donated by N. H. McArthur and P. G. Harms, TX A&M University, College Station TX), was used to isolate a $p75^{NTR}$ cDNA.

The PCR reactions were started with an initial denaturation at 95 C for 5 min ($p75^{NTR}$ and COX-2) or 7 min (all others) of the template (either 2 μl RT reactions or 5–10 ng plasmid NGF or cyclophilin 350 cDNAs) in the presence of 1 × *Taq* buffer, 1.25 mM $MgCl_2$ (Promega Corp., Madison, WI), and deoxynucleotides [200 μM each of deoxy (d)-ATP, dCTP, dGTP, and dTTP; Amersham Pharmacia Biotech, Inc., Piscataway, NJ]. After the initial denaturation step, the primers (see Table 1 for sequence and amount) and *Taq* polymerase (2.5 U; Promega Corp.) were added to the reaction tube in a final reaction volume of 100 μl. There were three PCR protocols used: 1) NGF and cyclophilin 107 (94 C for 4 min, followed by 35 cycles of 94 C for 15 sec, 55 C for 1 min, and 72 C for 2 min), 2) trkA and cyclophilin 350 (95 C for 2 min, 55 C for 3 min, and 72 C for 10 min; followed by 35 cycles of 92 C for 1 min, 55 C for 2 min, and 72 C for 3 min), and 3) $p75^{NTR}$ and COX-2 (95 C for 45 sec, 57 C for 10 min, and 72 C-10 min, followed by 35 cycles of 95 C for 45 sec, 57 C for 2 min, and 72 C for 2 min; the annealing temperature for COX-2 was 55 C). The PCR products were cloned into the pGEM-T vector (Promega Corp.) and sequenced (trkA, $p75^{NTR}$, COX-2, and cyclophilin). In the case of NGF three internal restriction enzyme sites were used to confirm the identity of the cDNA.

As in rats and humans, bovine trkA appears to occur in two forms, either with or without an 18-bp insert in the extracellular domain (47). The neuronal isoform, which includes the insert (isoform II) (47), has been shown to exhibit greater responsiveness to NT-3 (48). The neuronal form of the receptor was cloned from thecal cells and used in these experiments. The complementary RNA (cRNA) transcripts generated from these templates were radiolabeled with [^{32}P]UTP for RNase protection assay and with [^{35}S]UTP for hybridization histochemistry (trkA and NGF). Preparation of templates for transcription and the transcription procedure itself were performed as previously reported (49, 50).

RNase protection assay (RPA)

Total RNA for RPA was prepared by the phenol-extraction method for tissues (51) or cells (52). The RPA was carried out according to the method of Gilman *et al.* (53), as previously described (54). Sense RNA standards were prepared using the templates described above. The sense RNAs were transcribed, purified, and quantified according to published procedures (54). The ^{32}P-labeled cRNAs of interest were simultaneously hybridized to total RNA extracted from ovaries or cells. After RNase digestion, the protected species were isolated by PAGE, visualized, and analyzed as previously reported (54).

Hybridization histochemistry

The procedure employed was based on the method of Simmons *et al.* (55) with modifications as previously reported (56). Cellular expression of trkA and NGF mRNAs was determined in ovaries collected at the abattoir and immediately fixed by immersion in 4% paraformaldehyde-0.1 M sodium borate buffer, pH 9.5 (overnight at 4 C). The ovaries were embedded/frozen in OCT compound (Miles, Inc., Elkhart, IN). The

TABLE 1. Primer sequences used for PCR of cDNAs

Probe	Primer	Primer sequence	pmol	Species
NGF	5′-Sense	ACAGGAGCAAGCCCTCCT	50	Bovine
	3′-Antisense	GCCTGCTTGCCGTCCAT	50	Bovine
TrkA	5′-Sense	GAGGTGCTGGGCAGAGAA	100	Human/rat
	3′-Antisense	CTTCCGCATTTGTTGAGCA	100	Rhesus
$p75^{NTR}$	5′-Sense	AGCCAACCAGACCGTGTGTGA	50	Human/rat
	3′-Antisense	GTCCTGGCAGGAGAACACGAG	50	Human/rat
Cyclophilin (350 bp)	5′-Sense	CTTTGCAGACGCCGCTGTCTCTTTTCGCCG	100	Rat
	3′-Antisense	GCATTTGCCATGGACAAGATGCCAGGA	100	Rat
Cyclophilin (107 bp)	5′-Sense	GTTCCAAAGACAGCAGA	25	Bovine
	3′-Antisense	CCCTGGCAGATAAATCC	25	Bovine
COX-2	5′-Sense	AGCAAATCCTTGCTGTTCCAA	100	Rat
	3′-Antisense	CAATGTTCCAGACTCCCTTGA	100	Rat

TABLE 2. Description and percent similarity of bovine cDNAs to human and rat cDNA

Bovine cDNA	Size (bp)[a]	Accession no. (nucleotides)[b]	Similarity to human/rat[c]					
			Human (%)	nt[d]	Accession no.	Rat (%)	nt[d]	Accession no.
NGF	304	M26809 (2–305)	90	519–822	X52599	84	639–942	M36589
TrkA	540	AF228019 (I-503)	82	927–1414	M23102	80	874–1382	M85214
$p75^{NTR}$	247	AF228020 (1–205)	93	308–512	M14764	84	311–515	X05137
Cyclophilin	350	AF228021 (1–288)	93	8–295	Y00052	90	36–323	M19533
Cyclophilin	107	AF228021 (92–198)	94	99–205	Y00052	94	127–233	M19533
COX-2	212	AF031698 (181–350)	88	169–338	M90100	85	196–365	U03389

[a] cRNA probe size, includes cross-species primers (see Table 1).
[b] Sequences exclude cross-species PCR primers.
[c] Calculated using the bovine sequences without the cross-species primers (see Table 1).
[d] Position in reported mRNA sequence.

hybridization was performed on 10-μm cryostat sections (56). Control sections were incubated with sense trkA or NGF RNA probes.

Immunohistochemistry

TrkA and NGF. Immunohistochemical detection of trkA and NGF was performed in 14-μm cryostat sections from ovaries collected and fixed at the abattoir. The ovaries were fixed by immersion in Zamboni's fixative and embedded/frozen in OCT compound as previously described (7) and processed for trkA and NGF immunohistochemistry using the polyclonal antisera trk 763 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and K-596 (6, 9) to identify trkA and NGF, respectively. According to the manufacturer, antibody trk 763 specifically recognizes trkA, without cross-reacting with other trk receptors. We have previously shown that antibody K-596 preferentially recognizes NGF (6). Tissue sections were incubated overnight at 4 C with the antibodies at 2 μg/ml (trk 763) or at a 1:500 dilution (K-596), and the immunoreaction was developed the next day with diaminobenzidine (Sigma) as previously described (6).

BrdU

Rat ovaries were immersion-fixed in Carnoy's fixative (57) for 15–18 h, transferred to 70% ethanol, embedded in paraffin, and serially sectioned at 4 μm. Paraffin was removed from the sections by immersion in xylene, followed by rehydration in a graded series of ethanol before a 30-min treatment at 37 C with 2 N HCl to increase the accessibility of the DNA to the antibody (58). The sections were then incubated with a monoclonal antibody to BrdU (diluted at 1:1000; Sigma) overnight at 4 C, and the immunoreaction was developed the next day as outlined above. The sections were lightly counterstained with Gill's hematoxylin.

Data analysis

The differences in mRNA levels, PGE_2 release, and luciferase activity (relative light units) were analyzed using one-way ANOVA and Student-Newman-Keuls multiple test for individual means. Percentages were first subjected to the arcsine transformation before applying a one-way ANOVA followed by the Student-Newman-Keuls multiple test for individual means or the least significant difference test for multiple comparisons.

