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Modulation of peroxisome proliferator-activated receptor δ activity affect NCAM and ST8Sia lV (PST1) induction by teratogenic VPA-analogues in F9 cell differentiation

Alfonso Lampen, Paul A. Grimaldi, and Heinz Nau

Institut für Lebensmitteltoxikologie, Stiftung Tierärztliche Hochschule Hannover, Germany (A.L.,H.N.); and Inserm U636, Centre de Biochimie, University of Nice-Sophia Antipolis, France (P.A.G.)

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Running title: Effects of PPARδ alteration on expression of NCAM and PST1 in F9 cells

Address for correspondence:

Priv.-Doz. Dr. Dr. Alfonso Lampen
Institut für Lebensmitteltoxikologie
Stiftung Tierärztliche Hochschule Hannover
Bischofsholer Damm 15
D-30173 Hannover, Germany
E-Mail: Alfonso.Lampen@tiho-hannover.de

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Abbreviations:

cPGI: carbaprostacyclin; VPA: valproic acid; 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; PPAR: peroxisomal proliferator-activated receptor; PPRE: PPAR responsive element; HDAC, histone deacetylase.

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ABSTRACT

It has been suggested that the teratogenic effects of the antiepilepitic 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 (HDAC). 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 non-teratogenic VPA-derivatives had no effect at all. The polysialyltransferases (ST8SiaIV [PST1], ST8SiaII) are responsible for the addition of polysialyic acid (PSA) to NCAM. The mRNA expression of PST1 was highly induced by only teratogenic VPA-derivatives and TSA. As shown by FACS analysis the level of PSA was higher after treatment of F9 cells with teratogenic VPA-derivatives. Interestingly, 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 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 signalling pathway.

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Introduction

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 due to VPA itself and not one of its metabolites (Ehlers et al., 1992). The teratogenic effects in vivo and in vitro depend on structural requirements (described in (Nau et al., 1991; Bojic et al., 1996 and Lampen et al., 1999). In vitro only teratogenic VPA-derivatives induce cell differentiation of embryonic F9 stem cells (Lampen et al., 1999) which is believed to reflect early events in the embryonal development (Alonso et al., 1991; Werling et al., 2001), whereas non teratogenic 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; Lampen et al., 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 non-teratogenic compounds had no effect at all (Lampen et al., 2001a).

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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 (Phiel et al., 2001; Gottlicher et al., 2001). Trichostatin (TSA) is an anti-cancer compound and a well characterized HDAC inhibitor able to induce cell differentiation. Interestingly, TSA is also a teratogenic compound that induces neural tube defects very similar to 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 posttranslational modifications of glycosylation, sulfation, and phosphorylation have been described (Goridis and Brunet, 1992)). Potenital 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 neuritogensis and NCAM expression. VPA is reported to increases 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. Due to its

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steric properities, PSA attenuates cell-cell adhesion and is generally considered to be a promoter of neural plasticity, allowing cell movements and changes in cell interactions (Rutishauser and Landmesser, 1996). There are two enzymes responsible for the addition of oligosacharides to the NCAM protein, the two closely related polysialyltransferases ST8SiaII (STX) and ST8SiaIV (PST1). To the best of our knownledge, it is not known if VPA, VPA-derivatives or TSA have 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 additon, we analysed the role of the PPARδ activity in expression of NCAM and PST1.

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Materials and methods

Cell culture and reagents

Mouse F9 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in Ham´s F12/DMEM (GIBCO/BRL) 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 cPGI was obtained from Cayman Chemical (Ann Arbor, U.S.A.).

Plasmids

The pBV-NCAM expression vector contains the NCAM promoter (Barton et al., 1990) and was a generous gift from Professor C.H. Barton (Department of Biochemistry and Molecular Biology, University of Southampton, UK). Interestingly, the NCAM promoter contains according to the web-based software tool ConSite® 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 Professor R. Gerardy-Schahn (Medizinische Hochschule Hannover). Interestingly, this promoter contains according to the web-based software tool ConSite® also responsive elements of c-fos and AP2. The pSG5-mPPARα and the pSG5-mPPARγ expression vector contains mouse PPARα or mouse PPARγ and were kindly provided by Professor G. Perdew, Penn State University, U.S.A. and described in Sumanasekera et al. (2003). The pcDNA3-PPARδ expression plasmid and the pcDNA3-PPARδE411P mutant cDNA of PPARδ were derived

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from pSG5-FAAR (Amri et al., 1995) as described in Bastie et al. (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 (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 previously described by Hauck and Nau (Hauck and Nau, 1992) and Bojic et al. (Bojic et al., 1996)).

Transfection and drug treatment

Gene transfer was carried out using the calcium phosphate preciptation 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 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 hr of exposure the medium was removed, cells were washed twice in PBS without Ca^{2+} and Mg^{2+}, and harvested in 200 µl lysis buffer (0.1 M Tris acetate pH 7.5, 2 mM EDTA, 1-% Triton X100). For measurement of luciferase activity the samples were pipetted into transparent reading tubes and transferred to a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany). There, the samples were mixed automatically with 100 µl luciferin-containing buffer (20 nmol/test) and 300 µl assay buffer (25 mM glycylglycerine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 2

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mM ATP, pH 7.8). We used a CMV-ß-gal plasmid as a control for transfection efficiencies and to standardize luciferase activities.

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, 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 chemiluninescence using an ECL advanced reagent (Amersham, Braunschweig, Germany).

Transfection of F9 cells and treatment with indomethacin

Expression vector (RSV-Luc), transfection, and reporter gene assay are described previously (Lampen et al., 1999). Interestingly, the RSV-promoter contains according to the web-based software tool ConSite® responsive elements of c-fos and AP2. The concentration of DMSO 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 to known data in the literature. Induction of RSV-Luc by 1 mM VPA was used to normalize the interassay variabilities. After 20 hr of exposure the medium was removed, cells were washed twice in PBS without Ca^{2+} and Mg^{2+} and harvested

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in 200 µl lysis buffer (0.1 M Tris acetate pH 7.5, 2 mM EDTA, 1 % Triton X100). For measurement of luciferase activity the samples were pipetted into transparent reading tubes and transferred to a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany) 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. (Auboeuf 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 GAP-DH. 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 of 50-80 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, Hilden, Germany), denaturated, and a second fusion-PCR was

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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 about 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® Biorad, Munich, Germany) using a bluecript plasmid (pBR32) as a DNA-quantification marker. A regression analysis was performed to quantificate the competitor cDNA. Different dilutions of the competitor were used in the competitive PCR together with different dilutions of the wildtype 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, France). The primer flanking sequences covers exons 2-9 and reacts with all known NCAM splice forms.

