Journal of Cellular Biochemistry 64:447–457 (1997)

Retinoic Acid Abolishes the Calcitonin Gene-Related Peptide Autocrine System in F9 Teratocarcinoma Cells

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Abstract Calcitonin gene-related peptide (CGRP), expressed predominantly in F9 embryonal carcinoma cells, is both a potent chemotactic agent and an autocrine growth factor for these cells. We analyzed the effect of retinoic acid (RA)-induced differentiation of F9 cells into primitive parietal endoderm-like cells, on CGRP production and the CGRP responsiveness of these cells. Poly(A) RNA extracted from F9 cells and analysed by Northern blotting and hybridization with a CGRP probe showed a specific band of about 1200 bases corresponding to mature CGRP mRNA. This band was not detected in F9 cells treated for 6 days with RA (differentiated primitive parietal endoderm-like cells) or in PYS cells (established parietal endoderm-like cell line). During RA-induced differentiation of F9 cells, CGRP mRNA levels fell within 24 h after treatment and were almost undetectable after 2 days. RA treatment also reduced CGRP secretion by F9 cells; the effect was maximal at 3 days and remained stable thereafter. Similarly, RA rapidly reduced adenylate cyclase responsiveness to chicken CGRP (cCGRP) and human CGRP (hCGRP). An 80% fall in cAMP release into the culture medium in the presence of CGRP was observed after 24 h of RA treatment. These results demonstrate that RA rapidly abolishes the CGRP autocrine system involved in the proliferation of F9 cells, at the same time inducing their differentiation into primitive parietal endoderm. They point to the interaction between retinoic acid and growth factors in the regulation of cell proliferation and differentiation. J. Cell. Biochem. 64:447–457.

Key words: retinoic acid; teratocarcinoma cells; calcitonin gene-related peptide

Retinoic acid (RA) exerts drastic effects on vertebrate development and cell differentiation [de Luca, 1991; Gudas, 1992; Holdener, 1993; Chambon, 1994]. In addition, retinoids can inhibit or reverse the malignant transformation of some cell types. RA has recently been used with success to treat or prevent various cancers and dysplasias [Hong et al., 1986; Alberts et al., 1986; Weiner et al., 1986; Meng-er et al., 1988; Kraemer et al., 1988; Hong et al., 1990]. In acute promyelocytic leukemia, for example, large doses of RA can result in complete remission [Warrel et al., 1993]. The biological actions of RA or its derivatives are mediated at the cellular level by two different types of specific nuclear receptor, RARs (RARα, RARβ, and RARγ) and RXRs (RXRα, RXRβ, and RXRγ), which belong to the superfamily of steroid/thyroid hormone receptors. These receptors function as ligand-activated transcription factors and regulate gene expression by binding to DNA response elements associated with their target genes [for reviews see Leid et al., 1992; Petkovitch, 1992; Chambon, 1993, 1994; Giguère, 1994; Glass, 1994]. Numerous studies have pointed out the interactions between RA and growth factors in regulating cell growth and differentiation [Tabin, 1991]. RA modulates growth factors and their receptor expression during cell differentiation [Thompson et al., 1989; Zheng et al., 1992; Roulier et al., 1994]. In addition, we recently showed that growth factors such as epidermal growth factor increase the expression of RXRα in human trophoblastic cells [Roulier et al., 1996].

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide produced by tissue-specific alternate splicing of the primary transcript [Amara et al., 1982; Russo et al., 1988]. CGRP has neurotropic and neurotrophic activ-

Contract grant sponsors: Comité de Paris de la Ligue Nationale contre le Cancer, Association pour la recherche contre le Cancer.

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Received 11 June 1996; Accepted 16 September 1996

ity and is widely distributed in numerous tissues. We have demonstrated that CGRP, which is predominantly expressed in F9 cells [Leff et al., 1987], is both a potent chemotactic agent [Gerbaud et al., 1991] and an autocrine growth factor for F9 cells [Segond et al., 1992]. F9 cells derived from OTT 6050 embryonal carcinoma cells resemble the inner cell mass of the early (day 4–5) postimplantation mouse embryo and thus offer an in vitro model to study early biochemical events in embryonic development [Martin, 1980] and cell differentiation. Indeed, F9 cells are nullipotent cells with a limited capacity to differentiate spontaneously [Bernstine et al., 1973]. Exposure to retinoic acid induces the formation of primitive endoderm cells, while the addition of cAMP leads to complete differentiation into a parietal endoderm [Strickland and Madhavi, 1978; Plet et al., 1986; Liapi et al., 1987]. It was therefore of interest to study the effect of retinoic acid-induced differentiation on the production of CGRP by F9 cells and their response to the autocrine function of this peptide. We examined the effect of retinoic acid on F9 cells in terms of the expression of CGRP mRNA, CGRP secretion into the culture medium, and CGRP-stimulated adenylate cyclase by measuring cAMP synthesis and secretion.

MATERIALS AND METHODS

Human CGRP (hCGRP) and chicken CGRP (cCGRP) were from Peninsula Laboratory (England). [α^{32}P] ATP, [α^{32}P] dCTP, and [α^{32}P] UTP were from Amersham International (Buckinghamshire, UK).

Cell Culture

F9 cells, kindly provided by Dr. Jetten (NIH, Maryland, USA), were grown in 100-mm tissue culture dishes (Costar, Brumath, France) at 37°C in Dulbecco-Vogt's modified Eagle's medium (Gibco, Paisley, UK) containing 10% fetal calf serum (Biological Industries, Israel), antibiotics (50 units/ml penicillin and 50 mg/ml streptomycin), and 4 mM glutamine (Gibco) in a humidified atmosphere containing 5% CO_2. The growth medium was changed every 48 h.

