[CANCER RESEARCH 63, 4984–4989, August 15, 2003]

Procaine Is a DNA-demethylating Agent with Growth-inhibitory Effects in Human Cancer Cells[1]

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ABSTRACT

Methylation-associated silencing of tumor suppressor genes is recognized as being a molecular hallmark of human cancer. Unlike genetic alterations, changes in DNA methylation are potentially reversible. This possibility has attracted considerable attention from a therapeutics standpoint. Nucleoside-analogue inhibitors of DNA methyltransferases, such as 5-aza-2′-deoxycytidine, are able to demethylate DNA and restore silenced gene expression. Unfortunately, the clinical utility of these compounds has not yet been fully realized, mainly because of their side effects. A few non-nucleoside inhibitors of DNA methyltransferases have been reported, including the anti-arrhythmia drug procainamide. Following this need to find new demethylating agents, we have tested the potential use of procaine, an anesthetic drug related to procainamide. Using the MCF-7 breast cancer cell line, we have found that procaine is a DNA-demethylating agent that produces a 40% reduction in 5-methylcytosine DNA content as determined by high-performance capillary electrophoresis or total DNA enzyme digestion. Procaine can also demethylate densely hypermethylated CpG islands, such as those located in the promoter region of the *RARβ2* gene, restoring gene expression of epigenetically silenced genes. This property may be explained by our finding that procaine binds to CpG-enriched DNA. Finally, procaine also has growth-inhibitory effects in these cancer cells, causing mitotic arrest. Thus, procaine is a promising candidate agent for future cancer therapies based on epigenetics.

INTRODUCTION

In the last decade, transcriptional silencing of tumor suppressor genes (such as $p16^{INK4a}$, *hMLH1*, *BRCA1*) associated with the hypermethylation of the CpG islands located in their promoter regions has been accepted as a common feature of human cancer (1, 2). In recent years, a CpG island hypermethylation profile of human primary tumors has emerged, showing specific gene promoter hypermethylation of these genes that is dependent on tumor type (3, 4). However, all human neoplasms have multiple bona fide and candidate tumor suppressor genes affecting different cellular pathways that are simultaneously inactivated in the same tumor, and that contribute to the neoplastic phenotype (1–4).

The tumor suppressor genes silenced by promoter hypermethylation provide very attractive targets for the development of drugs to "wake-up" these dormant genes in the fight against cancer. In cancer cell lines, the inhibition of DNA methylation and reactivation of these genes can be accomplished by the nucleoside inhibitors 5-azacytidine and DAC, also known as decitabine (Fig. 1; Ref. 5). The re-expression of these silent genes through the use of these drugs completely restores their functionality, as has been demonstrated for *hMLH1* and $p14^{ARF}$ (6, 7). The release of the repression of tumor suppressor and cell cycle genes then leads to the inhibition of tumor growth. The same drastic reduction of cell growth has also been described in a colorectal cancer line genetically disrupted at the two major DNA methylatransferases (DNMT1 and DNMT3b), leading to demethylation and reactivation of the cell cycle inhibitor $p16^{INK4a}$ (8).

One of the limitations of the nucleoside analogues in the clinical trials has been the side effects, such as thrombocytopenia and neutropenia, which are probably caused by cytotoxic effects associated with the drug's incorporation into the DNA independently of their DNA hypomethylation value. This has encouraged the search for inhibitors of DNA methylation that are not incorporated into DNA. The drug procainamide, approved by the FDA[3] for the treatment of cardiac arrhythmias, has been proposed as being a non-nucleoside inhibitor of DNA methylation (9, 10). Procainamide causes global DNA hypomethylation (9, 10) and restores expression of the detoxifier gene *GSTP1* in prostate cancer cells in which it has been silenced by hypermethylation (11). This action is thought to be mediated by the binding of procainamide to GC-rich DNA sequences (12, 13). We decided to test the putative DNA hypomethylation and growth-inhibitory actions of PCA, a drug approved by the FDA for use as a local anesthetic. Both PCA and procainamide are derivatives of 4-aminobenzoic acid, but the former is the ester with 2-(diethylamino)ethanol and the latter is the amide with 2-(diethylamino)ethylamine. These distinct compounds have different hydrogen-bonding behavior, and it is thought that their interactions with proteins, DNA, and other biomolecules are not the same.

Our results demonstrate that PCA acts as an inhibitor of DNA methylation in breast cancer cells, causing global genomic DNA hypomethylation and demethylation and reactivation of tumor suppressor genes with hypermethylated CpG islands. We observed that this effect is associated with, and possibly mediated by, PCA binding strongly to CpG-rich DNA. Finally, we found that PCA suppresses growth in these breast cancer cells simultaneously with the occurrence of demethylating events. These findings support the possible use of PCA and its derivatives in epigenetics-based cancer therapies.

