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Modulation of Peroxisome Proliferator-Activated Receptor δ Activity Affects Neural Cell Adhesion Molecule and Polysialyltransferase ST8SiaIV Induction by Teratogenic Valproic Acid Analogs in F9 Cell Differentiation

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ABSTRACT

It has been suggested that the teratogenic effects of the antiepileptic drug valproic acid (VPA) is reflected in vitro by the differentiation of F9 cells, activation of peroxisome proliferator-activated receptor δ (PPARδ), and inhibition of histone deacetylases (HDACs). The aim of this study was to identify genes involved in the differentiation of F9 cells induced by VPA, teratogenic VPA derivatives, or the HDAC inhibitor trichostatin A (TSA) and to characterize the role of PPARδ. Treatment of the cells with teratogenic VPA derivatives or TSA induced differentiation of F9 cells, mRNA, and protein expression of the neural cell adhesion molecule (NCAM) as well as activated the 5′-flanking region of the NCAM promoter, whereas nonteratogenic VPA derivatives had no effect at all. The polysialyltransferases [ST8SiaIV (PST1) and ST8SiaII] are responsible for the addition of polysialic acid (PSA) to NCAM. The mRNA expression of PST1 was highly induced by only teratogenic VPA derivatives and TSA. As shown by fluorescence-activated cell sorting analysis the level of PSA was higher after treatment of F9 cells with teratogenic VPA derivatives. It is interesting that overexpression of the PPARδ but not PPARα or PPARγ in F9 cells resulted in higher induction of NCAM mRNA and protein expression and of PST1 mRNA expression (and a higher PSA level) than in mock-transfected F9 cells. Furthermore, repression of PPARδ activity in F9 cells inhibited these effects. We conclude that NCAM and PST1 are molecular markers in F9 cell differentiation caused by treatment with teratogenic VPA compounds or TSA and suggest that in addition to HDAC inhibition PPARδ is involved in the signaling pathway.

Valproic acid (2-*n*-propylpentanoic acid; VPA) has a remarkable antiepileptic activity, but it is teratogenic in humans and in mice when given during early organogenesis of the embryo (Nau et al., 1991). The mechanism of interference of VPA with embryonic development is unknown. In humans, VPA can cause spina bifida, a posterior neural tube defect. In the mouse, the predominant neural tube defect after single VPA injection on day 8 of gestation is exencephaly, an anterior neural tube defect. Repeated treatment on day 9 of gestation induces posterior neural tube defects (spina bifida aperta and occulta) (Ehlers et al., 1992). In the search for new drugs with selective anticonvulsant activities and less toxicity, numerous derivatives and various metabolites of VPA have been investigated and found to exert anticonvulsant activity in rodents (Nau et al., 1991; Ehlers et al., 1992). It has been shown that the teratogenic effects are caused by VPA itself, not one of its metabolites (Ehlers et al., 1992). The teratogenic effects in vivo and in vitro depend on structural requirements (Nau et al., 1991; Bojic et al., 1996; Lampen et al., 1999). In vitro only teratogenic VPA derivatives induce cell differentiation of embryonic F9 stem cells (Lampen et al.,

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ABBREVIATIONS: VPA, valproic acid; PPAR, peroxisomal proliferator-activated receptor; HDAC, histone deacetylase; TSA, trichostatin A; NCAM, neural cell adhesion molecule; PSA, polysialic acid; STX, polysialyltransferase ST8SiaII; PST1, polysialyltransferase ST8SiaIV; cPGI, carbaprostacyclin; AP-2, activator protein-2; PBS, phosphate-buffered saline; DTT, dithiothreitol; RT-PCR, reverse transcription-polymerase chain reaction; RSV, Rous sarcoma virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s); PCR, polymerase chain reaction; RT, reverse transcription; FACS, fluorescence-activated cell sorting; ANOVA, analysis of variance; butyl-4-yn-VPA, 2-(2-propinyl)-hexanoic acid; pentyl-4-yn-VPA, 2-(2-propinyl)-heptanoic acid; hexyl-4-yn-VPA, 2-(2-propinyl)-octanoic acid; heptyl-4-yn-VPA, 2-(2-propinyl)-nonanoic acid; isobutyl-4-yn-VPA, 2-(2-methylpropyl)-4-pentynoic acid; 4-yn-VPA, 2-*n*-propyl-4-pentynoic acid; E-2-en-VPA, 2-*n*-propyl-2-pentenoic acid; 4-en-VPA, 2-*n*-propyl-4-pentenoic acid; ethyl-4-yn-VPA, 2-ethyl-4-pentynoic acid.

1999), which is believed to reflect early events in the embryonal development (Alonso et al., 1991; Werling et al., 2001), whereas nonteratogenic VPA derivatives did not induce F9 cell differentiation.

VPA has been shown to interact with an intracellular receptor, the peroxisome proliferator-activated receptor (PPARδ; Lampen et al., 1999, 2001a; Werling et al., 2001). Three PPAR isotypes have been identified: α, β (also called δ and NUC1), and γ. Structure-activity investigations have shown that only teratogenic VPA derivatives activate PPARδ, whereas nonteratogenic compounds had no effect at all (Lampen et al., 2001a).

Acetylation and deacetylation of histones play significant roles in the regulation of gene transcription in many cells. There are two classes of enzymes involved in the acetylation state of histones, histone acetyl transferases, and histone deacetylases (HDACs). It was recently shown that VPA inhibits HDAC activity in F9 teratocarcinoma cells and that PPARδ is derepressed by HDAC inhibition (Gottlicher et al., 2001; Phiel et al., 2001). Trichostatin (TSA) is an anticancer compound and a well characterized HDAC inhibitor able to induce cell differentiation. It is interesting that TSA is also a teratogenic compound that induces neural tube defects very similar to those induced by VPA (Svensson et al., 1998; Phiel et al., 2001), suggesting that VPA and TSA may act in a similar manner.

The neural cell adhesion molecule (NCAM) plays a major role in the development and plasticity of the nervous system (Rutishauser et al., 1988). Three different isoforms of NCAM that are encoded by a single-copy gene and generated via alternative RNA splicing as well polyadenylation, and post-translational modifications of glycosylation, sulfation, and phosphorylation have been described previously (Goridis and Brunet, 1992). Potential inhibition effects of VPA on tumor metastasis are currently under investigation, and some in vitro and in vivo studies indicate a close relationship not only between tumor metastasis and NCAM expression but also between neuritogenesis and NCAM expression. VPA is reported to increase membranous expression of NCAM in human glioma cell lines as well as in Ntera-2 cells (Cinatl et al., 1996; Skladchikova et al., 1998), but it is not clear how VPA controls NCAM.

Polysialic acid (PSA) is a unique polysaccharide consisting of α-2,8-linked sialic acid residues attached to *N*-glycosylation sites on the fifth immunoglobulin-like domain of NCAM (Muhlenhoff et al., 1998). PSA modifies the adhesive potential of NCAM. Because of its steric properties, PSA attenuates cell-cell adhesion and is generally considered a promoter of neural plasticity, allowing cell movements and changes in cell interactions (Rutishauser and Landmesser, 1996). Two enzymes are responsible for the addition of oligosaccharides to the NCAM protein: the two closely related polysialyltransferases ST8SiaII (STX) and ST8SiaIV (PST1). To the best of our knowledge, it is not known whether VPA, VPA derivatives, or TSA has an effect on PST1 or STX. The present study was undertaken to identify genes involved in the VPA-induced differentiation of F9 cells as valuable model for early events in the embryonal development and to compare the effects with treatment of the cells with a typical HDAC inhibitor (TSA). In addition, we analyzed the role of the PPARδ activity in expression of NCAM and PST1.

Materials and Methods

Cell Culture and Reagents. Mouse F9 cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in Ham's F-12/Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) medium supplemented with 2 mM glutamine, 0.0012% (w/v) mercaptoethanol, and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. The carbaprostacyclin (cPGI) was obtained from Cayman Chemical (Ann Arbor, MI).