Retinoic Acid Treatment of Cells

All-trans retinoic acid (diluted in absolute ethanol and stored for up to 2 weeks at −80°C) was added 24 h after plating to give a final concentration of 0.1 μM (30 ng/ml). The final concentration of ethanol in the medium (0.01%) did not alter cell morphology or hormone responsiveness (not shown). Cells were treated either with retinoic acid alone for 1 to 6 days, or with retinoic acid for 4 days followed by 1 mM dibutyryl cyclic AMP plus retinoic acid for two days. All-trans retinoic acid and dibutyryl cAMP were from Sigma (St. Louis, MO).

CGRP Radioimmunoassay

24-h conditioned medium (10 ml) was collected on each day of culture and stored at −20°C; cells were scraped free with a rubber policeman, extracted in 0.1 M acetic acid, lyophilised, and stored at −20°C. CGRP was measured by means of a sensitive and specific radioimmunoassay [Segond et al., 1992] after purification of the culture supernatants on an AMPREP column (Amersham, les Ulis, France). Eluted CGRP was lyophilised and dissolved in 500 μl of RIA phosphate buffer. Serial dilutions (10 to 100 μl) were incubated for 4 days with sheep antiserum against hCGRP (Bachem, Bâle, Switzerland) at a final dilution of 1/250000 in 0.05 M phosphate buffer containing 0.3% albumin and 10 mM EDTA in a final volume of 400 μl. Then 100 μl of [^{125}I]-CGRP (Amersham) was added and incubation was continued for 3 days. Free and bound hormone were separated by charcoal adsorption in RIA buffer in the presence of 0.25% gelatine.

mRNA Extraction

Total RNA were extracted from F9 cells by a guanidium thiocyanate method [Chomczynski and Sacchi, 1987]. Total poly(A)-rich RNA were purified on oligo-dT cellulose (Boehringer Manheim, Germany).

Northern Blot Analysis

Ten μg of poly(A) RNA extracted from control F9 cells, from F9 cells treated with retinoic acid, and from PYS cells was denaturated with glyoxal, subjected to electrophoresis on 1% agarose gels [Thomas, 1980] and transferred to GeneScreen membranes. The membranes were then dried and baked for 2 h at 80°C. Ten micrograms of mRNA extracted from rat thyroids were run in the same conditions. The membranes were prehybridized for 16 h at 50°C in 50% formamide, 50 mM sodium phosphate, pH 6.5, 0.8 M NaCl, 1 mM EDTA, 0.1% SDS,

2.5× Denhardt's solution, 250 µg/ml denaturated fetal calf thymus DNA, 500 µg/ml yeast RNA and 10 µg/ml poly(A). Hybridization was carried out for 18 h at 50°C in the same buffer containing a [^{32}P]-labelled specific CGRP riboprobe (10^8 cpm/µg DNA) obtained from pSP-CGRP plasmid (a gift from Pr. Craig). The membranes were then washed four times for 20 min at 60°C with 50 mM NaCl, 20 mM sodium phosphate, pH 6.5, 1 mM EDTA and 0.1% SDS, then exposed to Hyperfilm MP (Amersham) for 4 days at −80°C. Before rehybridization in the same conditions with a ^{32}P-labelled specific 18S rRNA cDNA probe, the same northern membrane was dehybridized in 0.05 M Tris-HCl (pH 8), 0.1× Denhardt's, 0.2 mM EDTA, 0.05% sodium pyrophosphate, 3 h at 68°C and prehybridized.

RT-Polymerase Chain Reaction

cDNA was synthesized from 4 µg of total RNA. The reaction mixture had a final volume of 20 µl and contained 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM MgCl2, 10 mM DTT, 10 U of RNAsin, 200 U of reverse transcriptase (Life Technologies, Cergy Pontoise, France), 1 mM each dNTP and 50 pmol of a 3′ oligo dT primer. Annealing was performed for 10 min at 23°C and primer extension for 1 h at 37°C. The reaction mixture was half-diluted, and 36 µl was made up to 95 µl with Taq polymerase buffer containing 0.5 units of Taq polymerase (Appligene, Illkirch, France); after heating to 90°C (hot-start), 50 pmol of each specific CGRP primer, described in Table I, was added in a volume of 5 µl. For the actin control, 4 µl of the diluted RT reaction mixture was treated in the same manner, in presence of 50 pmol of each specific actin primer described below. Amplification was run for 30 cycles: 30 sec at 95°C (denaturation), 30 sec at 55°C (annealing), and 30 sec at 72°C (extension). The reaction was finished after 5 min of elongation.

TABLE I. Nucleotide Sequence of PCR Primers*

	5′-3′ Sequences of primers		Amplified sequence
rat CGRP	AGAAGAAGCTCGCCTACTG GGGGCTATTATCT- GTTCAAGCCTG	s as	274 bp
mouse actin	TGTATGCCTCTGGTCGTACC CAACGTCACACTTCATGATGG	s as	438 bp

*The amplified sequence was specific for CGRP I.