Results

The bovine ovary contains all components of the NGF ligand receptor system and preferentially expresses the neuronal form of trkA

The presence in the bovine ovary of each of the mRNAs encoding the three components of the NGF ligand receptor system was determined by RPA using total RNA from granulosa cells and thecal cells of small (< 5 mm), medium (5–10 mm), and large (>10 mm) follicles. Control tissues consisted of brain tissue from the cerebral cortex (negative control for trkA) and basal forebrain (positive control for trkA). The basal forebrain showed abundant levels of the neuronal form of trkA mRNA (form II) (47) and $p75^{NTR}$ mRNA, but contained much lower levels of NGF mRNA (Fig. 1, *left panel*). As expected, the cerebral cortex showed no trkA mRNA, low levels of $p75^{NTR}$ mRNA, and a NGF mRNA content similar to that present in the basal forebrain.

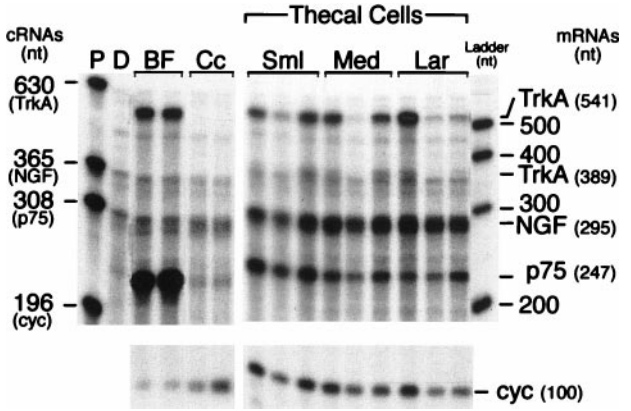

FIG. 1. Detection of NGF, trkA, and $p75^{NTR}$ mRNAs in thecal cells from bovine ovarian antral follicles of different sizes. Total RNA samples from the basal forebrain (BF) and cerebral cortex (Cc) were used as positive controls. Four ^{32}P-labeled cRNAs (trkA, 630 nucleotides; NGF, 365 nucleotides; $p75^{NTR}$, 308 nucleotides; cyclophilin; 196 nucleotides) were hybridized simultaneously with samples containing 10 μg total RNA. The protected fragments are as follows: neuronal trkA mRNA, 541 nucleotides; nonneuronal trkA mRNA, 389 nucleotides; NGF mRNA, 295 nucleotides; $p75^{NTR}$ mRNA, 247 nucleotides; and cyclophilin (cyc) mRNA, 100 nucleotides. P, Undigested cRNA probes; D, digested probes; Sml, small follicles (<5 mm); Med, medium follicles (5–10 mm); Lar, large follicles (>10 mm).

All three (NGF, trkA, and $p75^{NTR}$) mRNAs were present in the ovary (Fig. 1, *right panel*). Thecal cells from small, medium, and large follicles showed considerable variation in their content of trkA mRNA, whereas NGF and $p75^{NTR}$ mRNAs were more uniformly expressed. The neuronal form (form II) was detected as a 541-nucleotide band, and the nonneuronal form (form I) as a 389-nucleotide fragment (Figs. 1 and 2). Surprisingly, not only thecal cells but also granulosa cells contained trkA mRNA, which, as in thecal cells, appeared to be predominantly expressed as the neuronal form (Fig. 2). In contrast to trkA, NGF and $p75^{NTR}$ were selectively expressed in thecal cells (Fig. 2). No apparent differences in trkA, NGF, and $p75^{NTR}$ mRNA expression

related to either follicle size or the ovarian compartment in which the mRNAs were detected (Figs. 1 and 2). Likewise, the three mRNAs were equally expressed in the theca interna and externa of small, medium, and large follicles (Fig. 2).

Hybridization histochemistry confirmed the selective expression of NGF mRNA in the thecal compartment (Fig. 3A, *arrowheads*) and the presence of trkA mRNA in both thecal (*arrowheads*) and granulosa cells (*arrows*; Fig. 3C). The expression of both mRNAs did not appear to be limited to a subpopulation of thecal cells, but instead it was diffusely present throughout the thecal compartment. Adjacent sections incubated with the respective sense RNA probes showed no specific hybridization (Fig. 3, B and D, respectively). In contrast to the exclusive localization of NGF mRNA to thecal cells, immunohistochemical detection of the NGF protein showed its presence in both granulosa (*arrows*) and thecal cell (*arrowheads*) compartments of antral follicles (Fig. 4A). Preabsorption of the antibody with purified mouse NGF decreased, but did not eliminate, the immunoreactivity (Fig. 4B). In agreement with the *in situ* hybridization results, trkA immunoreactive material was detected in both granulosa (*arrows*) and thecal cells (*arrowheads*, Fig. 4C). Preabsorbing the trkA antibody with the peptide used to generate it resulted in a substantial reduction in immunoreactive material in both follicular compartments (Fig. 4D).

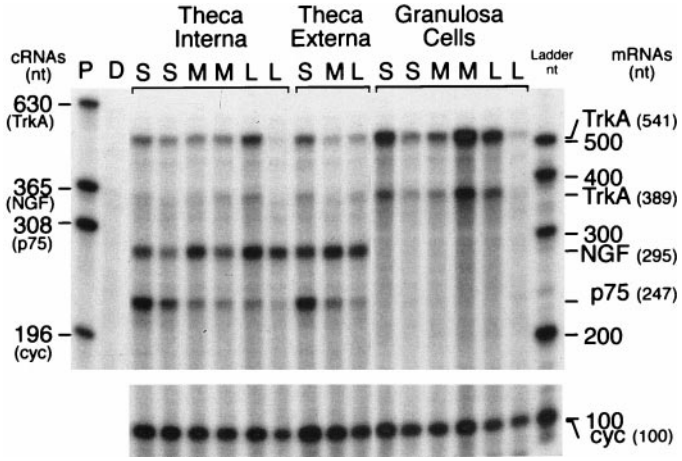

FIG. 2. The mRNAs for NGF, trkA, and $p75^{NTR}$ are expressed in both the theca interna and externa of bovine ovarian follicles of different sizes, whereas granulosa cells only contain the neuronal and nonneuronal forms of trkA mRNA. P, Undigested probes; D, digested probes; S, small follicles; M, medium follicles; L, large follicles; cyc, cyclophilin mRNA.

Expression and inducibility of the NGF ligand-receptor system in thecal cells in vitro

When thecal cells were placed in culture, there was a precipitous loss of trkA mRNA, which decreased substantially after only 24 h in culture, becoming undetectable thereafter (Fig. 5). Although the level of $p75^{NTR}$ mRNA declined gradually after plating, it was clearly detectable even after 18