Plasmids. The pBV-NCAM expression vector contains the NCAM promoter (Barton et al., 1990) and was a generous gift from Prof. C. H. Barton (Department of Biochemistry and Molecular Biology, University of Southampton, Southampton, UK). It is interesting that, according to the Web-based software tool ConSite (http://www.phylofoot.org/consite), the NCAM promoter contains responsive elements of c-fos and AP-2. These two genes have been recently identified as markers of VPA-induced F9 cell differentiation (Werling et al., 2001). The pGL2-PST2 plasmid contains the ST8SiaIV promoter (Eckhardt and Gerardy-Schahn, 1998) and was a generous gift from Prof. R. Gerardy-Schahn (Medizinische Hochschule Hannover). It is interesting that this promoter also contains, according to ConSite, responsive elements of c-fos and AP-2. The pSG5-mPPARα and the pSG5-mPPARγ expression vector contains mouse PPARα or mouse PPARγ and were kindly provided by Prof. G. Perdew (Penn State University, University Park, PA) and described in Sumanasekera et al. (2003). The pcDNA3-PPARδ expression plasmid and the pcDNA3-PPARδE411P mutant cDNA of PPARδ were derived from pSG5-FAAR (Amri et al., 1995) as described in Bastie et al. (2000). The dominant-negative PPARδ was generated by substitution of a glutamate residue by a proline in the loop preceding the AF-2 domain. Receptors mutated in or near the AF-2 region are inactive and neither release corepressors nor interact with coactivators.

Synthesis of VPA Derivatives. VPA was obtained from Sigma Chemie (Deisenhofen, Germany). E-2-en-VPA was obtained from Desitin (Hamburg, Germany). The other VPA derivatives and the pure enantiomers (*R*)- and (*S*)-4-yn-VPA were synthesized as described by Hauck and Nau (1992) and Bojic et al. (1996).

Transfection and Drug Treatment. Gene transfer was carried out using the calcium phosphate precipitation technique following standard protocols. The final DNA content was 0.2 μg of one expression plasmid (pBV-NCAM or GL2-PST2) per well in 1 ml of medium. Six hours after transfection, the medium was changed, cells were washed with phosphate-buffered saline (PBS), and new medium containing the test compounds was added. After 24 h of exposure, the medium was removed, cells were washed twice in PBS without Ca^{2+} and Mg^{2+}, and harvested in 200 μl of lysis buffer (0.1 M Tris-acetate, pH 7.5, 2 mM EDTA, and 1% Triton X-100). For measurement of luciferase activity, the samples were pipetted into transparent reading tubes and transferred to a luminometer (Lumat LB9507; Berthold Technologies, Bad Wildbad, Germany). There, the samples were mixed automatically with 100 μl of luciferin-containing buffer (20 nmol/test) and 300 μl of assay buffer (25 mM glycylglycerine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, and 2 mM ATP, pH 7.8). We used a cytomegalovirus-β-Gal plasmid as a control for transfection efficiencies and to standardize luciferase activities.

To study transfection of F9 cells with pSG5-mPPARα, pSG5-mPPARγ, pcDNA3-mock, pcDNA3-PPARδ, or pcDNA3-PPARδE411P, overexpression of F9 cells or repression of PPARδ was determined by RT-PCR and Western blotting. Total cell extracts were prepared from the cells in a buffer containing 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 1 mM vanadate, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40. The extracts were separated on 10% polyacrylamide SDS gels and blotted to nitrocellulose membranes. PPARδ and PPARδE411 mutant proteins were detected using a polyclonal antiserum raised against the A/B domain of mouse PPARδ; this antiserum recognizes native and mutated PPARδ. Immunodetection was performed by chemiluminescence using an enhanced chemiluminescence

advanced reagent (Amersham Biosciences Inc., Braunschweig, Germany).

Transfection of F9 Cells and Treatment with Indomethacin. Expression vector (RSV-Luc), transfection, and reporter gene assay were described previously (Lampen et al., 1999). It is interesting that the RSV-promoter contains, according to ConSite, responsive elements of c-fos and AP-2. The concentration of dimethyl sulfoxide in the cultures did not exceed 0.5%. Exposure was made in triplicate, and for each assay a positive control containing 1 mM VPA as well a negative control containing 1 mM 2-en-VPA (Lampen et al., 1999) was measured. This concentration of VPA was used in all experiments to ensure the comparison with known data in the literature. Induction of RSV-Luc by 1 mM VPA was used to normalize the interassay variabilities. After 20 h of exposure, the medium was removed, cells were washed twice in PBS without Ca^{2+} and Mg^{2+}, and harvested in 200 μl of lysis buffer (0.1 M Tris-acetate, pH 7.5, 2 mM EDTA, and 1% Triton X-100). For measurement of luciferase activity, the samples were pipetted into transparent reading tubes and transferred to a luminometer (Lumat LB9507; Berthold Technologies) and assayed as described previously (Lampen et al., 1999).

Total RNA Preparation. Total RNA was extracted from F9 cells as described in Lampen et al. (1999). In addition to absorbance measurements, the concentration of RNA was verified on agarose gel colored with ethidium bromide. The amount of RNA was quantified using Ribogreen kit (MobiTec, Göttingen, Germany) for the competitive RT-PCR.

Oligonucleotides Used for Amplifications. The competitive quantitative RT-PCR was performed as described in Aubeouf et al. (1997). The various primers for the construction of internal standards and for competitive quantitative RT-PCR were derived from the mouse cDNA-sequences of NCAM, PST1, or GAPDH. If possible, they were designed to span a product that consists of two exons.

Construction and Use of the Competitors. We used PCR to generate an internal deletion within the target cDNA sequence of each gene selected for quantification. We designed four primers (P1–P4) spanning two products (A and B) with a deletion between 50 and 80 bp. The reversed primer P2 of fragment A and the forward primer P3 of the fragment B contained a linker of 22 bp complementary to each other. After the PCR of fragments A and B, these two products were cleaned using PCR purification kit (QIAGEN GmbH, Hilden, Germany), denaturated, and a second fusion-PCR was performed with fragment A and B using primer P1 and P4. The resulting product contained primer sites of P1 and P4 and a deletion between fragment A and B of approximately 50 bp. This product was amplified in a next PCR and quantified after agarose gel electrophoresis and visualized by ethidium bromide staining and densitometric analyses (Molecular Analyst; Bio-Rad, Munich, Germany) using a Bluescript plasmid (pBR32) as a DNA quantification marker. A regression analysis was performed to quantify the competitor cDNA. Different dilutions of the competitor were used in the competitive PCR together with different dilutions of the wild-type cDNA.

Primer for NCAM. cDNA of NCAM (GenBank accession no. X15049) (Barthels et al., 1987) was a kind gift of Dr. Christo Goridis (Institute de Biologie du Development de Marseille, Marseille, France). The primer-flanking sequences cover exons 2 to 9 and react with all known NCAM splice forms: P1–P4, 622 bp; P3–P4, 518 bp (deletion of 104 bp); P1, TGAGGGTACTTACCGCTGTG; P2, GGATCCGTTCACAAGCTCGTCCCATCAGCATCACACACCAG; P3, GACGAGCTTGTGAACGGATCCTCTGCATCGCAGAGAACAAG; and P4, GTTGCTGGCAGTGCACATGT.

Mouse glyceraldehyde 3-phosphate dehydrogenase primer: GenBank accession no. M32599 (Sabath et al., 1990): P1–P2, 133 bp; P3–P4, 255 bp (deletion of 122 bp); P1, TGGTGAAGGTCGGTGTGAAC; P2, GGATCCGTTCACAACTGAGGTCAATGAAGGGGTCG; P3, GTTGTGAACGGATCCACCATCTTCCAGGAGCGAGA; and P4, GTGCAGGATGCATTGCTGAC.

Primer for ST8SiaIV (PST), accession no. Y09486 (Takashima et al., 1998). cDNA was a kind gift from Prof. R. Gerardi Schahn (Medizinsche Hochschule Hannover, Hannover, Germany). P1, ACCGCAGGTTTAAGACCTGTGC; P2, GGATCCGTTCACAAGCTCGTCCACATCAGCAGCGAACTCCA; P3, GACGAGCTTGTGAACGGATCCTCCTGCCTTCATGGTCAAAG; and P4, GCCAGTATCCTCTGACTGCATG.

