Different expression of adenylyl cyclase isoforms after retinoic acid induction of P19 teratocarcinoma cells

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Abstract We have investigated the adenylyl cyclase (AC) activity and gene expression in retinoic acid (RA)-primed murine P19 teratocarcinoma cells, which recapitulate in vitro the first stages of neuroectodermal formation. Here we show that the P19 stem cells possess a basal Ca^{2+}/CaM-stimulated AC activity, which increases about 10-fold after RA induction. The rise of AC activity is associated with a stage-specific up-regulation of AC2, AC5 and AC8 mRNAs and a down-regulation of AC3 mRNA. P19 cells provide a powerful model to investigate the role and specific regulation of AC isoforms during neuronal differentiation.

Key words: Adenylyl cyclase isoform; Cell differentiation; Retinoic acid

1. Introduction

The vitamin A derivative retinoic acid (RA) is an important natural morphogen in vertebrates, involved in specifying the anteroposterior axis and the central nervous system [1,2]. Severe congenital vitamin A deficiencies result in a spectrum of malformations including defects of eye, lung, cardiovascular and urogenital systems [3]. Endogenous RA applied to embryos during an early stage of development causes severe malformations, particularly within the hindbrain and branchial region of the head [2].

It is well established that retinoids exert their biological activity via three types of proteins: the cellular RA-binding proteins CRABP-I and II, the cellular retinol-binding proteins CRBP-I and II, and the nuclear RA receptors (RARs) or retinoid X receptors (RXRs). The latter are ligand-inducible transcriptional *trans*-regulators modulating the transcription of target genes via *cis*-acting DNA response elements (RAREs) [4,5]. Among the RA-responsive genes, some encode transcription factors or putative transcription factors such as OCT3 or HOX genes, which are known to be implicated in the patterning of the central nervous system [6].

Neuronal differentiation in vivo as well as in vitro requires elevation of the intracellular cyclic AMP (cAMP) concentration and is mediated by the cAMP/protein kinase A (PKA) cascade [7,8]. The intracellular level of cAMP is controlled mainly by the activity of adenylyl cyclases (AC). ACs form an enzyme superfamily that consists of at least nine isoforms (AC1–9) with distinct basal activity, regulation, tissue distribution and specific regulation during postnatal development [9,10]. These cyclases can be distributed in various subfamilies according to their sensitivity to calcium: AC1, AC3 and AC8 are activated by the calcium/calmodulin (CaM) complex; AC2, AC4 and AC7 are activated by βγ subunits and PKC phosphorylation; AC5 and AC6 are inhibited by low concentrations of calcium, and AC9 is insensitive to either calcium or βγ subunit. The complexity and diversity of AC regulation suggest that the AC isoforms could play a major role in controlling the level of cAMP, a critical determinant of embryonic development.

However, no data are currently available concerning the isoform-specific regulation of AC during embryonic development. The aim of this study was to characterize the isoform-specific regulation of AC during neuronal induction, using as a model murine embryonic carcinoma P19 cells since they can mimic in vitro cellular differentiation along the neuroectodermal pathway, when aggregated and exposed to 1 μM RA [11,12].

Here we report that RA induction of P19 cells leads to a marked increase of Ca^{2+}/CaM-stimulated AC activity, accompanied by a specific up-regulation of AC2, AC5 and AC8.

2. Materials and methods

2.1. Materials

ATP, GTP, cAMP, phosphocreatine, creatine phosphokinase, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide·HCl (W7) and forskolin (FSK) were purchased from Sigma Chemical. [α-^{32}P]ATP (30 Ci/mmol), [8-^{3}H]cyclic AMP (30 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (5000 Ci/mmol) and UTP (800 Ci/mmol) were obtained from Amersham. All other chemicals were of the highest purity commercially available.

2.2. Cell culture and growth conditions

Embryonal carcinoma P19 cells were kindly provided by Dr. M. Nemer (University of Montreal). The cells were cultured as monolayers in DMEM supplemented with 5% fetal calf serum at 37°C and in 5% CO_2. Neuronal differentiation was achieved as described elsewhere [13]. Differentiation was induced by plating cells into bacteriological grade Petri dishes at 10^5 cells/ml in DMEM supplemented with 10% fetal calf serum and 1 μM RA. Under these conditions, cells aggregate. After 4 days the aggregates were plated into tissue grade Petri dishes, whereupon morphological differentiation occurred. Neurons were identified as rounded cells with elongated axonal or dendritic processes, glial and other non-neuronal cells were seen as flat fibroblast shaped cells. Morphologically identifiable neurons and glial cells were observed in the cultures starting from day 5 after RA induction. Days of differentiation were numbered consecutively after the first day of RA treatment.