P1 -P4 = 622 bp; P3 - P4 bp= 518 (deletion of 104 bp);

P1= TGAGGGTACTTACCGCTGTG;

P2= GGATCCGTTCACAAGCTCGTCCCATCAGCATCACACACCAG;

P3= GACGAGCTTGTGAACGGATCCTCTGCATCGCAGAGAACAAG

P4= GTTGCTGGCAGTGCACATGT;

Mouse glyceraldehyde 3-phosphate dehydrogenase (mGAP-DH) primer: Genebank Accession No.: M32599; (Sabath et al., 1990).

P1 –P2 = 133 bp; P3 - P4 bp= 255; (deletion of 122 bp)

P1: TGGTGAAGGTCGGTGTGAAC

P2: GGATCCGTTCACAACTGAGGTCAATGAAGGGGTCG

P3: GTTGTGAACGGATCCACCATCTTCCAGGAGCGAGA

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P4: GTGCAGGATGCATTGCTGAC

Primer for ST8Sia IV (PST); Accession No.: Y09486 (Takashima et al., 1998). cDNA was a kind gift from Prof. R. Gerardi Schahn (MHH Hannover).

P1: ACCGCAGGTTTAAGACCTGTGC

P2: GGATCCGTTCACAAGCTCGTCCACATCAGCAGCGAACTCCA

P3: GACGAGCTTGTGAACGGATCCTCCTGCCTTCATGGTCAAAG

P4: GCCAGTATCCTCTGACTGCATG

RT-competitive PCR

Reverse transcription (RT) of 0.1 µg total RNA using oligo $(dT)_{15}$ was performed for 120 min at 42 °C with two units Super script II-reverse transcriptase (GIBCO/BRL, Karlsruhe, Germany) in Super script 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 were transferred to microtubes containing a different, but known, amount of competitor. After 120 s at 95 °C, the tubes were subjected to thirty cycles (60 s at 95 °C, 60 s at 57 °C, 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 (Biorad Munich, Germany). 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

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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. (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 to mouse GAP-DH is shown in the Figures.

Validation of the RT-PCR assay

To validate the RT-competitive PCR assay, RNAs corresponding to a part of mouse NCAM, PST1, or GAP-DH were synthesized by in vitro transcription (Promega Riboprobe system, 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).

Semi quantitative RT-PCR

A well established semi quanititative 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

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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 Acc. No.:Mm 001101).

Flow cytometry (FACS Analysis)

F9 cells were treated two days with or without VPA-derivatives. Afterwards, cells were separated from culture bottles with acutase® (Biochrom, Berlin, Germany) and 250000 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 mAnti 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 % BSA) buffer. The second antibody was either a fluorescein isothiocyanate (FITC)-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 MIF-buffer and the pellet was resuspended in a conic tube. After the addition of 100 µl PBS buffer and 200 µl PBS buffer containing 2 µg/ml propidium iodide the probes were measured in a FACscan (Becton Dickinson, Heidelberg, Germany). The propidium iodide-stained (dead) cells were excluded from the analysis. H28 mouse α Anti-NCAM and 735 mAnti PSA (Muhlenhof et al., 1998) were a generous gift of Professor. R. Gerardy-Schahn, Medizinische Hochschule Hannover, Germany).

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Statistics

Values for concentrations and concentration ratios were expressed as means ± standard deviations (SD). Statistical analysis for comparison of two means was performed using the analysis of variance (ANOVA).

Remarks

We investigated 13 antiepileptic VPA derivatives with almost comparable antiepileptic potency but different toxic effects, 8 known teratogenic and 5 known non-teratogenic VPA derivatives (Fig 1A). The teratogenic derivatives were S-4-yn-VPA; R,S-4-yn-VPA; 4-en-VPA; and five teratogens with increasing C-chain length: butyl-4-yn-VPA; pentyl-4-yn-VPA; hexyl-4-yn-VPA; and heptyl-4-yn-VPA. The non teratogenic 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).

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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, R,S-4-yn-VPA) induced differentiation of F9 cells that reflected the structure activity relationship seen in vivo well (Lampen et al., 1999; Lampen et al., 2001a; Lampen et al., 2001b).

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. Interestingly, 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 neural cell adhesion molecule (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. Non-teratogenic VPA derivatives (R-4-yn-VPA, 2-en-VPA, isobutyl-4-yn-VPA) did not induce NCAM gene expression at all. Furthermore, the

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induction of NCAM gene expression was also 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 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. Non-teratogenic 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-deriatives in vivo (Lampen et al., 1999; Lampen et al., 2001a). Interestingly, 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 analogues induced NCAM protein expression, whereas non-teratogenic 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

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luciferase reporter gene (Fig. 3). Interestingly, treatment with VPA and teratogenic VPA analogues induced reporter gene activity, whereas non-teratogenic VPA analogues 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 analogues on mRNA expression of PST1.

As PST1 is important for the addition of PSA to NCAM, we further investigated the effect of VPA analogues on the mRNA expression of PST1. Interestingly, we also determined an induction of PST1 mRNA expression after treatment of the F9 cells only with teratogenic VPA analogues, while non-teratogenic compounds had no effect at all. Here, too, the stereospecific effect of S-4-yn-VPA and R-4-yn-VPA was reflected (see 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 about 4.5. The HDAC-inhibitor TSA (in nM 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 analogues, the PSA level in F9 cells should be higher due to the induction of the PST1

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enzyme than in the cells treated with non-teratogenic 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 analogue, the higher the level of PSA in F9 cells (Fig. 5). Again, heptyl-4-yn-VPA was the most potent analogue, resulting in the greatest enhancement of the PSA level in F9 cells. In addition, TSA enhanced also 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). Interestingly, treatment with VPA and teratogenic VPA analogues induced the reporter gene, whereas non-teratogenic VPA analogues 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 non-teratogenic 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.

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Effect of sulindac and indomethacin on cell differentiation.

It has been shown that sulindac and indomethocin are able to inhibit the PPARδ-RXR heterodimerisation in colon cells ((He et al., 1999)). Using anti-sense contructs 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 the factor of 2.2 of 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 indomethacin (100 µM) 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. A similar effect we observed in regard to the mRNA expression of PST1 (data not shown). The same effect we observed 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.

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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. (Bastie et al., 2000) to make sure that we have F9 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 to mock-tranfected F9 cells (Fig. 10A). Again, 2-en-VPA had no effect.

The NCAM protein expression induced in mock-transfected cells was comparable to 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.