MATERIALS AND METHODS

Cell Culture. The human breast cancer cell line, MCF-7, obtained from the American Type Culture Collection, was grown in DMEM with 4.5 g/liter of glucose (Invitrogen), supplemented with 10% fetal bovine serum, penicillin/streptomycin, and amphotericine B (Invitrogen) as recommended by the supplier.

Drug Treatments. Twenty-four h after seeding, cells were washed with PBS (Sigma), the medium was replaced, and drug solutions were added to the desired final concentration. Except as otherwise specified, 72 h later, cells were washed and harvested. DAC (Sigma) was dissolved in water to a final concentration of 1.0 mM and was filtered for sterilization, aliquoted, and stored at −80°C. PCA hydrochloride and procainamide hydrocloride (Sigma) were dissolved in water to a final concentration of 0.27 M, filtered for sterilization, and stored at −20°C. Whenever needed, an aliquot of PCA solution was diluted to a final concentration of 10 mM.

Received 1/7/03; revised 5/22/03; accepted 6/6/03.

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[1] Supported by I+D Grant SAF2001-0059 and the International Rett Syndrome Association. A. V-G. is a Comunidad Autonoma de Madrid Fellow.

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[3] The abbreviations used are: FDA, Food and Drug Administration; DAC, 5-aza-2′-deoxycytidine (decitabine); DAPI, 4′,6-diamidino-2-phenylindole; dsDNA, double-strand DNA; GA, synthetic CpG-rich oligonucleotide; PCA, procaine; dmC, 2′-deoxy-5-methylcytidine; RARβ2, retinoic acid receptor β2; ssDNA, single-strand DNA; TUNEL, terminal deoxynucleotide transferase dUTP nick end labeling; CE, capillary electrophoresis; HPCE, high-performance CE; RT-PCR, reverse transcription-PCR.