Analysis of PCR Products

Twenty µl of CGRP amplified products and 10 µl of actin amplified products were analysed by electrophoresis on 1.2% agarose gels, visualized with ethidium bromide, and transferred to membranes (GeneScreen, NEN) after denaturation in 0.4 N NaOH. The amplified cDNA was hybridized with a CGRP- or actin-specific cDNA probe labelled with 32P using a random priming method (specific activities $> 10^8$ cpm/µg DNA were obtained). After prehybridization for 1 h at 42°C in 50% formamide, 1% SDS, 2 × SSC, 10% dextran sulfate, the membranes were hybridized at 42°C in the same buffer containing the specific probe, and were washed in 2 × SSC for 10 min at room temperature, twice in 2 × SSC 1% SDS for 20 min, twice in 0.2 × SSC 1% SDS for 20 min at the hybridization temperature, and for 2 min in 0.2 × SSC at room temperature. Autoradiography was done with intensifying screens for 1 h. The autoradiographs were scanned on a densitometer. CGRP data were divided by actin data and are expressed as % of control values. The results are the mean + SEM of three experiments. Data were subjected to analysis of variance (ANOVA) and also checked using a nonparametric test (Kruskal-Wallis).

Adenylate Cyclase Assay

Cells (after 2 days of culture in 90-mm dishes) were gently washed with ice-cold homogenization buffer (50 mM Tris-HCL, pH 7.8, 0.33 M sucrose, 1 mM $MgCl_2$), harvested by scraping, and homogenized (20 strokes) with a Dounce homogenizer. Microscopic examination showed almost complete disruption of each cell type in these conditions. Homogenates were centrifuged at 12000*g* for 10 min. The pellets were washed twice in the same buffer and protein content was determined by fluorometric assay [Bohlen et al., 1973]. Less than 3% of total adenylate cyclase activity remained in the supernatant. Adenylate cyclase activity was assayed by measuring the conversion of [α^{32}P]-ATP to cyclic [^{32}P]-AMP in crude membranes (12000 g pellet) at 37°C [Anderson et al., 1974]. The standard incubation mixture (final volume 50 µl) contained 25 mM $MgCl_2$, 0.2 mM ATP, 0.5 mM EGTA, 4×10^6 cpm [α^{32}P]-ATP, and 30 to 50 µg of membrane protein. In addition, the

reaction mixture contained GTP (10 μM) and CGRP at the indicated concentrations. Each experiment was repeated at least twice, and the results of triplicate determinations in each experiment agreed to within ±10%.

cAMP Determination

As cAMP secretion closely reflects its synthesis, we measured the amount of cAMP in the culture medium. The medium was removed and the cells (60,000/60 mm dish) were washed in serum-free medium, then incubated for 1 h in 4 ml of serum-free medium containing 0.1 mM isobutylmethylxanthine, an inhibitor of phosphodiesterase. CGRP was added at the indicated concentrations and the cells were incubated for a further 90 min. The incubation medium was frozen until use. The protein content of the cells was measured by fluorometric assay.

cAMP concentrations in the incubation medium were estimated by means of radioimmunoassay in 100 μl aliquots, using a rabbit anti-cAMP antiserum (kindly donated by J. Saez, INSERM U 418, France). The antibody-bound cAMP was separated from free cAMP by the use of a second antibody (Read-PR-1000, CIS, France). Nonspecific binding was determined by the addition of excess cAMP. The results are expressed as pmoles of cAMP per mg of cell protein. Each experiment was repeated at least twice, and the results of triplicate determinations in each experiment agreed to within ±10%.

Statistical Analysis

Statistical significance was defined using Student's *t*-test for comparison of two groups with a threeshold of $P < 0.05$.

RESULTS

Effect of Retinoic Acid on CGRP Synthesis and Secretion

The addition of 0.1 μM retinoic acid to growing F9 cells led to growth arrest and differentiation into a primitive parietal endoderm cell type [Strickland and Madhavi, 1978; Plet et al., 1986]. Exposure to retinoic acid for 4 days, followed by treatment with dibutyrylcyclic AMP for an additional 2 days, resulted in complete differentiation into a parietal endoderm [Strickland et al., 1980]. Poly(A) RNA extracted from F9 cells after 6 days of culture and analyzed by Northern blotting and hybridization with a CGRP probe in stringent conditions (60°C and 0.3 × SSC) showed a specific band of 1200 bases corresponding to mature CGRP mRNA (Fig. 1).