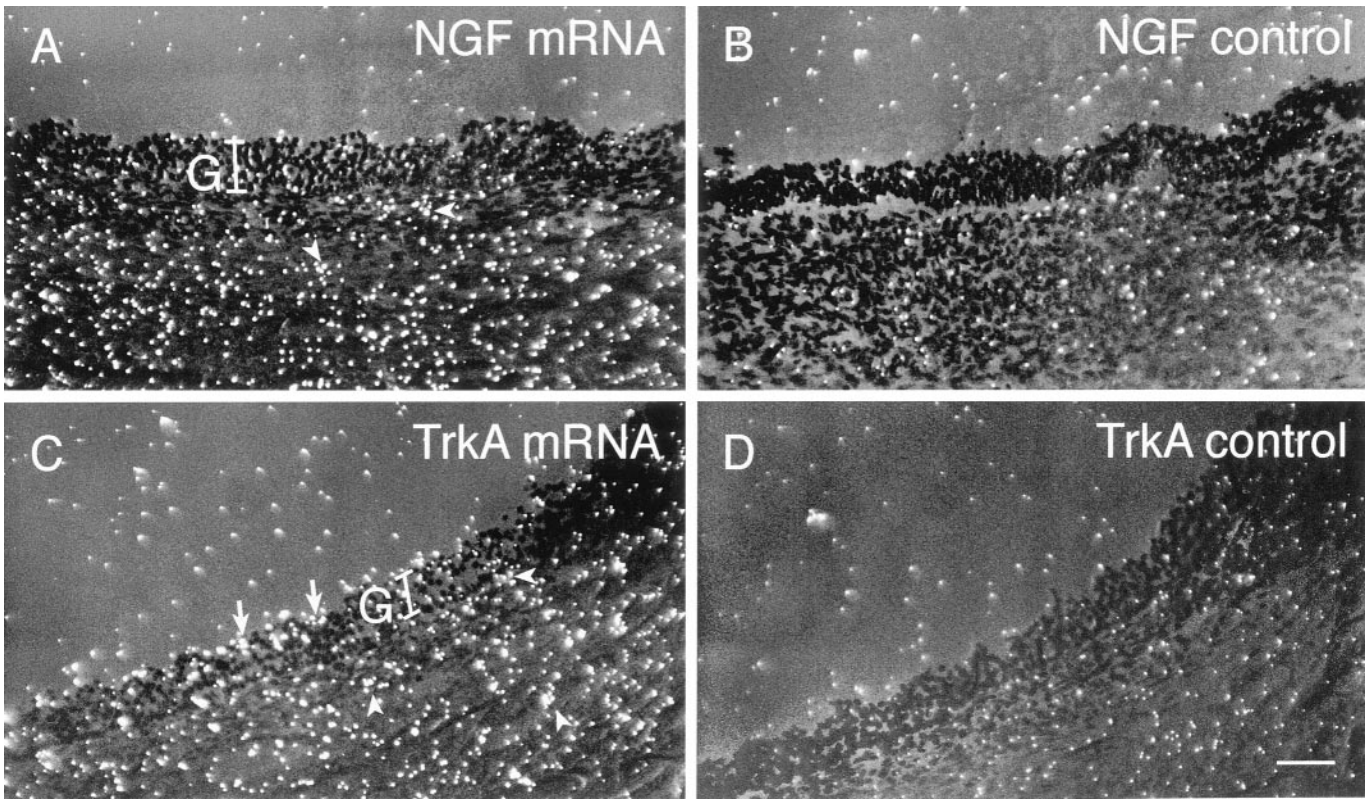

FIG. 3. Localization of NGF and trkA mRNA in bovine ovarian follicles by hybridization histochemistry. A, NGF mRNA is only present in the thecal compartment (denoted by *arrowheads*). B, Adjacent control section hybridized with the ^{35}S-labeled sense NGF RNA. C, TrkA mRNA is present in both the theca (examples denoted by *arrowheads*) and granulosa cell (examples denoted by *arrows*) compartments of the follicle. D, Control adjacent section hybridized with the ^{35}S-labeled sense trkA RNA. G, Granulosa cell layer. *Bar*, 50 μm.

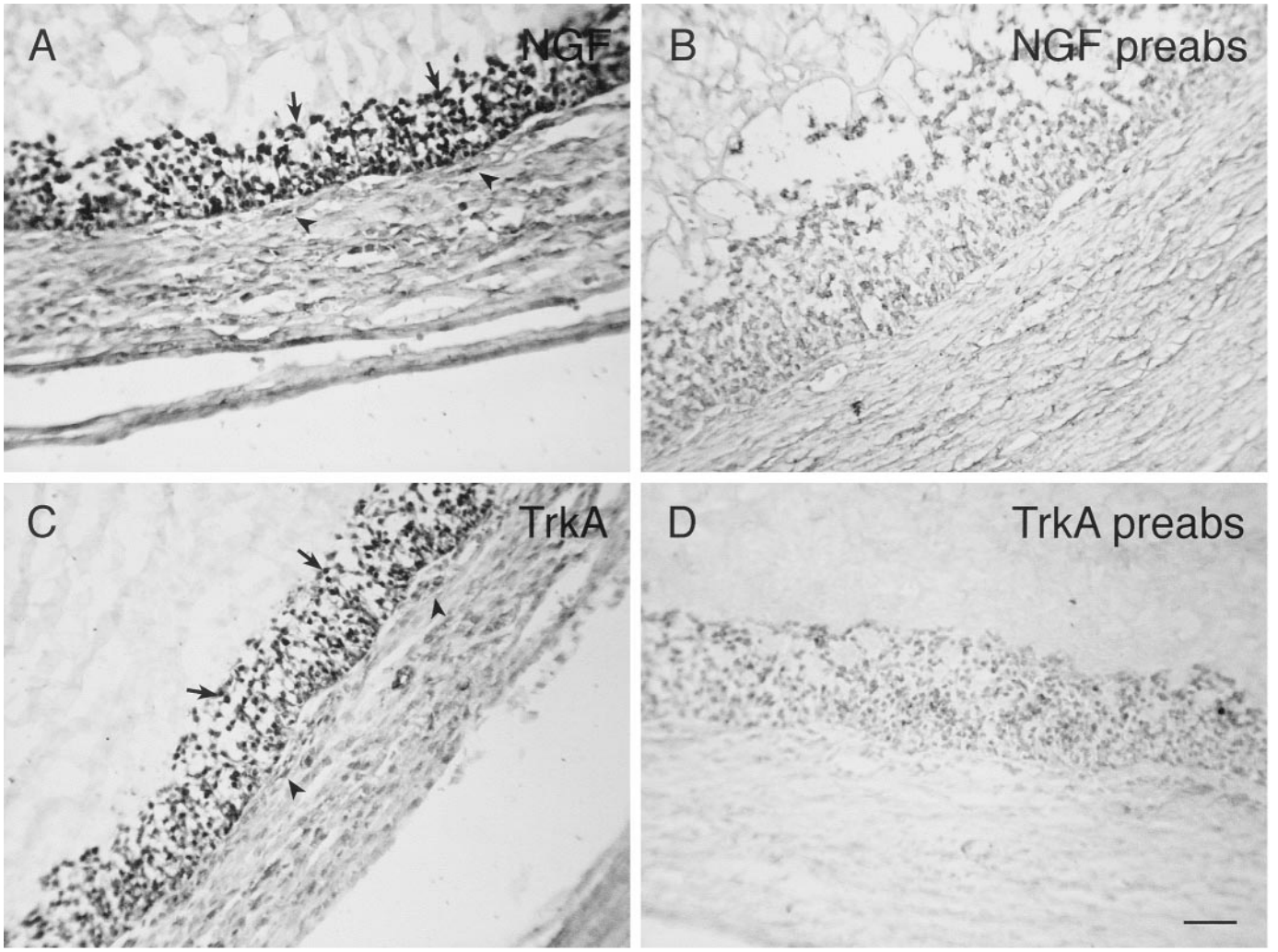

FIG. 4. Localization of NGF and trkA by immunohistochemistry in bovine ovarian follicle. A, NGF immunoreactive material is more abundant in granulosa than thecal cells (*arrowheads*). B, Control section incubated with antigen-preabsorbed NGF antiserum. C, TrkA receptor-like immunoreactive material is present in both thecal (*arrowheads*) and granulosa (*arrows*) cell compartments of the follicles. D, Control section incubated with antigen-preabsorbed trkA antiserum. *Bar*, 50 μm.

days of culture. In contrast, NGF mRNA remained at high levels throughout the duration of the culture (18 days; Fig. 5). The ability of thecal cells to make and release NGF was demonstrated by its presence in the culture medium, as measured by enzyme-linked immunosorbent assays. As shown in Fig. 6, the amount of NGF released to the medium was directly proportional to the number of cells plated per well.

To determine whether the *in vitro* loss of trkA mRNA expression is due to withdrawal of gonadotropin support, thecal cells in culture were treated with hCG, which in rats is an effective stimulator of trkA gene expression (6). Exposure to hCG 24 h after replating 3-day-old primary cultured bovine thecal cells, increased trkA mRNA within 3 h of exposure to the gonadotropin (Fig. 7). The levels remained elevated at 6 h, but returned to basal values by 24 h. In contrast to this increase, no up-regulation of NGF mRNA levels was observed in the same cultures (Fig. 7). Likewise, hCG did not influence the expression of $p75^{NTR}$ mRNA (not shown).

NGF stimulates androstenedione and progesterone secretion in freshly plated, high density, thecal cell cultures

Cell-cell contact and an unaltered thecal cell phenotype appear to be necessary for thecal cells to respond to NGF with steroid release. Freshly plated, high density cultures responded to NGF with an increased release of both androstenedione and progesterone after 72 h of treatment (Fig. 8, A and C). In contrast, cells plated at low density did not respond to NGF treatment with steroid release (Fig. 8, B and D). Both high and low density cultures responded equally well to hCG (Fig. 8). Thecal cells treated with NGF or hCG for 8 and 24 h 6 days after plating failed to release either progesterone or androgens in response to either challenge (not shown).