2.3. Assay of adenylyl cyclase activity

AC activity of purified P19 plasma membranes was measured as described previously [14] with some modifications. To obtain purified membranes, the cells were washed twice with ice-cold PBS, homogenized in 10 volumes of ice-cold lysis buffer (10 mM Tris-HCl (pH 7.6), 0.1 mM EGTA, 5 mM 2-mercaptoethanol and 0.5 mM PMSF (phenylmethylsulfonyl fluoride)) and centrifuged at 500×*g* for 5 min at 4°C. The supernatant was centrifuged at 17000×*g* for 30 min at

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4°C and the pellet washed in the same buffer three times. For analysis of the calcium effect on AC activity, the membranes were washed twice with 1 mM EGTA. The final pellet corresponding to the membrane fraction was resuspended in the lysis buffer to a final protein concentration of 2–4 mg/ml and stored at −80°C.

The standard AC assay contained 70 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM ATP (disodium salt), 1×10^6 cpm/assay [α-^{32}P]ATP (tetra(triethylammonium) salt) (Amersham), 1 mM cAMP containing [8-^{3}H]cAMP ($10–15\times10^3$ cpm/assay), 5 mM phosphocreatine (disodium salt), 60 U/ml creatine phosphokinase and 20–40 μg of membrane proteins in a final volume of 60 μl. The reactions were initiated by addition of the membranes, conducted for 10 min at 35°C and terminated by addition of 200 μl of 0.5 M HCl, followed by boiling for 7 min, and neutralized by 200 μl of 10 mM imidazole (pH 7.6). cAMP formed during the incubation was separated by Aluminia column and corrected for recovery of added [^{3}H]cAMP [15]. Data points are presented as mean activities ± S.E.M. of triplicate determinations.

Free concentrations of Ca^{2+} were calculated as described [16].

2.4. Probes

Probes specific for each of the AC subtypes were chosen from regions where the sequences are the most divergent. The 700 bp *Hin*dIII-*Bam*HI fragment used for the AC1 probe was isolated from the bovine AC1 sequence [17]. Probes specific for rat AC2, AC3, and mouse AC8 were obtained by reverse-transcription PCR (RT-PCR) using mRNA extracted from brain and specific following oligonucleotides: 5′-GGGAAGATTAGTACCACGGT-3′ and 5′-AGGAGAAGCCAAGGATGGACG-3′ for 345 bp rat AC2 probe [18]; 5′-ATGAGCACGAACTGAACCAGCT-3′ and 5′-GTCCCATGTAGTACTGGAGACAGCTC-3′ for the 455 bp rat AC3 [19,20]; and 5′-CAGTCTGGGCCTGAGGAAATT-3′ and 5′-AAGTCAGGTTCTTCAAGGGTA-3′ for the 477 bp mouse AC8 probe. The amplified fragments were subcloned in the *Sma*I site of pGEM3 (Promega). A 722 bp fragment derived from the mouse AC7 cDNA [21] was inserted in the TA cloning site of pCRII. Probes specific for AC4, AC5, AC6 and AC9 were obtained as described [22]. A mouse β-actin fragment (*Sma*I-*Kpn*I; 822 bp) was used as a control in all Northern blots [23]. A chicken calmodulin cDNA fragment, cross-hybridizing with CaMI, II and III, was used as a probe for CaM [24].

The probes were labeled with [α-^{32}P]dCTP by the Megaprime labeling system (Amersham) according to the manufacturer's instructions.

2.5. RNA preparations and Northern blotting

Total RNA was extracted by the guanidinium thiocyanate/phenol extraction method [25]. Poly(A)$^+$ RNA was selected by binding to oligo(dT) cellulose. For Northern blots, 10 μg of poly(A)$^+$ RNAs or 30 μg of total RNAs were electrophoresed on 1% agarose gel containing 2.2 M formaldehyde and transferred overnight onto Hybond N$^+$ membrane (Amersham) by capillary blotting. Hybridization with specific cDNA probes was carried out using Rapid-hyb buffer (Amersham) according to the manufacturer's instructions. Hybridization with the β-actin probe was used as an internal control to estimate the amount of total or poly(A)$^+$ RNA loaded in each well and the efficiency of the transfer. The membranes were washed for 15 min in 2×SSC, 0.1% SDS at room temperature, twice 0.2×SSC, 0.1% SDS at 65°C and exposed to Kodak XAR film at −80°C. Instant Imager analysis of the Northern blots was used for quantification of the signal intensity.