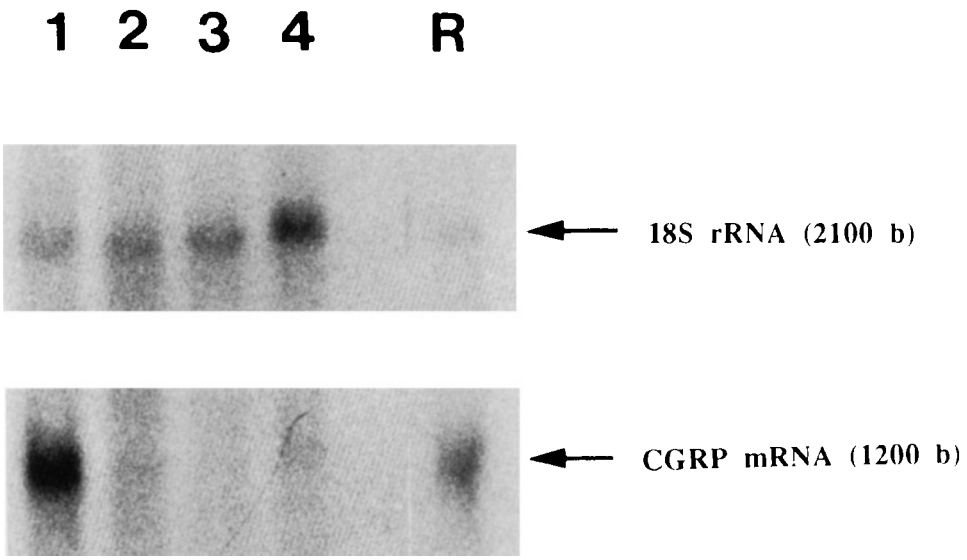

Fig. 1. Northern blot analysis of CGRP mRNA in teratocarcinoma cells. Hybridization of CGRP mRNA with 10 μg of poly(A) RNA extracted from F9 cells in the following conditions: **(lane 1)** 6 days in culture without retinoic acid (RA); **(lane 2)** 6 days in culture with RA (10^{-7} M); **(lane 3)** 4 days in culture with RA followed by 2 days with dibutyryl cAMP (10^{-3} M). **Lane 4** contained 10 μg of poly(A) RNA extracted from parietal endoderm PYS cells. **Lane R** contained 10 μg of poly(A) RNA extrated from rat thyroids. The analysis was performed on 1% agarose gel, with transfer to a GeneScreen membrane, hybridization with a specific [^{32}P] labelled CGRP RNA probe, and autoradiography on Hyperfilm MP for 4 days (Amersham). 18S rRNA was used as internal control.

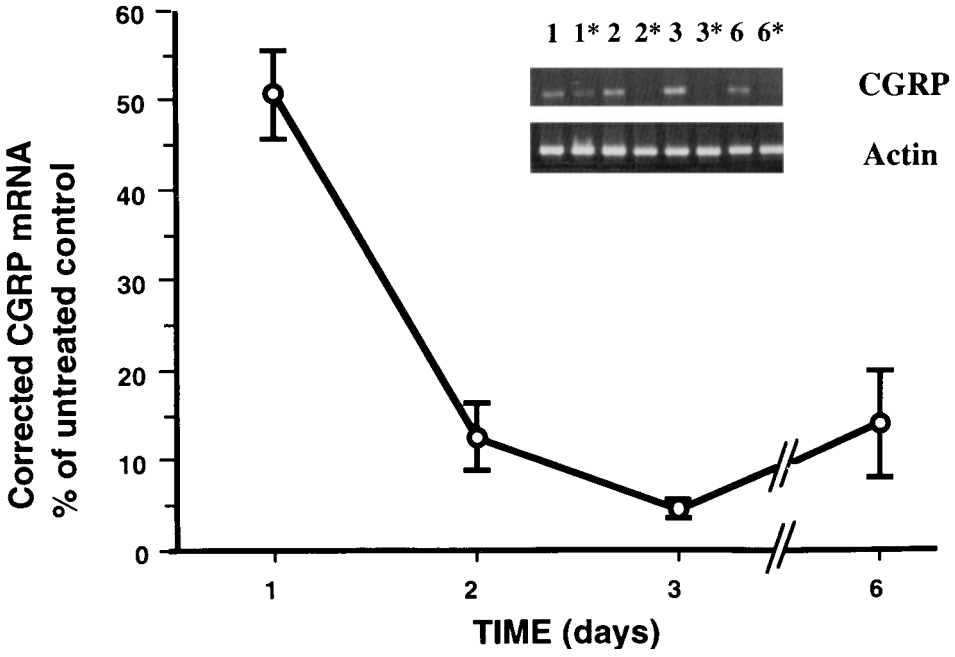

Fig. 2. Effect of retinoic acid treatment of F9 cells on CGRP mRNA levels. **Inset:** Agarose gel electrophoresis of amplified products of RT-PCR using CGRP- and actin-specific primers (respectively 20 µl and 10 µl) and 4 µg of RNA extracted from F9 cells treated with retinoic acid for 1 to 6 days **(Lanes 1*, 2*, 3*, 6*),** and from control F9 cells cultured for the same times **(Lanes 1, 2, 3, 6).** DNA was vizualized with ethidium bromide. **Graph:** Corrected CGRP mRNA as a percentage of untreated control values. Amplified products were transferred from agarose gel to GeneScreen (NEN) membranes. The DNAs were hybridized with a specific CGRP probe or with a specific actin probe. Autoradiographs were scanned with a densitometer after 1 h of exposure. CGRP data were divided by actin data and are expressed as % of control values. The results are the mean ± SEM of three experiments.