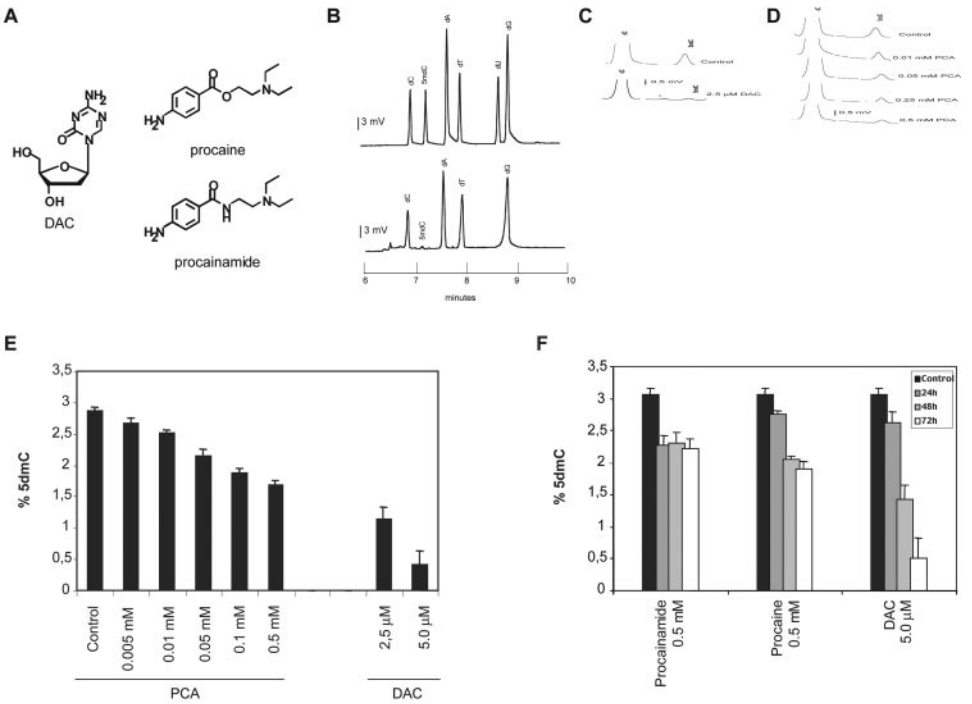


Fig. 1. *A*, chemical structure of the demethylating agents DAC, procainamide, and PCA. *B*, quantification of global DNA methylation by HPCE; *upper panel*, a representative electropherogram for standard nucleosides (*dC*, *5mdC*, *dA*, 2′-deoxythymidine, and 2′-deoxyguanosine) dissolved (5 mM) in Milli-Q grade water; *lower panel*, an electropherogram obtained after enzymatic hydrolysis of 4 μg of genomic DNA from the MCF-7 human cancer cell line. *C*, detail of the 5-dmC peak in electropherograms for control (*upper panel*) and treated with 2.5 μM DAC (*lower panel*) MCF-7 cell lines. *D*, detail of the decrease of the 5-dmC peak in electropherograms for MCF-7 cell lines treated with increasing concentrations of PCA. *E*, measurement of 5-dmC content in MCF-7 cell lines treated with increasing concentrations of PCA and DAC as a percentage of the total cytosine pool. *F*, content of 5-dmC of MCF-7 cells after 24, 48, and 72 h of treatment with PCA 0.5 mM, procainamide 0.5 mM, and DAC 5.0 μM. Results in *E* and *F* are expressed as mean ± SD. Analytical conditions for *B*, *C*, *D*, *E*, and *F* were 60.2 cm × 75-μm inner-diameter capillary (effective length, 50 cm) pressure injection (0.3 p.s.i.) for 3 s; 25°C; 17 kV voltage; buffer, 48 mM $NaHCO_3$ (pH 9.6) and 60 mM SDS; detection at 254 nm.

Quantification of the Genomic 5-Methylcytosine DNA Content. This was carried out as described elsewhere (14, 15). Briefly, DNA samples (5 μl, 0.2–1 μg/μl) extracted according to standard methods were heated for 3 min in a boiling water bath and cooled rapidly in ice. Ten mM $ZnSO_4$ (0.75 μl) and 1.25 μl of nuclease P1 (Sigma; 200 units/ml) in 30 mM $C_2H_3O_2Na$ were added and mixtures were incubated for 16 h at 37°C. Tris (1.25 μl; 0.5 M; pH 8.3) and 0.75 μl of alkaline phosphatase (Sigma; 50 units/ml) in 2.5 M $(NH_4)_2SO_4$ were then added, and mixtures were incubated for an additional 2 h at 37°C. Samples were centrifuged and stored at 4°C. For the CE procedure, an uncoated fused-silica capillary (Beckman-Coulter; 60.2 cm × 75 μm; effective length, 50 cm) was used in a CE system (P/ACE MDQ; Beckman-Coulter) connected to a data-processing station (32 Karat software). The running buffer was 14 mM $NaHCO_3$ (pH 9.6) containing 20 mM SDS. Running conditions were 25°C with an operating voltage of 17 kV. On-column absorbance was monitored at 254 nm. Before each run, the capillary system was conditioned by washing with 0.1 M NaOH for 3 min and was equilibrated with the running buffer for 3 min. Buffers and washing solutions were prepared with Milli-Q water and filtered throughout 0.45-μm filters. Hydrolyzed samples, previously filtered through 0.45-μm pore filters, were injected under pressure (0.3 p.s.i.) for 3 s. All of the samples were analyzed in duplicate, and three analytical measurements were made per replicate. The relative methylation of each DNA sample was taken as the percentage of dmC in total cytosine: dmC peak area × 100/(dmC peak area + dmC peak area).

DNA Digestion with Methylation-Sensitive Restriction Enzymes. One μg of each DNA sample was treated either with 5 units of *Msp*I, *Hpa*II, *Mcr*BC (New England Biolabs Ltd.) or a reaction mixture with no enzyme (control) in a total volume of 50 μl at 37°C for 3 h.

Bisulfite Genomic Analysis of the RARβ2 CpG Island. DNA samples were treated with sodium bisulfite as described previously (16), and primers spanning the CpG island of the RARβ2 promoter were used for bisulfite genomic sequencing (17, 18). At least three different sequencing reactions, in both strands, were carried out. Methylation-specific PCR (MSP) analysis of the RARβ2 CpG island was performed as described previously (19).

RT-PCR Analysis. RNA was extracted with TRIzol LS reagent (Invitrogen) following the supplier's instructions. The RT-PCR primers used to amplify the RARβ2 transcript have been described previously (20). Primers for the β-actin transcript were used as control. RT-PCR was performed in a one-step procedure with an Enhanced Avian RT-PCR kit (Sigma) under the following conditions: 50°C for 45 min; 94°C for 3 min; then 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min for 5 cycles; then 94°C for 30 s, 51°C for 30 s, 72°C for 1 min for 30 cycles; and a final extension (72°C for 5 min).

PCA-DNA Binding Affinities. PCA-DNA binding affinities were studied by CE mobility shift assay, as described elsewhere (21, 22). In brief, a neutral coating capillary (Beckman-Coulter; 32.5 cm × 50 μm; effective length, 20 cm) was used in a P/ACE MDQ CE system (P/ACE MDQ, Beckman-Coulter) connected to a Karat Software data-processing station. The running buffer [40 mM Tris-borate and 0.95 mM EDTA (pH 8.0)] was chosen to provide a low current when working at high voltage (30 kV, 923 V/cm) to maintain the stability of PCA-DNA complexes during separation. Laser-Induced Fluorescence (LIF) was detected by excitation at 488 nm (3-mW Argon ion laser provided by Beckman-Coulter S.A., Madrid, Spain), and emissions were collected through a 520-nm emission filter (Beckman-Coulter). Samples were injected under pressure (0.2 p.s.i.) for 2 s and the run temperature was maintained at 20°C. Before each run, the capillary was conditioned by washing with running buffer for 2 min. Buffers and running solutions were filtered through 0.2-μm pore-size filters. Three replicates of each concentration were prepared and each was run twice.

