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Effect of phosphodiesterase antagonists on glucocorticoid mediated growth inhibition in murine skin cell lines

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ABSTRACT

The effects of two cyclic nucleotide phosphodiesterase type 4 (PDE4) inhibitors on proliferation of cell lines representing different stages of mouse skin tumorigenesis were studied. Skin papillomas and carcinomas become resistant to the growth inhibition by glucocorticoids. Their control of cellular functions is mediated by a well-known transcription factor, glucocorticoid receptor. The primary aim of the present study was to determine whether the PDE4 inhibitors, that raise intracellular cAMP levels, can increase the sensitivity of mouse skin papillomas and carcinomas to the glucocorticoids. We sought to establish the effect of cAMP signaling on the glucocorticoid receptor function using well-known model representing non-tumorigenic keratinocyte cell line (3PC), papilloma (MT1/2) and squamous cell carcinoma cell line (Ca3/7). These cells were treated with the glucocorticoid fluocinolone acetonide (FA) alone or in concert with PDE4 inhibitors – rolipram or YM976. Results of our study revealed that both PDE4 inhibitors may increase the sensitivity of transformed cell lines to the growth inhibitory effect of FA. In the transformed cell lines, changes in the viability of cells were accompanied by an increase in mRNA level of two negative regulators of the cell cycle – p21 and p27 proteins. Co-treatment with PDE4 inhibitors and FA caused inhibition of an endogenous glucocorticoid-responsive gene (MT-1) expression. Thus, the PDE4 inhibitors exerted a differential effect on non-transformed and transformed keratinocytes and on glucocorticoid receptor signal transduction. These findings warrant further studies to clarify the mechanism by which PDE4 inhibitors modulate glucocorticoid receptor signal transduction in transformed cells.

1. Introduction

Glucocorticoids are very effective in preventing carcinogen- and tumor promoter-induced skin inflammation, hyperplasia, and mouse skin tumor formation when applied to skin together with a carcinogen (Schwarz et al., 1977; Slaga, 1984). During tumor promotion mouse keratinocytes become resistant to these hormones (Strawhecker and Pelling, 1992). Many rodent and human tumors become resistant to glucocorticoids mediated growth inhibition (Li et al., 1996; Moalli and Rosen, 1994). The reason of this resistance is caused by a low expression level of glucocorticoid receptor or expression of its altered form (Okret et al., 1991; Ray et al., 1996). No genetic alternation has been found in transformed mouse keratinocytes and glucocorticoid receptor mRNA and protein levels remain not affected in most mouse skin tumors (Spiegelman et al., 1997; Budunova et al., 1997). The cellular response to glucocorticoids is mediated through highly specific glucocorticoid receptor which belongs to a superfamily of nuclear hormone receptors (Schwarz et al., 1977; Mangelsdorf et al., 1995) and as in other family members, glucocorticoid receptor consists of several functional domains responsible for ligand and DNA binding, dimerization and transactivation (Tsai and O'Malley, 1994). Once within the nucleus, glucocorticoid receptor binds to DNA sequences known as glucocorticoid response elements (GREs) and activates transcription of responsive genes (Beato et al., 1996). It may also directly interact with other nuclear transcription factors such AP-1 and NFκB (Caldenhoven et al., 1995; Yang-Yen et al., 1990; Ray and Prefontaine, 1994; Scheinman et al., 1995).

Type 4 phosphodiesterases (PDE4) from a superfamily of at least 11 isozymes catalyze the breakdown of cyclic (cAMP) and/or cyclic GMP (cGMP) function in cells to control the resting level of cyclic nucleotides and to restore the steady-state levels following stimulatory events that utilize cAMP or cGMP as second messengers (Beavo, 1995). Their inhibition suppresses the release of inflammatory signals, e.g., cytokines, and inhibits the production of reactive oxygen species. Also, inhibition of the phosphodiesterase 4 activity could increase glucocorticoid receptor sensitivity, induce growth arrest in lymphoid cells and enhance glucocorticoid-mediated apoptosis (Miller et al., 2002; Ogawa et al., 2002; Meyers et al., 2007). Inhibitors of PDE4 can also influence the cell growth of murine carcinoma cells (Marko et al., 1998).

