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Phosphodiesterase IV Inhibition by Piclamilast Potentiates the Cytodifferentiating Action of Retinoids in Myeloid Leukemia Cells

CROSS-TALK BETWEEN THE cAMP AND THE RETINOIC ACID SIGNALING PATHWAYS*[S]

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Inhibition of phosphodiesterase IV by *N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyloxy-4-methoxybenzamide (piclamilast) enhances the myeloid differentiation induced by all-*trans*-retinoic acid (ATRA), retinoic acid receptor α (RARα), or retinoic acid receptor X agonists in NB4 and other retinoid-sensitive myeloid leukemia cell types. ATRA-resistant NB4.R2 cells are also partially responsive to the action of piclamilast and retinoic acid receptor X agonists. Treatment of NB4 cells with piclamilast or ATRA results in activation of the cAMP signaling pathway and nuclear translocation of cAMP-dependent protein kinase. This causes a transitory increase in cAMP-responsive element-binding protein phosphorylation, which is followed by down-modulation of the system. ATRA + piclamilast have no additive effects on the modulation of the cAMP pathway, and the combination has complex effects on cAMP-regulated genes. Piclamilast potentiates the ligand-dependent transactivation and degradation of RARα through a cAMP-dependent protein kinase-dependent phosphorylation. Enhanced transactivation is also observed in the case of PML-RARα. In NB4 cells, increased transactivation is likely to be at the basis of enhanced myeloid maturation and enhanced expression of many retinoid-dependent genes. Piclamilast and/or ATRA exert major effects on the expression of cEBP and STAT1, two types of transcription factors involved in myeloid maturation. Induction and activation of STAT1 correlates directly with enhanced cytodifferentiation. Finally, ERK and the cAMP target protein, Epac, do not participate in the maturation program activated by ATRA + piclamilast. Initial *in vivo* studies conducted in severe combined immunodeficiency mice transplanted with NB4 leukemia cells indicate that the enhancing effect of piclamilast on ATRA-induced myeloid maturation translates into a therapeutic benefit.

All-*trans*-retinoic acid (ATRA)[1] and retinoids exert their antileukemic activity through activation of three processes (*i.e.* cytodifferentiation, growth inhibition, and apoptosis). ATRA induces complete clinical remission in the majority of acute promyelocytic leukemia (APL) patients (1, 2) and is part of the standard protocol used for the management of this type of leukemia (3). Paradoxically, APL is characterized by the prototypical t15:17 translocation, which involves the nuclear retinoic acid receptor RARα and leads to the expression of the aberrant fusion protein PML-RARα (1). The success of ATRA in APL represents proof of principle that cytodifferentiation therapy (4) can be applied clinically. However, a more general use of ATRA at the clinical level is limited by toxicity and natural or induced resistance (5–7).

With this in mind, it would be desirable to devise strategies aimed to increase the efficiency of the intracellular signals regulating the cytodifferentiating, growth-inhibitory, and apoptotic action of retinoids. The identification of agents capable of augmenting the therapeutic index of ATRA may lead to the development of useful pharmacological combinations in the treatment of APL and other acute myeloid leukemias (AMLs). In addition, dissection of the molecular mechanisms underlying the interactions between these agents and retinoids in AML cells is likely to cast light on the process of myeloid maturation.

Analogs of cAMP (8–12), interferons (13–15), granulocyte colony-stimulating factor (16), and a novel class of experimental compounds, bis-indols (17), enhance the cytodifferentiating activity of ATRA in AML cell lines. Enhanced cytodifferentia-

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[S] The on-line version of this article (available at http://www.jbc.org) contains an additional figure.

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[1] The abbreviations used are: ATRA, all-*trans*-retinoic acid; APL, acute promyelocytic leukemia; ERK, extracellular signal-regulated kinase; EMSA, electromobility shift assay; 8-CPT-cAMP, 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphate; *O*-Me cAMP, 8-(4-chlorophenylthio)-2′-*O*-methyladenosine 3′,5′-cyclic monophosphate; Rp-8Br-cAMP, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer; AML, acute myeloid leukemia; PDEIV, phosphodiesterase IV; NBT-R, nitro blue tetrazolium reductase; PKA, cAMP-dependent protein kinase; MCP-1, monocyte chemotactic peptide 1; PKA-rIα and -rIβ, regulatory subunits I α and β of PKA; PKA-rIIα, regulatory subunit II α of PKA; PKA-cIα, catalytic subunit I α of PKA; TNF, tumor necrosis factor α; CREB, cAMP-responsive element-binding protein; RAR, retinoic acid receptor; RXR, retinoic acid receptor X; CAT, chloramphenicol acetyltransferase; RARE, retinoic acid-responsive element; PRAM, protein regulated by retinoic acid and PML-RAR; ctsD, cathepsin D; pLym, putative lymphocyte G_0-G_1 switch gene; BTG, B cell translocation gene 2 protein; THBS, thrombospondin; EGR1, early growth factor-regulated 1; cEBPα, -β, and -γ, CAAT element-binding proteins α, β, and γ, respectively; STAT1, signal transducer and activator of transcription 1; SCID, severe combined immunodeficiency; Epac, exchange protein directly activated by cAMP; MAP, mitogen-activated protein; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; RT, reverse transcriptase; MST, median survival time.

tion by combinations of bis-indols and ATRA translates into a therapeutically significant effect, as assessed in animal models of APL (17). The cross-talk between the cAMP signaling pathway and ATRA is particularly attractive in terms of the development of pharmacological combinations to be used in the cytodifferentiating treatment of AML. In fact, the cAMP pathway is modulated by various pharmacological agents (18–20), some of which are already in the clinics for the treatment of asthma (21, 22). In this report, we demonstrate that inhibition of phosphodiesterase IV (PDEIV), the enzyme that hydrolyzes cAMP to the corresponding nucleoside monophosphate, by piclamilast, potentiates the retinoid-dependent granulocytic maturation of the APL-derived NB4 and other AML cell lines. The molecular mechanisms underlying this potentiating effect are investigated in the NB4 model. Furthermore, we present initial evidence that the combination of ATRA and piclamilast is more effective than the single components in prolonging the life span of SCID mice transplanted with APL cells.

EXPERIMENTAL PROCEDURES

Chemicals—ATRA, rolipram, H89, 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphate (8-CPT-cAMP), dibutyryl-cAMP, H89, and 8-bromoadenosine-3′,5′-cyclicmonophosphorothioate-Rp-isomer (Rp-8-BrcAMP) were from Sigma. AM580, CD2809, and CD2915 were obtained from CIRD-Galderma (Sophia Antipolis, France). *N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyloxy-4-methoxybenzamide (piclamilast) was synthesized by Altana Pharma GmbH (Konstanz, Germany). 8-(4-chlorophenylthio)-2′-*O*-methyladenosine 3′,5′-cyclic monophosphate (*O*-Me cAMP) was from BIOLOG Life Science Institute (Bremen, Germany).

Cell Cultures, Intracellular cAMP, cAMP-dependent Protein Kinase (PKA) Activity, Electromobility Shift Assay (EMSA), Nitro Blue Tetrazolium Reductase (NBT-R) Activity, and Transfections—NB4 (23), NB4.R2 (24), U937 (25), and HL-60 (ATCC, Manassas, VA) cells and blasts from one AML patient (FAB classification M5) were cultured as described (16). Cell number and viability were determined following staining with erythrosin (Sigma) (26).

Intracellular cAMP and PKA activity were determined with the cAMP Biotrak EIA (Amersham Biosciences) and PKA PepTag Assay (Promega, Madison, WI) kits.

EMSAs were conducted as described (26) with an oligodeoxynucleotide representing the DNA sequence for the binding of cAMP-responsive element-binding protein (5′-GAGAGATTGCCTGACGTCAGAGAGGTAG-3′).

Nitro blue tetrazolium reductase (NBT-R) activity was measured in cell extracts, and the number of NBT-R^+ cells was determined as detailed (17). To determine the morphology of NB4 cells quantitatively, cytospins were stained with May-Grunwald-Giemsa. Approximately 100 cells/microscopic field were scored for the presence of morphologically differentiated cells (27).

COS-7 cells (ATCC) were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and transfected with RARα, PML-RARα, or the mutant RARαS369A cDNAs (28) in the presence of the CREB-dependent Som-CAT plasmid (containing 1.4 kb of 5′-flanking region of the somatostatin gene promoter) (29) or the RARE-containing β_2RARE-CAT, DR5-tk-CAT, or TRE-tk-CAT reporter constructs and the normalization plasmid pCH110 (containing the bacterial β-galactosidase cDNA) (17). CAT and β-galactosidase activities were measured in cell extracts as detailed (17).

RNA Purification, Nuclear Extracts, RT-PCR, Northern Blot, ELISA, Western Blot, and Fluorescence-activated Cell Sorting (FACS) Analyses—For the amplification of the cDNAs coding for human PDEIV, total RNA (1 μg) was subjected to reverse transcription. cDNAs were amplified by PCR with the following couples of amplimers: PDEIVA, 5′-TGAGCTGACGCTGGAGGAGG-3′ (nucleotides 1735–1754 of the PDEIVA cDNA, NCBI accession number BC038234) and 5′-CCACCAGGACGTGGGAGTGC-3′ (complementary to nucleotides 2222–2241); PDEIVB, 5′-GACCTGAACAAATGGGGTCT-3′ (nucleotides 985–1004 of the PDEIVB3 cDNA, U85048) and 5′-TTCAGGTCTGCCAGCAGGCT-3′ (complementary to nucleotides 1525–1544); PDEIVC, 5′-AGGCAGAGAACTGGCATATC-3′ (nucleotides 1520–1539 of the PDEIVC cDNA, NM_000923) and 5′-GATTGTCCTCCAGCGTGTCC-3′ (complementary to nucleotides 1984–2003); PDEIVD, 5′-ATGGTGAGTCAGACACGGAA-3′ (nucleotides 1753–1772 of the PDEIVD cDNA, BC036319) and 5′-TTGTCAGCTCTACCAAGCTG-3′ (complementary to nucleotides 2189–2208).

Northern blot analysis was performed as detailed (8) with specific human cDNA probes obtained following RT-PCR amplification of the relevant transcripts. The couples of amplimers used for the amplification of the cDNAs were as follows: vinculin (5′-ATGCCAGTGTTTCATACGCG-3′, nucleotides 51–70; 5′-CTAAGATCTGTCTGATGGCC-3′ complementary to nucleotides 940–960 of the vinculin cDNA, M33308); thrombospondin (THBS) (5′-CAGCTTTCCGCATGCAGGAT-3′, nucleotides 281–300; 5′-AGGAGCCCTCACATCGGTTG-3′ complementary to nucleotides 1341–1360 of the THBS cDNA, NM_003246); early growth factor-regulated 1 (EGR1) (5′-AGCAGCAGCAGCACCTTCAA-3′, nucleotides 511–530; 5′-CCGCAAGTGGATCTTGGTAT-3′ complementary to nucleotides 1511–1530 of the EGR1 cDNA, NM_001964); B cell translocation gene 2 protein (BTG) (5′-TTTTGGGACCCAAAGAGTATCCAC-3′, nucleotides 1356–1379; 5′-GGATTGTACTGAGACAGACAGCAA-3′ complementary to nucleotides 1877–1900 of the BTG cDNA, NM_006763); CD38 (5′-ACTGCCAAAGTGTATGGGATGCTT-3′, nucleotides 311–334; 5′-CAGATGTGCAAGATGAATCCTCAG-3′ complementary to nucleotides 941–964 of the CD38 cDNA, NM_001775); sialoadhesin (5′-CACATGGCTCTGTTCATCTGCAC-3′, nucleotides 2431–2453; 5′-CATCCCGATACCAACGATATGAG-3′ complementary to nucleotides 2778–2800 of the sialoadhesin cDNA, NM_023068); protein regulated by retinoic acid and PML-RAR (PRAM) (5′-GCAGAGATGAAGCGCCCTCAGTT-3′, nucleotides 1778–1800; 5′-CAGAAGTCGACATCATCGTACAC-3′ complementary to nucleotides 2158–2180 of the PRAM cDNA, NM_032152); cathepsin D (ctsD) (5′-GCACAAGTTCACGTCCATCC-3′, nucleotides 211–230; 5′-ACACCTTCTCACAGGGGATC-3′ complementary to nucleotides 1111–1130 of the ctsD cDNA, NM_001909); pLym (5′-GAGATGATGGCCCAGAAGCGCA-3′, nucleotides 199–221; 5′-ACCGTGTCCTGCTGCTTGCCTTT-3′ complementary to nucleotides 409–431 of the pLym cDNA, NM_015714); CAAT element-binding protein α (cEBPα) (5′-CCAAGAAGTCGGTGGACAAGAACA-3′, nucleotides 820–843; 5′-TAGAGACCCTCCACCTTCATGTAG-3′ complementary to nucleotides 1637–1660 of the cEBPα cDNA, NM_004364); CAAT element-binding protein β (cEBPβ) (5′-AAACCAACCGCACATGCAGATGGG-3′, nucleotides 1417–1440; 5′-GATTCCCAAAATATACAGACGCCT-3′ complementary to nucleotides 1751–1774 of the cEBPβ cDNA, NM_005194); CAAT element binding protein epsilon (cEBPϵ) (5′-GCTAGGGGACATGTGTGAGCATGA-3′, nucleotides 261–284; 5′-CTCTGCCATGTACTCCAGCACCTT-3′ complementary to nucleotides 887–900 of the cEBPϵ cDNA, NM_001805). The granulocyte colony-stimulating factor receptor probe is a full-length human cDNA (16).

