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Activation of both Protein Kinase A (PKA) type I and PKA type II isozymes is required for retinoid-induced maturation of acute promyelocytic leukemia cells

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Running title: PKA-I and PKA-II interplay in retinoid-induced maturation

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Abbreviations:

PKA, cAMP-dependent protein kinase A; PKA-I/II, Protein kinase type I/II; APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; RAR, retinoic acid receptor-α; 8-CPT-cAMP, 8-(4-Chlorophenylthio)adenosine3',5'-cyclic adenosine mono phosphate; 2-Cl-8-MA-cAMP, 2-Chloro-8-methylamino-cAMP; 8-Pip-cAMP, 8-Piperidino-cAMP; N6-MBC-cAMP, N^6-Mono-tert-butylcarbamoyl-cAMP; Sp-5,6-Di-Cl-cBIMPS, 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole-3',5'-cyclic monophosphorothioate, Sp-isomer; 2-Cl-8-AHA-cAMP, 2-chloro-8-Aminohexylamino-cAMP; N^6-Bz-8-PipcAMP, N^6-benzoyl-8-piperidino-cAMP; CM-H_2DCFDA, 5-(and 6)-choromethyl-2',7'-dichlorohydrofluorescein diacetate acetyl ester; NBT, p-Nitro-Blue Tetrazolium; DCF, dihydrodichlorofluorescein diacetate.

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ABSTRACT

Acute promyelocytic leukemia (APL) is characterized by granulopoietic differentiation arrest at the promyelocytic stage. In most cases this defect can be overcome by treatment with all-*trans*-retinoic acid (ATRA) leading to complete clinical remission. Cyclic AMP signaling has a key role in retinoid treatment efficacy: it enhances ATRA-induced maturation in ATRA-sensitive APL cells (including NB4 cells) and restores it in some ATRA-resistant cells (including NB4-LR1 cells). Here, we showed that the two cell types express identical levels of the Cα catalytic subunit and comparable global cAMP-dependent protein kinase A (PKA) enzyme activity. However, the maturation-resistant NB4-LR1 cells had a PKA isozyme switch: compared to the NB4 cells, they had decreased content of the juxtanuclearly located PKA-RIIα and PKA-RIIβ, and a compensatory increase of the generally cytoplasmically distributed PKA-RIα. Furthermore, PKA-RII subunit existed mainly in the less cAMP-responsive non-autophosphorylated state in the NB4-LR1 cells. We showed by using isozyme-specific cAMP analog pairs that both PKA-I and PKA-II had to be activated to achieve maturation in NB4-LR1 as well as NB4 cells. Therefore, special attention should be paid to activate not only PKA-I, but also PKA-II in attempts to enhance ATRA-induced APL maturation in a clinical setting.

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Introduction

Acute promyelocytic leukemia (APL) is characterized by an arrest of neutrophile granulocyte maturation at the promyelocytic stage and the reciprocal chromosomal translocation t(15,17), which fuses the promyelocytic leukemia (PML) and the retinoic acid receptor-α (RARα) (Rowley et al., 1977) resulting in the formation of a chimeric gene encoding the PML/RARα fusion protein (Borrow et al., 1990; de The et al., 1991). The unique sensitivity of APL cells to all-*trans* retinoic acid (ATRA)-induced granulocyte differentiation has been successfully exploited for the treatment of this leukemia**.** Despite the great success of ATRA combined with chemotherapy in APL clinical management, a significant number (5-30%) of patients develop resistance to ATRA and relapse (Ferrara, 2010). ATRA-induced APL differentiation can be enhanced by activators of cAMP signalling, and some primarily ATRA-resistant APL cells can be induced to differentiate if ATRA is combined with cAMP analogs (Guillemin et al., 2002; Kamashev et al., 2004; Ruchaud et al., 1994).

The major effector of cAMP is the cAMP-dependent protein kinase A (PKA). At low cAMP concentration, PKA exists as an inactive tetrameric holoenzyme composed of two regulatory (R) and two catalytic (C) subunits. Activation of PKA occurs when four molecules of cAMP bind to the R subunits promoting the dissociation of the PKA holoenzyme into two active catalytic subunits and a dimer of regulatory subunits (Tasken et al., 1997). There are two types of PKA differing in their regulatory subunits (R subunits): RIα or RIβ in PKA type I (PKA-I) and RIIα or RIIβ for PKA type II (PKA-II) (McKnight et al., 1988; Skalhegg and Tasken, 1997). The PKA regulatory subunits define the sensitivity to cAMP (Rannels et al., 1985; Robinson-Steiner et al., 1984; Woodford et al., 1989; Zhang et al., 2012) and localize PKA at various subcellular sites through interaction with A-kinase anchoring proteins (AKAPs) (Skroblin et al., 2010). Furthermore, only PKA-II but not PKA-I undergoes autophosphorylation (Rosen and Erlichman, 1975), which serves as a "feed-forward" signal by enhancing the cAMP responsiveness of PKA-II (Kopperud et al., 2003; Martin et al., 2007) .

The NB4 cell line, a model of APL (Lanotte et al., 1991), and its variant sublines, NB4-LR1 and NB4-LR2, resistant to ATRA-induced maturation, offer an *in vitro* model to study ATRA-induced differentiation signalling pathways in APL cells and the molecular mechanisms of ATRA resistance

(Duprez et al., 2000; Guillemin et al., 2002). The PKA activity appears to tune the ATRA-sensitivity of NB4 wild-type cells. NB4 cells, like APL cells in patients, do not differentiate at physiological (1.0-10 nM) concentrations of ATRA but they underwent differentiation in response to pharmacological doses (0.1-1.0 μM) of ATRA. The NB4 cells lost ATRA responsiveness when the basal PKA activity was inhibited by either R subunit directed competitive antagonists of PKA dissociation (Ruchaud et al., 1994), or by active site directed inhibitors of the C subunits (Zhao et al., 2004). Conversely, sustained increase of the endogenous level of cAMP rendered NB4 cells responsive to physiological levels of retinoids (3-10 nM) (Quenech'Du et al., 1998). In ATRA-induced maturation-resistant NB4-LR1 cells, maturation was achieved by sequential or simultaneous treatment with ATRA and cAMP analogs (Ruchaud et al., 1994). The *in vitro* studies on NB4 cell lines have been confirmed *in vivo* using PML-RAR transgenic mouse models and so far one APL patient (Guillemin et al., 2002). Recently, it has been proposed that cAMP/PKA, by inducing Ser873 phosphorylation of the PML-RARα fusion protein, could contribute to eradicate Leukemia Initiating Cells (LIC) *in vivo* (Nasr et al., 2008). These reports indicate not only a significant role of the cAMP-PKA pathway in the differentiation of granulocytic cells, but also in the eradication of LIC *in vivo*. Therefore, understanding the mechanism of action of cAMP/PKA signalling in APL cells is of interest not only for the treatment of APL, but also for the understanding of its etiology and progression.