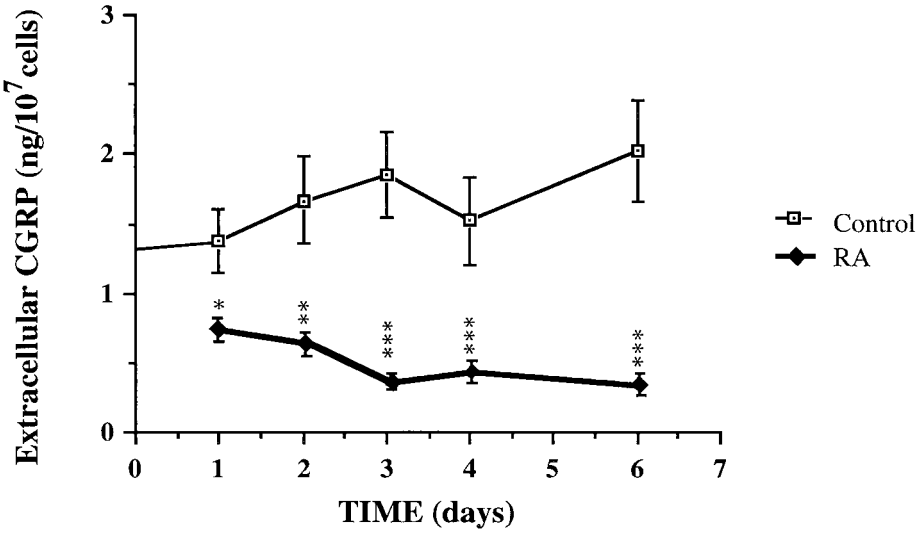

Fig. 3. Effect of retinoic acid treatment of F9 cells on CGRP secretion. Twenty-four h conditioned media were collected each day from cells treated (–◆–) or untreated (–⊡–) with retinoic acid. Serial dilutions of eluates obtained from the different culture supernatants on an AMPREP column (Amersham) were measured by using a specific CGRP radioimmunoassay with sheep antiserum against hCGRP at a final dilution of 1/250,000. The results are expressed as the mean of the three dilutions. (*) = $P < 0.05$, (**) = $P < 0.01$, (***) = $P < 0.001$. Statistical differences reflect differences at each time point for treated vs. control.

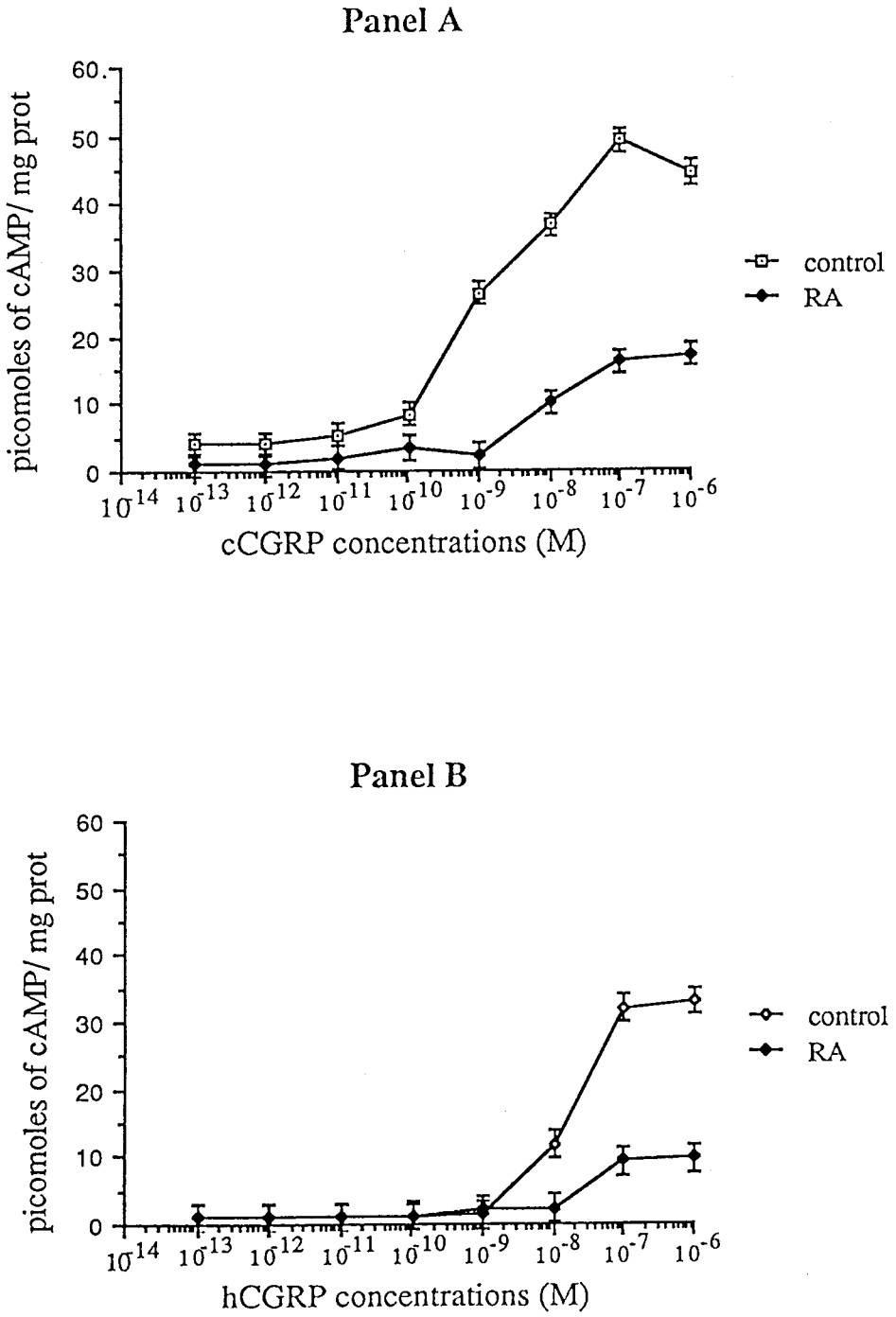

Fig. 4. Effect of increasing concentrations of CGRP on extracellular cAMP levels in F9 cell cultures treated or untreated with retinoic acid. F9 cells treated (–◆–) or untreated (–⊡–) with 10^{-7} M retinoic acid for 24 h were incubated for 90 min in serum-free medium in the presence of cCGRP **(A)** or h CGRP **(B).** Cyclic AMP was extracted and measured by radioimmunoassay as described in Materials and Methods. The results are expressed as means ± SEM of triplicate determinations.