Protein extracts of NB4 cells were prepared as described (17). Nuclear and cytosolic fractions were isolated with nuclear extract kit (Active Motif Europe, Rixensart, Belgium). The nuclear extracts are essentially free of cytosolic contamination. Similarly, cytosolic extracts (cytosol) are not significantly contaminated with cell nuclei. In fact, the protein band corresponding to the nuclear protein marker histone H3 is present only in the nuclear fraction, whereas the cytosolic marker caspase-3 is evident only in the cytosolic fraction (see Supplemental Fig. 1S).

EMSAs using a CREB-specific oligonucleotide were conducted on nuclear extracts as reported (17). Western blot analyses were performed on total or nuclear extracts (17). The antibodies directed against ERK-1/ERK-2, CREB, STAT1, and the corresponding phosphorylated forms were from Cell Signaling Technology (Beverly, MA). The antibodies recognizing cEBPα (sc-9315), cEBPβ (sc-150), cEBPϵ (sc-158), PKA-rIα (sc-18798), PKA-rIIα (sc-908), PKA-cα (sc-903), histone H3 (sc-10809), cathepsin D (sc-6486), and β-actin (sc-8432) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-RARα polyclonal antibodies were used as described (30). CD11b, CD11c, CD38, and CD33 fluorescein-conjugated antibodies (Becton Dickinson Europe, Erembodegem, Belgium) were used for FACS, as described (16). ELISAs were used to determine secreted monocyte chemotactic peptide 1 (MCP-1) (31) and TNF (32) (Endogen, Woburn, MA).

In Vivo Experiments—For the *in vivo* experiments, cells were suspended in 199 Hanks' medium, and 0.1 ml (1×10^6 cells/mouse) were intraperitoneally inoculated in SCID mice (17). Piclamilast was dissolved in 0.5% carboxyl methyl cellulose, 0.01% Tween 80 solution and injected at a dose of 10 mg/kg. ATRA (Sigma) was dissolved in the same solution and injected at a dose of 15 mg/kg. Drugs were administered intraperitoneally in a volume of 100 μl/animal 1 day after the inoculation, and the treatment continued for 13 days (5 daily injections/week). Data on the survival of animals were analyzed considering the following parameters: median survival time (MST) and percentage increase in life span (MST-treated/MST control $\times$ 100) $-$ 100). Statistical treatment of the results was conducted according to the Cox regression model (33).

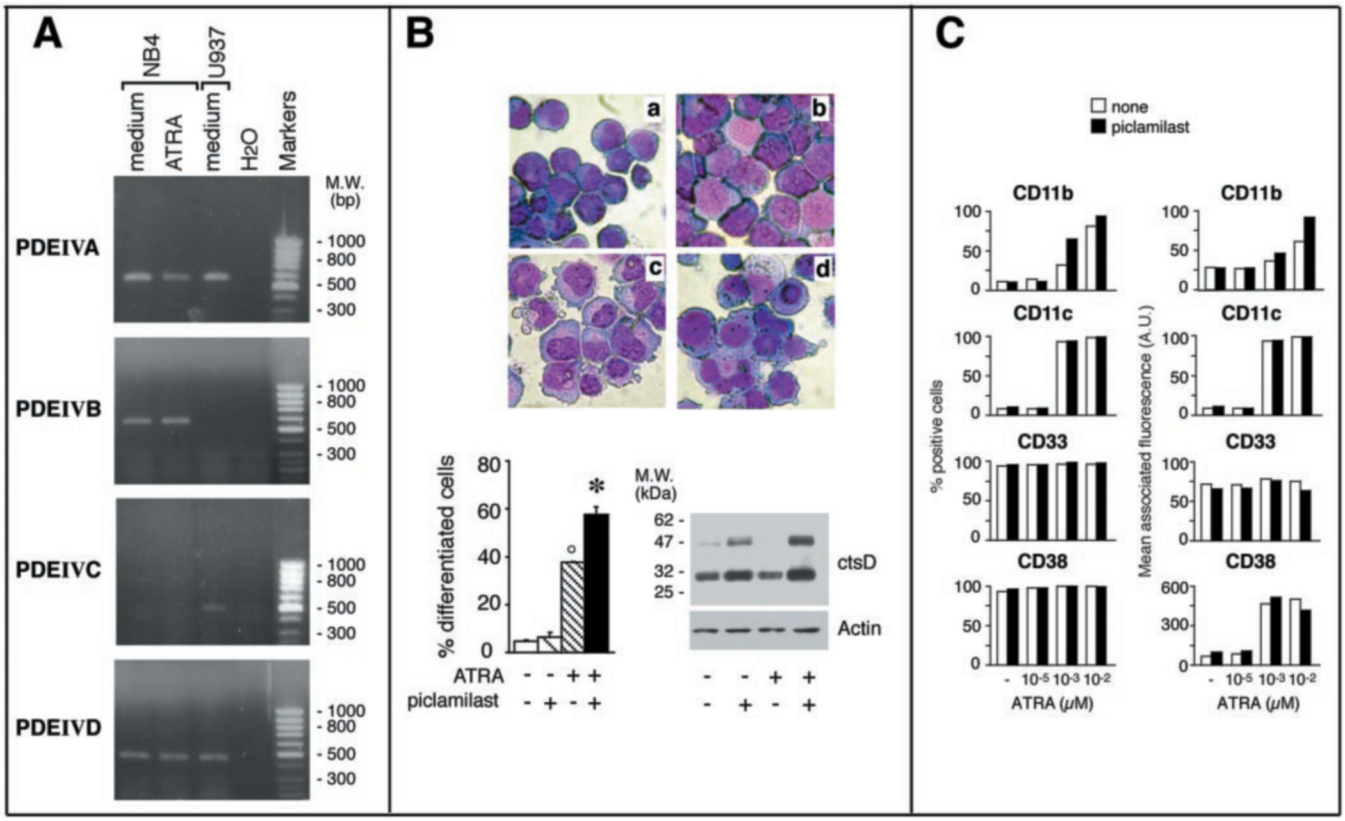

FIG. 1. **Expression of PDE IV isoenzymes and effect of the combination of piclamilast and ATRA on NB4 differentiation.** *A*, cells seeded at an initial concentration of 150,000/ml were treated for 4 days in medium alone (NB4 and U937) or 1 μM ATRA (NB4). Total RNA (1 μg) was extracted and subjected to RT-PCR for the amplification of the transcripts corresponding to the various PDEIV isoforms. *A* illustrates ethidium bromide staining of representative 1% agarose gels. The *lanes* indicated by H_2O represent negative controls obtained by running amplification reactions in the absence of the cDNA template. DNA molecular weight markers are shown on the *right* of each gel (100-bp DNA ladder). *B*, the morphology of NB4 cells treated for 3 days with Me_2SO vehicle (*a*), piclamilast (30 μM) (*b*), ATRA (0.1 μM) (*c*), or the combination of the two compounds (*d*) is shown. The images were obtained at the microscope following May-Grunwald-Wright staining of culture cytospins. The percentage of morphologically differentiated cells is shown in the *lower graph* (*left*). Granulocytic maturation was quantitatively determined following double blind scoring of the cell cytospins. A minimum of three independent fields (at least 150 cells/field) from each cytospin was considered. Each value represents the mean ± S.D. of three independent culture flasks. °, significantly higher than the corresponding vehicle treated control according to Student's *t* test ($p < 0.01$). *, significantly higher than the corresponding ATRA-treated sample according to Student's *t* test ($p < 0.01$). The expression of the two forms of ctsD observed in NB4 cells was determined in cell extracts by Western blot analysis using specific polyclonal antibodies (*right*). The filter was rechallenged with an anti-β-actin antibody. Protein molecular weight markers are indicated. *C*, NB4 cells were treated for 3 days with vehicle (Me_2SO) or the indicated concentrations of ATRA in the absence or presence of 30 μM piclamilast. The surface expression of the myeloid markers, CD11b, CD11c, CD33, and CD38, was determined in NB4 cells by FACS analysis. The *graphs* on the *left* indicate the percentage of marker-positive cells, whereas those on the *right* show the mean associated fluorescence for each marker. The results are representative of two independent experiments.

RESULTS

Inhibition of PDEIV Enhances and Accelerates the Differentiation of the ATRA-sensitive NB4 Cell Line—As illustrated in Fig. 1*A*, upon RT-PCR analysis, the APL-derived NB4 blast synthesizes detectable amounts of the transcripts coding for members of the PDEIV A, B, and D families. PDEIVC substitutes PDEIVB in the monoblastic cell line U937, which is used as a positive control (34) in these experiments. Treatment of NB4 cells with ATRA for 4 days does not affect the levels of the various PDEIV transcripts.

Challenge with combinations of ATRA and various PDEIV inhibitors, such as rolipram and piclamilast, enhances the ATRA-dependent maturation of NB4 cells (data not shown). Since piclamilast belongs to a chemical series of promising PDEIV inhibitors undergoing phase II/III clinical trials (22), all subsequent experiments were conducted with this molecule.

The effect of piclamilast on the morphological differentiation of NB4 cells is illustrated in Fig. 1*B*. Relative to what was observed with ATRA alone, NB4 cultures treated for 3 days with ATRA (0.1 μM) + piclamilast (30 μM) showed an increased proportion of cells with lobated nuclei, cytoplasmic granules, and/or elevated cytoplasm/nucleus volume ratios, three parameters that are associated with granulocytic maturation (27). No detectable myeloid maturation is evident upon treatment with the PDEIV inhibitor alone. Enhanced morphological maturation by ATRA + piclamilast is associated with a parallel stimulation of the synthesis of ctsD, a retinoid-regulated and lysosomal differentiation marker (35). The enhancing effect of piclamilast is not limited to ctsD and extends to the other surface differentiation marker, CD11b, as demonstrated by the FACS analysis shown in Fig. 1*C*. Maximal induction of CD11b is observed at the highest concentration of ATRA considered (0.01 μM), both in terms of the number of positive cells and mean associated fluorescence. At 0.001 μM ATRA, the addition of piclamilast results in a doubling of the number of CD11b-positive cells. At 0.01 μM ATRA, the PDEIV inhibitor maintains a significant stimulating effect on CD11b mean associated fluorescence. Similar effects are not evident in the case of CD11c and CD38, two other ATRA-regulated surface antigens. As expected (16), undifferentiated and ATRA-, piclamilast-, or ATRA + piclamilast-treated NB4 cells are positive for CD33.