The NB4-LR1 cell, which requires exogenous stimulation of the cAMP signalling in addition to ATRA treatment for maturation, provides a valuable tool to decipher the complex PKA/retinoid cross-talk required for APL cell maturation. A previous study suggested that the differential response of NB4 and NB4-LR1 cells to ATRA treatment was explained by a lower level of basal level of cAMP in NB4-LR1 than in NB4 wild-type cells (Zhao et al., 2004). Here, we showed that NB4-LR1 cells differed also from NB4 cells by having downregulated PKA-IIα and PKA-IIβ, resulting in a PKA-II to PKA-I isozyme switch. Moreover, the sustained activation of both types of PKA by cAMP analogs was required to allow ATRA-induced maturation of APL cells.

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Materials and Methods

Reagents. All-*trans* retinoic acid (ATRA), 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic adenosine mono phosphate (8-CPT-cAMP), PKA inhibitory peptide (Protein Kinase A inhibitor fragment 6-22 amide, PKI), p-Nitro-Blue tetrazolium chloride (NBT) and Phorbol 12-myristate 13-acetate (PMA) were from Sigma (St. Louis, MO). 2-Chloro-8-methylamino-cAMP (2-Cl-8-MA-cAMP), 8-Piperidino-cAMP (8-Pip-cAMP), N^6-Mono-tert-butylcarbamoyl-cAMP (N^6-MBC-cAMP), 5,6-Dichloro-1-β-D-ribofuranosyl benzimidazole-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-5,6-DiCl-cBIMPS), and the Epac specific cAMP analog (8-pMeOPT-2'O-Me-cAMP, #M0134) were from Biolog (Bremen, Germany). The new cAMP analogs 2-Chloro-8-Aminohexylamino-cAMP (2-Cl-8-AHA-cAMP) and N^6-benzoyl-8-piperidino-cAMP (N^6-Bz-8-Pip-cAMP) were synthesized as described (Huseby et al.).

Cell lines and cell culture. NB4, NB4-LR1 and NB4-LR2 cell lines were cultured as previously reported (Lanotte et al., 1991) in RPMI 1640 medium supplemented with 10 % foetal bovine serum (PAA Laboratories, Pasching, Austria), penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and sodium bicarbonate (0.75 mg/ml), and incubated at 37°C in a 5% CO_2 atmosphere.

In vitro PKA assay. PKA activity in NB4 cell lysates was measured using a PepTag non-radioactive protein kinase assay kit (Promega, Madison, WI). This assay is based on the *in vitro* phosphorylation of a fluorescent PKA-specific peptide substrate (Kemptide). Briefly, NB4 cells were washed twice with ice cold PBS, 10 mM potassium phosphate pH 7.2, 0.3 mM EGTA, 0.1 % Triton-X-100, 2.5 mM NaF, 0.2 mM Na_3VO_4, 0.5 mg/ml trypsin inhibitor (lysis buffer) supplemented with 1 % protease inhibitor cocktail (P8340) (Sigma, St. Louis, MO) and 1mM phenylmethanesulfonyl fluoride and incubated on ice for 15 min. Cells were then lysed with a Dounce homogenizer, nuclei were eliminated by centrifugation at 950 g for 3 min and the lysate was desalted through a Sephadex-G25 column equilibrated in the lysis buffer. The assay was performed according to manufacturer's

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recommendations in the presence or in the absence of cAMP analogs. Lysate samples (13 μg protein) were incubated for 45 min at 25°C and the reactions were stopped by heating at 95°C for 10 min. The catalytic C subunit provided in the kit was used as a positive control. Phosphorylated and nonphosphorylated kemptides were separated by gel electrophoresis and their fluorescence under UV illumination analysed using a CCD camera system (LAS 4000, GE Heathcare). The PKI peptide derived from the selective inhibitor of PKA (Cheng et al., 1986) was added at 180 μM to assess the contribution of non-PKA activity to total phosphotransferase activity. Basal and stimulated PKA activity was quantitated as the PKI-inhibited fraction of the phosphorylated kemptide.

Whole cell extracts and western blot analysis. Cells were washed twice with ice cold PBS and lysed with 1 ml of 8 % SDS, 0.25 M Tris pH 6.8, 1% protease inhibitor cocktail (P8340), 2.5 mM NaF and 0.2 mM Na_3VO_4. The lysates were vortexed and boiled twice for 5 min. Insoluble material was removed by centrifugation at 18,000 g for 1 h. Protein concentration was determined using BCA protein assay (Thermo Scientific, Rockford, IL). Total cellular proteins were separated by SDS-polyacrylamide gel electrophoresis according to Laemmli (Laemmli, 1970). Cell extraction and electrophoresis conditions for 2D-gels were as described before (Wu et al., 2006). Total cellular proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) were transferred onto polyvinylidene fluoride (PVDF) membranes. Blots were blocked with 5% non-fat dry milk, 0.5% Tween 20 in PBS pH 7.4 (PBS-Tween-milk), and then probed with one of the following primary antibody: anti-PKA-RIα, anti-PKA-RIIβ, anti-PKA-Cα, anti-PKA-RIIβ-pS114, anti-PKA-RIIα-pS99 (BD Biosciences, Franklin Lakes, NJ, 610609, 610625, 610980 612550 and 558244, respectively), anti-PKA-RIβ (Merk Millipore, Darmstadt, Germany, 539233), anti-PKA-RIIα (Santa Cruz Biotechnology, Santa Cruz, CA, sc908), anti-GAPDH (Abcam, Cambridge, UK, ab9485), anti-actin (Sigma, St. Louis, MO, A2066) or anti-vimentin (Novocastra Laboratories, Newcastle, UK, NCL-VIM-V9) diluted in PBS-Tween-milk. After washing, blots were incubated with the appropriate peroxydase-conjugated secondary antibody diluted in PBS-Tween-milk, washed again and developed by chemiluminescence (Western Lighting Plus-ECL, Perkin Elmer, Waltham, MA). When indicated,

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protein dephosphorylation was performed using λ protein phosphatase (Merk Millipore, Darmstadt, Germany). Dephosphorylation was performed by incubation of the blots for 4 h, at room temperature, in 50 mM Tris pH 7.4, 150 mM NaCl containing 1 % bovine serum albumin, 0.1 % Triton X-100, 2 mM $MnCl_2$ and 400 U/ml λ protein phosphatase during 1 h. The blots were then washed with 0.1 % Tween-20 in PBS and processed as above for immunological detection.

Conventional and confocal immuno-fluorescence microscopy. NB4 cells were fixed in 2% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature, permeabilized with 0.1% Triton X100 in PBS pH 7.4 for 10 min and blocked in 2% BSA in PBS pH 7.4 (PBS-BSA). Cells were incubated with the primary antibody diluted in PBS-BSA (anti-PKA-RI, BD Biosciences, 610165; anti-PKA-RIIα, Santa Cruz Laboratories, sc 908; Anti-PKA-RIIβ, BD Biosciences, 610625 or anti-β-COP, Sigma, G6160) overnight at 4°C, washed in PBS, incubated with Alexa Fluor 488 or Alexa Fluor 594-conjugated secondary antibody (Life Technologies, Carlsbad, CA, diluted in PBS-BSA for 1 h and washed again in PBS. Cells were mounted on slides with 5 µl of mounting medium containing 4,6-diaminidine-2-phenylindole (Dapi) (Vectashield H-1200, Vector Laboratories, Burlingame, CA) to counterstain nuclei. Cells were observed either by conventional fluorescence microscopy (Leica DMRD, equipped with 63x objective, Leica, Wetzlar, Germany) or laser scanning confocal microscopy (Zeiss LSM 510, equipped with 63x objective, Carl Zeiss, Oberkochen, Germany).