Binding reactions were performed in 50 mM Tris buffer (pH 7.5). The following GC-rich sequences were purchased as single-stranded oligonucleotides (Operon-Qiagen): GA, forward, GAT CCG ACG ACG ACG ACG AXG ACG ACG ACG ACG ACG ACG ATC; GA, reverse, GAT CGT CGT CGT CGT CGT CGT XGT CGT CGT CGT CGT CGG ATC; MLH1 forward, GAA XGT GAG CAX GAG GCA CTG AGG TGA TTG GCT GAA GGC ACT TCX GTT GA; MLH1 reverse, TCA AXG GAA GTG CCT TCA GCC AAT CAC CTC AGT GCC TXG TGC TCA XGT TG; RARβ forward, CXG AGA AXG XGA GXG ATC XGA GCA GGG TTT GTC TGG GCA CXG; RARβ reverse, G TGC CCA GAC AAA CCC TGC TXG GAT XGC TXG XGT TCT XGG; where X, in each case, is a 5-methylcytosine residue in the methylated oligonucleotides and unmethylated cytosine rings in the unmethylated ones. Forward oligonucleotides were labeled at their 5′ ends with 6-FAM. Complementary oligonucleotides were mixed at equimolar concentrations and were annealed by bringing the solution to 95°C and allowing it cool down slowly to room temperature. Increasing amounts of the drug were added to 6-FAM-labeled DNAs and incubated for 45 min. at 4°C. Dissociation constants were quantified by scatchard analyses using GraFit 3.1 software. The saturation of the oligonucleotide [R = complex/(complex + PCA)] was plotted against increasing quantities of the drug. The dissociation constant (K_d) was then calculated, seeking the best fit of the data to curves of different binding models.

Cell Cycle and Apoptotic Analysis. For cell counting, cells treated with 0.5 mM PCA hydrochloride; 0.5 mM procainamide hydrochloride; and serum-starved cells (72 h each treatment) were washed, harvested, fixed in formol/4%