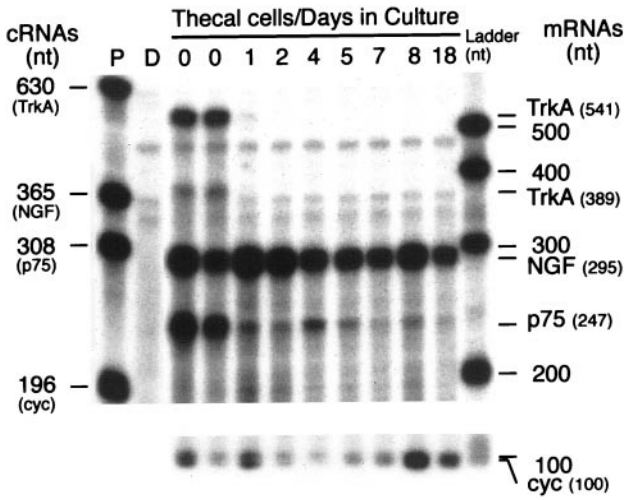

FIG. 5. The effect of time in culture on the content of NGF, trkA and $p75^{NTR}$ mRNAs of isolated thecal cells as assessed by RPA. Thecal cells were isolated from small, medium, and large follicles. P, Undigested probes; D, digested probes.

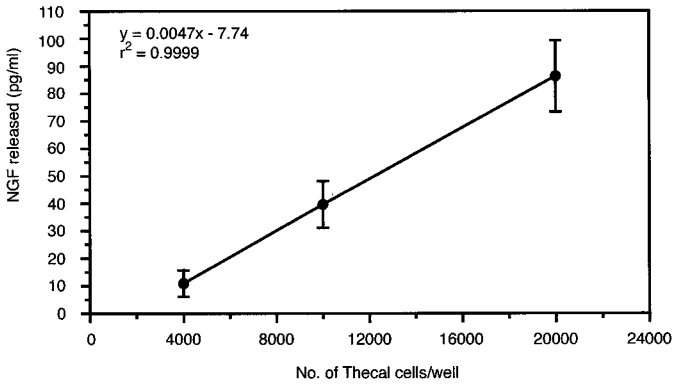

FIG. 6. Linear relationship between the amount of NGF released and the number of thecal cells plated per well. Thecal cells were isolated from small, medium, and large follicles. The medium was collected for NGF assay 24 h after cell plating. Each *point* represents the mean of eight independent observations ± SEM.

NGF stimulates PGE_2 release from thecal cells via activation of trkA receptors

The effect of NGF on PGE_2 release was determined in thecal cells transfected with pCMV, the control plasmid or with the pJM5 trkA-expressing plasmid. Control cells transfected with pCMV did not release PGE_2 in response to NGF (Fig. 9A, *left panel*). In contrast, the presence of the trkA receptor resulted in a rapid increase in PGE_2 release after 1 h of treatment (Fig. 9A, *right panel*). The effect was still evident by 2 h, but at 8 h basal PGE_2 release had increased to levels that were not further modified by NGF treatment. Control cells responded to hCG treatment with PGE_2 release only after 8 h of treatment, and NGF did not potentiate the hCG effect (Fig. 9B, *left panel*). In contrast, trkA-expressing cells responded to the gonadotropin within 1 h. PG levels remained elevated for the duration of the treatment (8 h; Fig. 9B, *right panel*). NGF added in conjunction with hCG potentiated the early effect of hCG on PGE_2 release, *i.e.* after 1 h of exposure, but not the effect at 2 and 8 h (Fig. 9B, *right panel*).

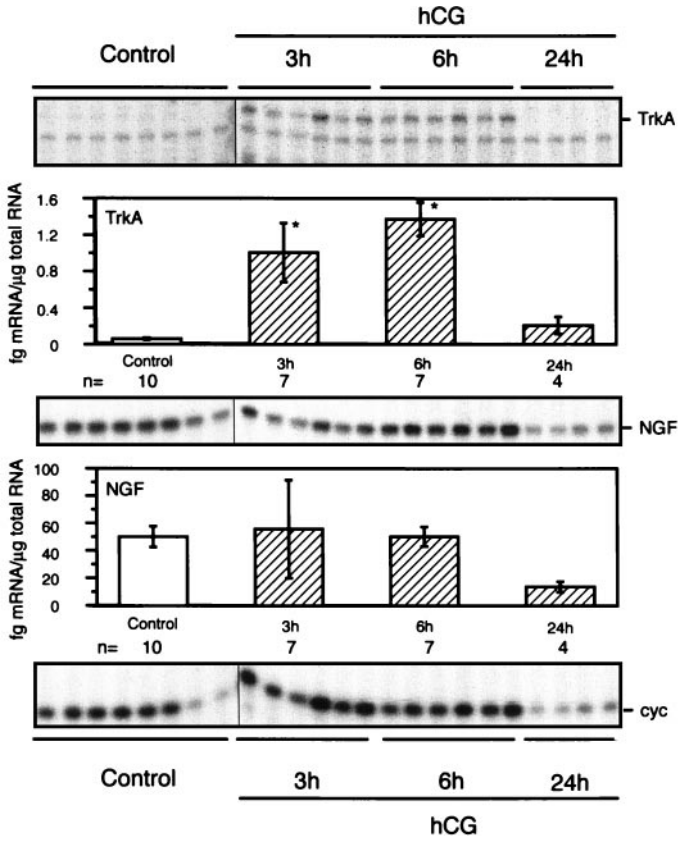

FIG. 7. hCG increases trkA, but not NGF mRNA levels in isolated bovine thecal cells. Thecal cells were isolated from small, medium, and large follicles. The cells were treated with hCG (1 µg/ml) in serum-free Ham's F-12 medium supplemented with insulin (5 µg/ml) for 3, 6, and 24 h. TrkA and NGF mRNA content was determined by RPA. The values obtained after comparing the hybridization signals with those of known amounts of sense RNA standards were normalized using the levels of the cyclophilin mRNA found in each sample. The sizes of the protected fragments were the same as represented in Figs. 1, 2, and 5.

NGF stimulates PGE_2 release regardless of changes in cyclooxygenase-2 gene expression

Cyclooxygenase is the rate-limiting enzyme in PG synthesis. Although COX-2, the inducible form of the enzyme (59) has been reported to be expressed only in granulosa cells of the bovine ovary (60), RT-PCR experiments in which COX-2 mRNA was amplified from both thecal and granulosa cells and sequenced to confirm identity (Table 2) demonstrated that the mRNA is present in thecal cells as well as granulosa cells. Although the COX-2 mRNA detected in thecal cells could be due to contamination with granulosa cells, the presence of COX-2 mRNA in bovine thecal cells has been previously reported by others (61).

To further characterize the role of trkA in NGF-induced PGE_2 release, NIH-3T3 cells ectopically expressing trkA receptors (trkA-3T3) (41) were used. Like thecal cells expressing trkA, trkA-3T3 cells responded to NGF with an increase in PGE_2 release within 1 h of exposure (Fig. 10). To determine whether this acute effect was accompanied by an NGF-dependent increase in transcriptional activity of the COX-2 gene, native NIH-3T3 cells and trkA-3T3 cells were transiently transfected with a