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However, Genebank analysis of the 5´-flanking promoter of NCAM and PST1 using web-based software tool ConSite (www.phylofoot.org/consite) showed no PPRE indicating that these genes are not direct target genes.

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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 analogues has previously been determined in vivo (Nau et al., 1991; Hauck and Nau, 1992; Bojic et al., 1996; Bojic et al., 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 analogues (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 analogues 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

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expression. Both genes were induced only by teratogenic VPA derivatives, whereas non-teratogenic VPA derivatives such as 2-en-VPA 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. Interestingly, this structure activity 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.

Recently, it has been shown that the well characterized HDAC inhibitor TSA mimics VPA effects on embryogenesis. Exposure of TSA to Xenopus embryos or mouse embryos causes developmental defects (including anterior neural tube defects) that are virtually identically to VPA-induced defects (Svensson et al., 1998; Phiel et al., 2001). Therefore HDAC inhibition seems to be involved in the teratogenic mechanism. Here we report for the first time that TSA also induce 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 inhibiton might be an additional important component in the signalling pathway of the VPA- or TSA-induced F9 cell differentiation.

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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 non-teratogenic 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 analogues and the enhanced levels of PSA lead to the conclusion 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 ß1 and collagen IV are expressed for example by parietal endoderm-like cells, (Alonso et al., 1991). These markers are induced by RA, but not by VPA (Werling et al., 2001).

Induction of differentiation by VPA thus defines a different type of differentiation which 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. Interestingly, an AP-2 site was found in the minimal promoter region of the

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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.

While NCAM mediates stable cell-cell contacts in the absense of PSA, the adhesion molecule is converted into an “anti-adhesive” factor by the presence of PSA (Yang et al., 1994). But due to its steric characteristics, 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 (Fukuda, 1996), and the reexpression of PSA has been observed in serveral tumors. Biosynthesis of PSA can be realized by ST8Sia II and ST8SiaIV, two closly related polysialyl transferases. PSA immunoreactivity has been shown to correlate closely with mRNA expression of polysialyl transferases (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 (see Michalik et al. (2003), for a review of the literature). In F9 cells, PPARδ has been shown to play an important role in differentiation, as inhibition of PPARδ by an anti-sense construct resulted in a reduced differentiation of F9 cells (Werling et al., 2001). Moreover,

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only teratogenic VPA derivates activate PPARδ and differentiation of F9 cells, whereas non-teratogenic VPA derivates neither activated PPARδ nor induced differentiation, although they activated PPARα and PPARγ (Lampen et al., 1999; Lampen et al., 2001a). To the best of our knowledge, there are no previous reports of interactions between PPARs and NCAM of PST1. However, additional mechanism such as HDAC inhibition are important since PPARδ agonists alone such as carbaprostacyclin (cPGI; Forman et al., 1997) did not induce NCAM protein expression or PSA level in F9 cells (Fig. 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 contruct 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, as no PPRE 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

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differentiation. However according to the results of Lee et al. (2003) it has to be taken in 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.

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Send reprint request to:

Priv.-Doz. Dr. Dr. Alfonso Lampen
Institut für Lebensmitteltoxikologie
Stiftung Tierärztliche Hochschule Hannover
Bischofsholer Damm 15
D-30173 Hannover, Germany
E-Mail: Alfonso.Lampen@tiho-hannover.de

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Legends

Fig. 1A. Structur of VPA and VPA-derivatives and their teratogenic potency in vivo; (+ indicates the teratogenic potency in comparison to VPA as standard substance).

Fig. 1B. Gene expression of NCAM in F9 cells after treatment with different VPA-derivatives. NCAM and GAP-DH mRNA expression were measured by competitive RT-PCR as described in the methods section. F9 cells were treated for 48 h with 1 mM 2-en-VPA, 1 mM VPA, and 0.25 mM 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 ± SD from triple determinations, with asterisks indicating a significant difference ($p < 0.005$; ANOVA) from untreated cultures.

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 0.25 mM 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. A typical result of an experiment. B. Structure activity relationships. Values represent means ± SD from four determinations, with asterisks indicating a significant difference ($p < 0.01$; ANOVA) from untreated cultures.

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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 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 ± SD from triple determinations, with asterisks indicating a significant difference ($p < 0.005$; ANOVA) from untreated cultures.

Fig. 4. Expression of PST1 in F9 cells after treatment with different VPA-derivatives. PST1 and GAP-DH mRNA expression were measured by competitive RT-PCR as described in the methods section. F9 cells were treated 48 h with 1 mM 2-en-VPA, 1 mM VPA and 0.25 mM 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 ± SD from triple determinations, with asterisks indicating a significant difference ($p < 0.005$; ANOVA) from untreated cultures.

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 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. A typcal result of an experiment. B. Structure activity-relationships. Values represent means ± SD from

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four determinations, with asterisks indicating a significant difference ($p < 0,005$; ANOVA) from untreated cultures.

Fig. 6. Teratogenic VPA derivatives activated the 5´-flanking region of PST1. F9 cells were transiently transfected with pBV-NCAM-RE and SV40 β-gal control reporter. F9 cells were treated 48 h with 1 mM 2-en-VPA, 1 mM VPA and 0.25 mM 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 ± SD 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 hours and cell extracts were subsequently assayed for luciferase activities. Values represent means ± SD from triple determinations, with asterisks indicating a significant difference ($p < 0,005$; ANOVA) from untreated cultures. (Indo = indomethacin).

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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 in the methods section. 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) the densitometric analysis of a gel is shown.

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 = indomethacin). NCAM expression and PSA level was measured by FACS analysis as described in the methods section. Values represent means ± SD 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.

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Fig. 10A. 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. Afterwards, 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 in the methods section. Values represent means ± SD from five determinations, with asterisks indicating a significant difference ($p < 0,01$; ANOVA) from untreated cultures.

Fig. 10B. 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. Afterwards, 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 in the methods section. Values represent means ± SD from five determinations, with asterisks indicating a significant difference ($p < 0,01$; ANOVA) from untreated cultures.

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Fig. 1A.