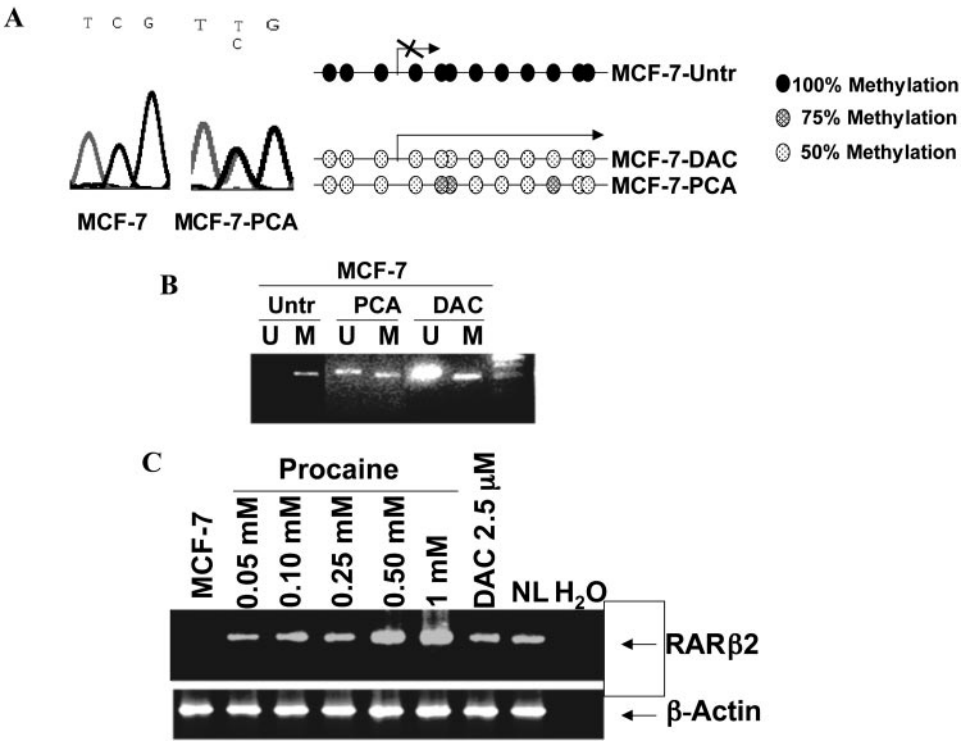


Fig. 2. *A*, bisulfite genomic sequencing of the CpG island of the *RARβ2* gene. *Left panel*, chromatograms shows retention of "C" preceding G, indicating DNA methylation in the MCF-7 control cell line (*left chromatogram*), whereas in MCF-7 treated with PCA 0.5 mM (*right chromatogram*) there is a 50% increase of "T" in the same position, indicating DNA demethylation. *Right panel*, graphical representation of DNA methylation status of the *RARβ2* CpG island under different treatments; *arrow*, transcription start site. *B*, methylation-specific PCR analysis of the CpG island of the *RARβ2* gene. *PCR band*: *Lane U*, unmethylated alleles; *Lane M*, methylated alleles. Untreated MCF-7 cells (*Untr*) show only methylated alleles, whereas treatment with PCA (0.1 mM) and DAC (2.5 μM) induces the appearance of unmethylated alleles of *RARβ2*. *On right*, molecular weight marker. *C*, analysis of the expression of *RARβ2* by RT-PCR. Untreated MCF-7 does not express the RARβ2 transcript, whereas PCA (in a concentration-dependent manner) and DAC restore RARβ2 expression. *Right*, normal lymphocytes (*NL*) and water.

PBS, and stored at 4°C until counting; to count, we used three random fields in two different experiments. To establish the mitotic index, understood as the relative number of cells in metaphase or anaphase with respect to the total number of cells, cells were stained with DAPI (Sigma), and the nuclei were visualized by microscopy. Again, three random fields in two different experiments were used. For apoptosis analysis, we performed the TUNEL assay according to the manufacturer's instructions (Roche Diagnostics).

RESULTS

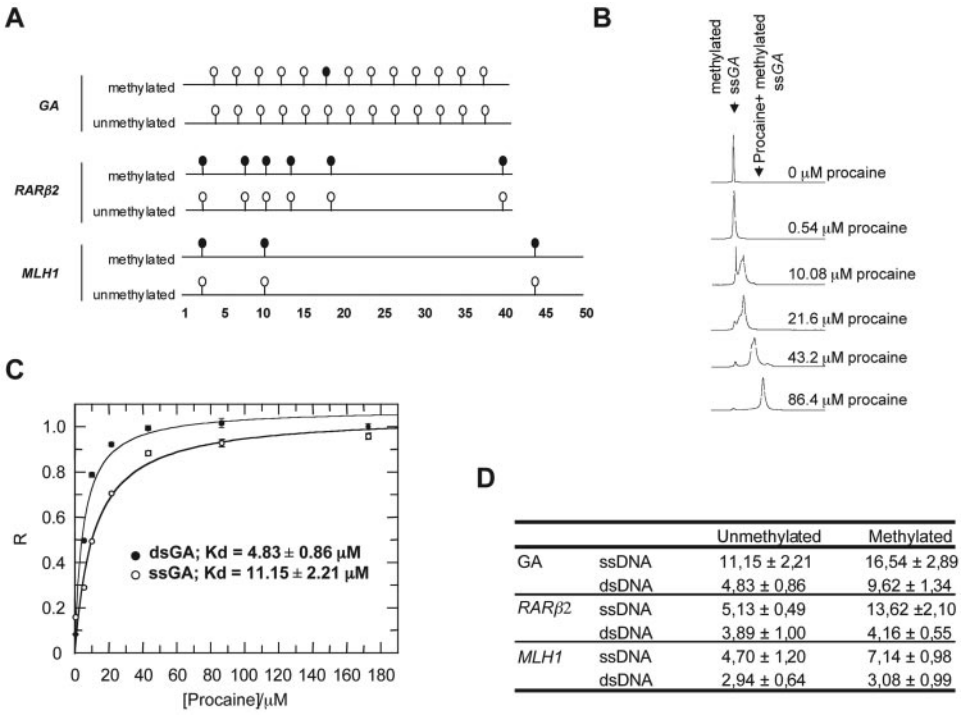
PCA Causes Global Genomic DNA Hypomethylation in the MCF-7 Breast Cancer Cell Line. The human breast cancer cell line, MCF-7, was treated with a range of concentrations of PCA (0.005, 0.01, 0.05, 0.1, and 0.5 mM) for 72 h and the 5-methylcytosine DNA content before and after each treatment was measured by HPCE, as described previously (14, 15). Examples of the analysis are shown in Fig. 1, *B–D*. Whereas the untreated MCF-7 cells had a 2.87 ± 0.04% 5-methylcytosine DNA content, we observed a directly proportional reduction of methylcytosine DNA groups according to increasing doses of PCA. The greatest reduction, of 41% (absolute value 1.68 ± 0.074), was attained at the highest concentration (Fig. 1*E*). We used MCF-7 cells treated with DAC as an internal control to validate the DNA-demethylating events; DAC was a much stronger DNA demethylating agent even at lower concentrations than was PCA (Fig. 1*C*). The global DNA demethylation induced by PCA and DAC was also corroborated by running gels of DNA digested, respectively, with the methyl-isoschizomer enzymes *Msp*I and *Hpa*II (which share the same target, although Hpa does not cut it if the CpG is methylated) and the *Mcr*BC enzymes, which only cut methylated CpGs (data not shown).