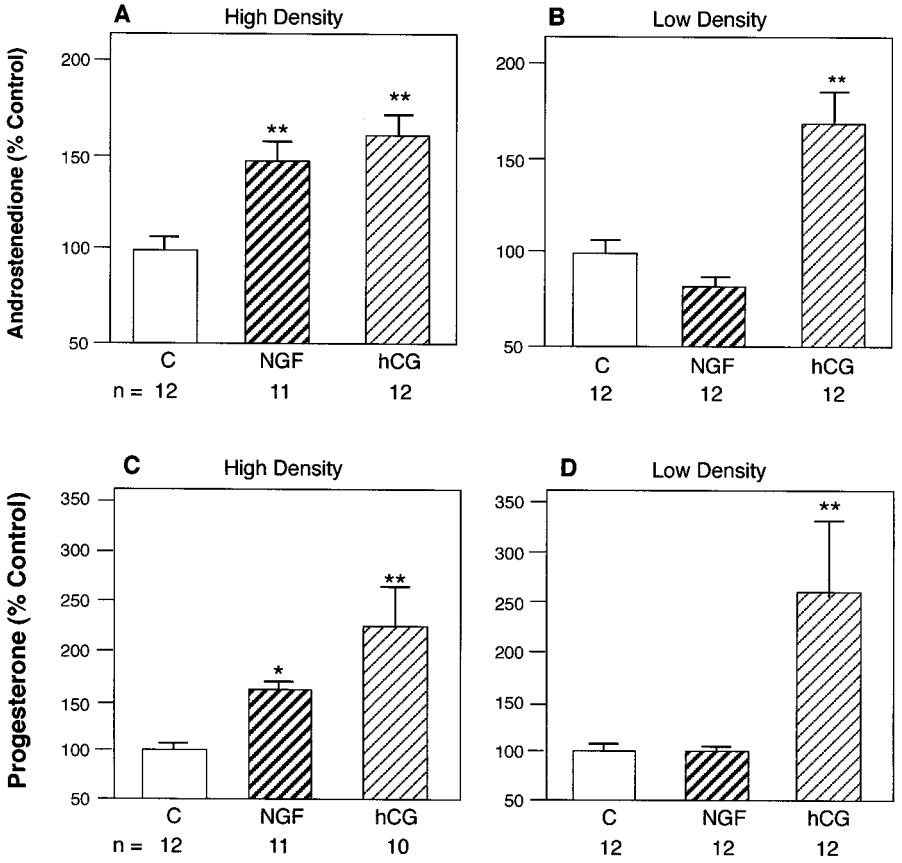

FIG. 8. Effect of NGF and hCG on androstenedione and progesterone production by freshly plated thecal cells seeded at high or low density. Thecal cells were isolated from small, medium, and large follicles. The steroid levels were normalized according to the DNA content of each well and expressed as the percent change from the values obtained in untreated controls (C). NGF, 25 ng/ml; hCG, 100 ng/ml. *, $P < 0.05$; **, $P < 0.01$ (*vs.* control).

luciferase reporter construct driven by the rat COX-2 promoter (COX-2-P) or the promoterless reporter plasmid alone (pGL2). Twenty-four hours later the cells were exposed to NGF for a short (1 and 2 h) or a long (24 h) period of time. As shown in Fig. 10, B and C, the COX-2-P was very active in 3T3 cells, inducing more than a 10-fold increase in luciferase activity 24 h after transfection and a 25-fold increase at 48 h. The short-term exposure to NGF did not alter COX-2-P activity. In contrast, after the 24-h exposure, basal COX-2-P activity was significantly augmented by NGF. The increase in basal COX-2-P activity may be due to endogenous NGF, which has been shown to be produced by these cells (12). This effect was specific, as it was obliterated by treating the cells with a neutralizing NGF antiserum (Fig. 10C). The stimulatory effect of NGF on COX-2-P activity was not seen in native NIH-3T3 cells (Fig. 10D) and was not reproduced by either NT-3 or BDNF (Fig. 10D) in trkA-3T3 cells (Fig. 10D).

Gonadotropin-induced ovulation in rats is preceded by an increased proliferative activity of thecal cells

To assess the hypothesis that some cells of the thecal compartment of periovulatory follicles undergo proliferation before ovulation (28, 29), immature rats were treated with PMSG followed by hCG 48 h later. The animals were then injected with a single dose of BrdU at several intervals after hCG and 2 h before removing the ovaries for histological examination. As shown by others (62), the nuclei of many granulosa cells in antral follicles of mature ovaries from

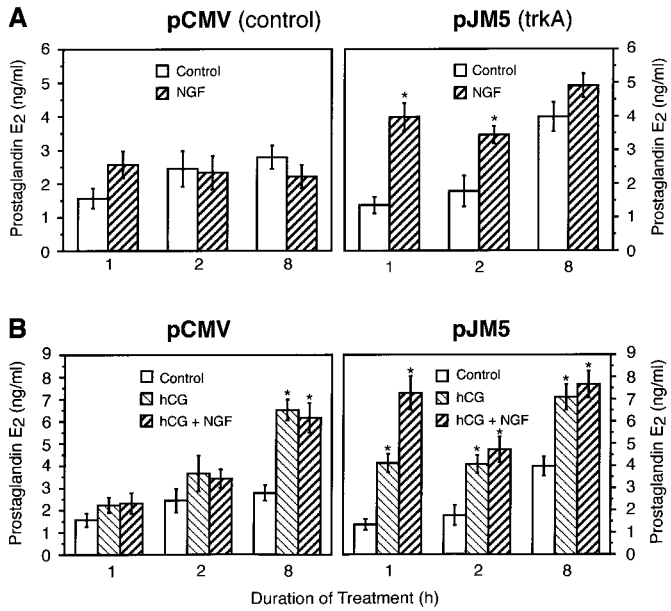

FIG. 9. NGF stimulates PGE_2 release from thecal cells isolated from small, medium, and large follicles expressing the trkA receptor. Thecal cells (plated at 500,000 cells/well in 6-well plates) were transfected with either pCMV or with pJM5. After 24 h the cells were treated with 100 ng/ml NGF (A), hCG (B; 1 μg/ml), or hCG in combination with NGF. In both A and B, PGE_2 levels were determined in the medium collected at 1, 2, or 8 h. *, $P < 0.05$ *vs.* control for that time period (n = 6 for all groups).

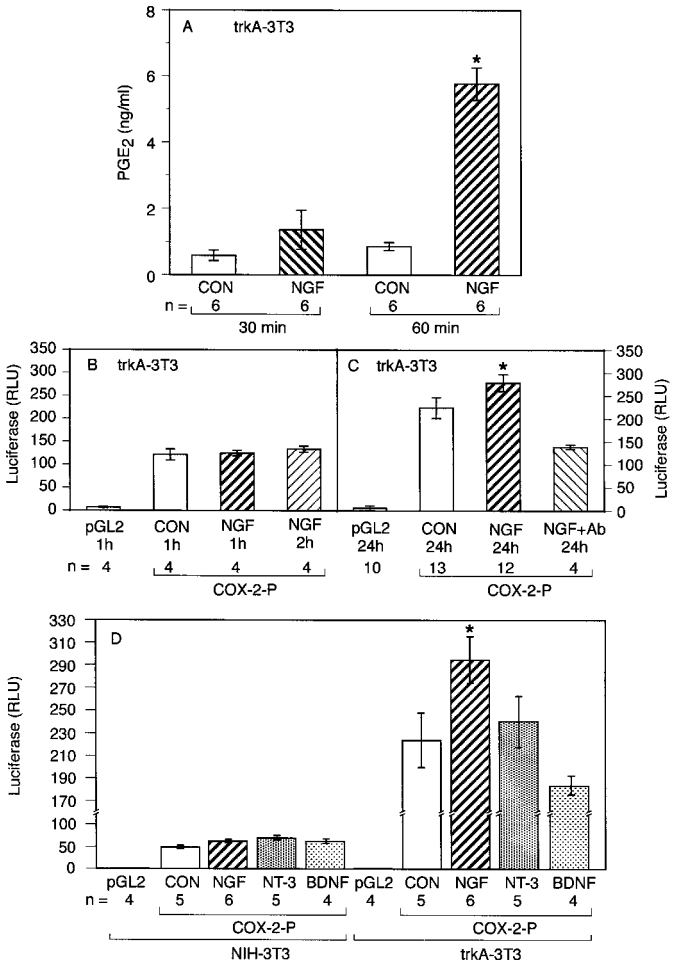

FIG. 10. A, NIH-3T3 cells stably expressing the trkA receptor (trkA-3T3) release PGE_2 in response to NGF (50 ng/ml) within 1 h of exposure to NT. B, Short-term exposure to NGF (100 ng/ml) did not alter COX-2-P activity in trkA-3T3 cells transiently transfected with a luciferase reporter gene (pGL2) driven by the rat COX-2-P. The cells were treated with NGF 24 h after transfection. C, Long-term (24-h) exposure to NGF *trans*-activates the COX-2-P, and the effect is blocked by NGF antibodies. Both treatments (NGF and NGF plus antiserum) were initiated 24 h after transfection with the reporter plasmid. D, NGF activates the COX-2-P in NIH-3T3 cells expressing the trkA receptor, but not in native NIH-3T3 cells. Native and trkA-expressing NIH-3T3 cells were transiently transfected with either plasmid alone (pGL2) or COX-2-P, and after 24 h they were treated with NGF, NT-3, or BDNF (at 100 ng/ml each for 24 h). Luciferase activity is reported as relative light units (RLU). *, $P < 0.05$ *vs.* TrkA-3T3, pCOX-2 control (CON).

saline-treated animals showed abundant BrdU immunoreactivity (Fig. 11, A and B, *arrowheads*). Treatment with hCG inhibited this activity for at least 8 h (Fig. 11, C and D). In contrast, a subset of thecal compartment cells, which exhibited modest proliferative activity in untreated rats (Fig. 11, A and B), become proliferative 10–12 h after hCG administration, near the time of ovulation (Fig. 11, E and F, *arrows*).