Induction of the cell membrane enzymatic complex, NBT-R, is another popular myeloid marker used to assess granulocytic maturation of APL cells. Piclamilast (30 μM) enhances the

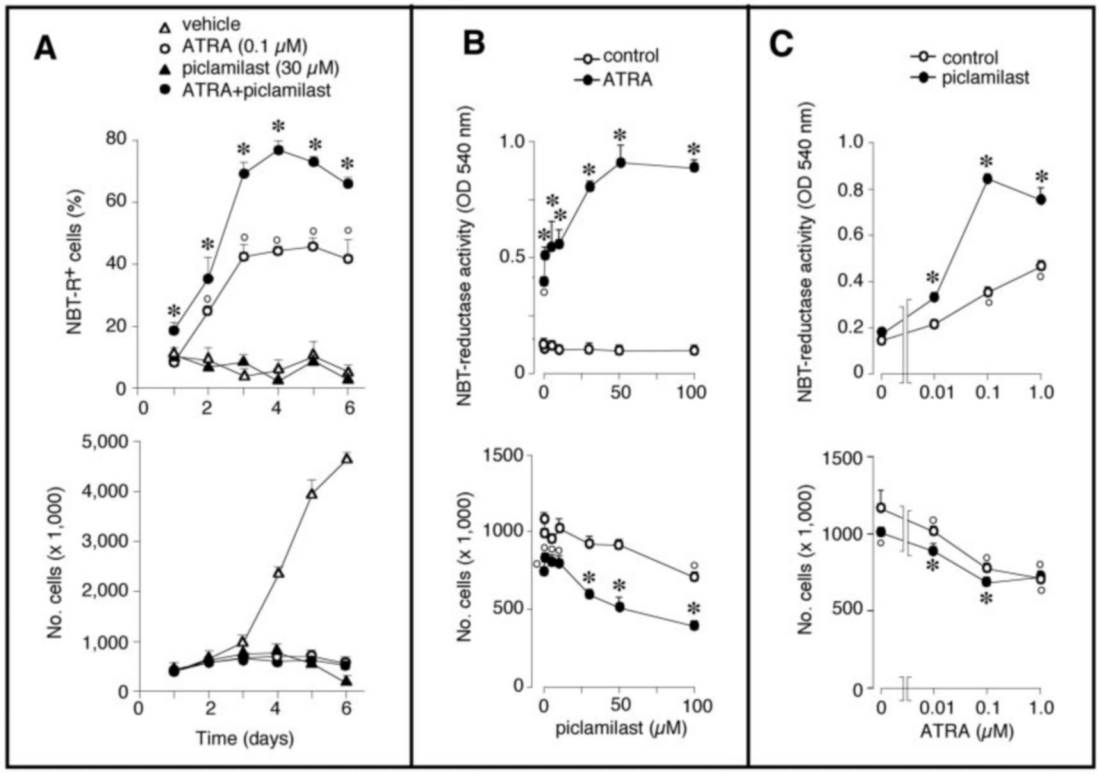

FIG. 2. **Time- and concentration-dependent effects of piclamilast and ATRA on NBT-R activity.** *A*, NB4 cells (150,000/ml) were treated with vehicle (Me_2SO), ATRA, piclamilast, or the combination of the two compounds as indicated for various lengths of time. Each day, the level of NBT-R positivity (*upper curves*) and the total number of cells (*lower curves*) were determined on aliquots of each culture flask. The results are representative of two independent experiments. *B*, NB4 cells (150,000/ml) were treated for 3 days with medium (control) or ATRA (0.1 μM) in the absence or presence of the indicated concentrations of piclamilast. NBT-R activity in cell homogenates (*upper graph*) and the total number of cells (*lower graph*) were determined. *C*, for these experiments, the concentration of piclamilast (30 μM) was kept constant, and the concentrations of ATRA were varied as indicated. NBT-R activity (*upper curves*) and the total number of cells (*lower curves*) were determined. Each experimental value represents the mean ± S.D. of three independent culture flasks. In all of the experiments considered, the percentage of viable cells was >90%. °, significantly higher or lower than the corresponding vehicle-treated control according to Student's *t* test ($p < 0.01$). *, significantly higher or lower than the corresponding ATRA-treated sample according to Student's *t* test ($p < 0.01$).

ATRA-dependent induction of NBT-R in NB4 cells. Enhanced NBT-R is evident both when the enzymatic activity is measured in cell extracts and when the number of NBT-R^+ cells is counted (data not shown). The enhancing effect of piclamilast is similar to that observed with the cAMP analogue, 8-CPT-cAMP (data not shown). Fig. 2*A* demonstrates that treatment with piclamilast (30 μM) does not result in a significant increase in the number of NBT-R^+ NB4 at any of the time points considered. However, when the PDEIV inhibitor is added to ATRA, the retinoid-dependent increase of NB4 positive cells is enhanced and accelerated. Enhanced NBT-R activity is not only time-dependent but also dose-dependent relative to ATRA (Fig. 2*B*) and piclamilast (Fig. 2*C*). When ATRA is present in the medium at a concentration of 0.1 μM, a significant increase in the levels of retinoid-induced NBT-R is already observed with 1 μM piclamilast, and the effect plateaus at 30 μM. In the absence of ATRA, even high concentrations of piclamilast (100 μM) are ineffective in inducing NBT-R. Piclamilast has a growth-inhibitory action at all of the concentrations tested, and the action is augmented by the presence of ATRA. When the concentration of piclamilast is kept constant at 30 μM, enhancement of the ATRA-dependent induction of NBT-R is evident at all of the concentrations of the retinoid. A similar dose-response curve is observed in the case of the antiproliferative effect of ATRA + piclamilast.

Piclamilast Interacts with ATRA, RAR, and RXR Agonists—Piclamilast enhances the ATRA-dependent induction of NBT-R activity in the retinoid-sensitive HL-60 myeloid leukemia cell line (Fig. 3*A*) and in primary cultures of blasts isolated from an AML patient (Fig. 3*B*). In both cell types, piclamilast + ATRA induces a significantly greater antiproliferative effect than ATRA or piclamilast alone (data not shown). In AML blasts, the enhancing action of the PDEIV inhibitor on NBT-R induction and growth inhibition requires long exposure times (7 days) and is associated with a reduction in cell viability. ATRA + piclamilast reduces the total number of cells observed in control conditions by 69 ± 5% (mean ± S.D., $n = 3$), and this compares to 37 ± 2 and 10 ± 1% in the case of ATRA and piclamilast, respectively. Exposure of the blasts to piclamilast for 7 days does not result in a significant cytotoxic effect relative to what observed in basal conditions (viability: 64 ± 5 *versus* 61 ± 3%), whereas the combination of ATRA + piclamilast is more cytotoxic than the retinoid alone (viability 37 ± 4 *versus* 50 ± 4%). In U937 myelomonocytic cells, piclamilast and ATRA induce NBT-R activity on their own, although the combination is no more effective than the single components (Fig. 3*C*). In NB4 cells, the RAR/RXR heterodimeric pathway is involved in the cross-talk between piclamilast and ATRA. In fact, the RARα agonist, AM580 (36, 37), can substitute for ATRA (Fig. 3*D*).

Granulocytic maturation of certain ATRA-sensitive and -resistant AML cells is induced by combinations of cAMP analogs and RXR agonists (38). Treatment of NB4 cells for 3 days with 0.01 μM CD2915, a selective RXR agonist, does not result in a significant increase in the percentage of NBT-R^+ cells, whereas a higher concentration (0.1 μM) augments the proportion of cells expressing the differentiation marker (Fig. 3*D*). With both concentrations of CD2915, the addition of piclamilast to the

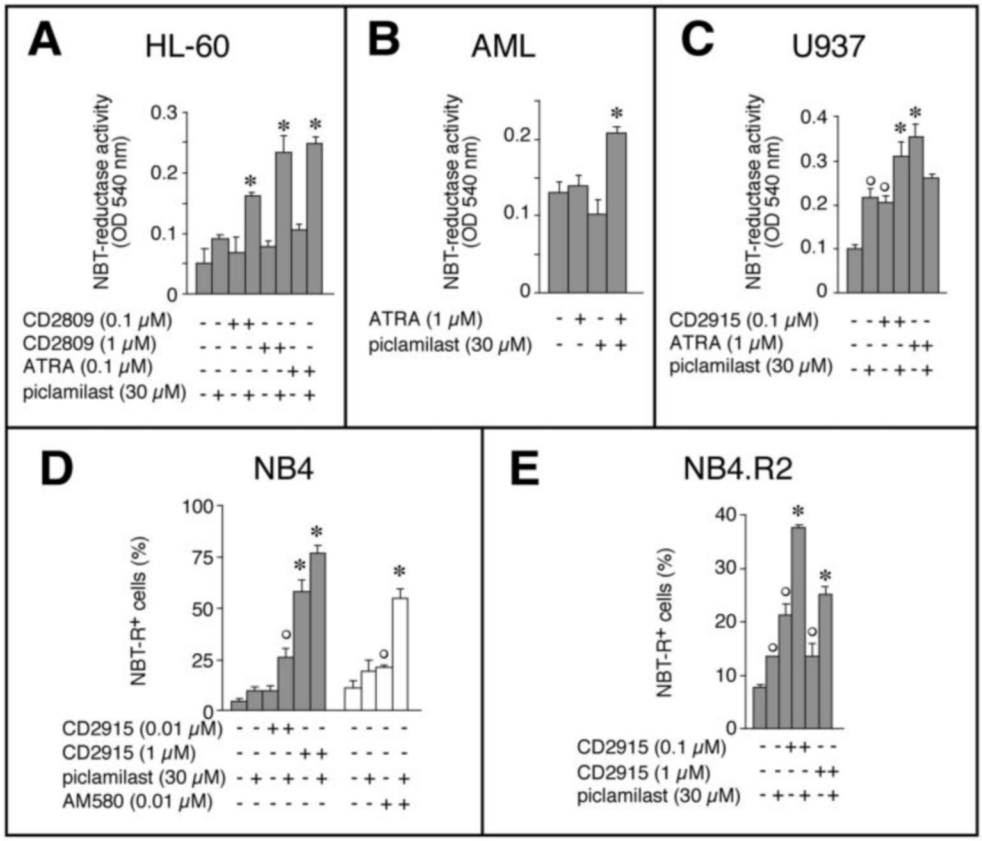

FIG. 3. **Effect of piclamilast and ATRA, the RARα agonist AM580, and the RXR agonists CD2915 and CD2809 on the level of NBT-R activity of ATRA-sensitive and -resistant myeloid cell lines.** HL-60 cells (*A*), freshly isolated blasts from the peripheral blood of one AML patient (*B*), U937 cells (*C*), NB4 cells (*D*), and the ATRA-resistant NB4-derived subline, NB4.R2 (*E*), seeded at an initial concentration of 150,000/ml, were treated for 3 days (7 days in the case of the AML blasts) with vehicle or the indicated concentrations of piclamilast, ATRA, the RARα agonist, AM580, the RXR agonists, CD2915 and CD2809, or combinations thereof. Differentiation was determined by measuring NBT-R activity in cell homogenates or by counting the percentage of NBT-R$^+$ cells. Each experimental value represents the mean ± S.D. of three independent culture flasks. °, significantly higher than the corresponding vehicle treated control according to Student's *t* test ($p < 0.01$). *, significantly higher than the corresponding ATRA-treated sample according to Student's *t* test ($p < 0.01$). The results are representative of at least two independent experiments.

medium causes a significant increase in the number of cells expressing NBT-R activity. Similar effects are observed in U937 cells (Fig. 3*C*) as well as in the ATRA-resistant NB4.R2 (Fig. 3*E*) but not in the different retinoid-resistant NB4/007 cell line (data not shown). Enhancement of NBT-R induction is reproduced in HL-60 cells when CD2915 is substituted by CD2809, another RXR agonist (Fig. 3*A*) (37). Two other myeloid leukemia cell lines, KG1 and Kasumi (a cell line with the 8:21 chromosomal translocation), are refractory to the differentiating action of piclamilast and ATRA or RXR agonists (data not shown).

PKA Mediates the Cytodifferentiation Induced by ATRA and Piclamilast—In NB4 cells, a dose-dependent suppression of the enhancing effect of piclamilast on retinoid-induced NBT-R activity is evident with Rp-8Br-cAMP, a competitive inhibitor of cAMP (Fig. 4*A*). At all of the concentrations tested, the cAMP analog does not alter the growth and viability of NB4 cells treated with vehicle, piclamilast, ATRA, or piclamilast + ATRA. Similar suppressive effects on the ATRA-dependent induction of NBT-R activity are caused by substitution of Rp-8Br-cAMP with the PKA inhibitor H89 (10 μM) (39) (Fig. 4*B*). Like Rp-8Br-cAMP, H89 does not revert the growth inhibition afforded by treatment of NB4 cells with ATRA or the combination of ATRA and piclamilast (data not shown). The concentration of H89 used is effective in inhibiting PKA activity as assessed in undifferentiated NB4 cells treated with the kinase inhibitor for 16 h (Fig. 4*B*, *inset*).