Treatments at 24, 48, and 72 h were developed to precisely define the optimum time points for demethylation (Fig. 1*F*). Parallel treatments with procainamide and DAC were also performed as positive controls. The maximum demethylating effect of PCA is observed at 72 h, although a similar level is observed at 48 h. For procainamide, the highest demethylation level was already evident after 24h of treatment, whereas for DAC, the demethylation is progressive, the strongest loss seen at 72 h.

PCA Induces Demethylation of the CpG Island of the Tumor Suppressor Gene *RARβ2* and Restores Its Expression. Once the overall DNA demethylation effect had been found, we decided to test the effects on a particular hypermethylated locus. We chose the CpG island of the RARβ2, which our group and many others have reported as being hypermethylated in this cell line, in association with its transcriptional silencing (9, 17, 20, 23, 24), and in which methylation-mediated silencing is a common feature of many human primary tumors (25–27). The treatment of MCF-7 cells with PCA in concentrations higher than 0.01 mM (data not shown) led to the demethylation of the RARβ2 promoter-associated CpG island and restored the expression of the RARβ2 transcript. This hypomethylation of the CpG island was demonstrated by bisulfite genomic sequencing around the transcription start site, comparing untreated MCF-7 cells with PCA-treated cells (Fig. 2*A*). These results were corroborated by methylation-specific PCR with primers covering the same area (Fig. 2*B*). Again, we used MCF-7 cells treated with the classical DNA demethylating agent DAC as positive controls for the hypomethylation events at the RARβ2 CpG island (Fig. 2, *A* and *B*).

The induction of demethylating events in the CpG island of the RARβ2 gene was associated with the re-expression of the mRNA of RARβ2, which RT-PCR revealed to be absent from the untreated MCF-7 cells (Fig. 2*C*). The degree of gene reactivation (similar to the total loss of 5-methylcytosine DNA content) was directly proportional to the dose of PCA administered to the cells (Fig. 2*C*). As a positive control of restoration of gene expression, we used MCF-7 cells, treated with DAC, that also re-expressed the transcript (Fig. 2*C*).

PCA Directly Binds to CpG-rich DNA. We wondered about the mechanism of action of PCA by which DNA is demethylated. In contrast to DAC, PCA is not a nucleoside inhibitor (see structure in Fig. 1*A*); thus, we thought that, rather than through incorporation into DNA, it might exert its effects by binding to GC-rich DNA, as do procainamide and *N*-acetyl-procainamide (12, 13). To test this hy-



		Unmethylated	Methylated
GA	ssDNA	11,15 ± 2,21	16,54 ± 2,89
	dsDNA	4,83 ± 0,86	9,62 ± 1,34
RARβ2	ssDNA	5,13 ± 0,49	13,62 ±2,10
	dsDNA	3,89 ± 1,00	4,16 ± 0,55
MLH1	ssDNA	4,70 ± 1,20	7,14 ± 0,98
	dsDNA	2,94 ± 0,64	3,08 ± 0,99

Fig. 3. Binding of PCA to DNA. *A*, scheme of the CpG distribution in the oligonucleotides used for the binding assay. ●, methylated CpG; ○, unmethylated CpG. *B*, electropherograms for mixtures of methylated single-strand GA (ssGA; 24 nM) and increasing concentrations of PCA in 50 mM Tris (pH 7.5) buffer. Analytical conditions: 32.5 cm × 50 μm capillary (effective length 20 cm); low pressure injection at 0.2 p.s.i. for 2 s; 20°C; 30 kV voltage; reverse polarity (anode at the detector end); buffer, 40 mM Tris-borate–0.95 mM EDTA (pH 8.0); laser-induced fluorescence detection: excitation at 488 nm, emission at 532 nm. *C*, single-site ligand-binding fit for unmethylated ssGA (○) and unmethylated double-strand (ds)GA (●) with PCA using GraFit 3.1 software. Saturation (R) = complex/(complex + PCA). *D*, table showing the values obtained for dissociation constants of the different DNA-procaine complexes studied. Values are expressed in μM. Results in *C* and *D* are expressed as means ± SD.