We have employed two PDE4 inhibitors: rolipram and YM976. Rolipram is a potent PDE4 inhibitor and since its discovery (Schneider

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et al., 1986), has been widely used as the reference drug for the characterization of this isoenzyme (Bertolino et al., 1988). YM976, 4-(3-chlorophenyl)-1,7-diethylpyrido [2, 3-d] pyrimidin-2(1H)-one, is a novel and selective PDE4 inhibitor whose structure is totally different from other PDE4 inhibitors (Aoki et al., 2000). Both compounds are selective inhibitors of PDE4 with proven anti-inflammatory properties although structurally different. We have examined whether these PDE4 inhibitors are able to increase the sensitivity of transformed keratinocytes cell lines, to the glucocorticoids. Analyzing the expression level of negative regulators of the cell cycle, we have tried to elucidate the effect of PDE4 inhibitors on the cell cycle regulation.

2. Materials and methods

2.1. Chemicals

The glucocorticoid, fluocinolone acetonide (FA) and PDE4 inhibitors, rolipram and YM976, were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell lines and cell culture conditions

Three murine keratinocyte cell lines, non-tumorigenic (3PC), papilloma (MT1/2), and squamous cell carcinoma (Ca3/7) cell lines, representing various stages of malignant transformation (Klann et al., 1989; Conti et al., 1988) were obtained from the University of Texas M. D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX. Note that the susceptibility of 3PC cells to different stimuli was, in general found to be comparable to that of primary keratinocytes (Klann et al., 1989; Jansen and Jongen, 1996). All cell lines were cultured at 37 °C in the 5% CO_2 in MEM Eagle Joklik Modification Medium with low calcium content (Sigma-Aldrich) supplemented with insulin (5 μg/ml), epidermal growth factor (5 ng/ml), transferrin (10 μg/ml), *O*-phosphoethanolamine (10 μM), ethanolamine (10 μM), penicillin (50 U/ml), streptomycin (50 ng/ml), gentamicin sulfate (50 μg/ml) and 8% fetal bovine serum.

2.3. Cell growth and cell cycle analysis

For the quantitative evaluation of proliferation of cultured cells we have initially used both the ATPLite Luminescence ATP Detection Assay System (PerkinElmer, Boston, MA) and the MTT assay. The ATPLite system is based on the measuring ATP concentration (ATP is a marker for cell viability because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis). Cells were plated (10^3 per well) in 96-well plates in medium containing charcoal stripped serum and were allowed to adhere overnight before being treated with vehicle or tested compounds for an appropriate time. The ATPLite assay was performed according to the manufacturer's instructions.

For the cell cycle distribution analysis, cells were plated in 10 cm ∅ plate, in medium serum containing FA at the concentration of 1 μM, rolipram and YM976, both at the concentration of 10 μM, or combination of FA (1 μM) and one of the PDE4 inhibitors (10 μM) for 72 h. After that, cells were trypsinized, counted using hemocytometer, fixed in 75% ethanol and stained with propidium iodide. Samples were analyzed using a FACSCalibur Analyzer (Becton Dickinson, San Jose, CA) at The Flow Cytometry Core of the University of Texas Health Science Center at San Antonio.

2.4. Determination of cAMP concentration

The cAMP concentration was analyzed using a cAMP kit from R&D systems (Minneapolis, MN). This assay is based on the competitive binding technique in which cAMP present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled cAMP for sites on a mouse monoclonal antibody.