The Rap-exchanging factor, Epac, mediates some of the biological effects of cAMP (40). Treatment of NB4 cells for 3 days with *O*-Me cAMP, a selective Epac activator (41), does not affect the percentage of NBT-R$^+$ NB4 cells regardless of the presence/absence of ATRA and/or piclamilast in the growth medium (Fig. 4*C*). Furthermore, the expression of NBT-R activity in NB4 cells treated with ATRA + piclamilast is not modified by the addition of *O*-Me cAMP. Finally, in the experimental conditions considered, the activator of Epac has no significant effect on the number and viability of cells (data not shown).

Piclamilast and ATRA Modulate the cAMP Signaling Pathway—Proliferating NB4 cells contain detectable amounts of intracellular cAMP (Fig. 5*A*). Treatment with piclamilast for 6 h leads to an almost 3-fold elevation in the cyclic nucleotide levels, which are back to base line by 18 h. ATRA has no major influence on the constitutive or piclamilast-induced levels of cAMP.

Detectable and relatively constant amounts of PKA are determined in unstimulated NB4 cells (Fig. 5*B*). A significant elevation of PKA activity is observed upon treatment with piclamilast for 6 h, whereas an over 4-fold increase (similar to that afforded by the PKA agonist dibutyryl-cAMP) is evident by 18 h. PKA activity is back to basal levels by 96 h. Unexpectedly, the addition of ATRA to the growth medium for 18 h results in a level of PKA activation that is similar to that observed with piclamilast. However, treatment of NB4 cells with ATRA + piclamilast for the same amount of time is no more effective than the PDEIV inhibitor or the retinoid alone.

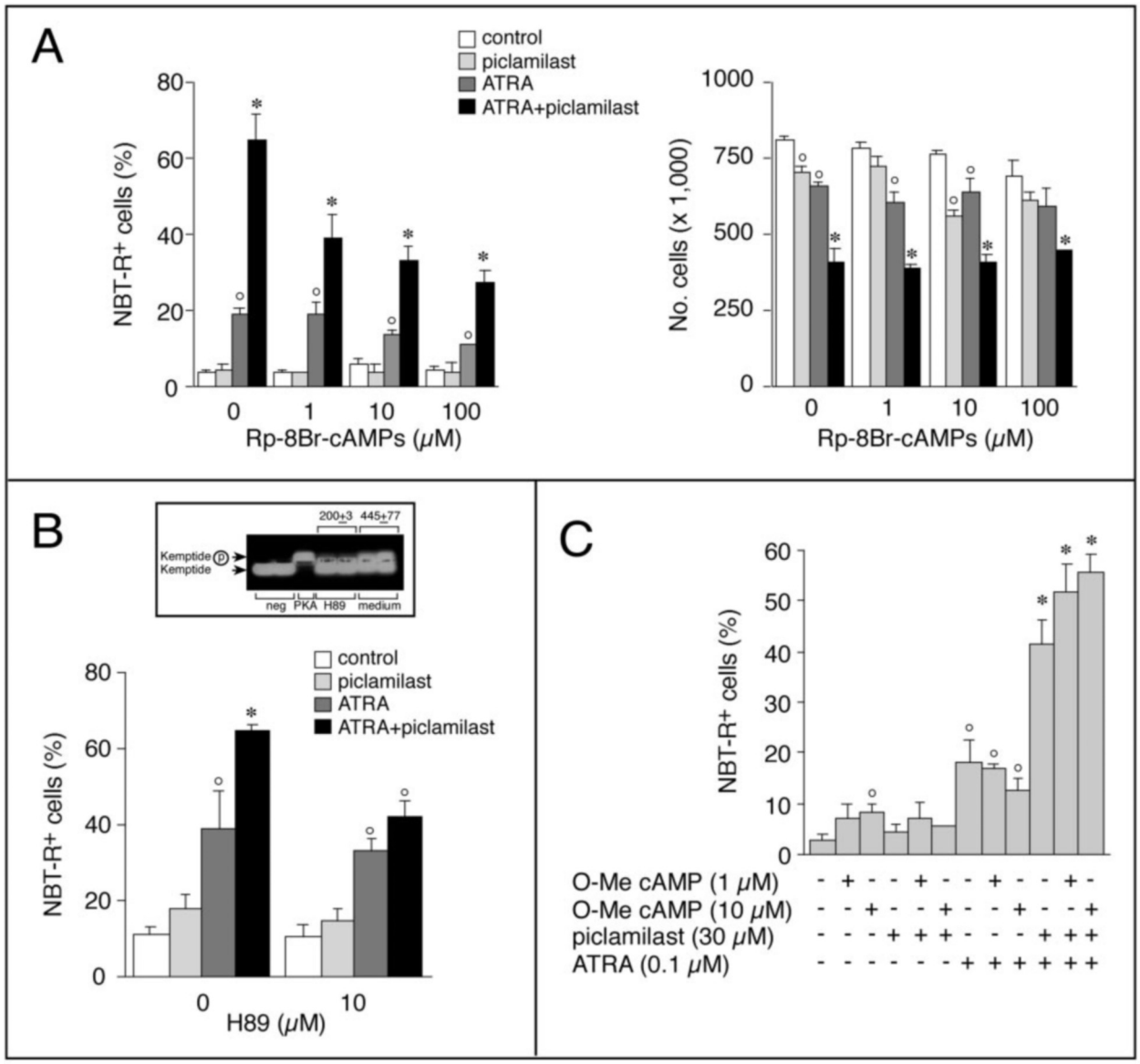

FIG. 4. **Effect of cAMP analogs and the PKA inhibitor H89 on the differentiation or growth of NB4 cells treated with ATRA, piclamilast, and the combination of the two compounds.** *A*, NB4 cells, seeded at an initial concentration of 150,000/ml, were treated for 3 days with Me_2SO vehicle (control), ATRA (0.1 μM), piclamilast (30 μM), or the combination of the two compounds in the presence and absence of the indicated concentration of the antagonistic cAMP analog, Rp-8Br-cAMP. Differentiation was determined by counting the percentage of NBT-R^+ cells (*left graphs*). The total number of cells was counted under the microscope following staining with erythrosine (*right graphs*). *B*, NB4 cells were treated with ATRA, piclamilast, or the combination of the two compounds in the absence or presence of 10 μM of the PKA inhibitor, H89, as in *A*. Differentiation was determined by counting the percentage of NBT-R^+ cells. The *inset* shows the inhibitory effect of H89 on the phosphorylation of a fluorescent derivative of Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide), acting as a specific substrate of the PKA. For this experiment, extracts of undifferentiated NB4 cells were treated with medium alone (*medium*) or medium containing H89 (10 μM). The figure represents a typical agarose gel electrophoresis in which the phospho-Kemptide can be separated from its nonphosphorylated form. The *lane* marked *PKA* is a positive control run with purified and activated PKA, whereas the *lane* marked *neg* is a negative control of the experiment run in the presence of buffer. The values (mean ± S.D. of two separate culture flasks) above the *H89* and *medium lanes* indicate the amount of fluorescent phospho-Kemptide isolated from the gel and are expressed in fluorescence arbitrary units. *C*, NB4 cells were treated with ATRA (0.1 μM), piclamilast (30 μM), or the combination of the two compounds in the absence or presence of the indicated concentrations of the selective Epac agonist, *O*-Me cAMP, as in *A*. The number of NBT-R-positive cells is shown. In all of the experiments shown, the percentage of viable cells was >90%. Each experimental value represents the mean ± S.D. of three independent culture flasks. °, significantly higher or lower than the corresponding vehicle-treated control according to Student's *t* test ($p < 0.01$). *, significantly higher than the corresponding ATRA-treated sample according to Student's *t* test ($p < 0.01$). The results are representative of at least two independent experiments.

Undifferentiated NB4 cells synthesize significant amounts of the PKA regulatory subunits, PKA-rIα and PKA-rIIα (Fig. 5*C*). Treatment of cells with piclamilast does not alter the intracellular levels of either PKA-rIα or PKA-rIIα. By contrast, down-modulation of PKA-rIIα is evident following incubation with ATRA for 24 and 72 h. At both time points, the addition of piclamilast to ATRA results in suppression of the retinoid-dependent down-modulation of PKA-rIIα.

Activation of PKA is accompanied by relocalization of the catalytic subunit to the nuclear compartment. The relocalization of the catalytic subunit was studied in NB4 cells treated with piclamilast, ATRA, and the combination of the two compounds following separation of nuclear and cytosolic fractions by differential centrifugation. Detectable amounts of the PKA catalytic subunit, PKA-cIα (42), are present in the cytosol of undifferentiated NB4 cells (Fig. 5*D*). Piclamilast, ATRA, and