RT-Competitive PCR. Reverse transcription (RT) of 0.1 μg of total RNA using oligo$(dT)_{15}$ was performed for 120 min at 42°C with 2 units of Superscript II reverse transcriptase (Invitrogen) in Superscript buffer (50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 20 mM DTT, and 0.2 mM each of dATP, dGTP, dCTP, and dTTP). The samples were then heated for 1 min at 99°C to terminate the reverse transcription reaction. The polymerase chain reaction was performed on 1 μl of a 1:10 dilution in water of a prepared cDNA. Then, 8 aliquots of the mixture was transferred to microtubes containing a different, but known, amount of competitor. After 120 s at 95°C, the

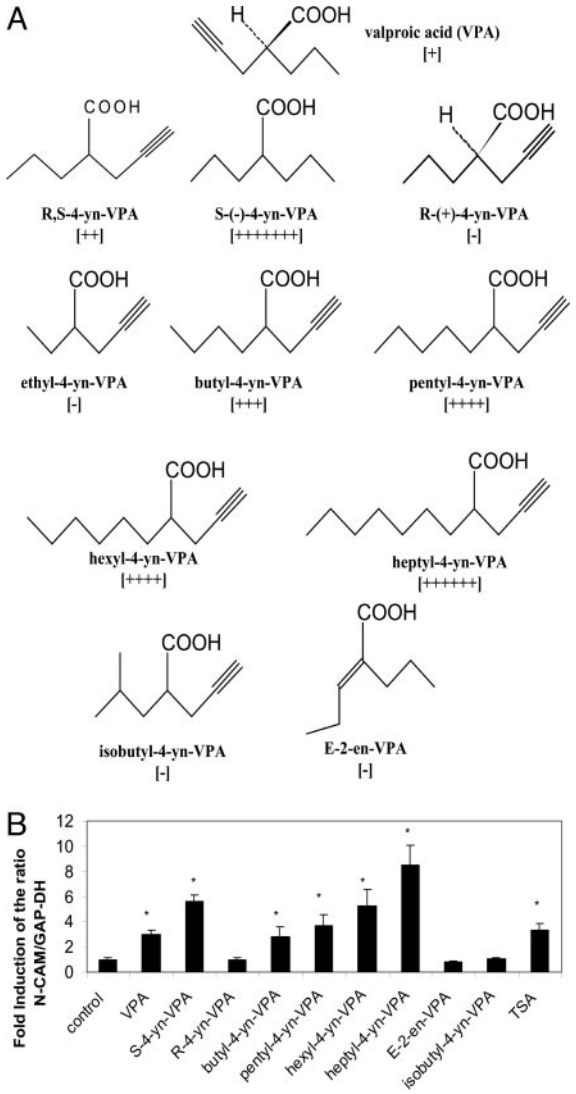

Fig. 1. A, structure of VPA and VPA derivatives and their teratogenic potency in vivo; + indicates the teratogenic potency in comparison with VPA as standard substance. B, gene expression of NCAM in F9 cells after treatment with different VPA derivatives. NCAM and GAPDH mRNA expression were measured by competitive RT-PCR as described under *Materials and Methods*. F9 cells were treated for 48 h with 1 mM 2-en-VPA, 1 mM VPA, and a 0.25 mM concentration of one of the following: (*S*)-4-yn-VPA, (*R*)-4-yn-VPA, butyl-4-yn-VPA, pentyl-4-yn-VPA, hexyl-4-yn-VPA, heptyl-4-yn-VPA, or 50 nM TSA. The analysis of the competitive RT-PCR is shown here. Values represent means ± S.D. from triple determinations, with asterisks indicating a significant difference ($p < 0.005$; ANOVA) from untreated cultures.

tubes were subjected to 30 cycles (60 s at 95°C, 60 s at 57°C, and 60 s at 72°C) of amplification and to a fill up step of 10 min at 72°C (MWG-Biotech, Ebersberg, Germany).

Analysis of the PCR Products. The PCR products on the ethidium-stained agarose gel were analyzed densitometrically with the Molecular Analysis software (Bio-Rad). The amount of PCR product was calculated by integration of the peak area using the Molecular Analysis software. To determine the concentration of the target cDNA, the logarithm of the peak surface ratio of competitor-to-target cDNA was plotted against the logarithm of the amount of competitor added to the PCR medium. The initial concentration of the target cDNA in the reaction was determined at the competition equivalence point as described by Auboeuf et al. (1997). The absence of genomic DNA amplification during the RT-competitive PCR assay was verified by performing the reactions without the reverse transcriptase in the RT step. The relative amount of mRNA expression in comparison with mouse GAPDH is shown in the figures.

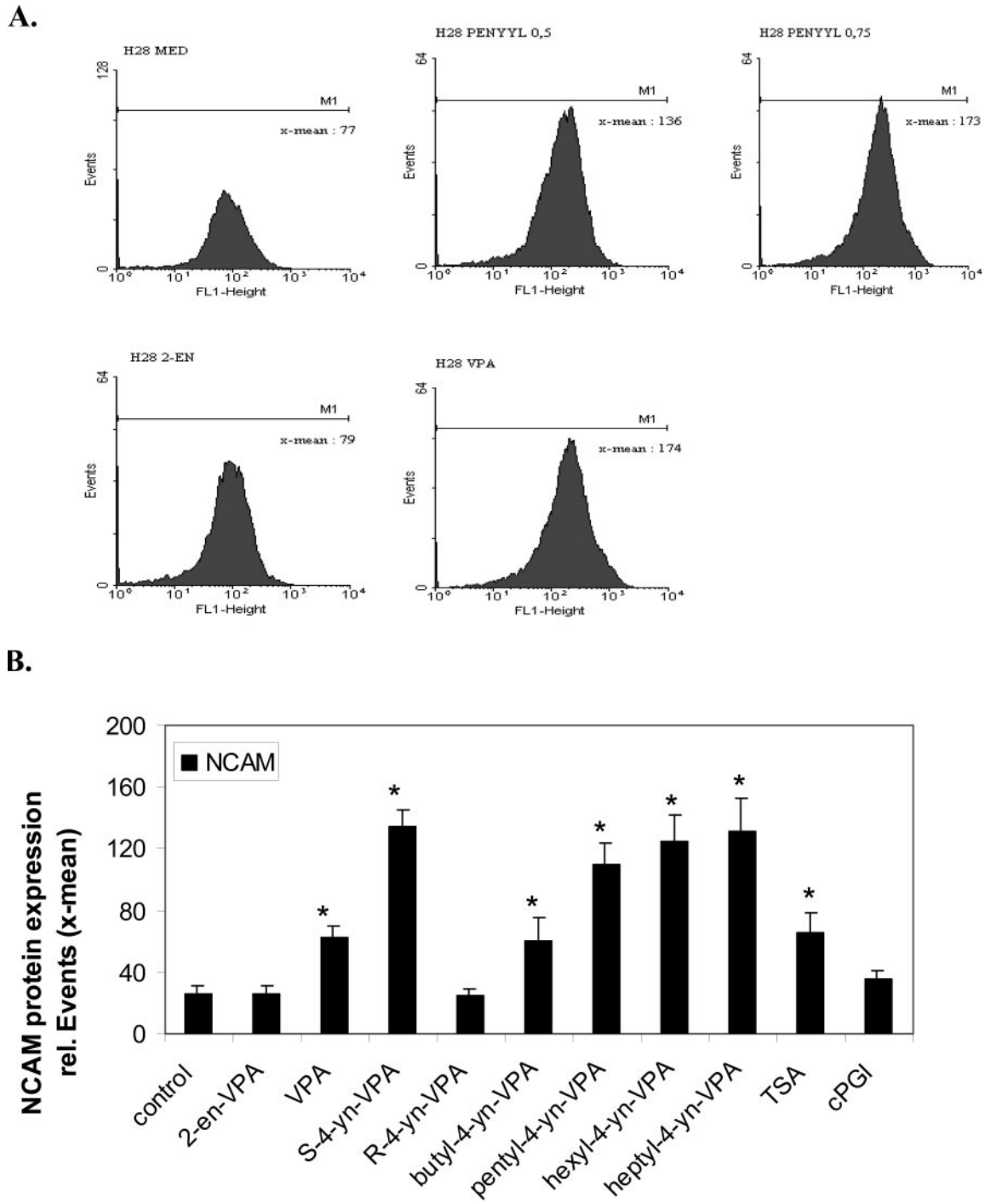

Fig. 2. Protein expression of NCAM in F9 cells after treatment with different VPA derivatives. F9 cells were treated 48 h with 1 mM 2-en-VPA, 1 mM VPA, and a 0.25 mM concentration of one of the following: (*S*)-4-yn-VPA, (*R*)-4-yn-VPA, butyl-4-yn-VPA, pentyl-4-yn-VPA, hexyl-4-yn-VPA, heptyl-4-yn-VPA, 50 nM TSA, or 5 μM cPGI. NCAM protein level was measured by FACS analysis. A, typical result of an experiment. B, structure-activity relationships. Values represent means ± S.D. from four determinations, with asterisks indicating a significant difference ($p < 0.01$; ANOVA) from untreated cultures.