3. Results

3.1. Adenylyl cyclase activity increases with time in RA-primed P19 cells. Ca^{2+} sensitivity

The evolution of AC activity in P19 plasma membranes as a function of time after RA induction is depicted in Fig. 1. After a 3 day lag period, basal AC activity increased 10-fold on day 7. The evolution of FSK-stimulated AC activity as a function of time was quite similar with a 5-fold increase at day 7.

AC activity was studied as a function of Ca^{2+} concentration in membranes from undifferentiated (UD) cells (Fig. 2A) and from RA-induced cells (day 7) (Fig. 2B). In the presence of 1 μM CaM added, the basal AC activities in both cases were stimulated by the Ca^{2+}/CaM complex by 5- and 3-fold in UD and RA-induced cells respectively (Fig. 2). Without CaM added, the basal AC activity in membranes from UD cells did not change markedly with the various concentrations of Ca^{2+} (Fig. 2A). The small increase in AC activity of differentiated cells observed in the presence of Ca^{2+} alone, without CaM added (Fig. 2B), is probably due to the large amount of endogenous CaM already present in neuronal cell membranes. Addition of W7, a drug which inhibits the binding of Ca^{2+} to CaM, totally abolished this stimulation of AC activity, confirming the specificity of the effect observed (Fig. 2B).

3.2. Determination of AC isoform mRNAs expressed in UD and RA-primed P19 cells

To determine the expression of AC genes in the course of RA-induced differentiation, Northern blot experiments were conducted with mRNAs isolated from UD and RA-induced cells (day 3 and day 7). Following hybridization of Northern blots with specific cDNA probes, undifferentiated P19 cells expressed mainly AC6 and AC3 mRNAs (Fig. 3A). mRNAs for AC2, AC5, AC7, AC8 and AC9 were detected in UD cells at very low levels, while mRNAs for AC1 and AC4 were undetectable (Fig. 3A and data not shown).

While the levels of mRNAs of AC7 and AC9 were stable during the course of differentiation, dramatic changes in the expression of AC2, AC3, AC5, AC6 and AC8 were observed (Fig. 3). AC2 mRNA increased 20-fold at day 3 and up to 30-fold at day 7. AC3 mRNA diminished markedly at day 3 and was even further reduced by day 7. AC6 mRNAs increased at day 3 (2.8-fold) and decreased in neuronally differentiated cells. AC5 and AC8 mRNAs were markedly increased by 8- and 10-fold respectively at day 7.

3.3. Isoform-specific increase of calmodulin mRNA in RA-primed P19 cells

The developmental increase of Ca^{2+}/CaM-stimulated AC activity in differentiated neuronal cells was accompanied by an isoform-specific increase of CaM itself (Fig. 4). Different

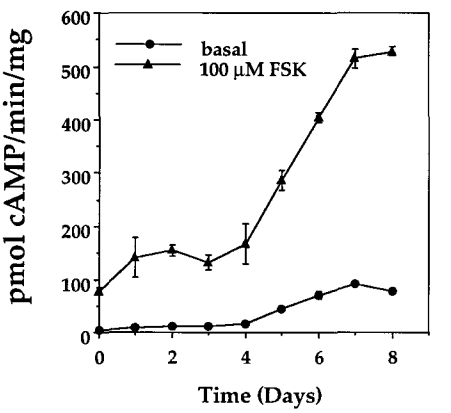

Fig. 1. AC activity in membranes prepared from P19 cells during the course of neuronal differentiation. P19 teratocarcinoma cells were induced to differentiate along the neuroectodermal pathway by aggregating in the presence of 1 μM RA. Each day AC activity was assayed on an aliquot in the absence (basal) or in the presence of 100 μM forskolin (FSK). Each point represents the mean ± S.E.M. (bars) value of triplicate determinations from a representative experiment.