RNA preparation and quantitative RT-PCR. Total cellular RNA was collected from samples using TRIzol reagent (Life Technologies, Carlsbad, CA) as described by the manufacturer. Reverse transcription using Transcriptor First Strand cDNA kit (Roche Diagnostics, Basel, Switzerland) and quantitative real-time PCR using the LightCycler technology and the LightCycler FastStart DNA MasterPLUS SYBR Green kit were performed as described before (Deville et al., 2011). PKA-RIIβ specific primers sequences were 5'-GGAGTTTCGGCGAACTGGC-3' for the sense primer located in exon 6 and 5'- TCTCCTGAAGGTTACCCTGTCC-3' for the antisense primer located in exon 7. PKA-RIIβ mRNA level was normalized to the expression of the ribosomal protein P2 mRNA

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measured in parallel using a sense primer 5'-GACCGGCTCAACAAGGTTAT-3' and an antisens primer 5'-CCCCACCAGCAGGTACAC-3' located in exon 5 and exon3-4, respectively.

Cell maturation assays. NB4 cells committed into the granulocytic differentiation pathway can be identified before morphological changes by probing their ability to undergo an oxidative burst after activation with the phorbol 12-myristate 13-acetate (PMA). This oxidative burst can be evaluated either by microscopy after performing the NBT reduction assay (Pick, 1986) or by flow cytometry after dihydrodichlorofluorescein diacetate (DCF) assay adapted from Eplin (Epling et al., 1992). For the DCF assay, we used 5-(and 6)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) (Life Technologies) instead of the H_2-DCF because it passes more easily through membranes, and its fluorescent oxidized product exhibits a much better retention in living cells. Briefly, NB4 cells were collected by centrifugation at 200 g and incubated in 1 µM CM-H_2DCFDA, 0.3 µM PMA, PBS during 20 min at 37°C. The cell suspension was then transferred in a cytometer tube containing cold PBS and incubated 20 min on ice. The fluorescence of the oxidized product of CM-H_2DCFDA was measured by flow cytometry (FacsCalibur, BD Biosciences, Franklin Lakes, NJ) on channel FL1.

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Results

NB4-LR1 cells are less sensitive than NB4 cells to cotreatment with 8-CPT-cAMP and ATRA. Previous studies have shown that the endogenous intracellular cAMP concentration is lower in maturation-resistant resting NB4-LR1 cells than in NB4 cells, and it has been proposed that this may contribute to their differential response to ATRA-induced maturation (Zhao et al., 2004). To investigate whether this lower cAMP level in NB4-LR1 cells fully explain their blunted ATRA responsiveness, we undertook a comparative NBT maturation assay of NB4 and NB4-LR1 cells treated with ATRA in combination with a maximally effective concentration (0.2 mM) of 8-CPT-cAMP. This potent exogenous cAMP analog, as expected (Quenech'Du et al., 1998), allowed robust maturation of NB4 cells at low ATRA concentrations, but was less efficient for the NB4-LR1 cells (Fig. 1A and B). This indicates that the deficient ATRA responsiveness cannot be explained by low cAMP level only. We searched therefore for an additional defect in the cAMP/PKA signalling downstream of cAMP for a possible explanation of this behaviour.

One explanation could be that the NB4-LR1 cells contained less PKA than the NB4 cells. Therefore PKA activity was determined in both cell lines in basal and 8-CPT-cAMP stimulated conditions using a non-radioactive cAMP-dependent protein kinase assay based on the phosphorylation of a kemptide. As the kemptide could be phosphorylated by kinase other than PKA, the addition of a selective inhibitor (PKI peptide) was used as a means to determine the actual contribution of PKA activity. Under basal condition (without 8-CPT-cAMP) the PKI sensitive portion of the kemptide phosphorylation was almost negligible in both NB4 and NB4-LR1. Upon PKA stimulation (addition of 8-CPT-cAMP) the phosphorylation of the kemptide significantly increased and was completely inhibited by the addition of the PKI demonstrating that the extract kinase activity was due mainly to PKA. When analysed comparatively, no significant difference in PKA activity was observed between extracts from NB4 and NB4-LR1 cells in the presence of PKA-saturating concentration of 8-CPT-cAMP, even after ATRA treatment (Fig. 1C). The similar overall content of PKA was further demonstrated by immunoblotting of the catalytic (C) subunit of PKA, which showed similar level in NB4 wild-type and NB4-LR1 cells (Fig. 2A). We concluded that the blunted ATRA responsiveness of NB4-LR1 cells was not due to lower overall PKA activity.

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ATRA-resistant NB4-LR1cells are deficient in PKA type II. We considered next whether NB4-LR1 cells differed from NB4 cells or NB4-LR2 cells with respect to PKA isoform expression or localisation. In NB4-LR2 cells ATRA-maturation resistance is caused by a PML-RARα mutation (Duprez et al., 2000) without impairment of cAMP signalling. Immunoblots analysis showed that NB4 and NB4-LR2 cells expressed the RIα regulatory subunit of PKA-I and both the RIIα (50 kDa) and RIIβ (52 kDa) subunits of PKA-II (Fig. 2A). Note that the blots probed with the anti-PKA-RIIβ antibody revealed an additional faint 50 kDa band (pointed out by an asterisk in Fig. 2A). This band was demonstrated by 2D-gel electrophoresis to be RIIα (Fig. 2B), indicating that the anti-PKA-RIIβ antibody has a slight cross-reaction with RIIα. No RIβ subunit was detected in any of the cell lines, using a specific anti-PKA-RIβ antibody (data not shown).

The NB4-LR1 cell extracts showed no detectable PKA-RIIβ protein band and less PKA-RIIα (Fig. 3A). The under-expression of PKA-RIIβ in NB4-LR1 compared to NB4 was reflected at the mRNA level in quantitative RT-PCR analysis using specific primers (Fig. 2C). Interestingly, NB4-LR1 cells expressed more PKA-RIα protein. Hence, the NB4-LR1 cells have a shift in isozyme composition, expressing more PKA-I and less PKA-II than NB4 and NB4-LR2 cells.