Validation of the RT-PCR Assay. To validate the RT-competitive PCR assay, RNAs corresponding to a part of mouse NCAM, PST1, or GAPDH were synthesized by in vitro transcription (Riboprobe system; Promega, Heidelberg, Germany) using plasmids containing the respective cDNA. Known amounts of these RNAs were quantified by RT-competitive PCR over a wide range of concentrations (0.25–50 amol) added to the RT medium. Standard curves were obtained. The linearity (with r between 0.98 and 1.00 for the different dose responses) and the slopes of the standard curves demonstrated that the RT-competitive assay developed in this work is indeed quantitative. The interassay variation of the RT-competitive PCR was estimated from at least eight separate determinations and found to be 3.8% when a small amount of target RNA was quantified (0.61 ± 0.04 amol) and 11% with a higher amount (13.2 ± 1.7 amol).

Semiquantitative RT-PCR. A well established semiquantitative RT-PCR method was used for the measurement of the mRNA expression of NCAM in F9 cells as described in Lampen et al. (2001c). The primers for NCAM were 5′-TGAGGGTACTTACCGCTGTG-3′ and 5′-GTTGCTGGCAGTGCACATGT-3′, with a product size of 622 bp. Primers for β-actin were GGCGGCACCACCATGTACCCT for sense and AGGGGCCGGACTCGTCATACT for antisense (GenBank accession no. Mm 001101).

Flow Cytometry (FACS Analysis). F9 cells were treated 2 days with or without VPA derivatives. Afterward, cells were separated from culture bottles with acutase (Biochrom, Berlin, Germany), and 250,000 cells in 100 μl were added in each microplate well of a 96-well plate. After centrifugation (1000 U/min for 5 min), one of the following was added to each well: the first antibody H28 mouse α-anti-NCAM (3.5 mg/ml) at a dilution of 1:50 (25 μl/well) or 735 m-anti-PSA (8.18 mg/ml) at a dilution of 1:50. After incubation for 20 min at 4°C, the wells were washed three times with MIF (PBS with 2.5% bovine serum albumin) buffer. The second antibody was either a fluorescein isothiocyanate-labeled anti-rat (for H28 NCAM) at a dilution of 1:10 or anti-mouse (for 735 PSA) at a dilution of 1:20. One of these antibodies was added to each well and incubated for another 20 min at 4°C. Finally, 100 μl of MIF buffer and the pellet was resuspended in a conical tube. After the addition of 100 μl of PBS buffer and 200 μl of PBS buffer containing 2 μg/ml propidium iodide, the probes were measured in a FACScan (BD Biosciences, Heidelberg, Germany). The propidium iodide-stained (dead) cells were excluded from the analysis. H28 mouse α Anti-NCAM and 735 m-anti-PSA (Muhlenhoff et al., 1998) were a generous gift of Prof. R. Gerardy-Schahn (Medizinische Hochschule Hannover, Germany).

Statistics. Values for concentrations and concentration ratios were expressed as means ± S.D. Statistical analysis for comparison of two means was performed using analysis of variance (ANOVA).

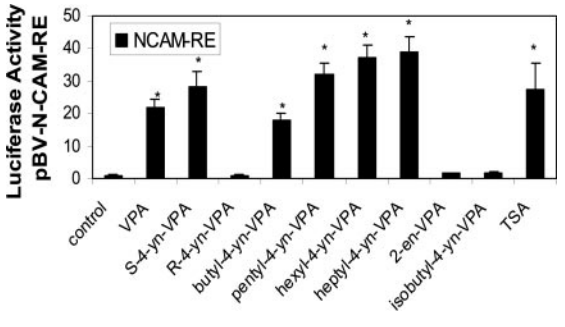

Fig. 3. Teratogenic VPA derivatives activated the 5′-flanking region of NCAM. F9 cells were transiently transfected with pBV-NCAM-RE and CMV-β-Gal control reporter. F9 cells were treated 48 h with 1 mM 2-en-VPA, 1 mM VPA, and 0.25 mM concentration of one of the following: (*S*)-4-yn-VPA, (*R*)-4-yn-VPA, butyl-4-yn-VPA, pentyl-4-yn-VPA, hexyl-4-yn-VPA, heptyl-4-yn-VPA, or 50 nM TSA. Cells were treated with or without test compounds 24 h, and cell extracts were subsequently assayed for luciferase activity. Values represent means ± S.D. from triple determinations, with asterisks indicating a significant difference ($p <$ 0.005; ANOVA) from untreated cultures.

Remarks. We investigated 13 antiepileptic VPA derivatives with almost comparable antiepileptic potency but different toxic effects, eight known teratogenic and five known nonteratogenic VPA derivatives (Fig. 1A). The teratogenic derivatives were (*S*)-4-yn-VPA, (*R*,*S*)-4-yn-VPA, 4-en-VPA, and four teratogens with increasing C chain length: butyl-4-yn-VPA, pentyl-4-yn-VPA, hexyl-4-yn-VPA, and heptyl-4-yn-VPA. The nonteratogenic derivatives were isobutyl-4-yn-VPA, isobutyl-ethyl-4-yn-VPA, ethyl-4-yn-VPA, E-2-en-VPA, and 5 methy-4-yn-VPA (Hauck and Nau, 1992).

Results

Teratogenic VPA Derivatives Induce F9 Cell Differentiation. Using the F9 cell in vitro model, we have shown that VPA and only teratogenic VPA derivatives induce F9 cell differentiation. VPA derivatives known to be teratogenic in vivo (butyl-4-yn-VPA, pentyl-4-yn-VPA, hexyl-4-yn-VPA, heptyl-4yn VPA, (*S*)-4-yn-VPA, and (*R*,*S*)-4-yn-VPA) induced differentiation of F9 cells that reflected the structure-activity relationship seen in vivo well (Lampen et al., 1999, 2001a,b).

The induction of differentiation increased with increasing length of the side chain up to heptyl-4-yn-VPA, which was the most potent drug. The relative potency of the tested compounds was heptyl-4-yn-VPA > hexyl-4-yn-VPA > pentyl-4-yn-VPA > (*S*)-4-yn-VPA > butyl-4-yn-VPA > (*R*,*S*)-4-yn-VPA, VPA. It is interesting that the well characterized HDAC inhibitor TSA also induced F9 cell differentiation similar to VPA with morphological changes to a polygonal shape typical of differentiated F9 cells (as described in Lampen et al., 1999).

Only Teratogenic VPA Derivatives Induced NCAM mRNA Expression. We tested the hypothesis that gene expression of the NCAM may be affected by VPA derivatives or TSA. As shown in Fig. 1B, the NCAM mRNA expression in F9 cells is strongly induced only by teratogenic VPA derivatives as measured by competitive RT-PCR. Nonteratogenic VPA derivatives [(*R*)-4-yn-VPA, 2-en-VPA, and isobutyl-4-yn-VPA] did not induce NCAM gene expression at all. Furthermore, the induction of NCAM gene expression also was stereoselective. (*S*)-4-yn-VPA induced NCAM mRNA expression very strongly (~6-fold), whereas the stereoisomer (*R*)-4-yn-VPA did not alter NCAM gene expression at all. VPA

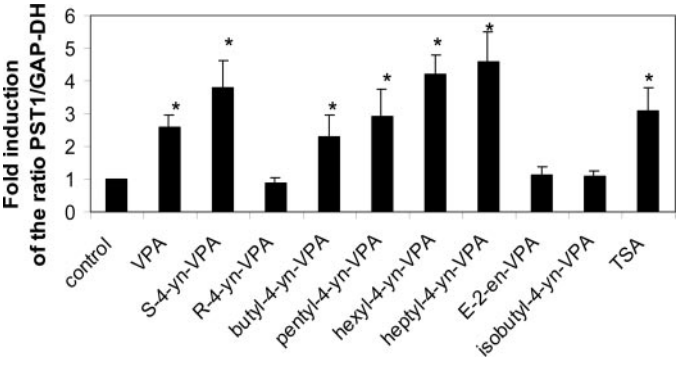

Fig. 4. Expression of PST1 in F9 cells after treatment with different VPA derivatives. PST1 and GAPDH mRNA expression were measured by competitive RT-PCR as described under *Materials and Methods*. F9 cells were treated 48 h with 1 mM 2-en-VPA, 1 mM VPA, and 0.25 mM concentration of one of the following: (*S*)-4-yn-VPA, (*R*)-4-yn-VPA, butyl-4-yn-VPA, pentyl-4-yn-VPA, hexyl-4-yn-VPA, heptyl-4-yn-VPA, or 50 nM TSA. The analysis of the competitive RT-PCR is shown. Values represent means ± S.D. from triple determinations, with asterisks indicating a significant difference ($p <$ 0.005; ANOVA) from untreated cultures.

induced the NCAM mRNA expression by a factor of 3. The longer the aliphatic side chain of the 4-yn-VPA derivative, the greater the induction of the NCAM gene expression. The strongest inducer was heptyl-4-yn-VPA, which increased NCAM gene expression by a factor of 8. Nonteratogenic compounds such as 2-en-VPA or isobutyl-4-yn-VPA did not induce NCAM gene expression in F9 cells at all. Thus, the specific induction of NCAM mRNA expression in F9 cells seems to correlate well with the induction of the differentiation, with activation of PPARδ, as well as with the teratogenic effects of the VPA derivatives in vivo (Lampen et al., 1999, 2001a). It is interesting that the HDAC-inhibitor TSA also induced NCAM mRNA expression.