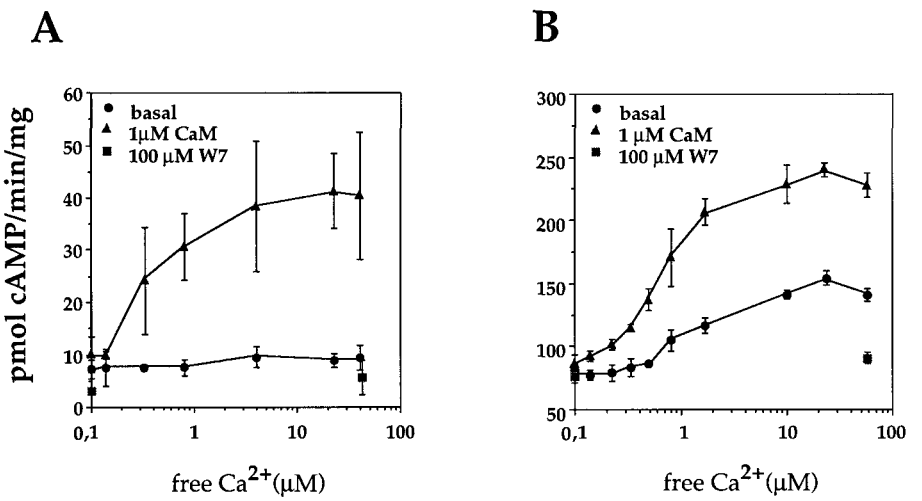

Fig. 2. Effect of Ca^{2+} and calmodulin on AC activity in membranes from UD (A) and differentiated (B) P19 cells. AC activities were assayed in the absence (basal) or in the presence of 1 μM calmodulin (CaM). W7, a specific inhibitor of CaM, was added to some assays to abolish the effect of endogenous CaM. Free concentrations of Ca^{2+} were adjusted using EGTA/$CaCl_2$ buffers. Each point represents the mean ± S.E.M. (bars) value of triplicate determinations from a representative experiment.

CaM mRNAs have been described [26], originating from a tree of different genes. Four species of calmodulin mRNAs (4.0 and 1.7 kbp for CaM-I, 1.4 kbp for CaM-II and 2.3 kbp for CaM-III) were detectable in P19 UD cells and an isoform-specific regulation of CaM expression occurred in the course of neuronal differentiation. CaM-I (1.7 kbp) and CaM-II (1.4 kbp) increased about 2-fold in aggregates on day 3, and subsequently remained so during the course of differentiation (Fig. 4). The two other CaM isoform mRNAs did not change.

4. Discussion

4.1. *The basal Ca^{2+}/CaM-stimulated AC activity in P19 cells increases upon RA-induced neuroectodermal differentiation*

In this paper, we show for the first time that the P19 stem cell line possesses a basal AC activity stimulated by the Ca^{2+}/CaM complex. This Ca^{2+}/CaM-stimulated activity, very low in UD cells, increases about 10-fold after RA induction. This result indicates that, at an early stage of development when ACs are apparently still loosely coupled to G-proteins, and when the repertoire of receptors, hormones and paracrine factors is still very limited, calcium might represent a key factor of regulation. Specific spontaneous Ca^{2+} transients, observed in developing neurons, have been reported to be sufficient and necessary to drive normal differentiation and are required for the normal expression of γ-aminobutyric acid or for the regulation of neurite extension [27,28]. Moreover, it has recently been shown that the calcium binding protein calreticulin can interfere with the RAR and RXR signaling pathways to disrupt retinoid-induced differentiation [29].

The specific AC isoform(s) responsible for this calcium sensitivity has not yet been identified. AC3 and AC8 are both expressed in UD cells at a low level, while AC1 mRNA was never detected. AC8 can be directly stimulated by Ca^{2+}/CaM [30,31], whereas AC3 is stimulated by the Ca^{2+}/CaM complex only when the enzyme is previously activated by other effectors, such as GppNHp or forskolin [32]. For this reason, AC3 is unlikely to be responsible for the calcium-stimulated activity. Furthermore, its mRNA completely disappeared in neuronally differentiated P19 cells. Thus, our data, together with the known biochemical properties of the different AC isoforms [30–33], suggest that AC8 is responsible for the Ca^{2+}/CaM-stimulated activity both in UD and RA-induced cells. However, a new isoform cannot be completely excluded.

The importance of the calcium signaling pathway in neuronal differentiation is supported by the isoform-specific induction of CaM gene expression after RA treatment. Three different calmodulin genes and at least five transcripts encode an identical, highly conserved protein [26], and exhibit a tissue-specific developmental pattern of expression related to calmodulin synthesis [34]. Our data fit well with this observation.

4.2. *Specific regulation of AC2, AC3, AC5 in course of RA-induced differentiation*

RA induction of P19 cells leads to specific up-regulation of AC2 mRNA and to down-regulation of AC3 mRNA. The AC6 mRNA increase could be considered non-specific, because it was also observed in cells aggregated without any inductor of differentiation (data not shown). One might hypothesize that the promoters of AC2 and AC3 contain RAREs or responsive elements for the RA-activated transcription factors, such as HOX, OCT or JUN.