The subcellular localisation of PKA-RI was qualitatively similar in the NB4 and NB4-LR1 cells, showing a diffuse slightly granular and mainly cytoplasmic distribution (Fig. 3). Although present at very low level throughout the cytoplasm, PKA-RIIα was mainly distributed in a perinuclear region in either cell type, suggesting a Golgi localisation (Fig. 3A). Double immunofluorescent labelling of PKA-RIIα and the Golgi marker β-COP followed by confocal microscopy analyses confirmed this localization (Fig. 3B). In NB4 cells, the labelling pattern of PKA-RIIβ was similar to that of PKA-RIIα. A Golgi localization of PKA-RIIα and PKA-RIIβ has been also reported in other cell types (McCahill et al., 2005; Rios et al., 1992). In NB4-LR1 cells, the expression of PKA-RIIα appeared slightly less intense compared to NB4 cells and little or no PKA-RIIβ protein was detected (Fig. 3), in agreement with the western blot studies (Fig. 2). Altogether, the data demonstrated a deficient expression of PKA type II, compensated by an over-expression of PKA type I in the ATRA

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maturation-resistant NB4-LR1 cell line, as compared to the ATRA-sensitive NB4 cell line and the NB4-LR2 subline. It could thus be hypothesised that the PKA isozyme switch from PKA-II to PKA-I could interfere with maturation of the NB4-LR1 cells.

PKA-RII is not induced by ATRA/cAMP maturation treatment in NB4-LR1 cells. Retinoids exert part of their anti-tumoral effects through reactivated expression of silenced genes (Abecassis et al., 2008; Villa et al., 2007). We wondered therefore whether ATRA/cAMP signalling cross-talk necessary for NB4-LR1 maturation could modulate the PKA-RII subunit expression. For this purpose, we determined the PKA regulatory subunit expressions in NB4-LR1 cells whose differentiation was triggered by a combination of ATRA (1μM) and 8-CPT-cAMP (0.2 mM), a potent PKA-I and PKA-II activator. The increased actin expression and concomitantly decreased vimentin expression with a constant expression of GAPDH (Fig. 4) confirmed immature NB4-LR1 leukemia blast differentiation towards neutrophilic granulocyte. We noted a slight increase in PKA-RIIα, but it never reached the level of expression observed in both NB4 and NB4-LR2 cells. Of note, PKA-RIα subunit expression was also slightly increased during this treatment. However, the differentiation was not accompanied by PKA-RIIβ protein expression. Therefore, a high expression of the PKA-RIIβ isoform is not obligatory for the ATRA/cAMP signalling cross-talk leading to maturation of NB4-LR1 cells.

Both PKA-I and PKA-II must be activated to achieve maximal ATRA-induced differentiation of NB4 and NB4-LR1 cells. In order to evaluate the contribution of each PKA isozyme, we exploited the fact that certain cAMP analogs prefer one of the two cAMP binding sites (A, B) of either the RI or RII subunit of PKA (Supplemental Table 1; (Huseby et al.; Ogreid et al., 1989)). In one series of experiments performed on NB4-LR1 cells treated with 0.1 μM ATRA, the DCF maturation assay showed that 8-Pip-cAMP, which selects site AI of PKA-I and site BII of PKA-II, was efficient only when combined with both 2-Cl-8-MA-cAMP (selects site BI of PKA-I) and the AII-directed N^6-MBC-cAMP (Fig. 5A). This result indicates that co-activation of both PKA-I and PKA-II is required to

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trigger the maturation response of NB4-LR1 cells. Therefore, the residual PKA-II in the NB4-LR1 cells had to be activated to achieve maturation.

We studied next the requirement of PKA-I and PKA-II in NB4 cell maturation. Since pharmacological concentration of ATRA alone induces NB4 cells maturation, the response of these cells to site-specific cAMP analogs can only be investigated at lower ATRA concentration. Indeed, it has been previously shown that NB4 maturation can be induced at near physiological concentrations (2-15 nM) of ATRA by either exogenous 8-CPT-cAMP treatment (Fig. 1) or a sustained increase in the endogenous level of cAMP produced by an autonomous bacterial adenylate cyclase (Quenech'Du et al., 1998). We observed (Fig. 5B) a significant synergistic effect on maturation only when combining cAMP analogs that complemented each other for binding to the cAMP sites (A, B) of both PKA-RI and PKA-RII, like 8-Pip-cAMP (AI, BII), 2-Cl-8-MA-cAMP (BI, BII) and N^6-MBC-cAMP (AII). This result indicates that co-activation of PKA-I and PKA-II is required to trigger the maturation response also of NB4 cells. This was confirmed in a second series of experiments with other site-specific cAMP analogs using the NBT maturation assay. The site AI-selective N^6-Bz-8-piperidino-cAMP was combined with the site BI-preferring 2-Cl-8-AHA-cAMP to achieve preferential activation of PKA-I, and the site AII-preferring N^6-MCB-cAMP combined with the site BII-preferring 5,6-DiCl-cBIMPS to achieve PKA-II activation. We found no differentiating effect of either cAMP analog pair alone at 2 nM ATRA (Fig. 5C, left) and only a moderate effect at 15 nM ATRA (Fig. 5C, right). However, the PKA-I and PKA-II directed pairs acted with strong synergy to produce a similar level of maturation as obtained with 0.2 mM 8-CPT-cAMP (Fig. 5C). The synergizing effect of adding the PKA-II directed analog pair to the PKA-I pair was not due to the activation of the exchange protein activated by cAMP (Epac), another intracellular target of cAMP, Indeed, the Epac specific cAMP analog 8-pMeOPT-2'O-Me-cAMP alone was unable to promote ATRA-induced differentiation of NB4-LR1 contrary to N6-benzoyl-cAMP, a poor Epac agonist and a full PKA activator (Supplemental Fig. 1). Furthermore, a strong synergy was observed also when the PKA-I pair was mixed with the site AII-directed analog N^6-MBC-cAMP (Fig. 5B), which does not activate Epac (Christensen et al., 2003).

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The PKA-I and PKA-II directed cAMP analog pairs were tested for their ability to activate PKA in extracts prepared from either NB4 or NB4-LR1 cells. Figure 6 shows that the PKA in the NB4 cell extracts was only partially activated when the cAMP analog 8-PIP-cAMP was combined either with N^6-MBC-cAMP to achieve selective PKA-II activation or with 2-Cl-MA-cAMP to achieve PKA-I activation. Only the triple combination activating both PKA-I and PKA-II achieved near full activation. For extracts from the NB4-LR1 cells that contain more PKA-I and less PKA-II (see Fig. 2-4) the PKA-I directed cAMP analog pair was more efficient than the PKA-II directed analog pair, but again the triple combination gave the highest PKA activity. These results support the idea that both PKA-I and PKA-II activation are required to achieve the high PKA activation that appears required for optimal stimulation of ATRA-induced cell differentiation.