NGF induces proliferation of thecal cells via activation of trkA receptors

The ability of NGF to induce proliferation of thecal compartment cells, was determined in cells collected from a mixed-sized population of antral follicles. The cells, which had lost their complement of trkA receptors after 6 days in culture, were transiently transfected with the trkA expression plasmid, stimulated with NGF for 22 h, and exposed to [^{3}H]thymidine for 4 h to estimate their proliferative activity. These experiments were repeated several times, each time with a new culture of cells. After transfection, the different batches of cells exhibited one of two different responses to the presence of trkA receptors. 1) Cells transfected with pJM5 and not treated with NGF did not increase their basal rate of proliferation over that of cells transfected with plasmid alone (Fig. 12A, *inset*). These cells responded to NGF or NT-3 with an increased rate of [^{3}H]thymidine incorporation (Fig. 12A). 2) Cells in which transfection with the trkA expression vector (pJM5) increased their basal rate of proliferation in the absence of added NGF (Fig. 12B, both panels). These cells did not respond to either NGF or NT-3 with a further increase in proliferation. Both sets of cells responded equally well to EGF stimulation (Fig. 12A, *left* and *right panels*), but failed to proliferate in response to NT-4 or BDNF. As before, native NIH-3T3 cells did not respond to any NT with proliferation (Fig. 12B). Both cell types responded equally well to the mitogenic peptide bFGF (Fig. 12B, *left panel*).

Discussion

This study describes the presence of NGF and its two receptors in the bovine ovary, and demonstrates that NGF can act on isolated thecal compartment cells to initiate three major events associated with the ovulatory process: progesterone secretion, PGE_2 production, and cell proliferation. Our results show that although each of these effects requires the presence of trkA, the high affinity tyrosine kinase NGF receptor, activation of steroid release also depends on the cytodifferentiation status of the target cell (see below).

A potential involvement of trkA receptors in ovulation was initially suggested by studies performed in rats, which demonstrated that trkA receptors are localized to the thecal-interstitial compartment of periovulatory follicles and that their expression increases more than 100-fold during the hours preceding the first ovulation (6). The ability of hCG to rapidly increase trkA receptor expression in ovarian cell dispersates (6) indicated that a significant fraction of the preovulatory increase in trkA mRNA abundance is a LH-dependent phenomenon. Activation of ovarian trkA receptors appears to contribute to the cascade of events leading to ovulatory rupture, as pharmacological blockade of trkA signaling or immunoneutralization of NGF actions reduced the incidence of ovulation in response to gonadotropins (6).

By showing that NGF can act directly on purified thecal cells containing trkA receptors to activate signaling pathways important for ovulation, the present results offer additional evidence in support of a facilitatory role for NGF in the ovulatory process. They are also in harmony with the view that the ovarian thecal compartment represents a physiological, nonneuronal target for NGF action (6, 34, 63) and emphasize the emerging concept that NTs exert pleiotropic actions throughout the organism, and that, within the endocrine system, they target discrete subpopulations of endocrine cells (5).

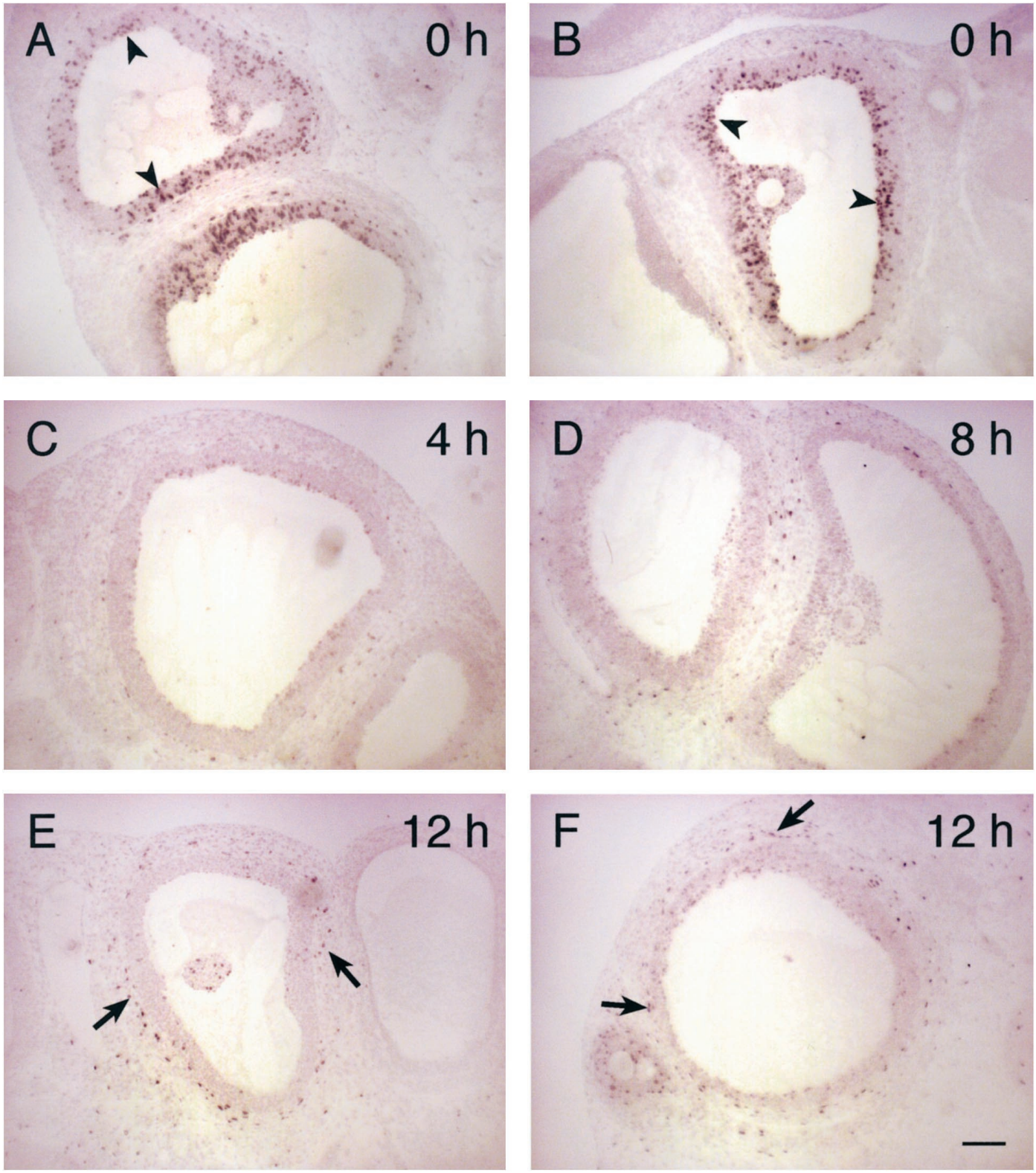

FIG. 11. Immunohistochemical detection of BrdU incorporated into the nuclei of follicular cells from PMSG-primed, hCG-treated rats. Collection of tissues was initiated 48 h after treatment with PMSG; BrdU was administered ip 2 h before collection of ovaries. Ovaries were collected at 0 h (A and B), 4 h (C), 8 h (D), and 12 h (E and F) after hCG. Notice the abundance of BrdU-positive granulosa cells before hCG administration (*arrowheads* in A and B) and the appearance of positive thecal cells 12 h after hCG (*arrows* in E and F). *Bar*, 100 μm.