It is possible that a part of the total AC activity increase is due to AC2 expression during the course of RA-induced differentiation. This isoform has a high basal activity and is stimulated by all known signaling molecules, such as Gαs, βγ as well as PKC [35–38]. The cells in which AC2 is the predominant isoform may then be able to integrate several positive signals received through multiple channels, and rapidly influence the intracellular cAMP level. Finally, the increase in AC5 mRNA that we observed is not surprising since, although it is a good marker of adult myocardium [39], it is enriched in the striatum where it shows a specific pattern of development [40].

Thus, a specific set of ACs is expressed in UD P19 cells and is specifically modified upon RA-induced differentiation, thus

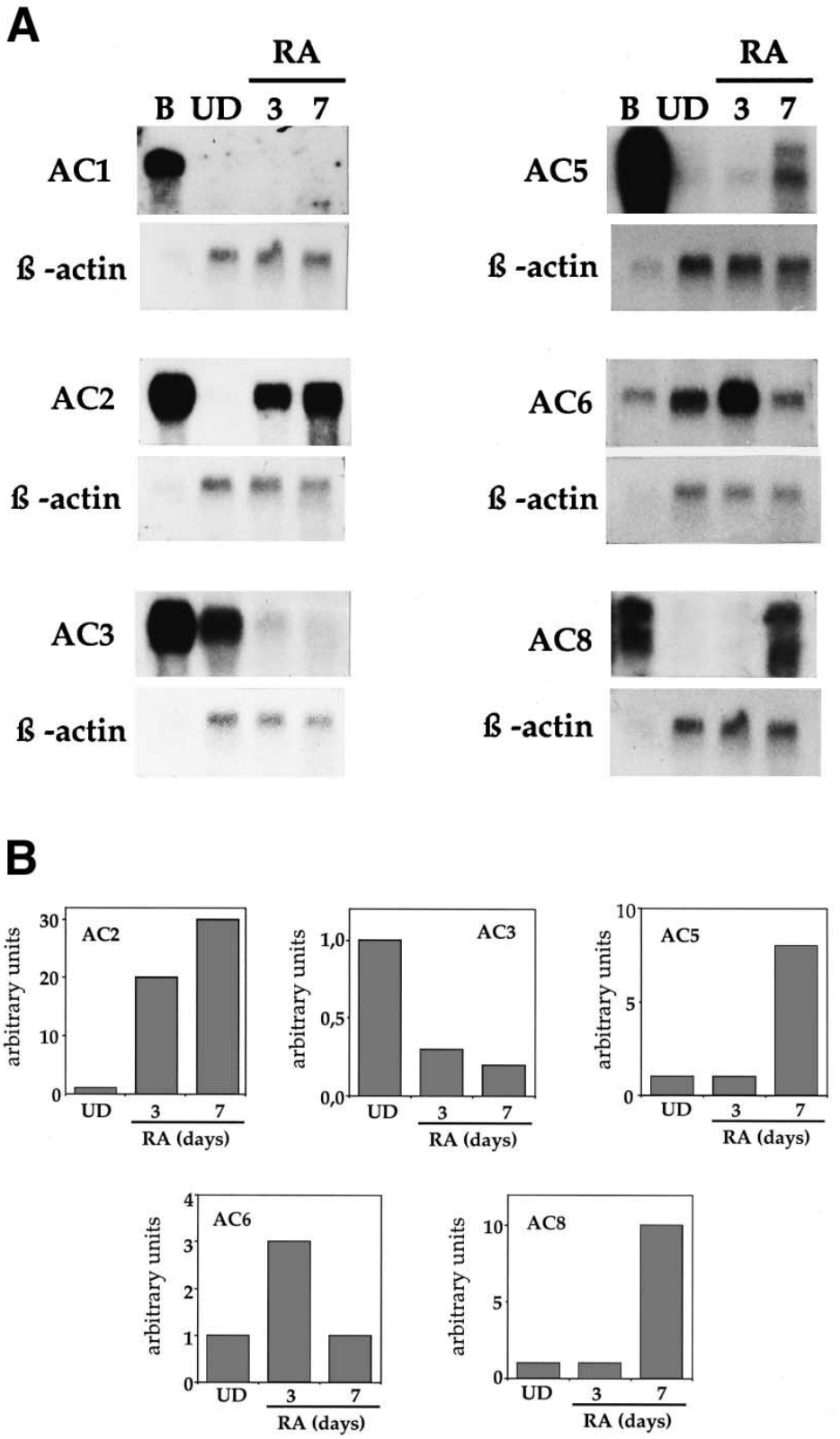

Fig. 3. Determination of AC isoforms expressed in UD and RA-induced P19. A: Northern blot analysis of poly(A)$^+$ RNA extracted from UD or RA-induced (day 3 and day 7) P19 cells. 10 μg of poly(A)$^+$ RNA obtained from different cell types or 2 μg of poly(A)$^+$ RNA from mouse brain was loaded in each line. Filters were hybridized to ^{32}P-labeled cDNA probes for the indicated AC isoforms, and afterwards for β-actin. Autoradiograms were obtained after exposure for 1 h (β-actin), 6 h (AC6) or 3 days (others ACs). B: Quantification of the changes in mRNA for the different AC isoforms during the course of RA-induced differentiation of P19 cells. Filters were scanned and analyzed using the Instant Imager system. The amount of mRNA for each AC was corrected according to the quantity of mRNA for β-actin and normalized to an arbitrary value of 1 for the UD sample.