Protein Expression of NCAM. We also measured the expression of the NCAM protein in F9 cells using FACS analysis. As shown in Fig. 2, only teratogenic VPA analogs induced NCAM protein expression, whereas nonteratogenic compounds had no effect. Structure-activity investigations showed that the prolongation of the aliphatic side chain of the 4-yn-VPA derivative enhanced NCAM protein expression. TSA also induced NCAM protein expression, whereas cPGI did not induce NCAM protein expression.

Interaction with 5′-Flanking Promoter of NCAM. To characterize the interaction of VPA derivatives with NCAM, we transfected F9 cells with an expression plasmid containing the 5′-flanking promoter of NCAM connected to a luciferase reporter gene (Fig. 3). It is interesting that treatment with VPA and teratogenic VPA analogs induced reporter gene activity, whereas nonteratogenic VPA analogs did not interact with the promoter at all. Furthermore, the stereospecific effect of (*S*)-4-yn-VPA and (*R*)-4-yn-VPA was reflected in that only the (*S*)-4-yn-VPA stereoisomer induced the reporter gene activity. The structure-activity investigation showed very similar effects as demonstrated in the mRNA expression study (Fig. 1B). In addition, TSA induced the reporter gene activity, suggesting that the induced type of differentiation by VPA derivatives and TSA could be similar.

Effect of VPA Analogs on mRNA Expression of PST1. Because PST1 is important for the addition of PSA to NCAM, we further investigated the effect of VPA analogs on the mRNA expression of PST1. It is interesting that we also determined an induction of PST1 mRNA expression after treatment of the F9 cells only with teratogenic VPA analogs,

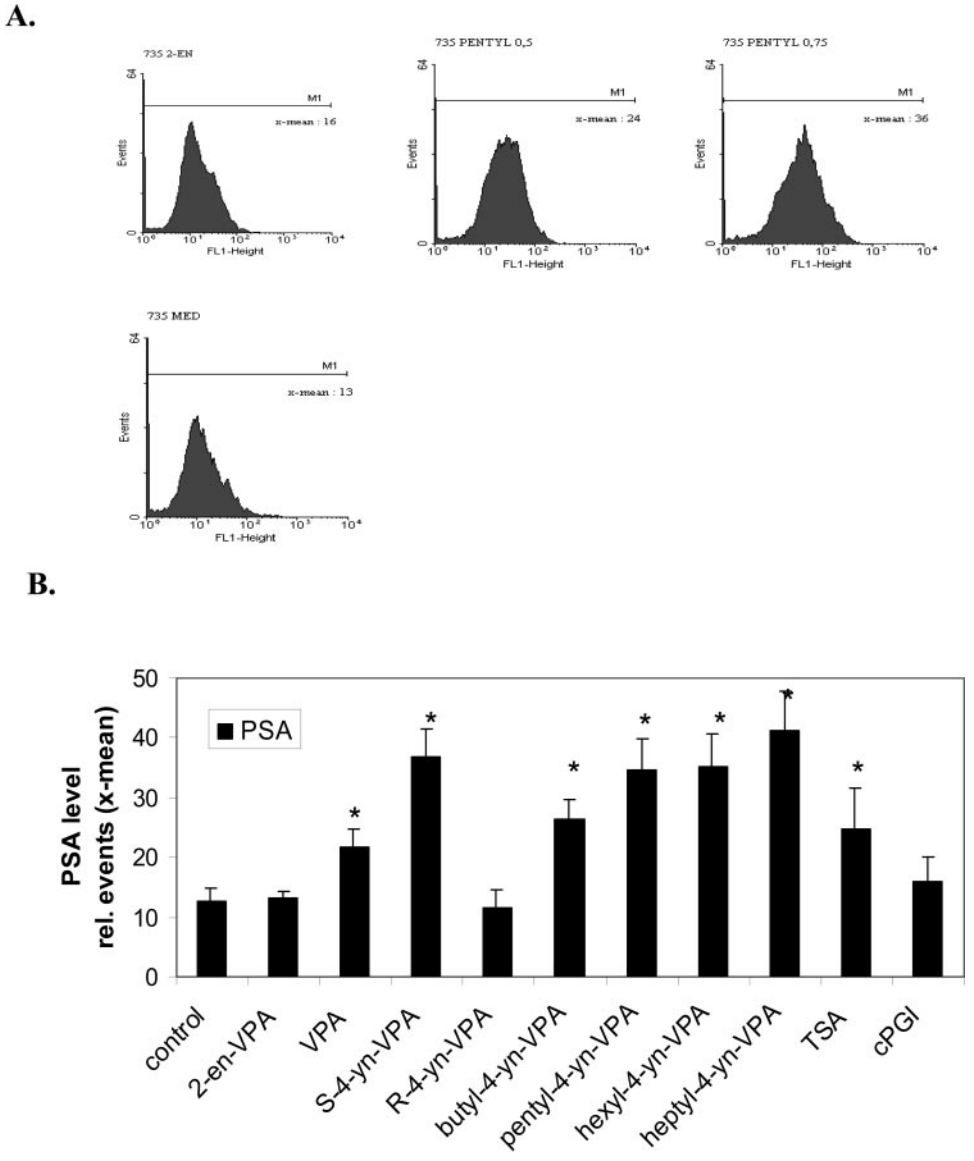

Fig. 5. PSA level in F9 cells after treatment with different VPA derivatives. F9 cells were treated with 1 mM 2-en-VPA, 1 mM VPA, and 0.25 mM concentration of either (*S*)-4-yn-VPA, (*R*)-4-yn-VPA, butyl-4-yn-VPA, pentyl-4-yn-VPA, hexyl-4-yn-VPA, heptyl-4-yn-VPA, 50 nM TSA, or 5 μM cPGI for 48 h. PSA level was measured by FACS analysis. A, typical result of an experiment. B, structure activity-relationships. Values represent means ± S.D. from four determinations, with asterisks indicating a significant difference ($p < 0{,}005$; ANOVA) from untreated cultures.

whereas nonteratogenic compounds had no effect at all. Here, too, the stereospecific effect of (*S*)-4-yn-VPA and (*R*)-4-yn-VPA was reflected (Fig. 4). Elongation of the aliphatic side chain resulted in a higher induction of PST1 mRNA expression. The strongest inducer, heptyl-4-yn-VPA (0.25 mM), increased PST1 expression by a factor of approximately 4.5. The HDAC inhibitor TSA (in nanomolar range) also induced PST1 mRNA expression, indicating that NCAM and PST1 are also molecular targets of TSA.

Polysialic Acid Level after Treatment with VPA Derivatives. If the mRNA of the PST1 enzyme is induced after treatment with teratogenic VPA analogs, the PSA level in F9 cells should be higher, as a result of the induction of the PST1 enzyme, than in the cells treated with nonteratogenic derivatives. Therefore, we measured the amount of PSA in F9 cells using a specific antibody against PSA. Only treatment of the F9 cells with teratogenic VPA derivatives enhanced the level of PSA, as shown in Fig. 6. Furthermore, structure-activity investigations showed that the longer the aliphatic side chain of the α-branched VPA analog, the higher the level of PSA in F9 cells (Fig. 5). Again, heptyl-4-yn-VPA was the most potent analog, resulting in the greatest enhancement of the PSA level in F9 cells. In addition, TSA also enhanced the PSA level, whereas cPGI did not enhance the PSA level.