No CGRP mRNA was detected in F9 cells treated with retinoic acid for 6 days (primitive parietal endoderm-like cells). CGRP mRNA was also undetectable in F9 cells treated with retinoic acid and dibutyryl cAMP (parietal endoderm-like cells), as well as in established parietal endoderm PYS cells. Time course analysis of the effect of RA on CGRP mRNA expression revealed that CGRP mRNA levels fell significantly ($P < 0.001$) by 50% after 1 day of treatment and were almost undetectable after 2 days and thereafter (Fig. 2).

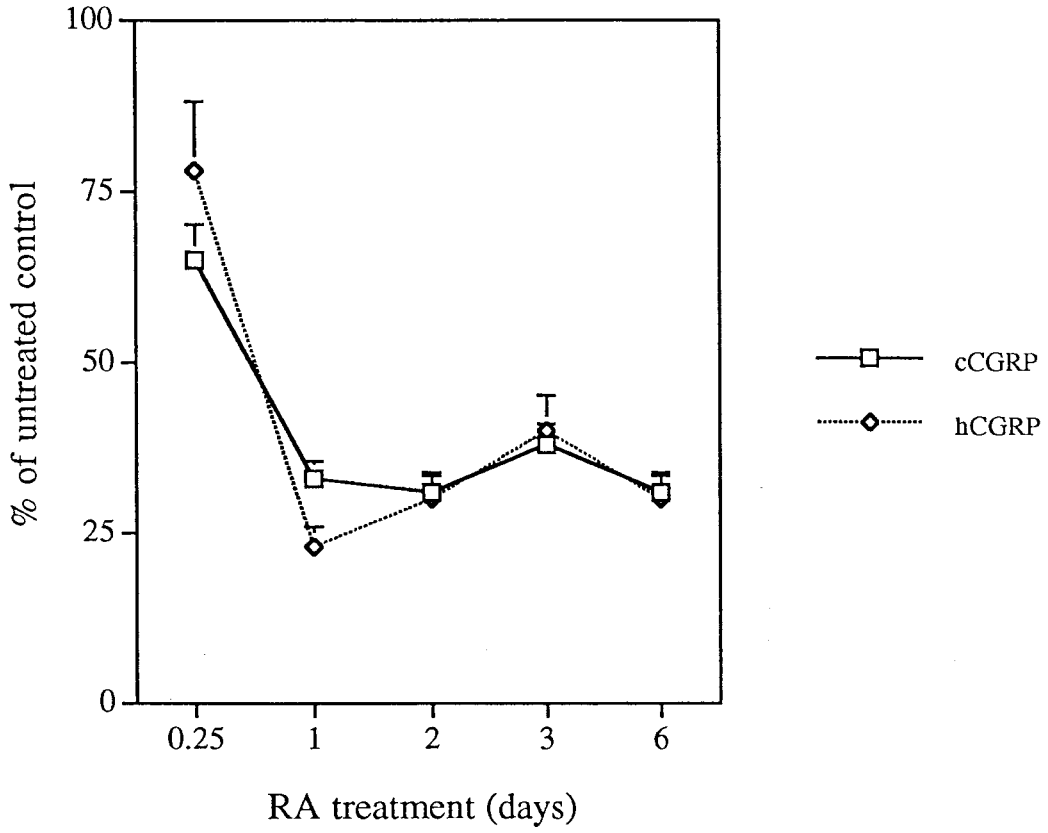

Fig. 5. Effect of retinoic acid treatment on cAMP levels in the culture medium of F9 cells incubated in the presence of CGRP. Intact cells were treated with 0.1 μM retinoic acid for the time indicated. F9 cells were incubated for 90 min in serum-free medium in the presence of 0.1 μM cCGRP (–□–) or 1 μM hCGRP (–◇–). Cyclic AMP was extracted and measured by radioimmunoassay as described in Materials and Methods. Cyclic AMP production is expressed as a percentage of that in control F9 cells incubated with CGRP. The values are the mean ± SEM of triplicate determinations.

Displacement of labelled human CGRP from its specific antibody by multiple dilutions of F9 cell medium ran parallel to that observed with increasing amounts of synthetic CGRP, allowing us to measure the immunoreactive CGRP secreted by F9 cells. F9 cells secreted CGRP (1.5 to 2 ng/10^7 cells/24 h) and no significant change in 24-h secretion was observed during the 6 days of cell culture (Fig. 3). CGRP (24 h) secretion was reduced during retinoic acid treatment: minimum values were reached after 3 days and remained stable for the following 3 days.

Effect of Retinoic Acid on cAMP Release

As shown in Figure 4, CGRP stimulated cAMP release in control F9 cells in a concentration-dependent manner. A maximal ten-fold stimulation was observed with 10^{-7} M cCGRP (panel A) and hCGRP (panel B). At low concentrations cCGRP was more effective than hCGRP in stimulating cAMP release. Retinoic acid treatment of F9 cells for 24 h led to a decrease in the amount of cAMP released in the presence of CGRP, but did not modify the concentration-response curve of the peptide. The stimulatory effect of cCGRP and hCGRP on cAMP release into the culture medium was gradually abolished by retinoic acid treatment (Fig. 5). The decrease (−30%) in cAMP release in the presence of CGRP was significant ($P < 0.01$) after 6 h of treatment with retinoic acid and maximal after 1 day. Differentiation into primitive parietal endoderm cells by exposure of F9 cells to retinoic acid for 4 days induced a 80% decrease in CGRP-stimulated release of cAMP. CGRP did not stimulate cAMP secretion in completely differentiated F9 parietal endoderm cells (data not shown).