In NB4-LR1 cells, PKA-RII is mainly in the non-phospho form at basal cAMP levels, but becomes autophosporylated at high cAMP concentration. The PKA-RII subunit, but not PKA-RI, can be autophosphorylated on a specific serine residue by the C subunit. The autophosphorylated RII subunit has about 10-fold decreased affinity for the C subunit of PKA, which leads to about 10 times higher sensitivity to activation by cAMP (Rangel-Aldao and Rosen, 1976; Rangel-Aldao and Rosen, 1977). The PKA-RII autophosphorylation state in APL cells could therefore give an important clue about the cAMP responsiveness of their PKA-II. We determined therefore the extent of PKA-RII autophosphorylation in non-stimulated and 8-CPT-cAMP stimulated NB4-LR1 and NB4 cells, using an anti-PKA-RIIα (pS99) phospho-directed antibody whose phospho-epitope specificity was confirmed by λ phosphatase treatment (data not shown). A strong constitutive phosphorylation of PKA-RIIα was visible in untreated NB4 cells and a slight increase was induced after treatment with 0.2 mM 8-CPT-cAMP combined or not with ATRA (Fig. 7). In constrast, PKA-RIIα phosphorylation was hardly detectable in untreated NB4-LR1 cells. However, and importantly, 8-CPT-cAMP treatment alone or combined with ATRA was able to reveal a significant enrichment of autophosphorylated PKA-RIIα. Note that even though the increase of phosphorylated PKA-RIIα could partly be related to the low increase in total RII levels in NB4-LR1 cells, the densitometric quantification of protein bands

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showed that in response to 8-CPT-cAMP, the mean level of phosphorylated-RIIα normalized to total PKA-RIIα is 2.7 fold higher than in non treated NB4-LR1 cells.

These results indicate that PKA-II is little autophosphorylated and thereby subresponsive to cAMP in NB4-LR1 cells with low cAMP level, but becomes more autophosphorylated and thereby more cAMP-responsive under strong stimulation of cAMP signaling.

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Discussion

The present study reveals an altered balance between PKA-I and PKA-II in the ATRA-maturation resistant NB4-LR1 APL cell line, whose ATRA resistance has been previously ascribed to lowered adenylate cyclase activity and less intracellular cAMP (Zhao et al., 2004). We showed that NB4-LR1 cells had a deficient ATRA response compared to wild type NB4 cells even when PKA was fully activated by a potent cAMP analog. In fact, these cells had a specific defect in PKA-RII, resulting from decreased expression of both PKA-RIIα and PKA-RIIβ isoforms. This decrease of RII subunits was compensated by an increase of the RIα subunit, the level of the PKA-Cα and the global PKA activity being unchanged. The isozyme switch led to a translocation of PKA since PKA-RII was concentrated in the perinuclear Golgi area, while PKA-RI had a general cytoplasmic distribution (Fig. 3). It is known that distinct cellular responses to cAMP can be elicited upon selective activation of one isozyme, and that the cAMP response can be influenced by PKA isozyme switching (Cho-Chung et al., 1995; Ji et al., 2008; Rohlff et al., 1993; Schwartz and Rubin, 1985). By using isozyme specific cAMP analogs, we demonstrated that the activation of the two PKA isoforms (PKA-I and PKA-II) pathways were required for retinoid dependent maturation of NB4-LR1 cells. The analog combinations used in the present study are the same as extensively validated for PKA-I and PKA-II selectivity *in vitro* (Ogreid et al., 1985), supplemented with second generation analogs with further enhanced *in vitro* PKA isozyme specificity as well as deficient ability to activate Epac. The first generation analog combinations were validated for cell use in fibroblasts with enforced expression of either RI or RII subunit (Otten et al., 1991). The improvements of the second generation analog 2-Cl-8-AHA-cAMP are a further enhanced specificity for cAMP site B of PKA-I and a very low affinity for Epac (Dao et al., 2006). The second generation analog N6-Bz-8-pip-cAMP has both improved selectivity for site AI and a complete lack of ability to activate Epac due to its N^6-substitution (Christensen et al., 2003). These observations point to a specific key role of the PKA-II for maturation even though PKA-I is upregulated probably due to a compensation mechanism. Therefore, PKA-I activation and PKA-II activation are essential to support the maturation of both NB4-LR1 and NB4 cells (Fig. 5). These conclusions were supported by the *in vitro* assay showing the contribution of each PKA isozyme to the total PKA activity. While a cAMP stimulation strong enough to activate both

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PKA-I and PKA-II will enhance the sensitivity of the NB4-LR1 cells to pharmacological ATRA concentrations, it cannot overcome the intrinsic sub-sensitivity of these cells to low ATRA concentrations. In fact NB4-LR1 cells remained maturation-resistant at physiological ATRA concentrations even at maximally stimulatory concentrations of cAMP agonists. However, even though PKA-I and PKA-II needs to be activated, it remains to be established whether they need to be activated at the same time or consecutively. Several genes that appear to be regulated by ATRA may be under the control of cAMP-PKA signalling pathway supporting the idea of complex signalling network. Therefore, it will be important to identify downstream targets of PKA-I/PKA-II co-activation.

The requirement for PKA-II activation to achieve NB4-LR1 cell maturation (Fig. 5A) led us to study whether the juxtanuclear PKA-RII could be sub-sensitive to cAMP in the NB4-LR1 cells and therefore could provide an efficient brake on PKA-dependent signaling unless exposed to high level of cAMP or cAMP analogs. In tissues like heart, cAMP stimulation converts the PKA-RII from the more cAMP-sensitive autophosphorylated state to the relatively cAMP-insensitive dephosphorylated state (Manni et al., 2008), which will lead to a "negative feed-back" of the cAMP-stimulated PKA activity (Kopperud et al., 2003). In contrast, in unstimulated NB4-LR1 cells, PKA-II was mainly in the non-autophosphorylated state, and became strongly phosphorylated in cells exposed to the strong cAMP agonist, 8-CPT-cAMP. The PKA-II will therefore exhibit a "feed-forward" response to increasing concentrations of cAMP agonists.

The low cAMP sensitivity of the nonphosphorylated PKA-II in the NB4-LR1 cells represents a challenge for the achievement of cAMP-stimulated maturation of NB4-LR1 cells at pharmacological ATRA concentrations, for which both PKA-II and PKA-I must be activated. This challenge is aggravated by the finding that the juxtanuclear cell compartment where PKA-II is anchored generally has lower cAMP concentration than in the general cytoplasm or close to the cell membrane (DiPilato et al., 2004). The juxtanuclear localization of PKA-RII is a key position to control the nuclear translocation of the free C subunit of PKA, whether it is derived from dissociation of PKA-II or PKA-I. In this connection, it is of interest that many potential PKA targets mediating cAMP-enhanced ATRA-induced APL cell differentiation are nuclear like RARs, RXRs, PML-RAR, CREB and its co-

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activators (Altucci et al., 2005; Harish et al., 2000; Mayr and Montminy, 2001; Nasr et al., 2009; Rochette-Egly et al., 1995; Santos and Kim, 2010). Recently, PML-RARα Ser873 phosphorylation (Nasr et al., 2008) was identified as an important target of retinoid-cAMP cross-talk in APL cells. This is a valid target also in the NB4-LR1 cells whose PML-RARα has an intact Ser873 (data not shown). Attempts to enhance the maturation of partially ATRA-resistant APL cells through stimulation of cAMP signaling will therefore be expected to be limited by the activation of PKA-II rather than PKA-I. Targeting the perinuclearly located PKA-II could be achieved by inhibitors of the co-localized cAMP phosphodiesterases (Houslay, 2010; Tasken et al., 2001) or by cAMP analogs with higher efficiency for PKA-II than PKA-I (Supplemental Table 1).