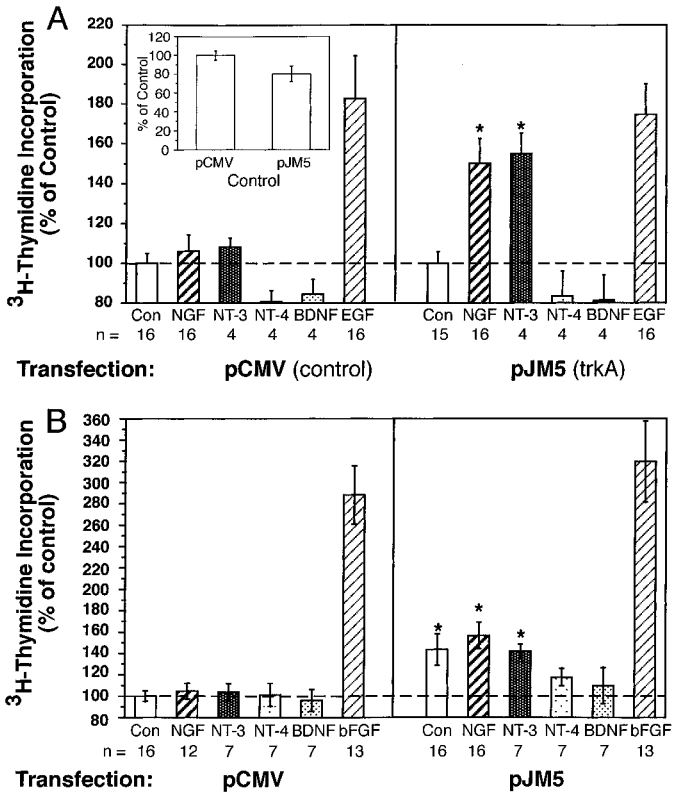

FIG. 12. NGF induces proliferation in thecal cells, isolated from small, medium, and large follicles, expressing trkA receptors. Thecal cells (plated at 10,000 cells/well in 24-well plates) were transiently transfected with either the pCMV control plasmid or the trkA-expressing plasmid pJM5 and immediately treated with the growth factors shown in the figure. Proliferation was assessed by measuring [^{3}H]thymidine incorporation. Epidermal growth factor and basic fibroblast growth factor, two known mitogenic growth factors, were used as positive controls. A, Summary of 4 experiments in which transient expression of trkA receptors in the absence of NTs did not result in increased proliferation (*inset*). To emphasize this result, the values obtained in cells transfected and treated with the different growth factors were expressed as a percentage of the basal mitogenic activity detected in cells transfected with either pCMV or pJM5, but otherwise left untreated. B, Summary of 4 experiments in which transient expression of trkA receptors resulted in an increased incorporation of [^{3}H]thymidine in the absence of exogenous growth factor treatment. To better demonstrate this observation all experimental values are expressed as a percentage of the values detected in cells transfected with the control pCMV plasmid. *, $P < 0.05$ *vs.* pCMV control.

In keeping with previous observations made in rat ovaries (6), bovine thecal cells were found to contain both trkA mRNA and trkA immunoreactive material. Separation of the thecal compartment into theca interna and externa revealed that both subcompartments of the follicular wall contain the receptors; RNase protection assays, complemented by *in situ* hybridization experiments, revealed the presence of trkA receptors in antral follicles of all sizes collected. Although this may indicate the existence of a fundamental difference between bovine and rat ovaries, in which trkA mRNA becomes abundant only during the hours preceding ovulation (6), we were not able to analyze bovine periovulatory dominant follicles in which such an increase would have been apparent. Thus, antral bovine follicles may simply express higher trkA receptor levels during development than rat follicles and still show a substantial increase at some point during the 22–30 h window elapsing between the LH surge and ovulatory rupture in this species (60, 61, 64). A surprising finding was the detection of trkA mRNA and immunoreactive trkA protein in granulosa cells of bovine follicles, and the presence of the receptors in the granulosa cell compartment of all size follicles examined. This localization, verified by RPA, *in situ* hybridization, and immunohistochemistry, is in contrast to the highly localized expression of trkA in the thecal-interstitial compartment of the rat ovary (6) and the apparent absence of trkA mRNA expression in granulosa cells of the rhesus monkey ovary, assessed by the sensitive method of RT-PCR (Dissen, G. A., C. L. Chaffin, and S. R. Ojeda, unpublished data). We do not know what the functions of NGF in bovine granulosa cells might be, but it is possible that they are particular to this species. NGF acting via trkA receptors in bovine granulosa cells may affect functions not specifically related to ovulation and/or required for ovulation to occur. Although it could be argued that trkA in granulosa cells may be relevant to the fact that cows are a monoovulatory species, nonhuman primates are also monoovulatory, and yet their ovarian granulosa cells do not contain the receptor. Alternatively, trkA may play a role in processes such as those underlying the regulation of granulosa cell differentiation by thecal cells in this species (65) and/or the maintenance of basal levels of PG synthesis within antral follicles (60). Further studies are required to resolve this issue, which is made more intriguing by the unexpected finding that the predominant trkA form expressed in bovine granulosa and thecal cells is the neuronal isoform, which has been shown in neuronal systems to confer the receptor responsiveness to NT-3 (47). The presence of this form in the bovine ovary is in contrast to the predominant expression of the nonneuronal trkA isoform in rat ovaries (6, 63, 66).

Regarding NGF itself, our findings show the presence of both NGF mRNA and NGF immunoreactive protein in most cells of the thecal compartment. We were not able to detect a high content of either TrkA or NGF in the theca interna, which would have suggested the preferential expression of this NT signaling complex in thecal steroidogenic cells. This localization, similar to that described in the rat ovary (6), suggests that NGF acts on thecal cells via paracrine/autocrine mechanisms to affect cellular functions. Although granulosa cells showed an abundance of NGF-like immunoreactivity, no NGF mRNA was detected in these cells, indicating that the immunoreactive material is either NGF bound to its trkA receptor or a cross-reacting protein. We favor the latter possibility, as it would be unusual for a ligand to remain so tightly bound to its high affinity receptor throughout the process to which the tissue is subjected before imunohistochemical analysis. The polyclonal antibodies to NGF were generated using a purified preparation of mouse 2.5S NGFβ extracted from mouse submaxillary glands, which was shown to be devoid of renin contamination (9). It is possible, however, that this preparation may have contained traces of NGFγ, a lysine-specific serine proteinase of the kallikrein family that reversibly associates with NGF (67). Submaxillary glands secrete a noncovalent multimeric complex com-

posed of three polypeptides, NGFα, the NT NGFβ, and NGFγ (68). Whereas the functions of NGFα remain to be elucidated, NGFβ is the NT shown to support the survival of neurons in the central and peripheral nervous system. NGFγ, on the other hand, has been implicated in the processing of NGFβ precursors (69, 70) and, more recently, in the cleavage of urokinase-type plasminogen activator (71); other functions have not been characterized. In considering that granulosa cells are a rich source of proteinases (72), it is conceivable that the NGF-like immunoreactivity detected in bovine granulosa cells represents cross-reaction of the antibodies with an NGFγ-related proteinase, probably of the kallikrein family, that may be produced in granulosa cells (73). Obviously, the epitopes recognized by our antiserum in such a proteinase would necessarily have to be different from those present in proteases synthesized by rat granulosa cells, because in no instance have we detected NGF-like material in these cells (6).