Effect of Retinoic Acid on Adenylate Cyclase Activity

This decrease in cAMP release following exposure of F9 cells to CGRP was associated with a decrease in cAMP synthesis, as shown by membrane adenylate cyclase activity studies. RA-

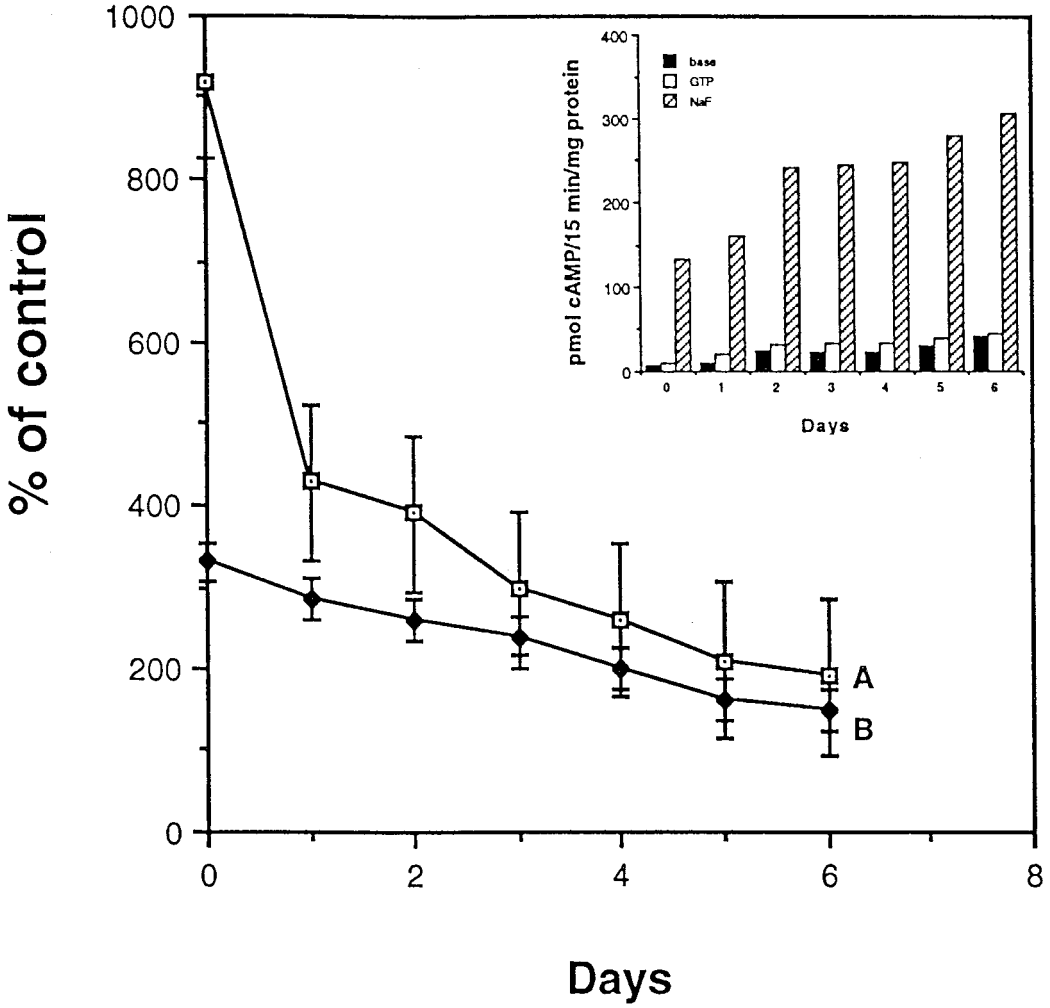

Fig. 6. Effect of retinoic acid treatment on the CGRP responsiveness of adenylate cyclase in F9 cells. Intact cells were treated with 0.1 μM retinoic acid for the time indicated, and crude membranes were prepared and assayed in triplicate for adenylate cyclase activity in the presence of 10 μM GTP with either 0.1 μM cCGRP **(A)** or 1 μM hCGRP **(B).** The results are the percentage stimulation of cyclic AMP production above that observed with 10 μM GTP alone. They represent the mean ± SEM of three different experiments. **Inset:** Adenylate cyclase activity of F9 cell membranes treated for the indicated time with retinoic acid and assayed in basal conditions, in the presence of 10^{-5} M GTP and 10^{-3} M sodium fluoride.

induced differentiation of F9 cells is associated with a gradual increase in membrane adenylate cyclase activity, measured in basal conditions, in the presence of GTP (nucleotide required to observed in vitro, hormonal stimulation of the adenylate cyclase activity) and sodium fluoride (chemical agent inducing maximal activation of the adenylate cyclase) [Evain et al., 1981]. These data were confirmed in this study (insert of Fig. 6). As shown in Figure 6, retinoic acid treatment induced a 2.5-fold decrease in adenylate cyclase responsiveness to chicken CGRP and a 1.5-fold decrease in responsiveness to human CGRP, within 24 h. A gradual decrease in CGRP-stimulated cAMP synthesis was then observed over the following days. As shown in Table II, CGRP activation of the cyclase was barely detectable (120% of the value in the presence of GTP alone) in F9 cells exposed for 4 days to RA, followed by treatment with dibutyrylcyclic AMP for an additional 2 days, conditions that resulted in complete differentiation into a parietal endoderm. Similarly, CGRP did not stimulate cAMP formation in established PYS parietal endoderm cells (Table II).