Altogether, our results could have general clinical implications in APL in that they mean that not only PKA activity but also individual variations of PKA-I versus PKA-II equipment could condition the susceptibility of tumor cells to therapeutic treatments. Indeed, varying outcomes of retinoid therapy in patients could be explained in relation to imbalanced PKA-I/PKA-II expression. Therefore, the knowledge of this ratio would help in the prediction of ATRA resistance in patients and in the definition of a strategy for ATRA/cAMP combinatorial therapy for them. The presently studied NB4-LR1 cells may serve as paradigm for the ATRA-resistant APL cell subpopulations responsible for the recurrence of APL in some patients treated with ATRA (Ferrara, 2010). Our results suggest that special attention should be paid to the anchored PKA-II if cAMP-based therapy is considered to enhance ATRA-induced APL cell maturation in a clinical setting. While PKA-I may be activated by mild stimulators of the endogenous cAMP level, PKA-II may be hard to activate without making use of PKA-II directed cAMP analogs. Our data should contributes to define a molecular basis for the design of combinatorial therapy and encourage either the integration of PKA-II directed analogs or the development of agents directed at releasing PKA-RII from its AKAP into *in vivo* testing in mice.

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Authorship Contributions

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Footnotes

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Figure legends

FIGURE 1. A. Effect of strong PKA stimulation (0.2 mM 8-CPT-cAMP) on the NB4 and NB4-LR1 cell maturation response to various concentrations of ATRA. NB4 and NB4-LR1 cells were exposed to ATRA (0 – 16 nM) for 48 h. Maturation was visualized by the NBT reduction assay. B. Quantification of maturation in NB4 (white bars) and NB4-LR1 (black bars) cells exposed to ATRA in the presence of 0.2 mM 8-CPT-cAMP. Data are given as mean ± SD, *n*=3. C. *in vitro* PKA catalytic activity in NB4 and NB4-LR1 cells treated or not with 100 nM ATRA. Kinase activities were quantified in cell lysates by measuring the phosphorylation of the fluorescent kemptide in the absence or in the presence of 1 µM 8-CPT-cAMP (CPT). The addition of a highly specific PKA inhibitory peptide (PKI, 180 µM) was used as a means to determine the actual level of PKA activity.

FIGURE 2. PKA subunits expression in NB4 cell lines. A. Expression of PKA subunits (RIα, RIIα, RIIβ and C) analysed by Western-blot in NB4, NB4-LR1 and NB4-LR2 cells. Actin was used as a control. * indicates an additional minor protein band corresponding to a cross-reaction of anti-PKA-RIIβ antibody with PKA RIIα subunit. B. 2D-western-blot analysis of PKA-RII subunits in NB4 and NB4-LR1. The region of the blot corresponding to proteins sizes between 35 and 75 kD was cut out and probed consecutively with specific PKA-RIIβ, PKA-RIIα and vimentin antibodies (arrow). The lower panel presents the merged picture. C. Expression of PKA-RIIβ mRNA in NB4 and NB4-LR1 cells was analysed by quantitative RT-PCR, normalized to the expression of ribosomal P2 mRNA and expressed as a percentage of that detected in NB4 cells. Data are given as mean ± SD, *n*=4.

FIGURE 3. Immunolocalization of PKA regulatory subunits in NB4 and NB4-LR1 cells. A. Conventional fluorescence microscopy. The nuclei were stained in blue. PKA-RI (upper panel, RI) and PKA-RIIβ (lower panel, RIIβ) were stained in green, PKA-RIIα (middle panel, RIIα) was stained in red. Note the prominent staining of the Golgi area by anti-PKA-RIIα (in NB4 and NB4-LR1) and anti-PKA-RIIβ (in NB4 only). Scale bar: 10 µm. B. Confocal microscopy analysis showing a cytoplasmic and nuclear localization of PKA-RI and the Golgi localization of PKA-RIIα in both NB4

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and NB4-LR1 cells. Double immunofluorescent staining of PKA RIIα (in red) and β-COP (in green) in NB4 and NB4-LR1 cells. Scale bar: 10 μm

FIGURE 4. PKA-RII is not induced by ATRA and 8-CPT-cAMP co-treatment in NB4-LR1 cells. NB4-LR1 cells were treated in conditions inducing cell maturation (1 μM ATRA; 0.2 mM 8-CPT-cAMP, CPT). PKA regulatory subunit expression (RIIβ, RIIα, RIα) was determined in cell lysates using specific antibodies. Actin, vimentin and GAPDH expressions were used as references. NB4 and NB4-LR2 cells were used as positive controls for PKA-RII subunits.

FIGURE 5. Activation of both PKA I and PKA II is required for APL cell maturation. NB4-LR1 (A) and NB4 cells (B) were cultured in the absence or in the presence of 100 nM or 2 nM ATRA, respectively, alone or in combination with site-specific cAMP agonists selective for PKA-I and PKA-II (see Supplemental Table 1) for 72 h. Maturation scores were estimated according to the DCF assay. The relative fluorescence of each sample (means of triplicates) corresponds to the ratio of the fluorescent signal measured versus the fluorescent signal of untreated NB4-LR1 cells. Induction of maturation was obtained with 0.2 mM 8-CPT-cAMP (positive control). Individually, site-selective analogs (0.1 mM) associated to ATRA treatment did not induce cell maturation. Pairs of cAMP analogs, triggering either PKA-I or PKA-II did not induce cell maturation. Maturation response was reached only when both PKA-I and PKA-II were activated by the combination of the three site-specific cAMP analogs. (C) NB4 cells were treated for 48 h with 2 nM (left) or 15 nM (right) ATRA together with a maximally effective concentration (0.2 mM) 8-CPT-cAMP (grey line indicated by the arrow) or in combination with site specific cAMP agonists selective for PKA-I (2-Cl-8-AHA-cAMP +N^6-Bz-8-Pip-cAMP) and PKA-II (N^6-MBC-cAMP or Sp5,6 DiCl-cBIMPS) (see supplemental Table 1). Maturation was assayed with NBT reduction assay. Data are given as mean ± SEM, *n*=2-3.

FIGURE 6. Activation of both PKA I and PKA II is required for full PKA activation. PKA activity was quantified in cell lysates by measuring the phosphorylation of the fluorescent kemptide in the

absence or in the presence of site-specific cAMP agonists selective for PKA-I and PKA-II. The relative PKA activity (measured as the PKI-sensitive portion of kemptide phosphorylation) was expressed as the percentage of the maximal activity measured when the cell lysates were incubated in the presence of 1 μM 8-CPT-cAMP. A near full PKA activation was reached only when both PKA-I and PKA-II were activated by the combination of the three site-specific cAMP analogs. Data are given as mean ± SEM, *n*=2-3.