pothesis, we carried out a CE mobility shift assay (CEMSA), as described previously (21, 22), to examine the binding affinity of PCA for three different CpG-rich DNA sequences either methylated or unmethylated (scheme shown in Fig. 3*A*). We observed that increasing concentrations of PCA retard peaks in single- and double-stranded CpG-rich DNA (Fig. 3, *B* and *C*), implying a strong interaction between PCA and DNA. From the values of the K_ds shown in Fig. 3*D*, it appears that PCA has more affinity for ddDNA than for ssDNA. The affinities (shown by the K_ds in Fig. 3, *C* and *D*) between PCA and CpG-rich DNA are similar to those between histone dimers and dsDNA (28). The binding affinity of PCA to the unmethylated or methylated forms of the three oligonucleotides used, CpG rich (GA), CpG medium (RARB2), and CpG poor (MLH1), demonstrates K_ds within the same micromolar range. Most interesting, the delay in migration time of the PCA-DNA complex with increasing concentration of PCA suggests that multiple molecules of PCA are able to bind simultaneously to CpG-rich DNA (Fig. 3*A*), as procainamide also does (12, 13).

PCA Exhibits Cell-Growth-inhibitory Effects. Finally, we examined the growth effects of PCA on MCF-7 cells, in addition to its DNA-demethylation properties. This is crucial to the future use of this drug in any putative cancer therapy based on epigenetics. We analyzed the effects of PCA and procainamide on MCF-7 at the cellular level by counting the number of cells, calculating the mitotic index by DAPI staining, and developing the TUNEL assay to measure the degree of apoptosis. As shown in Fig. 4*C*, PCA treatment (0.5 mM) induced an increase in the mitotic index, that is, the relative number of cells in M phase as indicated by an increased number of cells in mitotic metaphase and anaphase revealed by DAPI staining (Fig. 4, *C* and *D*) and by a concomitant decrease in absolute cell number (Fig. 4, *A* and *B*), which strongly suggested that PCA may promote cell cycle arrest in M phase. This effect was also observed with DAC (Fig. 4*A*) and, to a minor degree, with procainamide. No increase in the apoptosis rate measured by the TUNEL assay was observed as a consequence of administering PCA or procainamide (Fig. 4*D*). Thus, PCA has growth-inhibitory effects on human breast cancer cells that are associated with their mitotic arrest.

DISCUSSION

The inactivation of tumor suppressor genes is now recognized as being a major feature of all forms of human cancer. The re-expression in tumor cells of many of these genes can lead to suppression of cell growth (1, 2). Many of the demethylating agents are small versatile molecules that are in sharp contrast to the challenges of delivering gene therapy. As more methylation-mediated silenced genes are found in human neoplasms, there is increasing interest in the search for new demethylating agents of potential utility in cancer therapy. To this list, we may now add PCA, a drug that has been administered safely as a local anesthetic for many years.

We have found that PCA causes global DNA hypomethylation, demethylation and re-expression of a CpG-island-associated gene (*RARβ2*), and growth inhibition in breast cancer cells. In this way, it behaves very similarly to procainamide (both molecules are 4-aminobenzoic acid derivates), which restores the expression of the hypermethylated *GSTP1* gene in prostate cancer cells and diminishes xenograft tumor growth (11). Preclinical studies are now needed to ascertain whether PCA, in a similar manner to that of the classical demethylating agent DAC, synergizes with histone deacetylase inhibitors in the reactivation of dormant genes (29). One interesting aspect from a clinical standpoint is our observation that PCA stops the growth of cancer cells "*in vitro*." This observation can explain why PCA increases the antitumoral activity of several conventional anticancer drugs, such as cisplatin, mitomycin C, peplomycin, and doxorubicin (30–33). Other conventional strategies for cancer treatment could also benefit from the newly identified hypomethylating and growth-inhibiting actions of PCA. This is the case in radiotherapy, in which PCA has been shown to radiosensitize hypoxic cells and to increase their hyperthermic killing (34, 35).

Until now, one of the limitations of DNA hypomethylating agents such as DAC in the clinical setting has been the side effects (mainly myelotoxicity) of the treatments and the concern that its incorporation into genomic DNA might lead to mutations (36). These setbacks are characteristic of all nucleoside analogues in general, not only DNA-methyltransferase inhibitors. PCA is not incorporated into the DNA