DISCUSSION

CGRP, previously considered to be a neuropeptide acting principally on the nervous and

TABLE II. CGRP Responsiveness of Adenylate Cyclase Determined With Crude Membranes Prepared From F9 and Endodermal Cells

	Adenylate cyclase activity (picomoles/15 min/mg protein)		
Cell type	GTP [10^{-5} M]	cCGRP [10^{-7} M]	hCGRP [10^{-6} M]
Undifferentiated stem cells			
F.9	10 ± 1	92 ± 9	33 ± 4
Primitive parietal endoderm-like cells			
F.9 + 6 days RA	45 ± 4	83 ± 8	67 ± 6
Parietal endoderm-like cells			
F.9 + 4 days RA + 2 days [Dibutyryl cAMP + RA]	89 ± 9	100 ± 9	82 ± 8
PYS	127 ± 13	108 ± 9	90 ± 8

cardiovascular systems [Chatterjee et al., 1991; Fischer et al., 1987], may also be a growth factor modulating cell growth and differentiation. Indeed, CGRP stimulates the proliferation of various cell types [Wang et al., 1992; White et al., 1993; Cheng et al., 1995]. In addition, during development, CGRP regulates acetylcholine receptors [New et al., 1986; Fontaine et al., 1987], modulates dopaminergic phenotypes in the mouse olfactory bulb [Denis-Donini, 1989], and may act as a potentiating factor during the early phase of synaptogenesis [Lu et al., 1993]. Of particular interest in this context is our observation that CGRP, a chemotactic agent for F9 cells [Gerbaud et al., 1991], can also stimulate the growth of F9 embryonal carcinoma cells by an autocrine process [Segond et al., 1992].

F9 cells are one of the first models used to study the effect of retinoids on cell differentiation [Martin, 1975, 1980]. Resembling the inner cell mass of the embryo, F9 cells differentiate into primitive parietal endoderm cells when treated with retinoic acid. Later on, retinoids, the specific nuclear receptors for which have been recently isolated [Petkovich et al., 1987; Giguere et al., 1987; Brand et al., 1988], were shown to play a major role during embryogenesis, possibly by acting as morphogens and by regulating the expression of genes involved in cell growth and differentiation [Sulik and Alles, 1991]. Numerous studies [see for review Gudas, 1991; De Luca, 1991] have demonstrated the presence of retinoic acid receptors mRNA expression in F9 cells. Retinoic acid modulates CGRP mRNA expression during differentiation of the CATT thyroid C-cell line from a neuronal phenotype to a hormonal phenotype [Russo et al., 1988, 1992; Lanigan et al., 1993, 1994). Retinoic acid-mediated decrease in CGRP expression in these cells might be related to the ability of retinoids receptors to block HLH-octamer binding to an upstream enhancer element [Lanigan et al., 1993].

Our results clearly demonstrate that RA-induced differentiation of F9 cells is also associated with a marked fall in the levels of CGRP mRNA and peptide. Northern analysis of poly(A) RNA showed that it was not degraded after 6 days of culture, and the size of the mature CGRP mRNA in F9 cells was as previously reported [Lamari et al., 1994]. This CGRP mRNA is not detectable in differentiated cells. As we have already observed [Segond et al., 1992], and as shown in Figure 1, the level of CGRP mRNA increased with the time in culture. Retinoic acid treatment rapidly reduced the expression of this peptide mRNA, 10% of CGRP mRNA expression being observed after 2 days of treatment. This suggests that RA may modulate CGRP synthesis at the transcriptional level by directly or indirectly reducing CGRP mRNA transcription or stability.

Interestingly, with the same time frame and the same amplitude, RA induced a decrease in CGRP-stimulated cAMP synthesis and cAMP secretion by F9 cells. Indeed, the mitogenic effect of CGRP appears to be mediated by stimulation of the cAMP-dependent pathway [Haegerstrand et al., 1990]. This decrease could be due to modulation of the synthesis of the G protein involved in adenylate cyclase activation. This is supported by the following observations. Cross-linking of [^{125}I]-hCGRP to F9 cell membrane fraction with disuccinimidyl suberate revealed specific incorporation of [^{125}I]-hCGRP to five major protein bands of 59, 57, 48, 39, and 33 KD respectively. These bands were displaced by an excess of cold CGRP and not by salmon calcitonin. None of these bands was significantly modulated by retinoic acid treatment [Gerbaud P, personal data]. Similarly, CGRP receptor subtypes differ with the cell type and are coupled or uncoupled to the adenylate cyclase [Stangl et al., 1993; Aiyar et al., 1994].

Thus, retinoic acid-induced differentiation of F9 cells leads to a gradual disappearance of the autocrine CGRP system involved in the proliferation of these cells [Segond et al., 1992]. This suggests that abolishment of the mitogenic effects of the autocrine growth factor CGRP may be one of the mechanisms involved in the inhibition of F9 cell growth in presence of retinoic acid [Evain-Brion et al., 1981].

ACKNOWLEDGMENTS

This work was supported by a grant from the Comité de Paris de la Ligue Nationale contre le Cancer and the Association pour la recherche contre le Cancer.

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