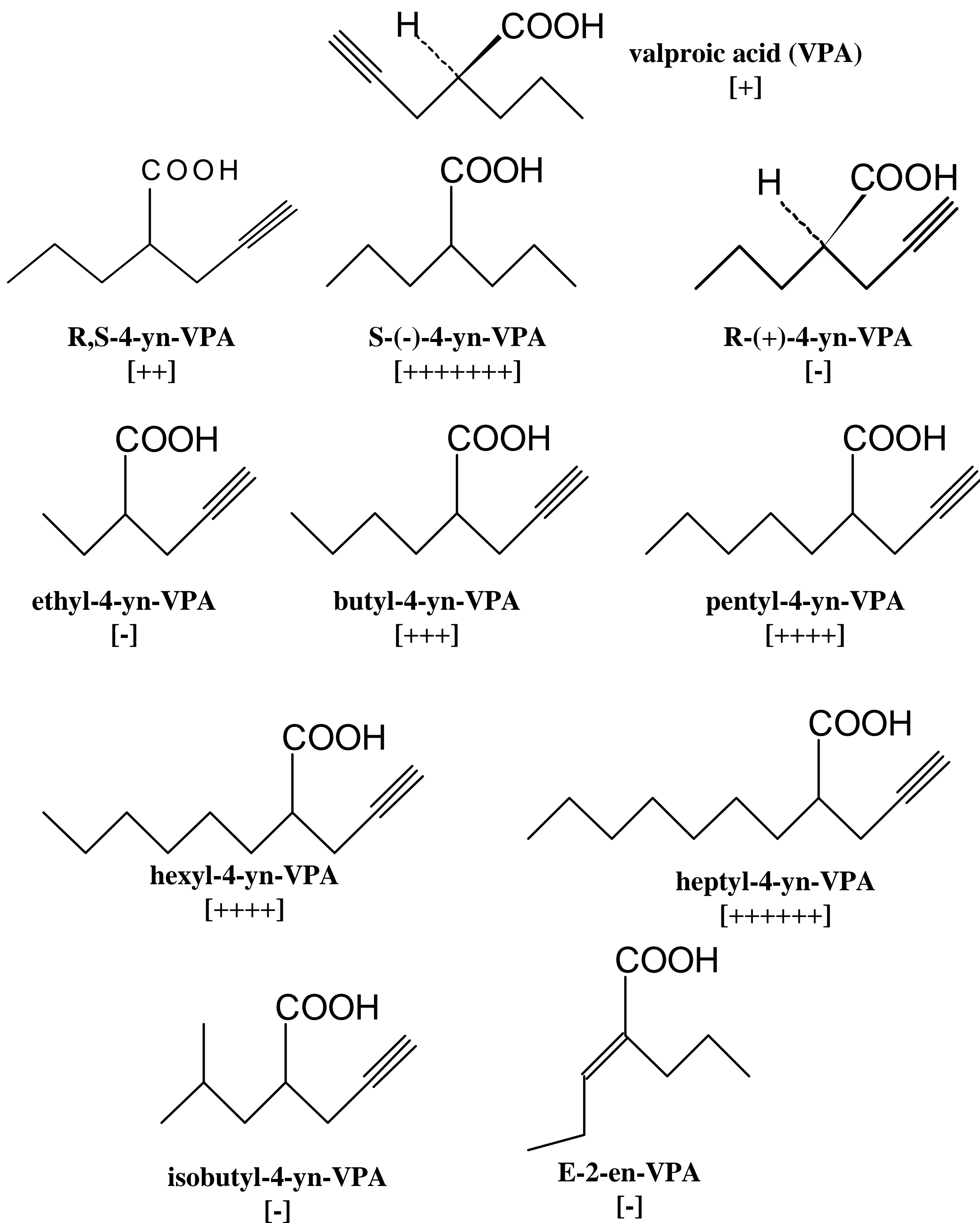

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Fig. 1B.