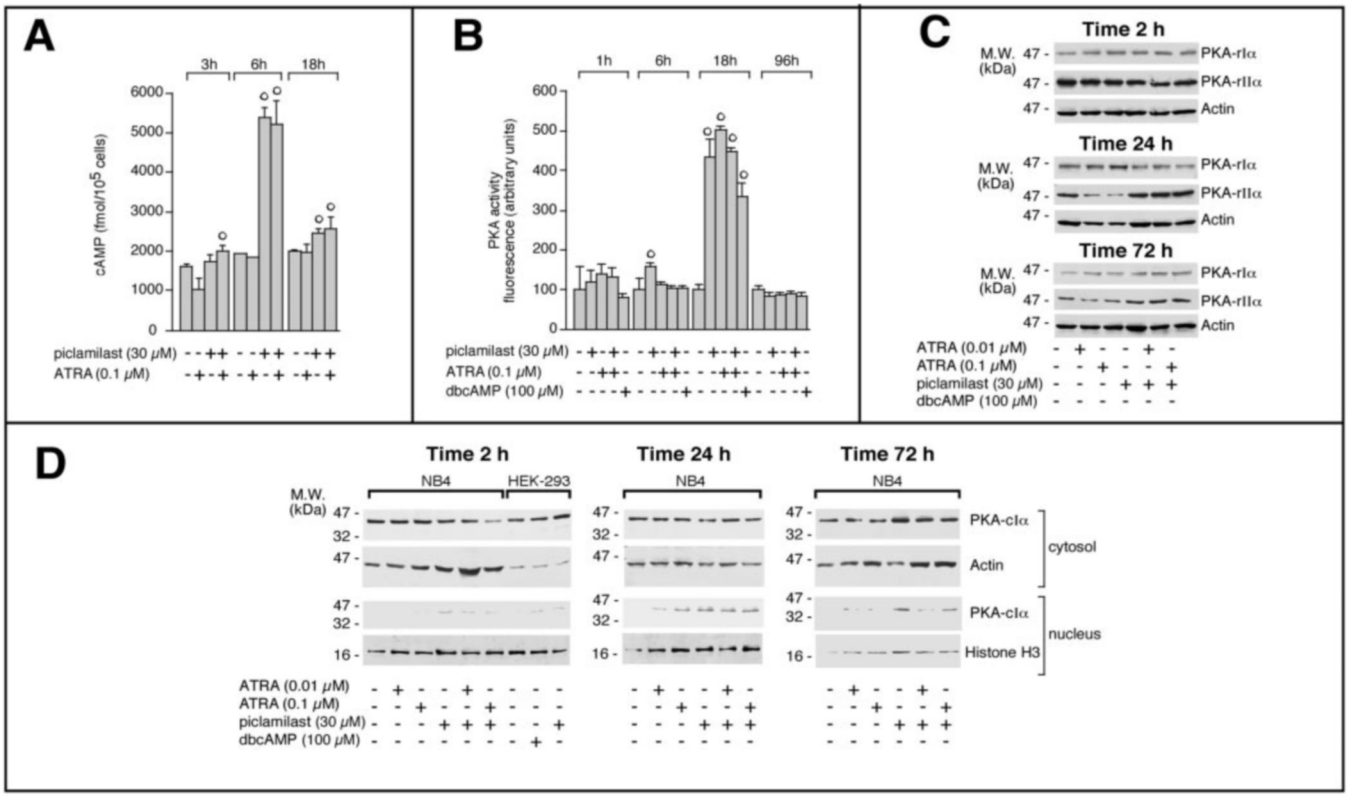

FIG. 5. **Effect of piclamilast and ATRA on cAMP and PKA.** *A*, NB4 cells seeded at an initial concentration of 150,000/ml were treated for 3 days with vehicle, ATRA (0.1 μM), piclamilast (30 μM), or the combination of the two compounds for the indicated amount of time. Cells were harvested and homogenized, and the levels of intracellular cAMP were determined. Each experimental value represents the mean ± S.D. of three independent culture flasks. °, significantly higher than the corresponding vehicle-treated control according to Student's *t* test ($p < 0.01$). *B*, NB4 cells seeded as in *A* were treated for 3 days with vehicle, ATRA (0.1 μM), piclamilast (30 μM), dibutyryl-cAMP (100 μM), or the indicated combinations of the compounds for various lengths of time, as shown. Cells were harvested and homogenized, and the levels of intracellular PKA were determined. Each experimental value represents the mean ± S.D. of three independent culture flasks. °, significantly higher than the corresponding vehicle-treated control according to Student's *t* test ($p < 0.01$). *C*, NB4 cells seeded at an initial concentration of 150,000/ml were treated with the indicated compounds and for the indicated amount of time. Cytosolic extracts (20 μg of protein) were subjected to Western blot analysis with specific antibodies recognizing the indicated proteins. The protein molecular weight markers are indicated on the *left*. Each experimental point represents a pool of extracts from three separate culture dishes. *D*, NB4 cells seeded as in *A* were treated as indicated. HEK-293 cells were used as positive controls for the effects of piclamilast and dibutyryl-cAMP (*dbcAMP*). Cytosolic (*cytosol*) and nuclear extracts (*nucleus*) were prepared, and identical aliquots (20 μg of protein) were subjected to Western blot analysis with antibodies recognizing the indicated proteins. PKAcIα, the Iα form of the catalytic subunit of cAMP-dependent protein kinase. The results are representative of two independent experiments.

the combination of the two compounds exert no significant effect on the levels of the cytosolic protein. Treatment of NB4 cells with piclamilast is associated with a rapid and long lasting translocation of PKA-cIα to the nucleus. This effect is not modulated by the addition of ATRA to the PDEIV inhibitor. Interestingly, significant translocation of PKA-cIα to the nucleus is observed also following challenge of NB4 cells with ATRA alone for 24 h. At 72 h, the level of nuclear PKA-cIα is still significantly above background in all the treatment groups. In these experiments, HEK-293 cells were used as positive controls for PKA translocation (see 2-h time point).

We determined the levels of the transcription factor, CREB (43), and its phosphorylation in the nucleus of NB4 cells (Fig. 6*A*) by Western blot as well as the amounts of CREB interacting with the corresponding DNA consensus sequence by EMSA (Fig. 6*B*). In EMSA, the retarded bands are specific for the CREB complexes, as demonstrated by competition experiments using wild type and mutated oligonucleotides as well as supershift with an anti-CREB antibody (data not shown). Fig. 6*A* demonstrates that undifferentiated NB4 cells synthesize large amounts of CREB constitutively. A proportion of the transcription factor is in its active state, as demonstrated by the level of phosphorylation. A rapid and transient increase in the phosphorylation and activity of CREB is observed after 2 h of treatment with piclamilast, ATRA, or ATRA + piclamilast. In all cases, activation of CREB is significant but minor (~2-fold induction). By 6 h, the stimulating effect of the PDEIV inhibitor is over. Stimulation by piclamilast is followed by progressive suppression of CREB phosphorylation (see 24-h time point) consequent to down-regulation of the CREB protein. Whereas the association of ATRA and piclamilast is no more efficient than the single components in activating CREB, it accelerates the down-regulation of the transcription factor afforded by the PDEIV inhibitor (see the 6-h panel). Regardless of the stimulus applied, CREB and the relative state of phosphorylation are back to control levels by 72 h. As illustrated in Fig. 6*B*, the constitutive activation of CREB in undifferentiated NB4 cells and the effects of piclamilast and/or ATRA on the levels and state of phosphorylation of the transcription factor are confirmed by EMSA at 24 and 72 h. Using the same assay (Fig. 6*B*, *bottom panel*), we demonstrate that treatment of NB4 cells with H89 for 24 h suppresses the activation of CREB observed in control and piclamilast-treated cells, suggesting PKA involvement. The PKA inhibitor does not affect ATRA or ATRA + piclamilast down-regulation of CREB activity.

The effect of piclamilast and/or ATRA on the expression of some CREB-dependent genes in NB4 cells (vinculin, THBS, EGR-1, and BTG) is complex, time-dependent, and not strictly related to the CREB activation pattern described above (Fig. 6*C*). Vinculin and BTG mRNAs are never up-regulated by

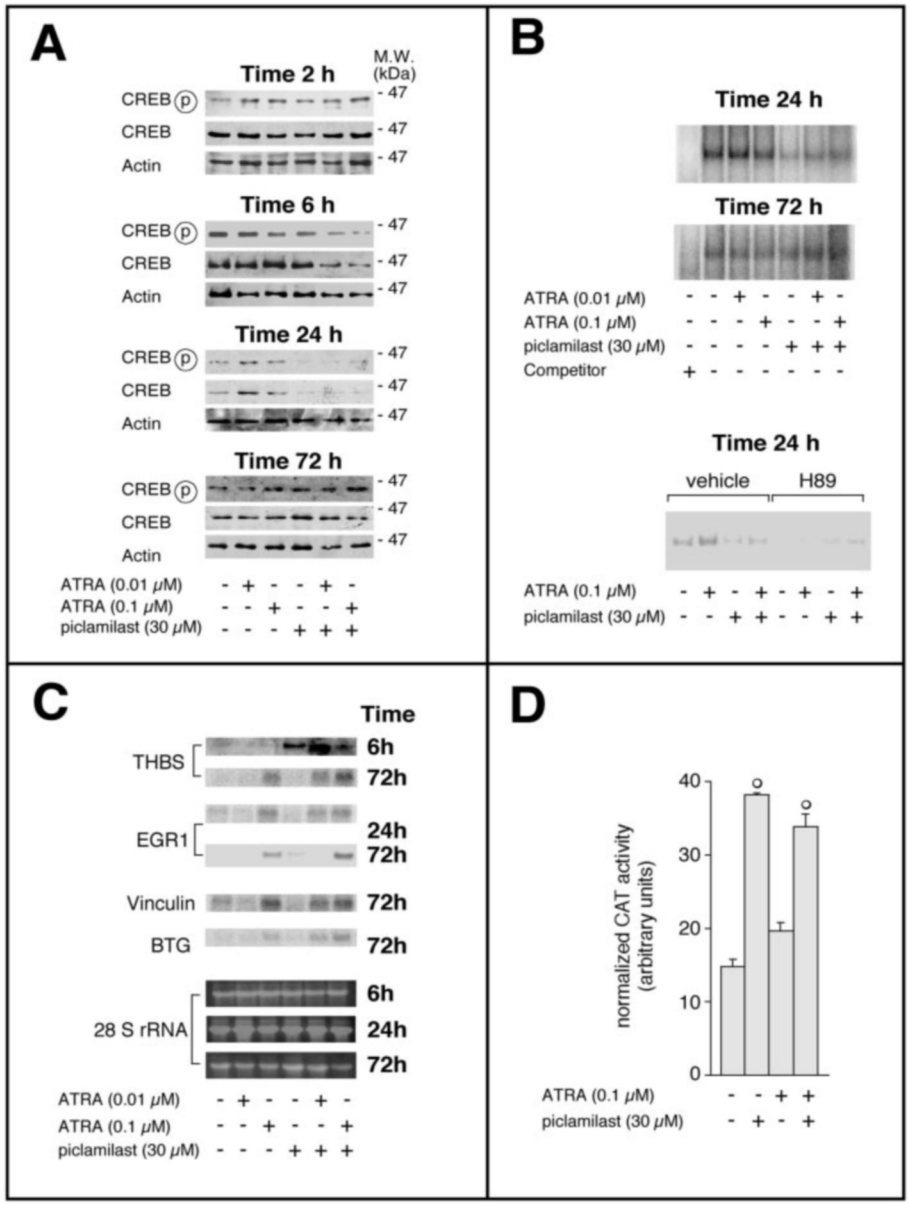

FIG. 6. **Effect of piclamilast and ATRA on CREB and CREB-responsive genes.** *A*, NB4 cells seeded at an initial concentration of 150,000/ml were treated with vehicle, ATRA (0.1 μM), piclamilast (30 μM), or the combination of the two compounds for the indicated amount of time. Equivalent amounts (20 μg) of nuclear cell extracts were subjected to Western blot analysis with antibodies recognizing CREB and the corresponding phosphorylated form (CREBp) as well as β-actin. *B*, NB4 cells seeded as in *A* were treated for the indicated amount of time. In the case of the experiments involving H89, the PKA inhibitor was present in the medium at a concentration of 10 μM. Nuclear extracts were prepared and subjected to EMSA with a radiolabeled double-stranded oligodeoxynucleotide corresponding to the DNA consensus sequence for the binding of CREB. *Competitor*, the presence of a 100-fold molar excess of the cold oligodeoxynucleotide in the binding reaction. The results are representative of at least three independent experiments. *C*, total RNA was extracted from NB4 cells treated with the indicated stimuli. Identical amounts of RNA (20 μg) were subjected to Northern blot analysis using specific THBS, EGR1, vinculin, and BTG cDNA probes radiolabeled with [^{32}P]dCTP. Ethidium bromide staining of representative RNA gels, illustrating the 28 S ribosomal RNA band, is shown at the *bottom*. These gels demonstrate that equal amounts of intact RNA were loaded in each lane of the gels used for Northern blot analysis. *D*, COS-7 cells were co-transfected with RARα (0.1 μg) and the cAMP-dependent gene reporter construct containing the somatostatin promoter as well as the normalization construct pCH110 coding for bacterial β-galactosidase. Eighteen hours following transfection, cells were treated with the indicated concentrations of ATRA and/or piclamilast. Thirty-six hours following the addition of the compounds, cell extracts were prepared and assayed for CAT and β-galactosidase activities. The data represent CAT activity in arbitrary units following normalization for the level of transfection using β-galactosidase. Each value is mean ± S.D. of three replicate culture dishes. °, significantly higher than the relative control sample according to Student's *t* test ($p < 0.01$). The results are representative of two independent experiments.

piclamilast, whereas THBS and EGR1 mRNAs are responsive to the PDEIV inhibitor. In the case of THBS, this effect is transient, being evident only at 6 h, whereas piclamilast-dependent induction of the EGR1 transcript is delayed (72 h). Interestingly, ATRA induces the four CREB-dependent mRNAs, albeit with different kinetics. However, this induction is enhanced by piclamilast only in the case of vinculin, BTG, and THBS, and the phenomenon is evident only when the retinoid is used at a concentration of 0.01 μM.