2.5. Western blot analysis

Equal amounts of proteins from differently treated cells were analyzed using specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). For Proliferating Cell Nuclear Antigen (PCNA), p21, p27 and β-actin detection, the primary antibodies were: mouse monoclonal anti-PCNA, anti-p21, anti-p27 and mouse monoclonal anti-β-actin IgG, used at 1:500 (for PCNA and β-actin) and at1:200 (for p21 and p27) dilution; the secondary antibody was goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA), used at 1:2000 dilution. Detection by a chemiluminescence reaction was carried out using a Western Blotting Luminol Reagent (Santa Cruz, CA), followed by exposure to a Kodak BioMax Light film (Kodak, Rochester, NY). The amount of PCNA was quantitated by using ImageQuant Software and normalized by β-actin.

2.6. Real time PCR analysis

Total RNA was extracted using TRI Reagent (MRC, Inc. Cincinnati, OH). RNA (1 μg) was reverse transcribed with oligo(dT), using a cMaster RT kit (Eppendorf North America, Westbury, NY) according to the manufacturer's protocol. Primer sets of:

5′-CTCAGACACCAGAGTGC-3′, 5′-GACAGTGAGCAGTTGCG-3′ for p21,
5′-TCAAACGTGAGAGTGTCTAACGG-3′, 5′-AGGGGCTTATG-ATTCTGAAAGTCG-3′ for p27, 5′-TGCTCCACCGGCGG-3′, 5′-TTTGC-AGACACAGCCCTGG-3′ for MT-1, and
5′-CATCCTGGCCTCGCTGTC-3′, 5′-CTCGTCGTACTCCTGCTTGGT-3′ for β-actin, were used and gave the unique products. Standard quantitative Real Time PCR (RT-PCR) was performed in triplicate using SYBR Green RealMaster Mix (Eppendorf North America, Westbury, NY) on the Realplex MasterCycler (Eppendorf,

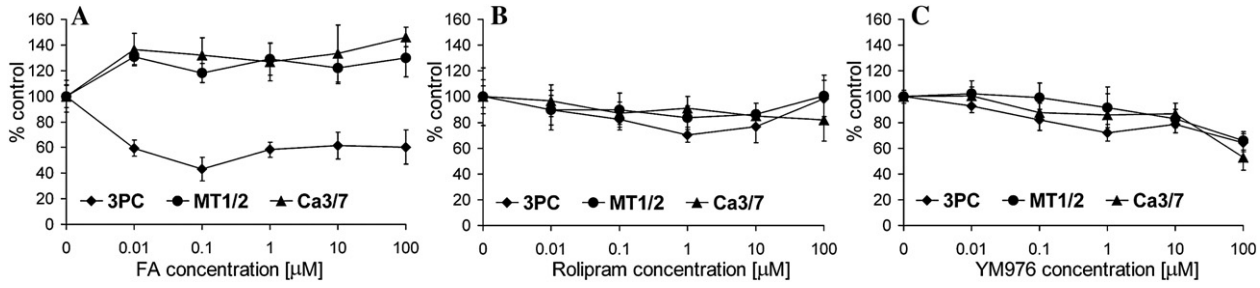

Fig. 1. Effect of FA (A), rolipram (B) and YM976 (C) on cell growth. Cultures of untransformed 3PC cells, papilloma-producing cells MT1/2, and carcinoma-producing cells Ca3/7 were treated with different concentrations of tested compounds for 72 h in 96 wells plate. Each point with vertical bar represents the average (±S.D.) of luminescence signal obtained using the ATPLite system from five wells per group. The average of luminescence signal is presented as percentage of cells treated with ethanol only.

Westbury, NY). RT-PCR cycle thresholds (Ct) of candidate genes were normalized to β-actin. The formula 2*Ct*(Candidate)/2Ct (Control) was used to calculate the normalized ratios.

2.7. Data analysis and statistic

The results were expressed as means ± standard deviation (S.D.). For comparison of the differences between the groups a two-tailed, unpaired, Student's *t* test was used. Differences were considered statistically significant at a *P* value <0.05 (*).