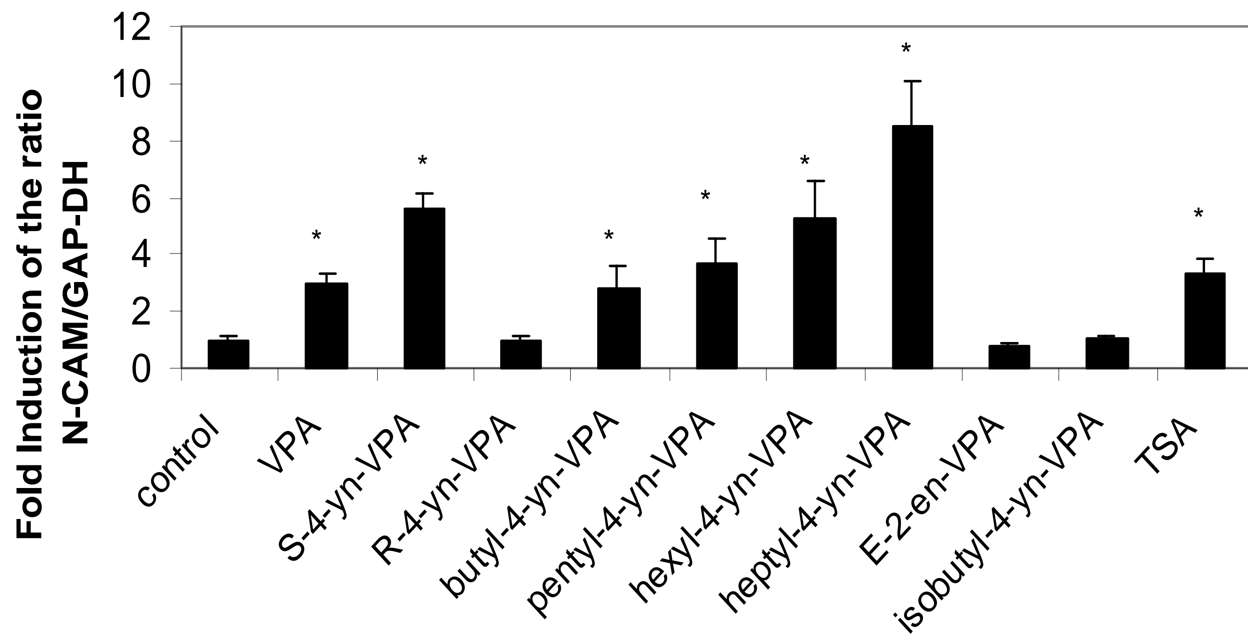

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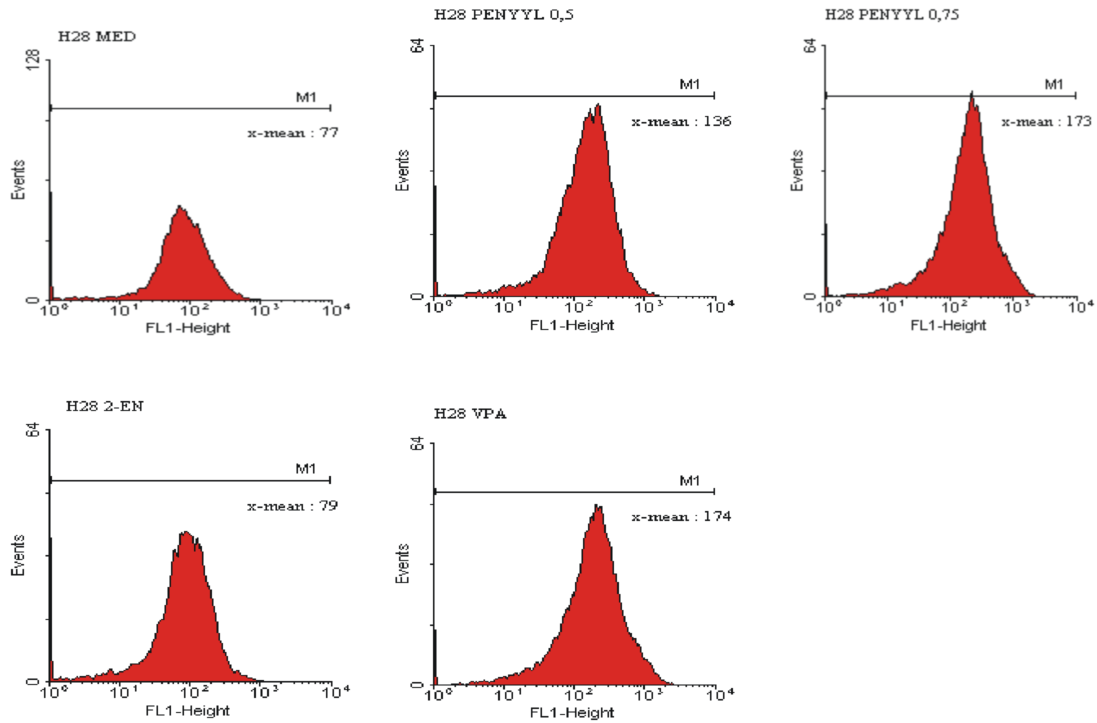
Fig. 2.

A.



B.

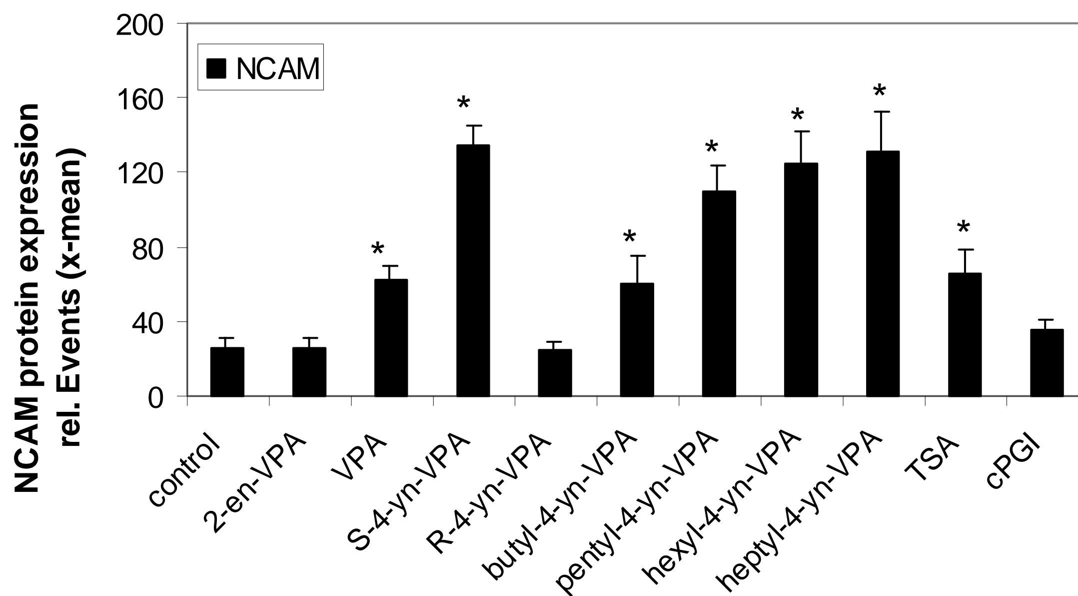


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Fig. 3.

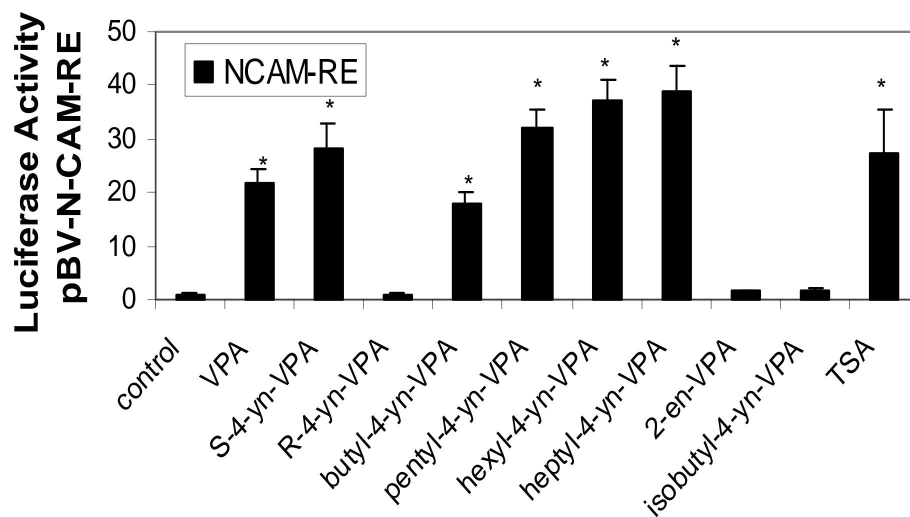

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Fig. 4.