Interaction with 5′-Flanking Promoter of PST1. To characterize the interaction of VPA derivatives with the regulative sequences of the PST1 gene, we transfected F9 cells with an expression plasmid (pGL2-PST2) containing a relevant part of the 5′-flanking promoter region of PST1 connected to a luciferase reporter gene (Eckhardt and Gerardy-Schahn, 1998). It is interesting that treatment with VPA and teratogenic VPA analogs induced the reporter gene, whereas nonteratogenic VPA analogs did not interact at all with the promoter (Fig. 6). The stereospecific effect of (*S*)-4-yn-VPA and (*R*)-4-yn-VPA was also reflected. The teratogenic (*S*)-4-yn-VPA induced the reporter gene, whereas the nonteratogenic (*R*)-4-yn-VPA did not interact at all. The longer the aliphatic side chain of the VPA derivative, the greater the interaction with the 5′-flanking promoter of PST1. Again, TSA was a potent inducer of the PST1 promoter, indicating that PST1 as well as NCAM are targets of VPA and TSA in F9 cell differentiation.

Effect of Sulindac and Indomethacin on Cell Differentiation. It has been shown that sulindac and indomethacin are able to inhibit the PPARδ-RXR heterodimerization in colon cells (He et al., 1999). Using antisense constructs, it has been also shown that PPARδ is a limiting factor in the F9 cell differentiation (Werling et al., 2001). Treatment of the cells with indomethacin repressed the induction of the differentiation by VPA as shown in Fig. 7. A similar repression was investigated after treatment of the cells with sulindac (data not shown).

Effect of Indomethacin on NCAM mRNA and Protein Expression. Treatment of F9 cells with VPA or the derivative *R*,(*S*)-pentyl-4-yn-VPA induced NCAM mRNA expression by a factor of 2.2 and 3.7, respectively, as measured by semiquantitative RT-PCR (Fig. 8). This result was confirmed by competitive RT-PCR. Treatment of the cells with the same compounds in the presence of 100 μM indomethacin repressed the mRNA induction of NCAM. The same effect was observed on the protein level Fig. 9A. Treatment with *R*,(*S*)-pentyl-4-yn-VPA induced NCAM protein expression. Treatment in the presence of indomethacin abolished the induction of NCAM protein expression. We observed a similar effect in regard to the mRNA expression of PST1 (data not shown). We observed the same effect in regard to the PSA level in F9 cells detected by FACS analysis (Fig. 9B) Again, treatment with (*S*)-pentyl-4-yn-VPA enhanced the PSA level, and treatment of the cells with the same compound in the presence of indomethacin repressed the PSA level.

Alteration of PPARδ Activity and Effects on NCAM Expression and PSA Content. PPARδ plays a critical role in differentiation in F9 cells (Werling et al., 2001). To evaluate the effect of PPARδ activity on the expression of NCAM and PST1 expression, we overexpressed PPARδ using an expression vector of PPARδ and repressed PPARδ by using a dominant-negative PPARδE411 mutant expression vector in F9 cells that impairs action of the endogenous nuclear receptor. First, we conducted control experiments to analyze PPARδ protein levels in the cells and characterized the activity of the dominant-negative mutation of PPARδ as described by Bastie et al. (2000) to make sure that we have F9

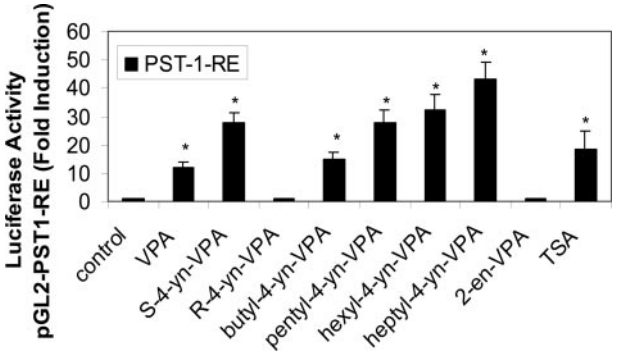

Fig. 6. Teratogenic VPA derivatives activated the 5′-flanking region of PST1. F9 cells were transiently transfected with pBV-NCAM-RE and simian virus 40 β-Gal control reporter. F9 cells were treated 48 h with 1 mM 2-en-VPA, 1 mM VPA, and 0.25 mM concentration of one of the following: (*S*)-4-yn-VPA, (*R*)-4-yn-VPA, butyl-4-yn-VPA, pentyl-4-yn-VPA, hexyl-4-yn-VPA, heptyl-4-yn-VPA, or 50 nM TSA. Cells were treated 24 h with or without test compounds, and cell extracts were subsequently assayed for luciferase activity. Values represent means ± S.D. from triple determinations, with asterisks indicating a significant difference ($p < 0.005$; ANOVA) from untreated cultures.

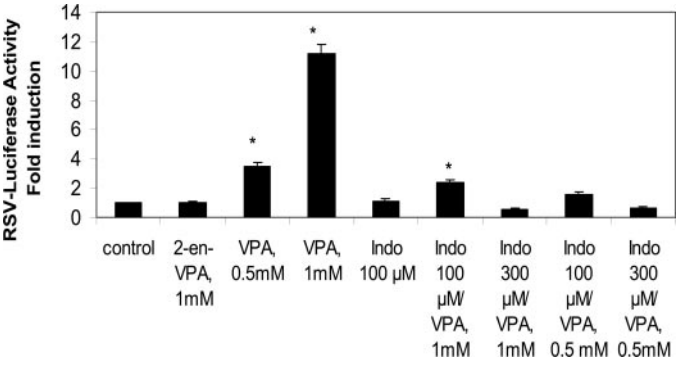

Fig. 7. Effects of indomethacin on the F9 cell differentiation. F9 cells were transiently transfected with pRSV-Luc or pUbi-Luc-GH (control) plasmids. Cells were treated with or without test compounds for 20 h, and cell extracts were subsequently assayed for luciferase activities. Values represent means ± S.D. from triple determinations, with asterisks indicating a significant difference ($p < 0.005$; ANOVA) from untreated cultures. Indo, indomethacin.

cells overexpressing PPARδ and F9 cells with repressed PPARδ activity.

Overexpression of PPARδ and treatment with a strongly teratogenic VPA derivative such as (*S*)-pentyl-4-yn-VPA resulted in an enhanced induction of NCAM protein expression in comparison with mock-transfected F9 cells (Fig. 10A). Again, 2-en-VPA had no effect.

The NCAM protein expression induced in mock-transfected cells was comparable with that of untransfected F9 cells. F9 cells transfected with the dominant-negative PPARδE411 mutant showed a diminished induction of NCAM protein expression (Fig. 10A).

After treatment with (*S*)-pentyl-4yn-VPA, PPARδ-overexpressed F9 cells were found to have higher PSA levels than mock-transfected F9 cells (Fig. 10B). After treatment with the highly teratogenic (*S*)-pentyl-4-yn-VPA compound, F9 cells transfected with the dominant-negative PPARδE411 mutant were found to have PSA levels (as measured by FACS analysis) lower than in mock-transfected cells (Fig. 10B). Overexpression of PPARα or PPARγ did not effect the expression of NCAM or PST1 (data not shown). We concluded that the impact of PPARδ activities on NCAM or PST1 seems to be specific. However, GenBank analysis of the 5′-flanking promoter of NCAM and PST1 using ConSite showed no peroxisomal proliferator-activated receptor-responsive element, indicating that these genes are not direct target genes.

Discussion

This work demonstrated that differentiation of F9 cells, only when induced by teratogenic VPA derivatives or by the well known HDAC inhibitor TSA, resulted in an induction of NCAM and PST1 on the mRNA and protein levels. Furthermore, to the best of our knowledge it has been shown for the first time that only teratogenic VPA compounds and TSA induced the promoters of NCAM and PST1. In addition, alterations of the PPARδ but not PPARα or PPARγ activity have an impact on the expression of NCAM and PST1 as well as on the level of PSA in differentiated F9 cells only after treatment with teratogenic VPA derivatives.