Given the complexity of the effects on CREB in NB4 cells, we evaluated the direct action of piclamilast and/or ATRA on the transcriptional activity of the CREB-containing promoter of the somatostatin gene. These experiments were conducted in COS-7 cells, since NB4 blasts are refractory to transient and stable transfection. As shown in Fig. 6*D*, the somatostatin reporter construct is activated by piclamilast. In contrast, de-

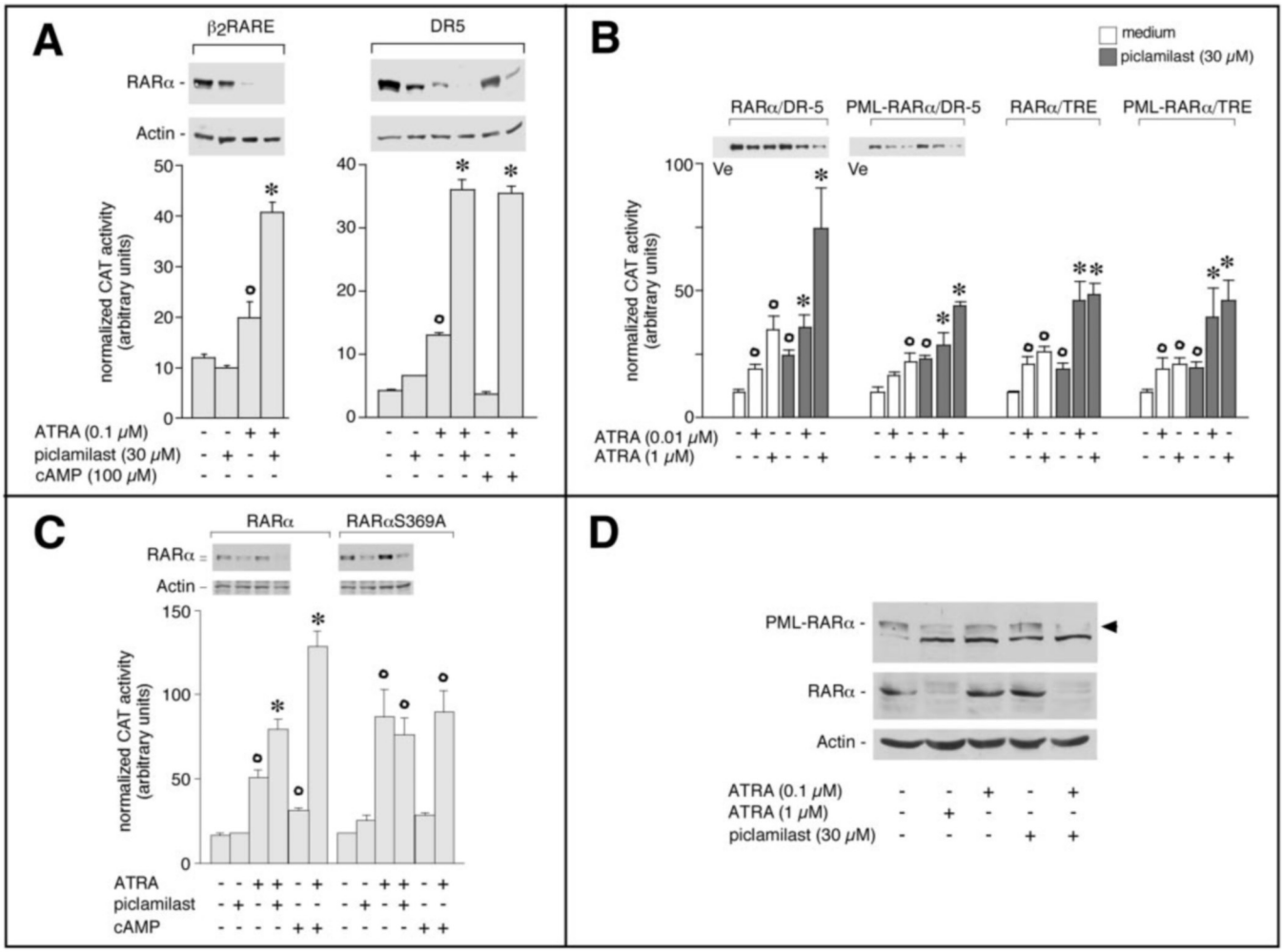

FIG. 7. **Effect of piclamilast and ATRA on RARα and PML-RARα transcriptional activity and degradation.** *A*, COS-7 cells were co-transfected with RARα (0.1 μg) and the ATRA-dependent gene reporter constructs β2RARE-CAT (1 μg; *β2RARE*) or DR5-CAT (1 μg; *DR5*) as well as the normalization construct pCH110. Eighteen hours following transfection, cells were treated with the indicated concentrations of ATRA, piclamilast, or 8-CPT-cAMP and combinations thereof. Thirty-six hours after the addition of the compounds, cell extracts were prepared and assayed for CAT and β-galactosidase activities. The data represent CAT activity in arbitrary units following normalization for the level of transfection using β-galactosidase. Each value is the mean ± S.D. of three replicate culture dishes. The results are representative of at least three independent experiments. Parallel Western blot analyses with anti-RARα and anti-β-actin antibodies are represented *above* the *bar graphs*. These experiments were performed on pooled extracts obtained from the same samples used for the determination of CAT activity and are normalized for the content of β-galactosidase. *B*, COS-7 cells were co-transfected with RARα (0.1 μg) or PML-RARα (0.1 μg) and the ATRA-dependent gene reporter constructs DR5-CAT (1 μg; *DR5*) or TRE-CAT (1 μg; *TRE*) as well as the normalization construct pCH110. Enzymatic measurements and Western blot analyses for PML-RARα and RARα were conducted as in *A*. The results are representative of at least three independent experiments. *C*, COS-7 cells were co-transfected with the wild type form of RARα (0.1 μg; RARα) or the mutant RARαS369A (0.1 μg), in which the PKA phosphorylation site has been inactivated by site-directed mutagenesis (Ser^{369} to Ala^{369}), and the ATRA-dependent gene reporter construct DR5-CAT (1 μg; *DR5*) as well as the normalization construct pCH110. Enzymatic measurements and Western blot analysis were conducted as in *A* or *B*. °, significantly higher than the relative control sample according to Student's *t* test ($p < 0.01$). *, significantly higher than the relative ATRA or piclamilast sample according to Student's *t* test ($p < 0.01$). *D*, NB4 cells were treated for 72 h with the indicated concentrations of ATRA, piclamilast, and ATRA + piclamilast. Cell extracts from pools of three separate culture dishes were subjected to Western blot analysis with anti-RARα and β-actin antibodies. The results are representative of at least two independent experiments. The *arrow* indicates the position of the band corresponding to PML-RARα.

spite co-transfection of the RARα cDNA, the CREB-regulated somatostatin promoter is not stimulated by ATRA. Furthermore, the addition of ATRA to piclamilast does not modulate the transcriptional activation of the PDEIV inhibitor. These data indicate that the activation of PKA and CREB induced by ATRA in NB4 cells may be limited to the myeloid cellular context. More importantly, what was observed in COS-7 cells indicates that the cross-talk between the retinoid and cAMP-dependent signaling pathways is unlikely to involve the activation of CREB.

Piclamilast Modulates the ATRA Signaling Pathway—PDEIV inhibition exerts modulatory effects on the nuclear retinoic acid receptors, and this is likely to be at the basis of the cross-talk between ATRA and piclamilast in AML cells. The effects of piclamilast on the transactivation properties of RARα and PML-RARα in COS-7 cells are shown in Fig. 7A. Piclamilast enhances the ligand-dependent activation of RARα regardless of the reporter construct, β2RAR-CAT or DR5-CAT, considered. The former consists of the chloramphenicol acetyltransferase (CAT) reporter gene controlled by the retinoic acid-responsive element (RARE) of the *β2RAR* promoter. In the latter, an artificial RARE controls the thymidine kinase promoter. In the case of DR5-CAT, the enhancing effect of piclamilast on the ligand-dependent transactivation of RARα is similar to that caused by 8-CPT-cAMP. Enhanced transactivation by piclamilast + ATRA is accompanied by decreased steady-state levels of RARα, as demonstrated by the Western blots shown. This is due to an increase in the proteasome-dependent degra-

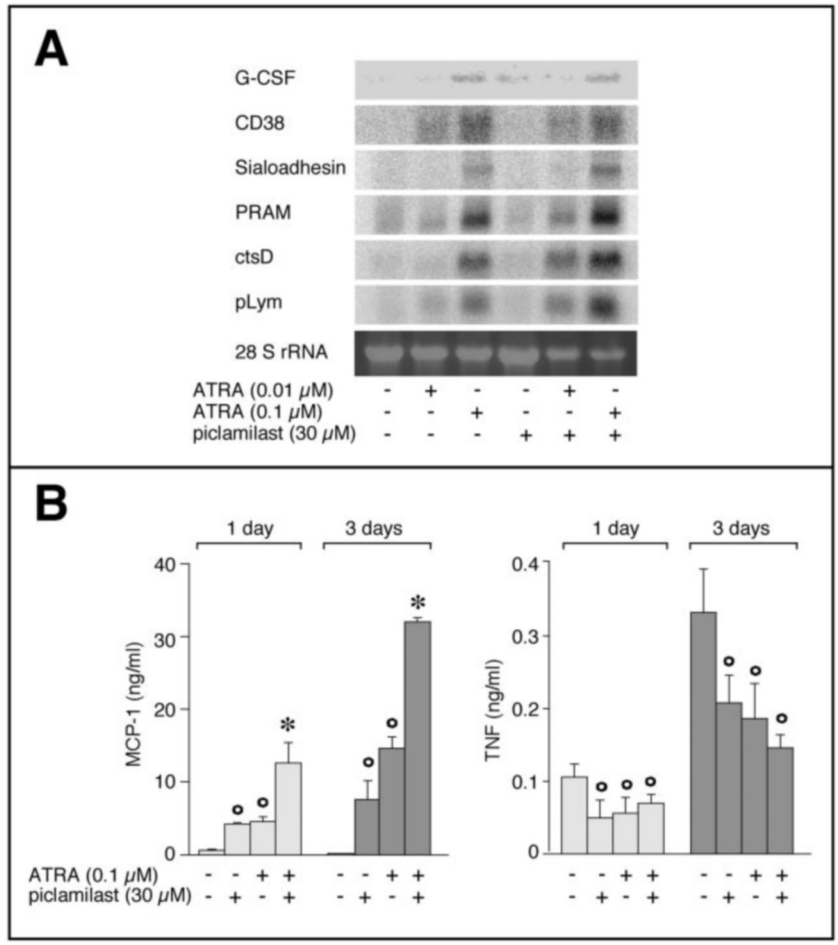

FIG. 8. **Effect of piclamilast and ATRA on the expression of retinoid-dependent genes and the secretion of MCP-1 or TNF.** *A*, NB4 cells were treated for 72 h with the indicated concentrations of ATRA, piclamilast, and ATRA + piclamilast. Cells (pools of three independent culture dishes) were harvested, and total RNA was extracted. Identical amounts of RNA (20 μg) were subjected to Northern blot analysis using the indicated cDNA probes labeled with [^{32}P]dCTP. The ethidium bromide staining of a representative RNA gel, illustrating the 28 S ribosomal RNA band, is shown at the *bottom*. *B*, NB4 cells were treated for the indicated amounts of time with ATRA, piclamilast, and ATRA + piclamilast. Culture medium was collected and used for the determination of MCP-1 and TNFα with specific ELISAs. °, significantly higher or lower than the relative control sample according to Student's *t* test ($p < 0.01$); *, significantly higher than the relative ATRA or piclamilast sample according to Student's *t* test ($p < 0.01$).

dation of retinoid receptors that accompanies ligand-dependent transactivation (44–46). Indeed, the phenomenon is blocked by the proteasome inhibitor MG132 (data not shown).

Piclamilast enhances the ligand-dependent transactivation not only of RARα but also of PML-RARα (Fig. 7*B*), although PML-RARα is a less sensitive target than RARα. These effects are observed with β2RAR-CAT (data not shown), DR5-CAT, and TRE-CAT, another thymidine kinase-based reporter construct containing a palindromic artificial RARE. As in the case of RARα, ligand-dependent activation of PML-RARα is associated with degradation of the receptor. However, this phenomenon is not modulated significantly by piclamilast.

The above results suggest a direct action of piclamilast on RARα and PML-RARα and demonstrate a direct correlation between the rate of degradation and the state of activation of RARα. To test this, we transfected RARαS369A, a receptor mutant whose PKA phosphorylation site has been inactivated (Fig. 7*C*). RARαS369A maintains responsiveness to ATRA-dependent transactivation but is refractory to piclamilast. Significantly, the PDEIV inhibitor has no detectable effect on the ligand-dependent degradation of RARαS369A. Consistent with this, inhibition of PKA by H89 suppresses the enhanced transactivation and degradation of RARα afforded by ATRA + piclamilast (data not shown).