3. Results

3.1. Effect of glucocorticoids and PDE4 inhibitors on cell growth and cell cycle distribution

Three murine keratinocyte cell lines, non-tumorigenic (3PC), papilloma (MT1/2), and squamous cell carcinoma (Ca3/7) cell lines were incubated in 96 wells plate (1000 cells per well) for 72 h in the presence of FA, rolipram and YM976 (Fig. 1). Initiated keratinocytes and cells corresponding to different stages of epidermal tumor development in mouse skin were analyzed with respect to their 3′,5′-cyclic adenosine monophosphate (cAMP) hydrolyzing activity. Treatment with concentrations >2 μM of rolipram, highly effective PDE inhibitor resulted in induction of apoptotic cell death.

As it was previously found, among tested cell lines only 3PC keratinocytes responded to the growth inhibition by FA (Spiegelman et al., 1997). Both MT1/2 and Ca3/7 cells were resistant to the growth inhibition by FA. For the cell cycle distribution analysis, cells were grown in 10 cm ∅ plate, in medium serum containing FA at the concentration of 1 μM, rolipram and YM976, both at the concentration of 10 μM, or combination of FA (1 μM) and one of the PDE4 inhibitors (10 μM) for 72 h after 72 h of treatment. After that, cells were trypsinized, counted using hemocytometer, stained with propidium iodide and analyzed by flow cytometry. Cell cycle analysis showed that FA treatment of 3PC cells resulted in G1 arrest (Fig. 2A) and FA treatment of MT1/2 and Ca3/7 cell lines stimulated cell proliferation (decrease cell number in G2 phase and increase in G1 phase) (Fig. 2B and C). Cell growth of all three cell lines remained nearly unaffected when grown in the presence of rolipram. YM976 treatment of all three cell lines resulted in a slight inhibition of growth at concentration of 10 μM (20% of inhibition), and 100 μM (40% of inhibition). Rolipram and YM976 presence (both at 10 μM) did not show any influence on the cell cycle distribution (Fig. 2) of all studied cell lines. We also did not find any signs of caspase-3 and caspase-9 activation during the treatment of the PDE4 inhibitors up to 72 h (data not shown).

In order to check the influence of PDE4 inhibitors on the effect mediated by FA, 3PC, MT1/2 and Ca3/7 cell lines were incubated in 96 wells plate (10^3 cells per well) for 72 h in the presence of FA (0.01, 0.1, 1.0 and 10 μM) and rolipram or YM976 at the concentration of 1 and 10 μM (Fig. 3). Both PDE4 inhibitors increased number of 3PC cells treated with FA (Fig. 3A and B), and at concentration of 10 μM reversed the FA effect decreasing cell percentage in G1 phase from 68.9% to 56.4% (rolipram) and 55.9% (YM976) (Fig. 2A). In fact, rolipram at the concentration of 10 μM completely reversed the growth inhibitory effect of FA (Fig. 3A). On the other hand, both rolipram and YM976 exerted a strong inhibitory effect on the growth of MT1/2 cells cultured in the presence of FA (Fig. 3C and D), significantly increasing number of cells in G1 phase (Fig. 3B). The effect of both PDE4 inhibitors on the growth of the Ca3/7cell line in the presence of FA was less pronounced. Thus, incubation of Ca3/7 cells with rolipram (10 μM) resulted in overcoming the stimulatory effect of FA, while incubation with YM976 (10 μM) resulted in decreasing the cell growth by 30%. Results obtained from manual cell counting fully confirmed data from ATP assay.