FIGURE 7. Activation of PKA-RIIα by 8-CPT-cAMP treatment.. A.NB4 and NB4-LR1 cells were treated with 1 μM ATRA and 0.2 mM 8-CPT-cAMP alone or in combination for 24 hours. Total PKA-RIIα and its serine 99 phosphorylated form were analysed by western blot using an anti-PKA-RIIα and an anti-PKA-RIIα (pS99) phospho-specific antibody, respectively. GAPDH served as a loading control. B. Phosphorylated and total PKA-RIIα bands were quantified by densitometry and normalized to GAPDH band. The results are expressed as percentage relatively to the control non treated NB4 cells arbitrary set to 100. Values are the means of three independent experiments.

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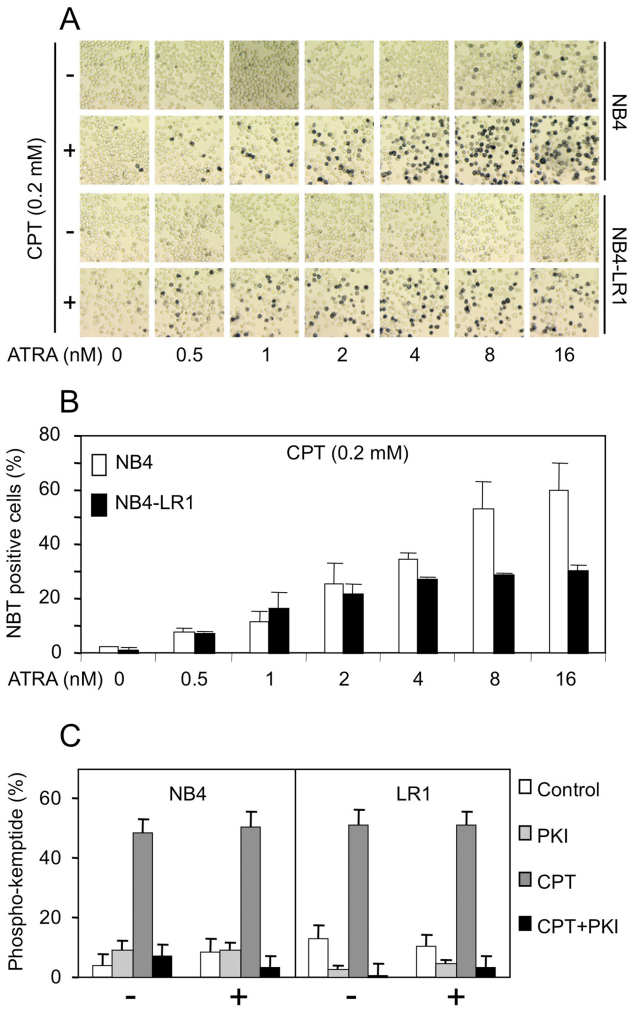

Figure 1

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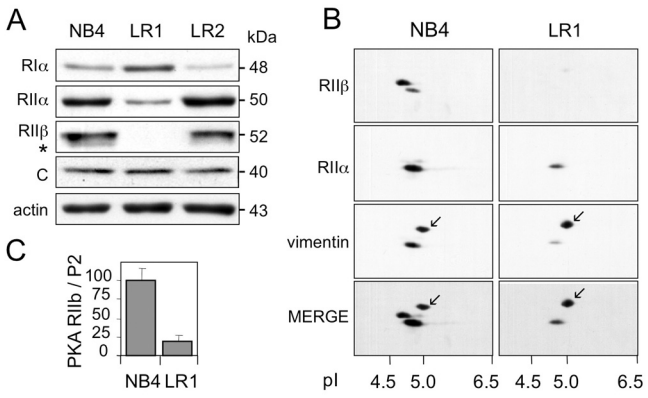

Figure 2

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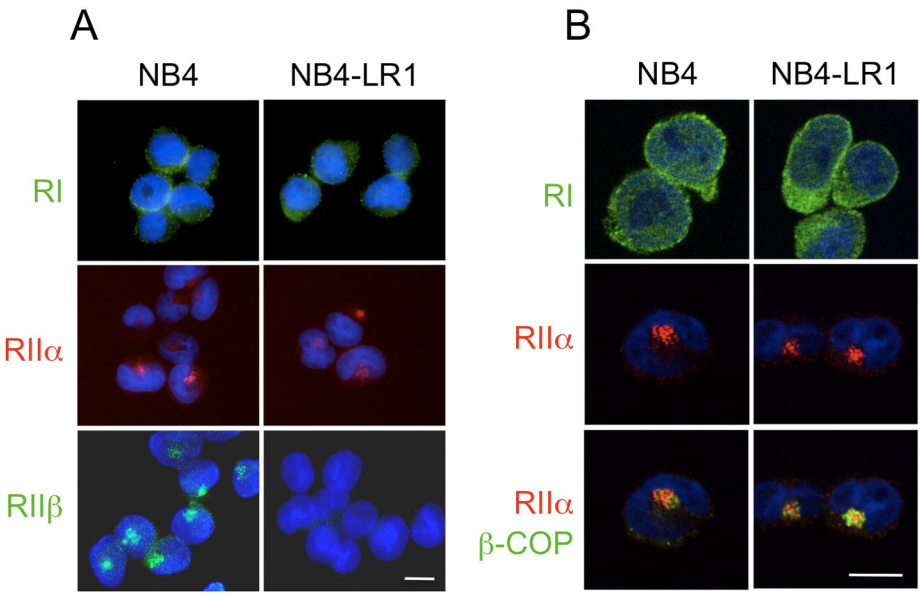

Figure 3

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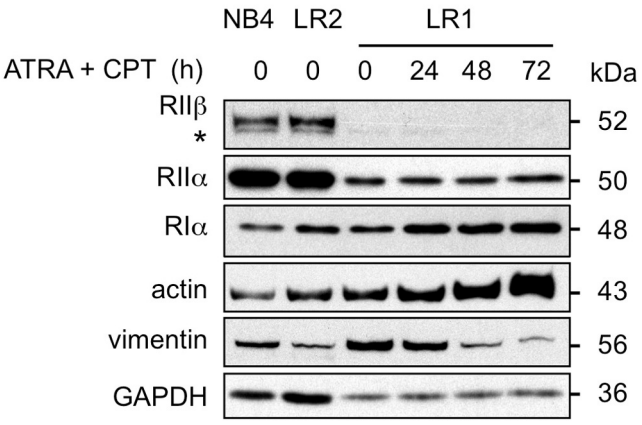