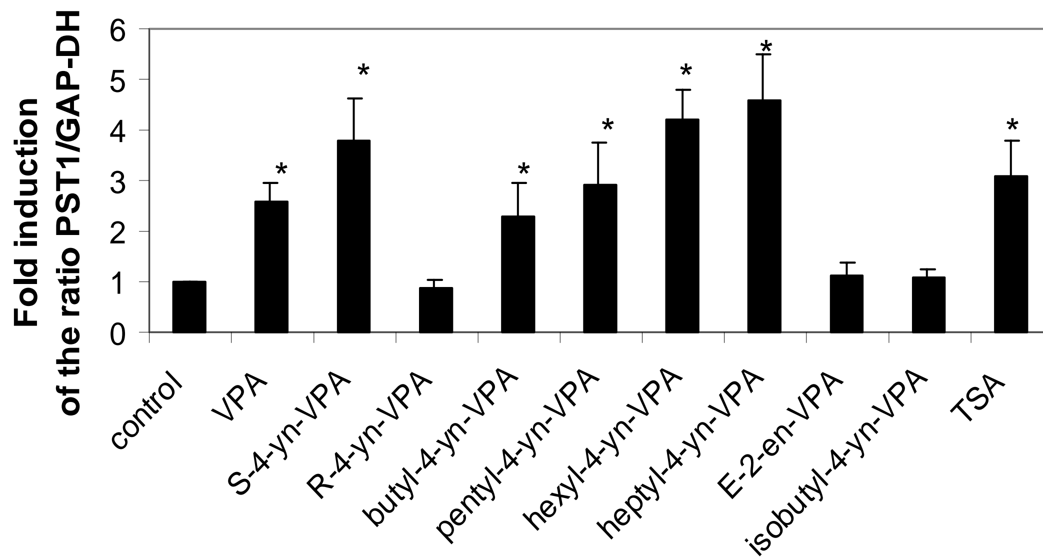

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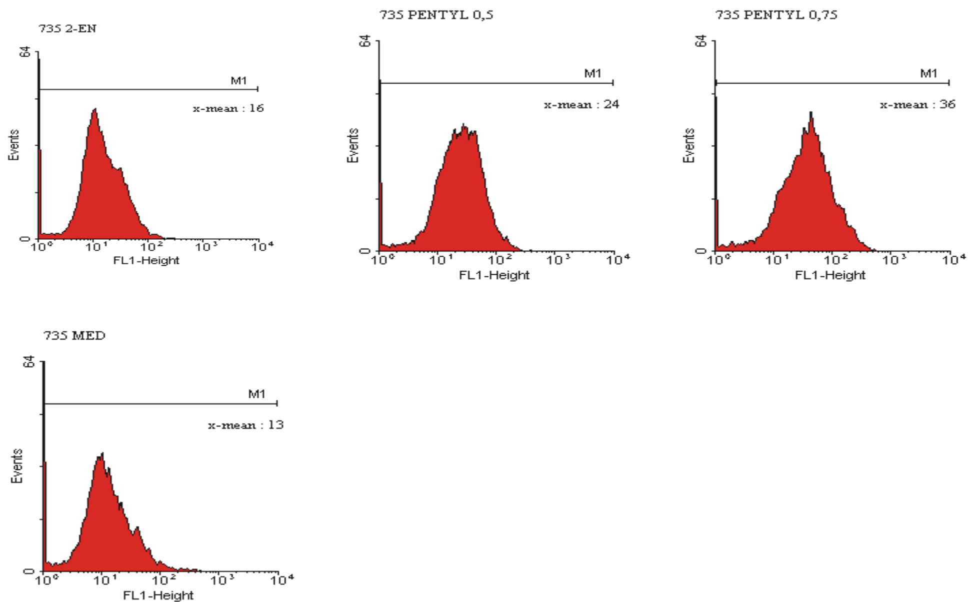
Fig. 5.

A.



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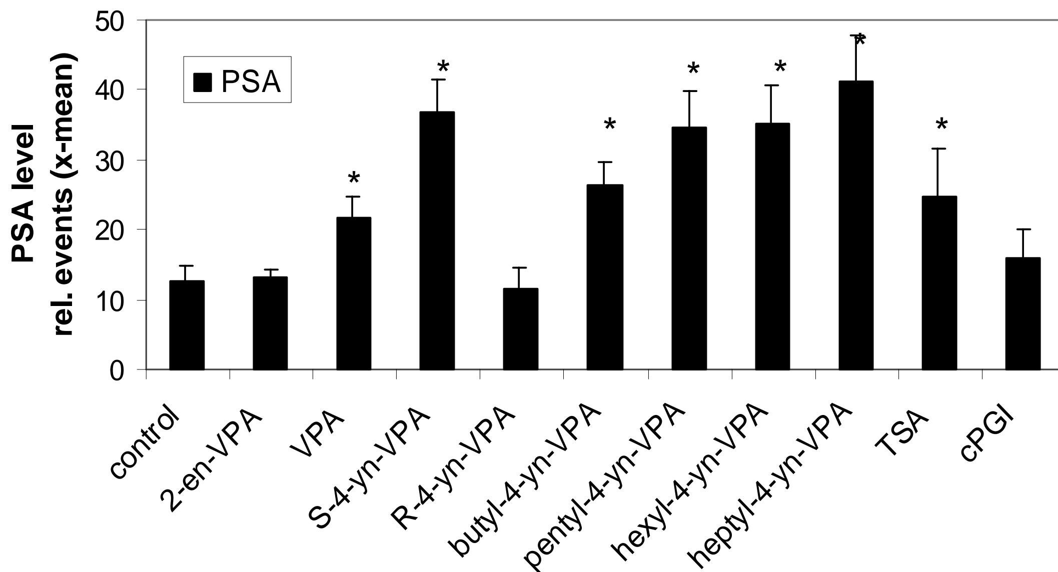

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Fig. 6.

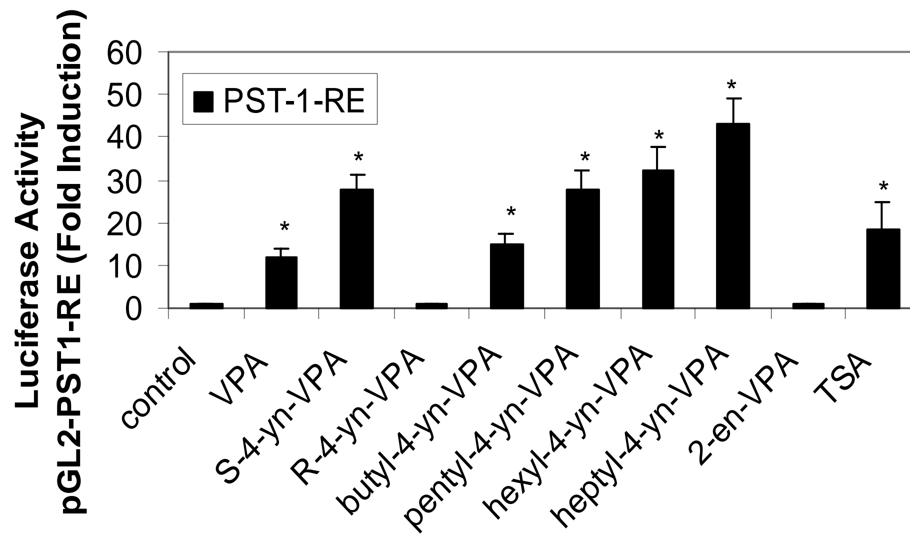

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Fig. 7.