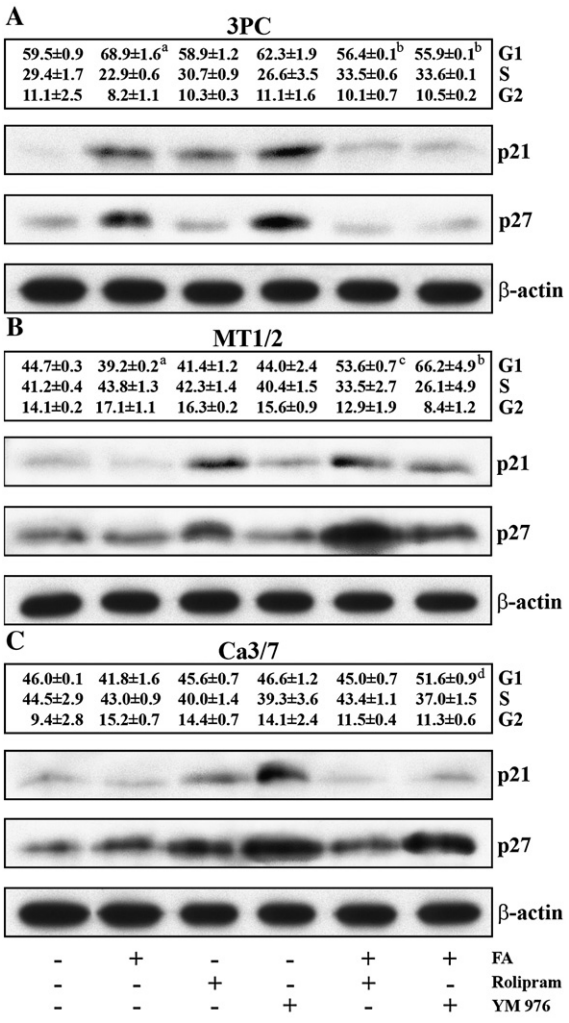

Fig. 2. Effect of FA and PDE4 inhibitors on the $p21^{CIP/WAF}$ and $p27^{KIP1}$ protein level and on the cell cycle distribution of 3PC (A), MT1/2 (B) and Ca3/7 (C) cell lines. Cells were incubated in the presence of FA (1 μM), rolipram (10 μM), YM976 (10 μM) or with a combination of FA (1 μM) and rolipram (10 μM) or YM976 (10 μM) for 72 h. The p21 and p27 proteins were detected by Western blot analysis. For normalization of differences in the amounts of protein loaded onto gel, the procedure was repeated with β-actin antibodies. For the cell cycle distribution analysis, after 72 h of treatment, cells were harvested, stained with propidium iodide and analyzed by flow cytometry. The quantitative data in each panel are average (±S.D.) from at least three repetitions, and were reproducible in an additional independent experiment. a, $P<0.01$ vs. non-treated group; b, $P<0.01$ vs. FA treated group; c, $P<0.001$ vs. FA treated group; d, $P<0.05$ vs. FA treated group.

3.2. Effect of FA and PDE4 inhibitors on the PCNA level in mouse keratinocytes

To examine the expression of the PCNA gene in 3PC, MT1/2, Ca3/7 cells, we used Western blot analysis. The cells were plated in 10 cm ∅ plate, in medium containing charcoal striped serum containing FA at the concentration of 1 μM, rolipram and YM976, both at the concentration of 10 μM, or combination of FA (1 μM) and one of the PDE4 inhibitors (10 μM) for 72 h. Results of this study revealed that FA did not influence the PCNA protein level in non-tumorigenic and papilloma cells while it caused a marked increase in the PCNA level in squamous carcinoma cells (Fig. 4). The PCNA protein level in 3PC cells was not significantly affected by both PDE4 inhibitors, even when they were applied together with FA. Meanwhile, YM976 caused a significant decrease of the PCNA level, *i.e.*, approximately by 30% in MT1/2 papilloma cells, and by 60% in Ca3/7 carcinoma cells. Co-treatment with YM976 and FA resulted in reversing of FA effect; PCNA