Ligand binding directs RARα and PML-RARα along the proteasome degradation pathway in the natural context of the NB4 blast (29, 47–50). As illustrated in Fig. 7*D*, treatment for 24 h with 1 μM ATRA results in a significant diminution of the levels of both RARα and PML-RARα, whereas similar phenomena are not observed with a lower concentration of the retinoid (0.1 μM). Whereas piclamilast has no effect on its own, the addition of the PDEIV inhibitor to 0.1 μM ATRA results in a degradation of the two RAR receptors similar to that induced by 1 μM ATRA.

Fig. 8*A* shows the effect of piclamilast, ATRA, and ATRA + piclamilast on the expression of ATRA-responsive genes (granulocyte colony-stimulating factor, CD38, sialoadhesin, PRAM, ctsD, and pLym) in NB4 cells. Whereas ctsD and granulocyte colony-stimulating factor are markers of granulocytic maturation, the functional significance of the other genes is unknown. Except for CD38 (see also Fig. 1*C*) and granulocyte colony-stimulating factor, expression of the mRNAs considered is enhanced by co-treatment with ATRA + piclamilast, although the PDEIV inhibitor has no effect on its own. The enhancing effect of piclamilast is more evident when ATRA is present at 0.01 μM, a concentration at which the retinoid does not increase the levels of sialoadhesin, PRAM, and ctsD and is marginally effective on pLym. MCP-1 and the cytokine, TNF, are two proteins regulated by ATRA at the gene level in opposite directions (51). MCP-1 is a myeloid differentiation marker (17), whereas TNF is a potential effector molecule in the APL-related ATRA syndrome (52). Fig. 8*B* demonstrates that piclamilast and ATRA increase the levels of MCP-1 and decrease those of TNF. The combination of piclamilast and ATRA is more effective than the single components in augmenting the production of MCP-1 but not in inhibiting TNF accumulation.

Effect of Piclamilast, ATRA, and the Association on cEBP, STAT1, and ERK—cEBP transcription factors are important mediators of myeloid maturation (53–56). Fig. 9*A* demonstrates that detectable amounts of cEBPβ and cEBPϵ are not present in undifferentiated NB4 cells. Treatment of NB4 cells with piclamilast or ATRA induces the 36–38-kDa (LAP) and the 23 kDa (LIP) forms of cEBPβ as well as cEBPϵ. In all cases, the phenomena are already detectable at 6 h and persist until 72 h.

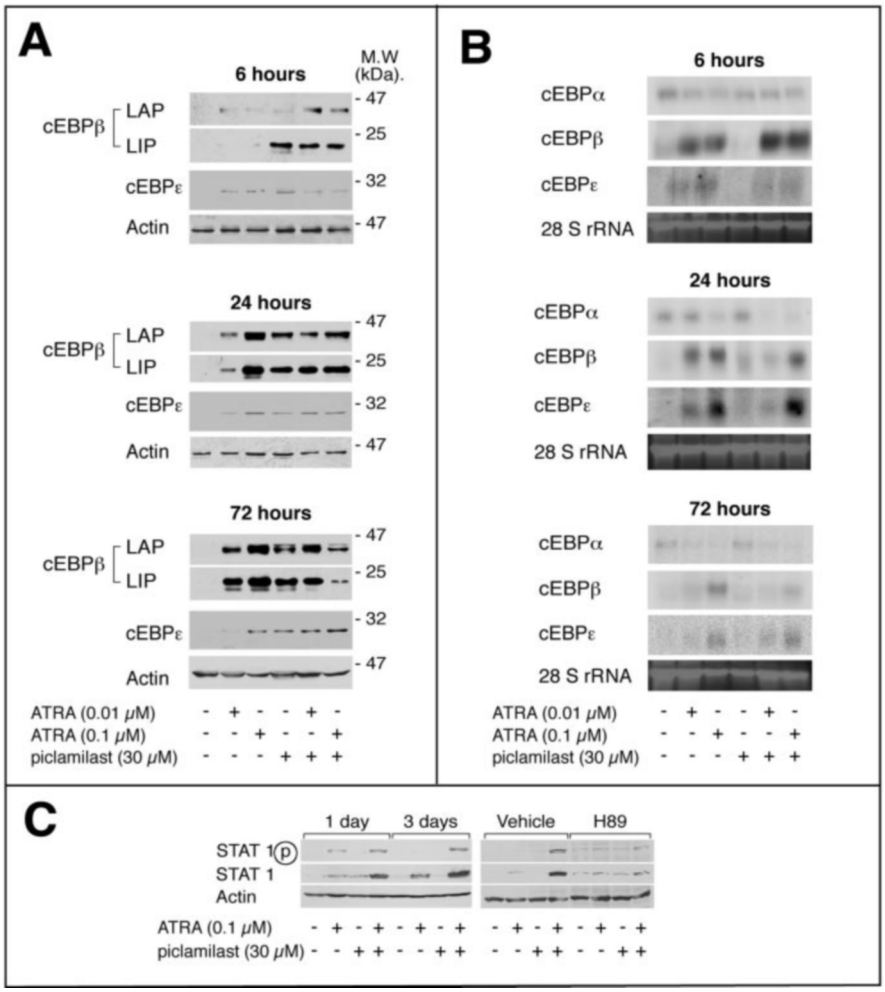

FIG. 9. **Effect of piclamilast and ATRA on the expression of members of the cEBP family of transcription factors as well as the levels and phosphorylation of STAT1.** NB4 cells (150,000/ml) were treated with the indicated compounds for various lengths of time. *A*, Western blot analysis was performed on total cell extracts, using anti-cEBPβ, anti-cEBPϵ, and anti-β-actin polyclonal antibodies. The results are representative of at least two independent experiments. *B*, Northern blot analysis were performed on total RNA. Filters were probed with ^{32}P-labeled cDNA probes specific for cEBPα, cEBPβ, and cEBPϵ amplified by RT-PCR. Ethidium bromide stainings of the 28 S ribosomal RNA (28 S rRNA) of the preparations used for the Northern blot analyses are shown. The results are representative of at least two independent experiments with each cDNA probe. *C*, for these experiments, cells were pretreated with H89 (10 μM) for 2 h prior to the addition of the other stimuli. Western blot analysis were performed on total cell extracts, using antibodies directed against β-actin, STAT1, and the corresponding tyrosine-phosphorylated form of the protein. The results are representative of at least two independent experiments.

Piclamilast exerts a particularly rapid and strong effect on LIP. Except for the enhancing effect of ATRA + piclamilast on the expression of LAP observed at 6 h, the combination is no more effective than the PDEIV inhibitor or the retinoid alone in inducing the various forms of cEBPβ and cEBPϵ. Indeed, long term treatment (72 h) with high concentrations of ATRA (0.1 μM) and piclamilast slightly inhibits the induction of cEBPβ. Induction of cEBPβ and -ϵ by ATRA is predominantly transcriptional, whereas the effect of piclamilast on these proteins is mainly translational or post-translational. In fact, a rapid and long lasting induction of the two transcripts is afforded by ATRA, whereas treatment with piclamilast is not associated with induction of cEBPβ or -ϵ mRNAs (Fig. 9*B*). The addition of piclamilast to ATRA has only marginal effects on the expression of the mRNAs coding for cEBPβ and -ϵ relative to what is observed with the retinoid alone. Interestingly, ATRA results in a dose-dependent diminution of the levels of the cEBPα mRNA, which is not affected by ATRA + piclamilast (see 24 h).

The transcription factor STAT1 is also involved in the process of myeloid maturation of leukemic cells (57–59). Piclamilast exerts a significant enhancing effect on the induction of STAT1 observed in ATRA-treated NB4 cells (Fig. 9*C*). This results in a detectable increase in STAT1 tyrosine phosphorylation, which is necessary for the activation of the protein. The PKA inhibitor, H89, blocks the positive effect of piclamilast on the regulation of STAT1 by ATRA.

Activation of the MAP kinase, ERK, has been involved in the process of myeloid maturation triggered by ATRA in the HL-60 myeloid cell line (60, 61). Piclamilast, ATRA, and ATRA + piclamilast do not affect the constitutive expression of ERK proteins in NB4 cells (Fig. 10*A*). However, treatment for 10 min and 1 h with the PDE IV inhibitor is associated with decreased ERK phosphorylation and activation. Inhibition of ERK phosphorylation by piclamilast is protracted if ATRA is added (see 1 day and 3 days). Fig. 10*B* demonstrates that co-treatment with U0126, a selective ERK inhibitor, does not have any detectable effect on the number of NBT-R$^+$ cells observed in NB4 cells challenged with vehicle, ATRA, piclamilast, and the combination. U0126 has a strong inhibitory action on the growth of undifferentiated NB4 cells and potentiates the antiproliferative and cytotoxic actions of ATRA. However, the addition of piclamilast to U0126 or U0126 and ATRA does not add to the magnitude of the observed effects.

In Vivo Activity of the Combination of Piclamilast + ATRA—To evaluate whether the potentiating effect of piclamilast on ATRA cytodifferentiating activity has therapeutic impact, we transplanted NB4 cells intraperitoneally in SCID mice. Animals were treated with vehicle, ATRA, piclamilast, and the combination of the two compounds and evaluated for survival. Fig. 11 illustrates the Kaplan-Meier survival curves of the animals belonging to the various experimental groups. The MST of vehicle-treated animals is 27 days (interval 24–30 days). Piclamilast does not affect the MST of leukemia-bearing animals (25 days; interval 20–26 days). As expected, ATRA has a significant effect on the survival of animals, increasing the MST to 37 days (interval 33–42 days, $p < 0.0001$ according to the Cox regression model). Piclamilast + ATRA is significantly ($p < 0.05$) more effective than ATRA alone in increasing the MST (40 days; interval 34–45 days) of leukemia-bearing animals. This translates into an increase in the life span over

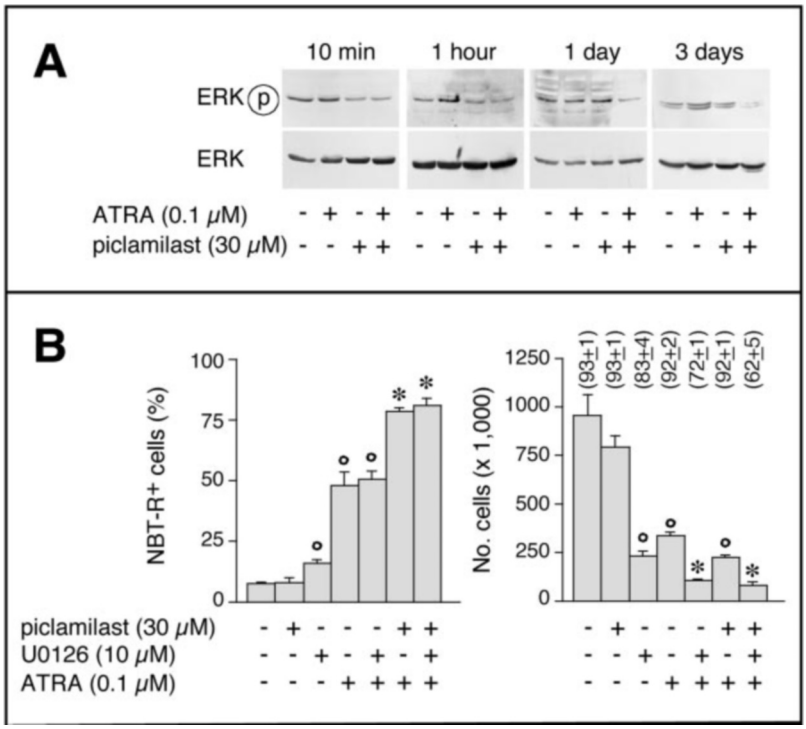

FIG. 10. **Effect of piclamilast, ATRA, and the combination on ERK levels and phosphorylation.** *A*, NB4 cells (150,000/ml) were treated with the indicated compounds for various lengths of time. Western blot analysis were performed on cell extracts, using antibodies directed against ERK and the corresponding phosphorylated form of the protein. The results are representative of at least two independent experiments. *B*, NB4 cells were treated for 3 days with the indicated compounds. The percentage of NBT-R^+ cells (*left graph*) and the total number of cells (*right graph*) were determined. The viability of cells is indicated in *parenthesis*. °, significantly higher or lower than the relative control sample according to Student's *t* test ($p < 0.01$). *, significantly higher or lower than the relative ATRA or piclamilast sample according to Student's *t* test ($p < 0.01$).