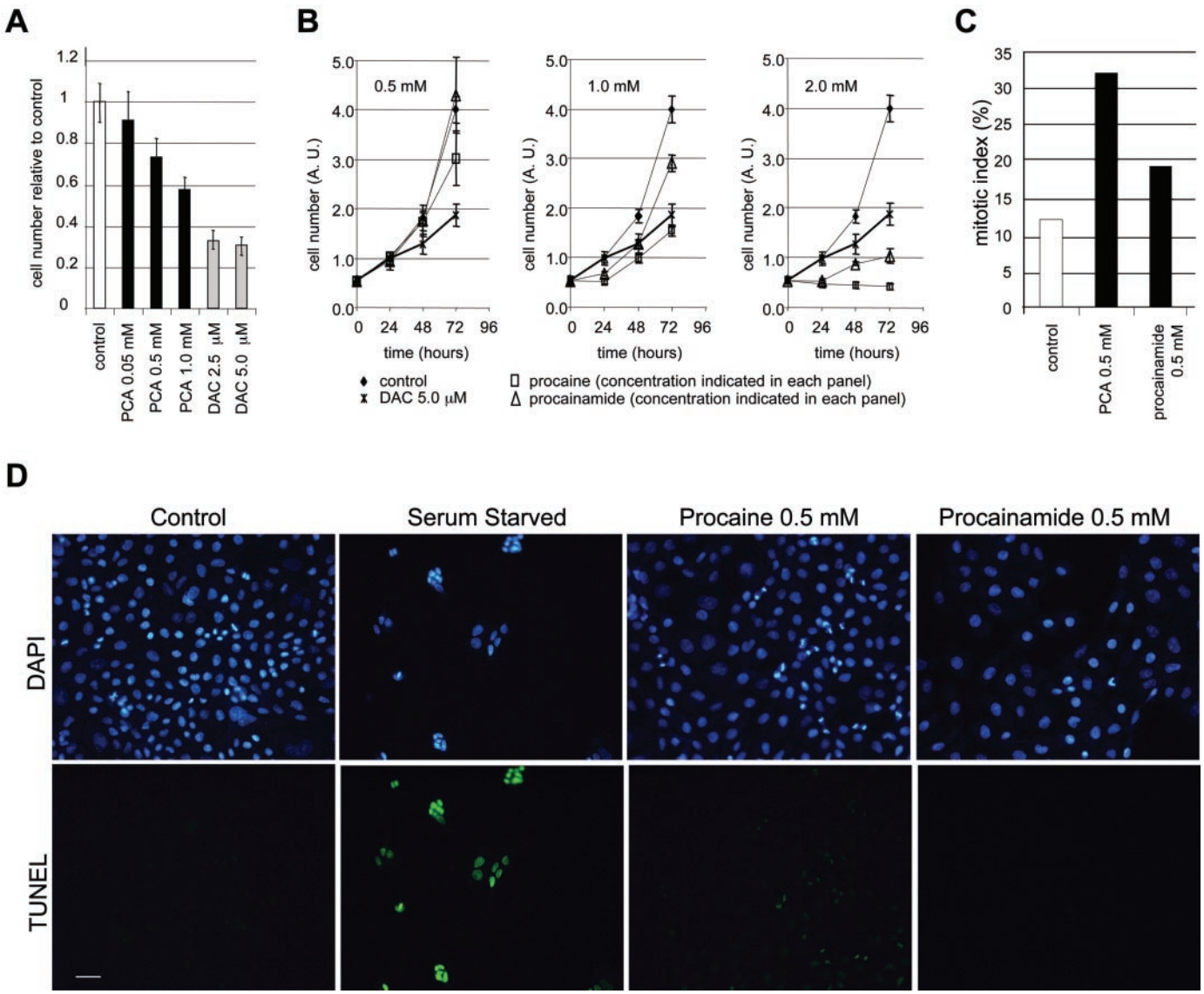


Fig. 4. PCA induces cell growth arrest in M phase. *A*, cell counting expressed as the number of cells relative to the untreated MCF-7 cells. *B*, cell counting (arbitrary units) of MCF-7 cells treated with procainamide (△) and PCA (□) at the indicated concentrations in each panel. DAC 5.0 μM [GRAPHIC] and untreated (♦) were included as controls in all cases. *C*, *mitotic index*, the number of cells in metaphase, anaphase, or telophase (mitotic figures) with respect to the total number of cells. *D*, apoptotic measurement by double staining with DAPI and TUNEL assay of MCF-7 control cells that were PCA treated (0.5 mM) and procainamide treated (0.5 mM). Cells undergoing serum starvation for 72 h were used as positive control for apoptotic induction. DAPI staining also shows an increase of mitotic figures in PCA-treated cells. *Bar*, 25 μm.

but, instead, binds to DNA. Thus, PCA may be an example of an agent that demethylates DNA and reactivates methylated genes with less potential side effects. It is important to mention that the doses of PCA that achieve significant demethylation and growth-inhibitory effects in our study are of the same order as those administered in conjunction with antineoplastic drugs (30–33) or radiotherapy (34, 35). Most important, PCA has even proved to protect against chemotherapy-related nephrotic and hepatic toxicities (31).

Our study supports a role for PCA as a promising DNA-hypomethylating drug with growth-inhibitory effects in cancer cells. Its long-established and safe use as a local anesthetic, with well-known pharmacological characteristics, may stimulate its prompt transition to preclinical and early clinical trials for epigenetics-based cancer treatments.

ACKNOWLEDGMENTS

We thank Dr. Esteban Ballestar for technical help and discussions.

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Cancer Res 2003;63:4984-4989.