The teratogenic potency of VPA and its analogs has previously been determined in vivo (Nau et al., 1991; Hauck and Nau, 1992; Bojic et al., 1996, 1998). The structural elements previously shown to be essential for teratogenicity have been confirmed by the results of the expression of NCAM and PST1: the molecule has to bear a carboxylic group, and the carbon atom adjacent to the carboxylic group has to have one hydrogen atom (Ehlers et al., 1992). In addition, the sp^3 configuration at carbon atom C-2 is essential, because analogs (E-2-en-VPA) in which carbon atom C-2 is sp^2-hybridized were not active either in vitro or in vivo. The teratogenic effect of VPA analogs has been shown in vivo to be stereoselective (Hauck and Nau, 1992). The present study demonstrates that the measurement of the expression of NCAM and PST1 reflects this specific effect. The structure-activity relationships of the VPA compounds tested in vitro and in vivo showed the same concentration-dependent response in each system. The prolongation of the aliphatic side chain of VPA derivatives from butyl-4-yn-VPA up to heptyl-4-yn-VPA was responsible for an interesting structure-activity relationship regarding the induction of NCAM and PST1 expression. Both genes were induced only by teratogenic VPA derivatives, whereas nonteratogenic VPA derivatives such as 2-en-VPA

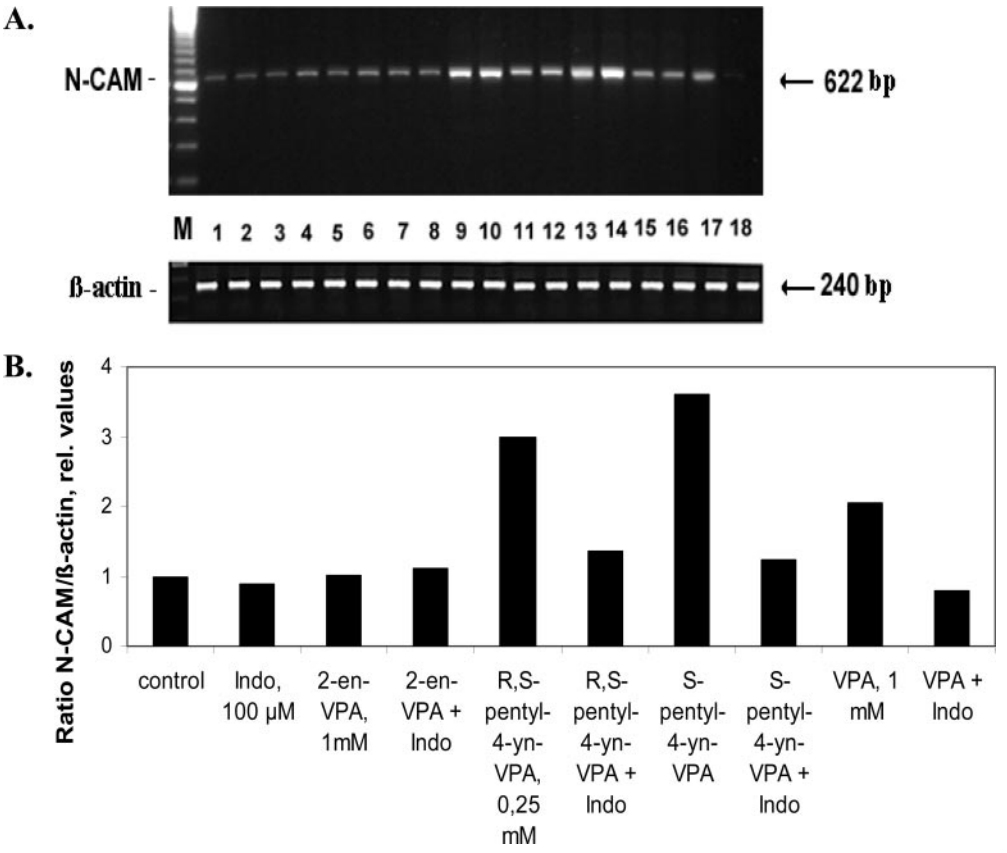

Fig. 8. Indomethacin repressed the induction of NCAM mRNA expression after treatment with VPA in F9 cells. NCAM and β-actin mRNA expression were measured by semiquantitative RT-RCR as described under *Materials and Methods*. F9 cells were treated with 2-en-VPA (as negative control), with (*R*,*S*)-pentyl-4-yn-VPA, (*S*)-pentyl-4-yn-VPA, or with VPA with or without indomethacin. 1 + 2, DMSO control; 3 + 4, 100 μM indomethacin; 5 + 6, 1 mM 2-en-VPA; 7 + 8, 1 mM 2-en-VPA and 100 μM indomethacin; 9 + 10, 0.25 mM (*R*,*S*)-pentyl-4-yn-VPA; 11 + 12 0.25 mM (*R*,*S*)-pentyl-4-yn-VPA and 100 μM indomethacin; 13 + 14, 0.25 mM (*S*)-pentyl-4-yn-VPA; 15 + 16, 0.25 mM (*S*)-pentyl-4-yn-VPA and 100 μM indomethacin; 17, 1 mM VPA; 18, 1 mM VPA and 100 μM indomethacin; M, molecular marker. Experiments were done at least in triplicate. A representative gel is shown. B, densitometric analysis of a gel is shown.

or (*R*)-4-yn-VPA had no effect at all on the expression of NCAM or PST1. Prolongation of the side chain resulted in enhanced induction of NCAM and PST1 expression as well as in induction of differentiated F9 cells. It is interesting that this structure-activity relationship reflects the potency of the teratogenic compounds in vivo. The teratogenic effect in vivo (the induction of exencephaly) is also enhanced from butyl-4-yn-VPA to heptyl-4-yn-VPA. Of the compounds tested here, heptyl-4-yn-VPA is the strongest teratogen in vivo (Hauck and Nau, 1992). In vitro, heptyl-4-yn-VPA also induced the greatest F9 cell differentiation, NCAM expression, and PST1 expression. These data suggest that there is a good correlation between F9 cell differentiation, NCAM and PST1 induction, and the teratogenic effect in vivo. Therefore, NCAM and PST1 may also play a significant role in the teratogenicity of VPA derivatives in vivo.

It has been shown that the well characterized HDAC inhibitor TSA mimics VPA effects on embryogenesis. Exposure of TSA to *Xenopus laevis* embryos or mouse embryos causes developmental defects (including anterior neural tube defects) that are virtually identical to effects induced by VPA (Svensson et al., 1998; Phiel et al., 2001). Therefore, HDAC inhibition seems to be involved in the teratogenic mechanism. Herein, we report for the first time that TSA also induces F9 cell differentiation and induction of NCAM as well as PST1 expression. In addition, TSA induced the promoter of NCAM and PST1, suggesting that there is a good correlation between the in vitro model (F9 cells) and the in vivo effects and that HDAC inhibition might be an additional important component in the signaling pathway of the VPA- or TSA-induced F9 cell differentiation.

Induction of NCAM expression by VPA has also been reported in other cell systems. In neuroblastoma cells, VPA induced NCAM expression, and the cells differentiated (Cinatl et al., 1996; Bojic et al., 1998). In addition, VPA also induced NCAM expression in human glioma cell lines and in Ntera-2 cells; however, it is not clear how VPA controls NCAM, but we have shown that only teratogenic VPA derivatives induced NCAM gene and protein expression in embryonic F9 cells. Furthermore, only teratogenic VPA derivatives interacted with the 5′-flanking region of the NCAM promoter, whereas nonteratogenic compounds had no effect on the expression of NCAM and did not interact with the NCAM promoter. The very similar induction pattern of PST1 in the mRNA expression after treatment with teratogenic VPA analogs and the enhanced levels of PSA lead to the conclusion

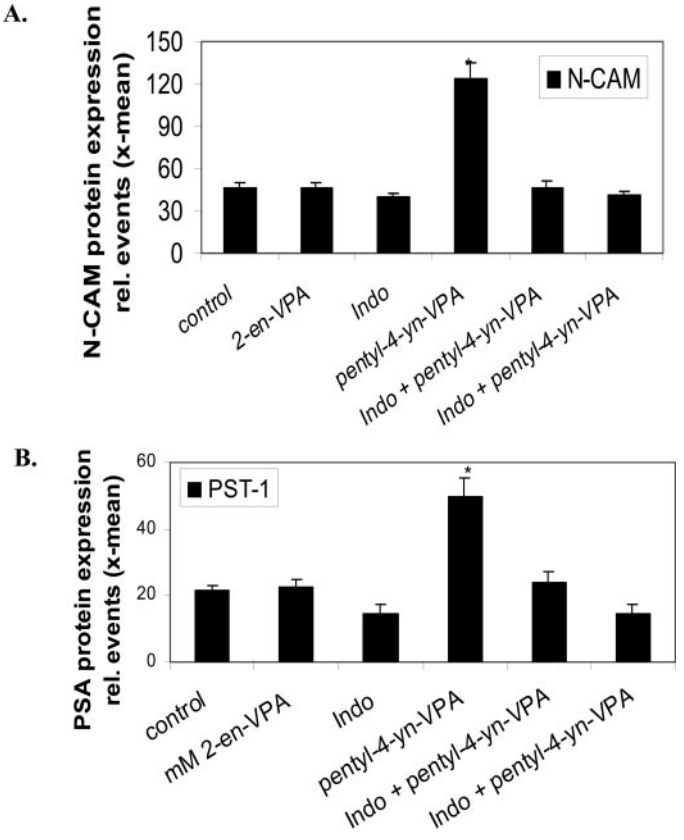

Fig. 9. Indomethacin repressed the induction of NCAM protein expression and PSA level by (*R*,*S*)-pentyl-4-yn-VPA in F9 cells. F9 cells were treated with 1 mM 2-en VPA (negative control), with 100 μM indomethacin alone, with 0.25 mM (*R*,*S*)-pentyl-4-yn-VPA alone, and with the combination of 50 μM indomethacin and 0.25 mM (*R*,*S*)-pentyl-4-yn-VPA or 100 μM indomethacin (indo). NCAM expression and PSA level was measured by FACS analysis as described under *Materials and Methods*. Values represent means ± S.D. from four determinations, with asterisks indicating a significant difference ($p < 0.005$; ANOVA) from untreated cultures. A, effects on NCAM protein expression. B, effects on the PSA level.