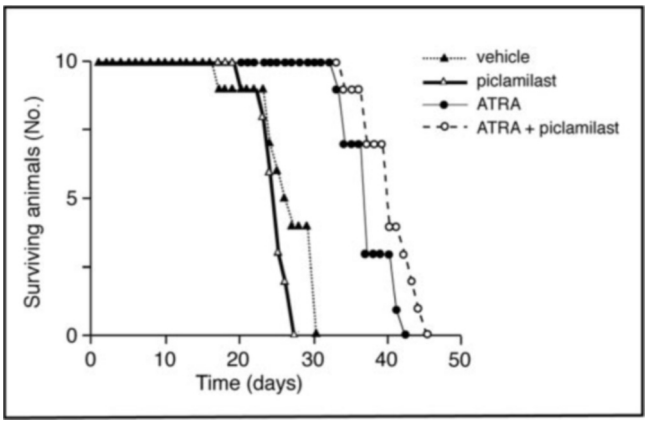

FIG. 11. ***In vivo* activity of piclamilast alone and in combination with ATRA.** SCID animals were transplanted intraperitoneally with NB4 cells (6×10^6 cells/animal). Animals were injected with NB4 cells on day 0. On day 3, animals were randomized in four experimental groups receiving one daily dose of vehicle, piclamilast (10 mg/kg), ATRA (15 mg/kg), and the combination of the retinoid and the PDE IV inhibitor. Treatment was continued for 13 days. The Kaplan-Meier's survivial curves of the various experimental groups are illustrated.

vehicle-treated animals of 37% in the case of ATRA and 48% in the case of ATRA + piclamilast. In no experimental group did we observe signs indicative of major systemic toxicity, such as treatment-associated lethality or significant body weight loss.

DISCUSSION

Inhibition of PDEIV by piclamilast enhances and accelerates the granulocytic maturation program activated in APL and other myeloid leukemia cells by ATRA, RARα, and RXR agonists through increases in the intracellular levels of cAMP. Cooperation between piclamilast and ATRA is likely to be mediated by the RAR/RXR pathway, since it is reproduced with the selective RARα agonist, AM580, and is not observed in the retinoid-resistant NB4.R2 and NB4/007 cell lines (data not shown), in which this pathway is disrupted (24, 62). Interestingly, not only ATRA-sensitive cell lines, but also the retinoid-resistant, NB4.R2 subline, respond to combinations of piclamilast and RXR agonists. This indicates that piclamilast facilitates myeloid maturation not only via retinoid-dependent RAR/RXR activation, but also through the recently described RXR/RXR pathway (8–11, 38). The data obtained *in vitro* are encouraging in view of the potential therapeutic application of combinations between selective PDE IV inhibitors and ATRA, RAR, or RXR agonists in the treatment of AML. Indeed, our initial *in vivo* studies in the APL model of the SCID mouse transplanted with NB4 cells demonstrate that treatment with ATRA and piclamilast is well tolerated and does not result in increased toxicity relative to ATRA and/or piclamilast alone. More importantly, the combination is more effective than ATRA alone in terms of increased survival of the leukemia-bearing animals. At present, our data demonstrate that the therapeutic efficacy of the combination of piclamilast and ATRA is only slightly superior to that of ATRA alone; however, the dosage and administration schedule need to be optimized.

The molecular mechanisms underlying the cross-talk between piclamilast and ATRA are summarized in Table I. Epac, is a direct cAMP target (40, 41) and is known to antagonize some of the cellular responses to PKA (63). However, Epac does not play any role in our differentiation model. Furthermore, the direct Epac activator, *O*-Me cAMP, does not disrupt the interaction between ATRA and piclamilast. Piclamilast stimulates PKA and translocates the catalytic subunit of the enzyme to the nucleus of NB4 cells. In the nucleus, the activated PKA subunit increases the constitutive phosphorylation of CREB and its DNA binding activity. CREB activation is transient and limited in magnitude and does not completely reflect PKA activation and export to the nucleus. The activation is followed by down-regulation of the entire CREB system by 24 h. The system is back to pretreatment levels by 72 h. The transient and limited activation of CREB is in line with the weak effects that piclamilast exerts on CREB-dependent genes. In NB4 cells, the early action of ATRA on the various components of the cAMP signal transduction system is similar to that of piclamilast in many respects. The retinoid activates PKA and translocates it to the nucleus. Furthermore and unlike piclamilast, ATRA down-modulates one of the two regulatory subunits of

TABLE I

Summary of the biological effects of piclamilast and ATRA

The table summarizes the results obtained in NB4 or COS cells treated with piclamilast, ATRA, and a combination of the two compounds on various components of the cAMP and the retinoid signaling pathways. The effects of ATRA and piclamilast, alone or in combination, on the myeloid maturation of NB4 cells are also summarized. The time frame of the various effects is indicated. The observed phenomena can be described as early or late events on the basis of their occurrence within or after the first 24 h. Plus and minus signs indicate an increase and a decrease in the observed parameter, respectively. If the combination of piclamilast and ATRA gives an increase or a decrease significantly higher or lower than that observed with ATRA, a double plus or double minus sign is shown. In the case of cEBPβ, +/− indicates attenuation of the increase observed with ATRA or piclamilast. ×, no variation in the parameter taken into consideration.

	Early events (<24 h)			Late events (>24 h)		
	Piclamilast	ATRA	ATRA + piclamilast	Piclamilast	ATRA	ATRA + piclamilast
CAMP pathway						
CAMP	+	+	+	×	×	×
Epac activation	×	×	×	×	×	×
PKA activation	+	+	+	×	×	×
PKA-cIα nuclear translocation	+	+	+	+	+	+
PKA-rIa	×	×	×	×	×	×
PKA-rIIa	×	−	×	×	−	×
CREB	×	×	×	−	−	−
CREB phosphorylation[a]	+	+	+	−	−	−
CREB-responsive genes						
THBS	+	×	+	×	+	++
EGR1	×	+	+	+	+	+
Vinculin	×	×	×	×	+	++
BTG	×	×	×	×	+	++
Retinoid pathway						
RARα degradation				×	+	++
PML-RARα degradation				×	+	+
RARα activation				×	+	++
PML-RARα activation				×	+	++
ATRA-responsive genes						
CD38				×	+	+
G-CSF				×	+	+
Sialoadhesin				×	+	++
PRAM				×	+	++
ctsD				×	+	++
pLym				×	+	++
MCP-1	+	+	++	+	+	++
TNF	−	−	−	−	−	−
Myeloid maturation						
Differentiation-associated transcription factors						
CEBPα	×	×	×	×	−	−
CEBPβ	+	+	++[b]	+	+	+
CEBPϵ	+	+	+	+	+	+
STAT1	×	+	++	×	+	++
Phosphorylated STAT1	×	+	++	×	×	+
MAP kinase						
ERK	×	×	×	×	×	×
Phosphorylated ERK	−	×	×	×	×	−
Differentiation markers						
Morphology				×	+	++
CD11b				×	+	++
NBT-R				×	+	++
CD11c				×	+	+

[a] CREB phosphorylation is an early and very transient event, since it is observed only during the first 2 h of treatment.
[b] Induction is observed only in the case of the LAP form of cEBPβ.

PKA. At present, the mechanisms underlying PKA activation by the retinoid are poorly understood. Indeed, at the time points considered, ATRA treatment does not lead to a detectable increase in intracellular cAMP. However, it was recently demonstrated that the retinoid causes a very rapid pulse of cAMP accumulation in NB4 cells (64). Regardless of the mechanism of PKA activation, this phenomenon is likely to be responsible for the transient and early activation of CREB observed upon treatment of NB4 cells with ATRA. Interestingly, activation of CREB by the retinoid seems to be limited to the NB4 and possibly the myeloid cellular context. In fact, in COS-7 cells transfected with RARα, ATRA does not activate the CREB- responsive promoter of the somatostatin gene.

A key question in the context of the cross-talk between piclamilast and ATRA concerns the point(s) at which the cAMP and the retinoid pathways converge, since this/these may be critical for enhanced myeloid maturation. Combining piclamilast and ATRA has no significant additive or synergistic effects on cAMP levels, PKA activation, and nuclear translocation relative to what is observed with the retinoid and the PDEIV inhibitor alone. In contrast, the association of ATRA and piclamilast accelerates the down-regulation of CREB afforded by piclamilast and prevents the retinoid-dependent down-modulation of PKA-rIIα. Accelerated CREB down-regulation by ATRA + piclamilast may lead to a more rapid arrest of cell growth, which, in turn, may accelerate the retinoid-dependent myeloid maturation program. Indeed, growth arrest and myeloid maturation of NB4 cells are tightly linked (37). The effect on PKA-rIIα may also be relevant in the context of the myeloid maturation of APL blasts, since this regulatory subunit is believed to be involved in cell differentiation, whereas PKA-rIα seems to transduce proliferative signals (65). Hence, tipping the intracellular balance of PKA-rIIα and PKA-rIα toward the former may facilitate the process of granulocytic maturation.

The action of piclamilast on the nuclear retinoid receptors is dramatic. Indeed, modulation of the primary targets of the

retinoid signaling pathway by piclamilast is likely to play a prominent role in the potentiating effect of the PDEIV inhibitor. Piclamilast enhances the ligand-dependent activation of both RARα and PML-RARα, probably through a direct action of PKA on the state of phosphorylation and activation of the two receptors. Stimulation of the ligand-dependent transactivation of RARα and PML-RARα is observed when two minimal artificial promoters are used, and the effect is suppressed in the case of the mutant RARαS369A receptor. Piclamilast enhances not only the retinoid-dependent transactivation but also the degradation of RARα. Transactivation and degradation of the RARγ receptor are intimately linked and possibly coupled processes (44, 45). Blocking degradation suppresses transactivation, and the opposite is also true. Mutation of the PKA phosphorylation site has no significant effect on ATRA-induced degradation, whereas it abrogates the increased proteolysis afforded by piclamilast + ATRA. This suggests that PKA activation and RARα phosphorylation on Ser^{369} by piclamilast facilitate the transactivation/degradation coupling. Interestingly, PML-RARα behaves differently from RARα. The aberrant fusion protein is degraded to the same extent by ATRA and ATRA + piclamilast. This suggests that the two receptors have different susceptibilities to ligand-dependent proteasomal degradation. Regardless of the mechanistic details, PKA activation results in enhanced expression of many, but not all, ATRA-regulated genes in NB4 cells. Enhanced activity of a large number of retinoid-activated genes may be at the basis of enhanced myeloid maturation.

STAT1, the transcription factors of the cEBP family, and the MAP kinase ERK are regulated by retinoids and have been implicated in the process of myeloid maturation (57–61). Our data are consistent with the idea that STAT1 plays a role in the process of granulocytic maturation set in motion by ATRA and enhanced by PDEIV inhibitors. In fact, the amounts as well as the activation state of STAT1 correlate with enhanced granulocytic maturation of NB4 cells by ATRA + piclamilast. Although piclamilast and ATRA induce cEBPβ and -ϵ through different molecular mechanisms, no significant interactions between the two compounds on these molecular targets are evident at the majority of the time points considered. The only exception is the enhanced induction of cEBPβ observed early (6 h) during the differentiation process. This effect may be of some significance for the granulocytic maturation of NB4 cells. In this context, it is relevant that piclamilast and ATRA induce not only the forms of cEBPβ that act as transcriptional activators (LAP1 and LAP2) but also the purportedly transcriptional inhibitor, LIP. Consistent with the idea that PKA modulates the ERK pathway in a negative fashion (66–68), phosphorylation and activation of the MAP kinase is reduced by piclamilast. Interestingly, prolonged down-regulation of ERK phosphorylation by the combination of piclamilast and ATRA may have relevance for the inhibition of cell growth, which is more evident upon treatment of NB4 cells with the combination than with piclamilast or ATRA alone. However, the data obtained with the U0126 inhibitor suggest that ERK activation (60, 61) is not a necessary event for the myeloid maturation of APL cells.

In conclusion, our results concur in defining the molecular mechanisms underlying the cross-talk between the cAMP and the retinoid signal transduction pathways. Furthermore, they indicate that PDE IV represents a valuable pharmacological target for future efforts aimed at the differentiation therapy of myeloid leukemia.

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