Previous studies demonstrated the ability of gonadotropins and growth factors to affect the steroidogenic output of isolated bovine thecal cells, using the same culture system we employed in the present experiments (20, 74). An important conclusion of these studies was that the response of thecal cells to either gonadotropins or growth factors is determined by the cytodifferentiation stage of the cells *in vitro*. Thus, androgen production was elevated during the first 3 days in culture and increased readily in response to hCG stimulation; androgens declined thereafter, and the cells became unresponsive to the gonadotropin (20). In contrast, progesterone secretion increased with time in culture and in response to hCG, at a time when the androgen response had been lost. A similar picture emerged from studies examining the effect of transforming growth factor-α (TGFα) and TGFβ on thecal cell steroidogenesis. TGFα suppressed androgen and progesterone secretion during the first 3 days in culture, but had no effect thereafter (74). Conversely, TGFβ stimulated progesterone secretion during the earlier days in culture, but also became ineffective at later times, as basal progesterone secretion increased (74). These and other observations led to the suggestion that thecal cells in culture undergo a differentiation (luteinization) process that profoundly affects their steroidogenic response to both gonadotropins and intraovarian growth factor regulators (74). The present observations are consistent with this interpretation, as they show the ability of NGF to stimulate androstenedione and progesterone secretion during the first 72 h after seeding, but not after a week in culture. Because trkA receptor expression declines precipitously 1 day after plating, the ineffectiveness of NGF to stimulate steroidogenesis in established cultures may be attributed to the rapid *in vitro* loss of functional high affinity receptors. This explanation is not, however, supported by the inability of NGF to affect steroidogenesis in thecal cells transfected with a trkA-encoding plasmid, a procedure that allowed the cells to respond to the NT with PG release and proliferation. It would then appear that NGF may facilitate thecal cell steroidogenesis, and thereby the biochemical differentiation of these cells, only before their *in vitro* steroidogenic capability becomes further differentiated. As shown in other cell systems (12, 41), the differentiating actions of NGF on thecal cells appear to be mutually exclusive with its proliferative effects. Thus, when applied to freshly seeded cells, NGF stimulated steroidogenesis in high density, presumably nonproliferating cultures, but not from low density, rapidly proliferating cells. Conversely, when administered to established 1-week-old cultures, it induced proliferation, but failed to affect steroidogenesis. Taken into an *in vivo* context, these findings suggests that NGF may contribute to increased thecal steroidogenic output during the early phases of the preovulatory period, particularly because at this time the ovulatory increase in LH secretion acts to simultaneously inhibit ovarian cell proliferation and stimulate steroidogenesis (75).

The results of experiments in which proliferation of follicular cells was examined in rats sequentially treated with PMSG and hCG confirmed the reported ability of hCG to inhibit cell proliferation (75) and suggested that this effect may occur very rapidly (within 4 h) after its administration. They also provided supportive evidence to the earlier observations that shortly before ovulatory rupture thecal fibroblasts of the follicular wall increase their proliferative activity (76, 77). Although we did not perform a detailed morphological analysis or a morphometric quantitation of these changes, BrdU-positive cells were consistently observed in the follicular wall 10–12 h after hCG. Interestingly, their spatial pattern of distribution indicated that this preovulatory increase in thecal cell proliferation is not limited to the apex of the follicular wall (76). That at least part of this enhanced proliferative activity may be due to an NGF-initiated, trkA-mediated signaling process was suggested by the ability of NGF to stimulate proliferation in isolated bovine thecal cells containing trkA receptors, but not from those lacking them. Unexpectedly, thecal compartment cells transfected with the trkA-encoding plasmid behaved in two different ways; in some experiments, basal proliferation remained at control values 48 h after transfection. These cells responded to NGF with a significant increase in proliferative activity, as defined by an increase in [^{3}H]thymidine incorporation. In other experiments, there was an increase in [^{3}H]thymidine incorporation after transfection, in the absence of NGF treatment; these cells did not respond to NGF with a further increase in proliferation. Because thecal compartment cells produce NGF, we interpret these findings as indicative of differences in responsiveness to endogenous NGF (which may be determined by the relative composition on small, medium, and large follicles in each preparation tested).

A stimulatory effect of NGF on thecal cell proliferation is in keeping with the early observation that ectopic expression of trkA receptors in fibroblastic cells did not result in differentiation or enhanced cell survival, but led to a proliferative response instead (12, 78). In fact, it is now well documented that NGF acts on nonneuronal and, in particular, endocrine cells to induce proliferation. Such an action has been observed in a variety of cellular systems, including normal undifferentiated hemopoietic cells (79), normal keratinocytes (80), thyroid (24) and pancreatic (25) cell lines, adrenal chromaffin cells (81), and more recently in a prostate adenocarcinoma cell line (26). The ability of NGF to induce proliferation in thecal cells expressing trkA receptors, the activation of thecal cell proliferation that occurs near the time

of ovulation (Refs. 76 and 77 and present study) and the striking increase in trkA receptor expression that takes place during the hours preceding the ovulatory rupture (6) suggest that activation of trkA receptors plays a significant role in inducing the proliferative activity of thecal compartment cells at the time of ovulation.

At least in rodents, an increase in intraovarian synthesis of PGE_2 is required for ovulation to occur (82). Mice carrying a null mutation of the COX-2 gene, which encodes the rate-limiting enzyme in PG synthesis, fail to ovulate in response to gonadotropins (23). The demonstration that the response is restored by the administration of PGE_2 (83) makes it evident that PGE_2 is the critical PG implicated in the ovulatory process in this species. Furthermore, mice lacking the PGE_2 receptor EP_2 exhibited a decrease in ovulation rate (84), whereas deletion of the $PGF_{2\alpha}$ receptor did not interfere with ovulation (85). Whether PGE_2 or $PGF_{2\alpha}$ is the most important PG involved in the ovulatory process of the ruminant ovary (86) remains to be established. Our results show that NGF induces PGE_2 release from isolated bovine thecal cells containing trkA receptors, but not from cells lacking the receptors. The effect is rapid, as it is already evident after 1 h of exposure to the NT, and is capable of amplifying the early increase in PGE_2 release elicited by hCG. As this amplification was no longer apparent after 2 h of simultaneous exposure to both secretagogues, it would appear that the initial amplification may reflect an NGF-dependent priming effect on the signaling pathway used by LH, preceding the productive coupling of LH receptors to this pathway.

The early stimulation of PGE_2 release by NGF was not accompanied by an increase in transcriptional activity of the COX-2 gene promoter, examined in 3T3 fibroblasts transfected with a trkA-encoding plasmid. Instead, the promoter activity was increased after 24 h of exposure to the NT (the earlier time assessed after the initial 2 h of exposure). That this increase is NGF dependent and NGF specific was demonstrated by the effectiveness of neutralizing antibodies to NGF to obliterate the increase and the ineffectiveness of related NTs such as NT-3 and BDNF to reproduce the effect of NGF. In all, the dynamics of the changes induced by NGF on PGE_2 release and COX-2 promoter activity in the present study are entirely consistent with that reported for the NT in neuronal cells. Exposure of PC-12 cells to NGF results in rapid ($<$1 h) stimulation of arachidonic acid metabolism (87), but causes a delayed activation of the gene encoding the constitutive cyclooxygenase isoform, COX-1 (88). It is plausible that this delayed genomic response represents a replenishment mechanism used by different cells to maintain an adequate level of COX proteins ready to be used upon ligand-induced stimulation of their enzymatic activity.

Inhibition of ovarian trkA tyrosine kinase-dependent signaling as well as immunoneutralization of ovarian NGF action have been shown to inhibit ovulation in rats (6). The present findings suggest that the actions of NGF in thecal cells at the time of ovulation involve at least three different components thought to contribute to the ovulatory process: stimulation of progesterone secretion, stimulation of PGE_2 production, and cell proliferation before ovulatory rupture. Although the exact contribution of trkA activation to the ovulatory cascade remains to be elucidated, it is important to note that in addition to PGE_2, several molecules recently shown to be required for ovulation were originally described as signaling molecules regulated by NGF, via activation of trkA receptors. Prominent among them are C/EBP-β and NGF-IA, two members of the basic leucine zipper family of transcriptional regulators. Both of them were identified in neuronal cells as direct downstream targets of the NGF high affinity receptor (89, 90) and shown to be present in preovulatory follicles, where they play an essential role in the ovulatory process (91–94).

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