Figure 4

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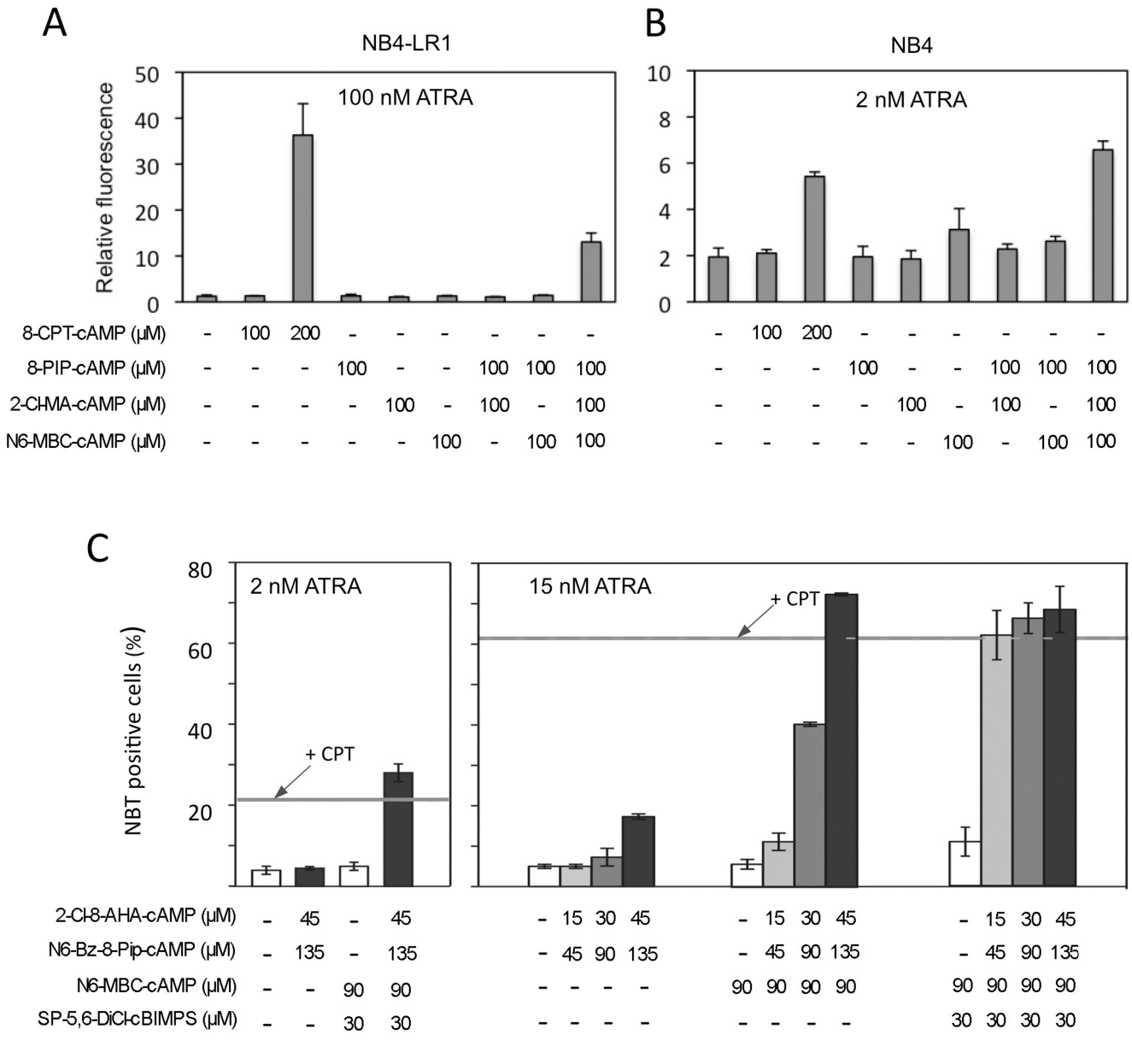

Figure 5

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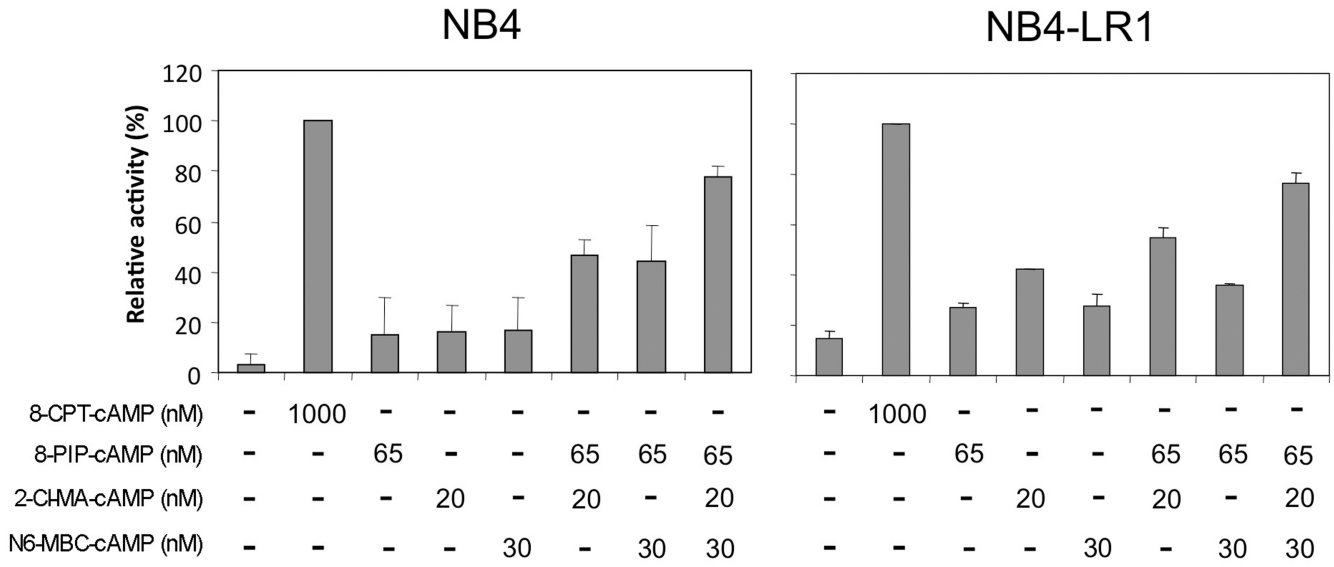

Figure 6

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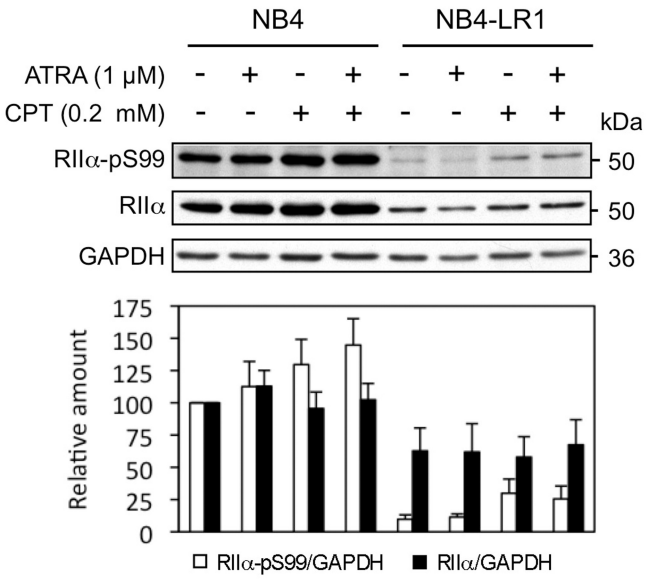

Figure 7