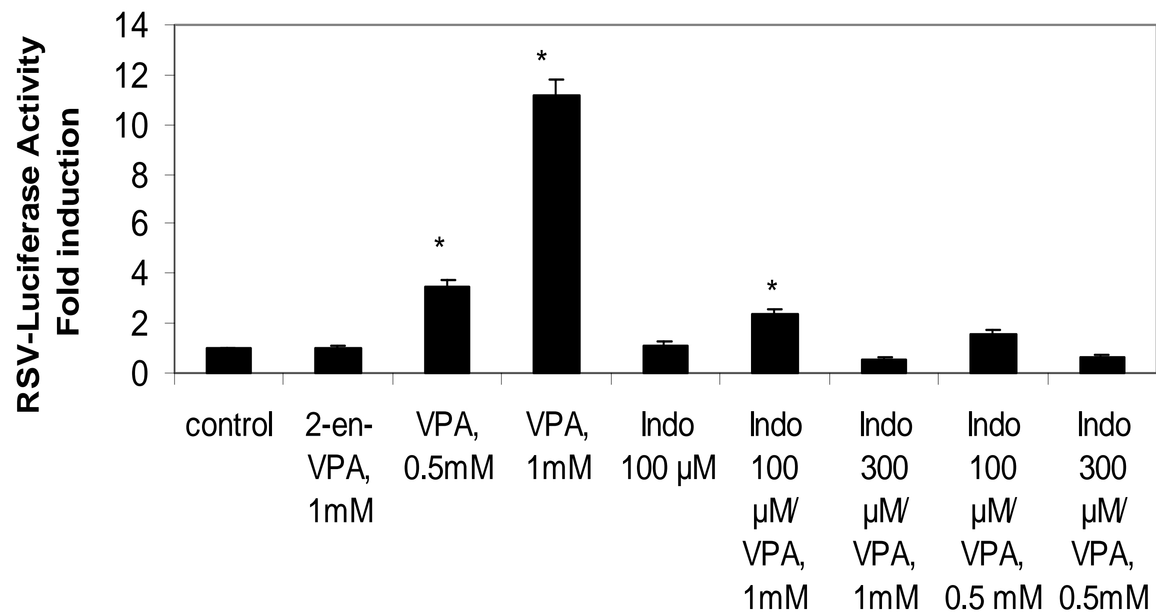

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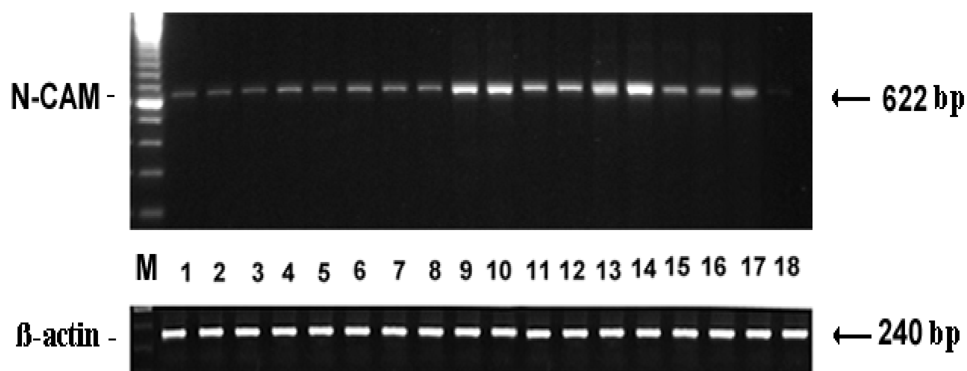
Fig. 8.

A.



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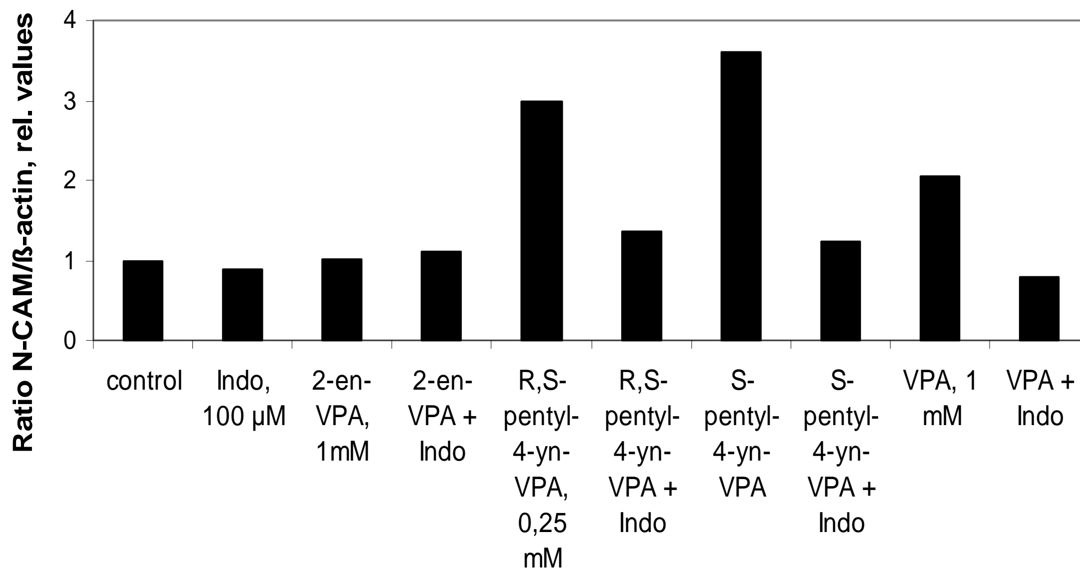

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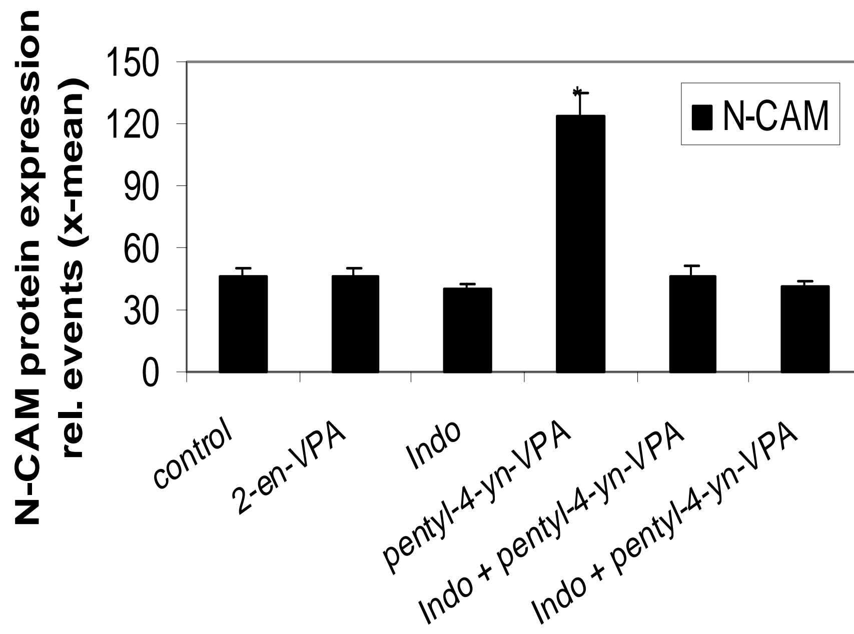
Fig. 9.