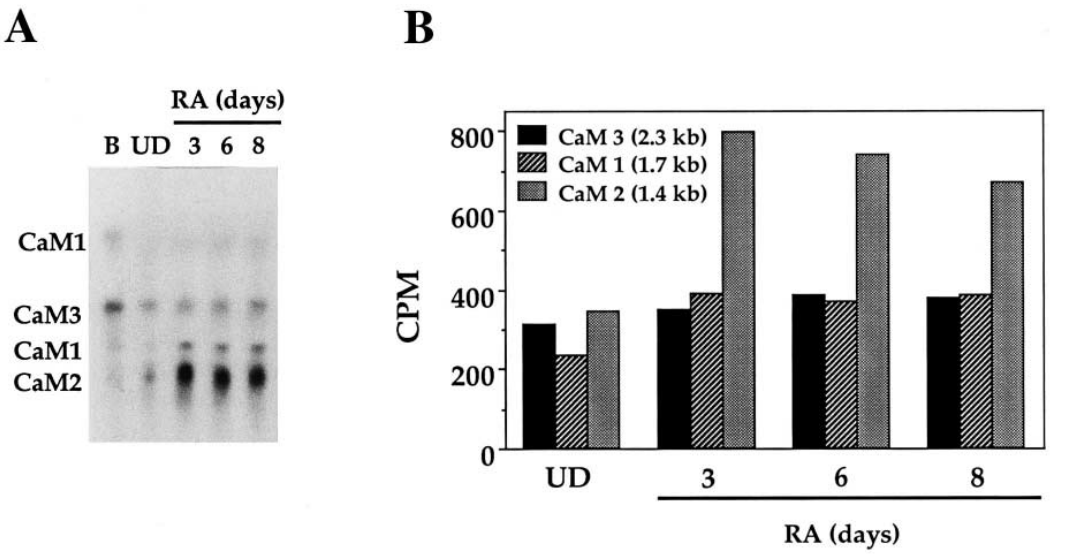

Fig. 4. Type-specific induction of calmodulin mRNA during RA-induced differentiation of P19 cells. A: Northern blot analysis of total RNA extracted from UD and RA-induced (day 3, day 6 and day 8) P19 cells. 40 μg of total RNA obtained from different cell types (10 μg for mouse brain) was loaded in each line. Filter was hybridized to ^{32}P-labeled chicken CaM cDNA probe. Autoradiograms were obtained after 2 h of exposure. B: Quantification of the changes in mRNA for the different isoforms of CaM during the course of RA-induced differentiation of P19 cells. Filter was scanned and analyzed using the Instant Imager System.

leading to the hypothesis that AC isoforms could play a very important role during cell differentiation.

In summary, the present study provides evidence for the importance of the Ca^{2+}/cAMP signaling pathway in neuroectodermal differentiation. Embryonic carcinoma P19 cells represent the first cell line expressing a basal Ca^{2+}/CaM-stimulated AC activity. After RA induction, the Ca^{2+}/CaM AC activity increases 10-fold and this increase is supported by a specific up-regulation of AC8 mRNA. RA induction also leads to specific down-regulation of AC3 and up-regulation of AC2 and AC5 mRNA. P19 teratocarcinoma cells provide a powerful model to investigate the role of AC in neuronal differentiation as well as in the elucidation of the specific regulation of AC2, AC3, AC5 and AC8 synthesis.

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