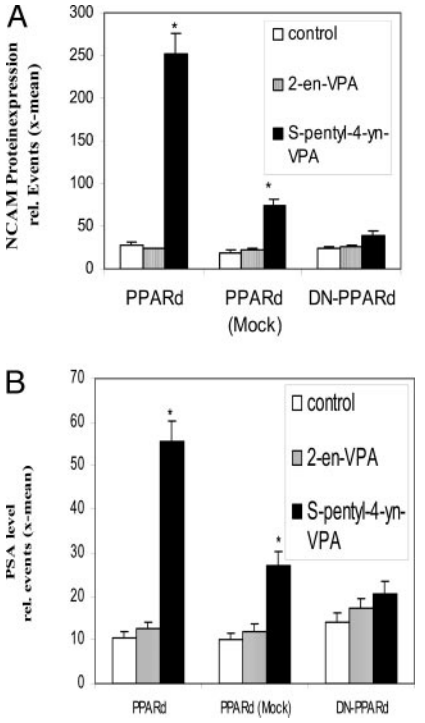

Fig. 10. A, effects of the modulation of PPARδ activity on the expression of NCAM. F9 cells were transiently transfected with pcDNA3-mock expression plasmid, pcDNA3-PPARδ (PPARd) or the pcDNA3-PPARδE411P (DN-PPARd) expression vector. Afterward, the cells were treated with 0.25 mM (*S*)-pentyl-4-yn-VPA, 2-en-VPA, or vehicle. After 36 h, the cells were subjected to FACS analysis as described under *Materials and Methods*. Values represent means ± S.D. from five determinations, with asterisks indicating a significant difference ($p < 0.01$; ANOVA) from untreated cultures. B, effects of the modulation of PPARδ activity on the level of PSA. F9 cells were transiently transfected with pcDNA3-mock expression plasmid, pcDNA3-PPARδ (PPARd), or with the pcDNA3-PPARδE411P (DN-PPARd) expression vector. Afterward, the cells were treated with 0.25 mM (*S*)-pentyl-4-yn-VPA, 2-en-VPA, or vehicle. After 36 h, the cells were subjected to FACS analysis as described under *Materials and Methods*. Values represent means ± S.D. from five determinations, with asterisks indicating a significant difference ($p <$ 0.01; ANOVA) from untreated cultures.

that PST1 is regulated by teratogenic VPA derivatives and TSA in the same manner as NCAM. This hypothesis is supported by the observation that only teratogenic compounds interacted with the 5′-flanking promoter of PST1. Both are induced only when F9 cells are differentiated, indicating that these genes are good markers of the differentiation induced only by teratogenic VPA derivatives. This differentiation is characterized by the induction of NCAM and PST1 expression because no markers of other types of differentiation were present. The markers laminin β1 and collagen IV are expressed, for example, by parietal endoderm-like cells (Alonso et al., 1991). These markers are induced by retinoic acid, but not by VPA (Werling et al., 2001).

Induction of differentiation by VPA thus defines a different type of differentiation that is characterized by the induction of viral promoter (Lampen et al., 1999), induction of the transcription factor AP-2 (Werling et al., 2001), and the induction of NCAM and PST1 as shown here. It is interesting that an AP-2 site was found in the minimal promoter region of the ST8SiaIV gene, and AP-2 has been implicated in the regulation of neural development (Zhang et al., 1996). Therefore, it is likely that AP-2 is involved in the signaling cascade.

NCAM is expressed at the blastoderm stage of embryonic development in the chick (Thiery et al., 1982) and is expressed in more than one germ layer. Qualitative and quantitative changes in NCAM expression have been observed during brain and muscle development. Modulation of NCAM expression can alter the adhesive nature of cell surfaces.

Whereas NCAM mediates stable cell-cell contacts in the absence of PSA, the adhesion molecule is converted into an "antiadhesive" factor by the presence of PSA (Yang et al., 1994). Because of its steric characteristics, however, PSA also interferes with other cell surface interactions (Rutishauser, 1998). PSA-NCAM is involved in promoting cell migration and axon guidance (Murakami et al., 2000), and its expression during development was found to be highest in phases of neuronal motility (Seki and Arai, 1991). Polysialylated NCAM represents an oncodevelopmental antigen, and the re-expression of PSA has been observed in several tumors. Biosynthesis of PSA can be realized by ST8SiaII and ST8SiaIV, two closely related polysialyltransferases. PSA immunoreactivity has been shown to correlate closely with mRNA expression of polysialyltransferases (Ong et al., 1998). Our data support this correlation, because we found induction of PST1 mRNA expression and an enhanced level of PSA only after treatment of F9 cells with teratogenic VPA derivatives.

The nuclear receptors PPARs are important factors in the differentiation of different cells (for review, see Michalik et al., 2003). In F9 cells, PPARδ has been shown to play an important role in differentiation, because inhibition of PPARδ by an antisense construct resulted in a reduced differentiation of F9 cells (Werling et al., 2001). Moreover, only teratogenic VPA derivatives activate PPARδ and differentiation of F9 cells, whereas nonteratogenic VPA derivatives neither activated PPARδ nor induced differentiation, although they activated PPARα and PPARγ (Lampen et al., 1999, 2001a). To the best of our knowledge, there are no previous reports of interactions between PPARs and NCAM of PST1. However, additional mechanisms such as HDAC inhibition are important because PPARδ agonists alone such as cPGI (Forman et al., 1997) did not induce NCAM protein expression or PSA level in F9 cells (Figs. 2 and 5).

However, this work demonstrated that a change in PPARδ activity alters the expression of NCAM and PST1 and consequently the differentiation of F9 cells. Overexpression of PPARδ resulted in an enhanced induction of NCAM and PST1 expression as well as in a higher level of PSA. Repression of PPARδ by expression of a PPARδ dominant-negative mutant that impairs action of the endogenous nuclear receptor as well a chemical inhibition of the heterodimerization (of PPAR and RXR) by indomethacin diminished these effects and inhibited differentiation. This is in agreement with observations by Werling et al. (2001). By using an antisense construct for PPARδ, they showed that VPA did not induce any sign of differentiation in F9 cells. The F9 cells do only express PPARδ (Lampen et al., 1999). Therefore, the most likely interpretation is that PPARδ is indirectly involved in the signaling network that controls F9 cell differentiation. PPARδ may also control the expression level of NCAM and PST1, most probably in an indirect manner, because no peroxisomal proliferator-activated receptor-responsive element was found in the promoter of NCAM or PST1, which indicates that these genes are indirect target genes of PPARδ. The fact that TSA, although structurally different to VPA, also induced F9 cell differentiation and induction of NCAM and PST1 suggests that HDAC inhibition is an additional important mechanistic module in the signaling pathway involved in F9 cell differentiation. However, according to the results of Lee et al. (2003), it has to be taken into account that PPARδ can also act independently of ligand by binding corepressors that would result in the derepression of NCAM and PST1 transcription.

In summary, it was shown here that NCAM and PST1 are new molecular markers of F9 cell differentiation induced only by teratogenic VPA derivatives or TSA. By using knockout mice for NCAM or PST1, it will now be possible to prove the role of NCAM and PST1 in the teratogenicity of VPAs in vivo. The in vitro results indicate that it is very likely that NCAM and PST1 may have a critical impact on the teratogenic effects of VPA derivatives in vivo.

Acknowledgments

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