A.



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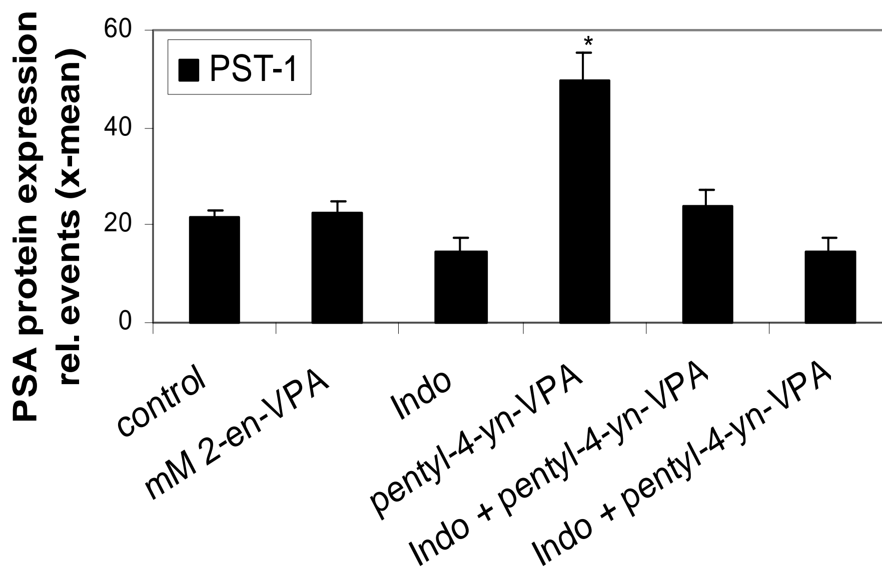

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Fig. 10A.

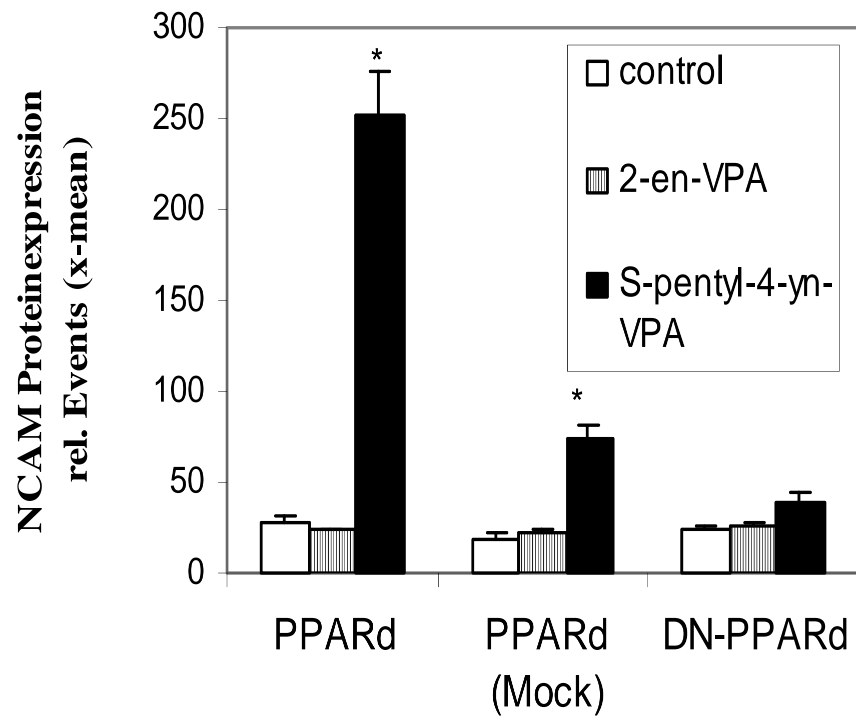

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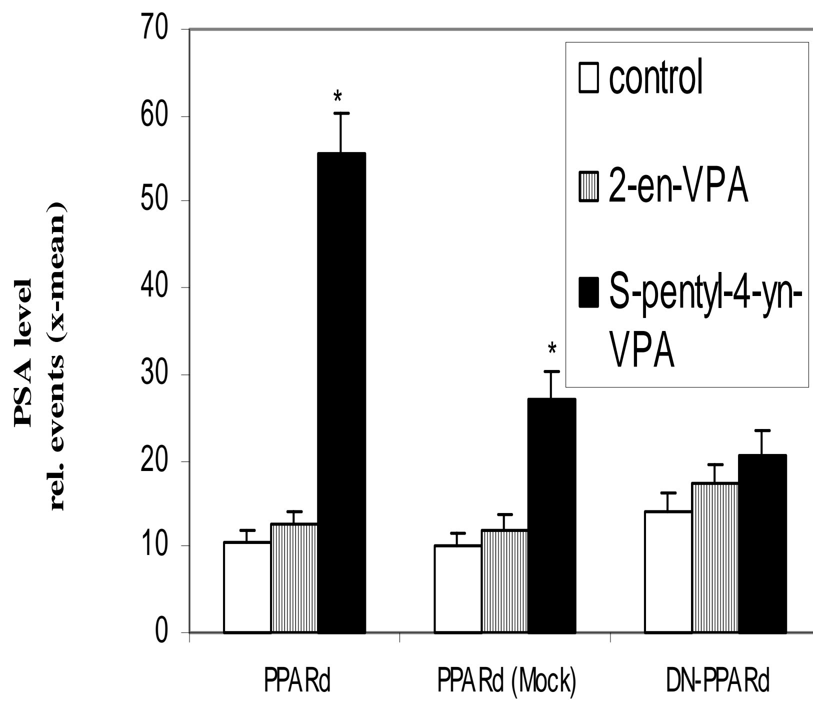
Fig. 10B.



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