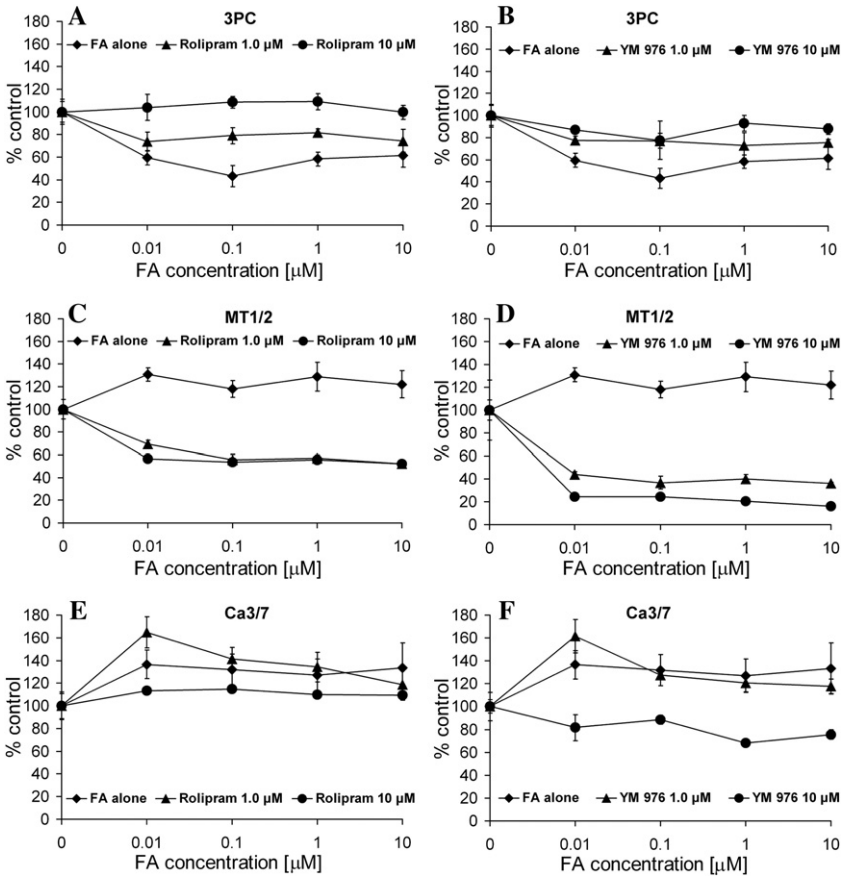

Fig. 3. Effect of co-treatment of PDE4 inhibitors with FA on cell growth. 3PC (A and B), MT1/2 (C and D) and Ca3/7 (E and F) cells were treated with different concentration of FA (0.01, 0.1, 1.0 and 10 μM) in the presence of rolipram (1.0 or 10 μM) or YM976 (1.0 or 10 μM) for 72 h in 96 wells plate. Each point with vertical bar represents the average (±S.D.) of luminescence signal obtained using the ATPLite system from five wells per group. The average of luminescence signal is presented as percentage of control cells treated with ethanol only.

level was lowered by YM976 in MT1/2 and Ca3/7 cell lines – in comparison to cells treated with FA only. The effect of rolipram was less pronounced. It did not significantly influence PCNA expression when applied alone and appeared to cause only a slight decrease of the PCNA level in MT1/2 when applied together with FA (comparing to FA only). These results seem to correlate with the results obtained using the ATPLite assay (see Figs. 1 and 3).

3.3. Rolipram and YM976 treatment up- regulates both $p27^{KIP1}$ and $p21^{CIP/WAF1}$ CDK inhibitors

To take a deeper look inside the molecular mechanism of the effect exerted by PDE4 inhibitors on murine keratinocytes, we have analyzed mRNA and protein levels of p21 and p27, two negative regulators of the cell cycle. Analysis of RNA and protein samples (by real time PCR and Western blot) isolated from 3PC, MT1/2 and Ca3/7 cell lines treated with rolipram and YM976 showed the elevated level of p21 mRNA and protein in all studied cell lines (Figs. 2 and 5A). Both inhibitors influenced also the expression of p27 gene but in 3PC and MT1/2 lines the effect was not so spectacular as in the case of p21 (Figs. 2 and 5B). On the other hand, the effect of both PDE4 inhibitors, on the p27 expression in Ca3/7 cells was very distinct (Figs. 2C and 5B).

3.4. Differential effects of PDE4 inhibitors on $p27^{KIP1}$ and $p21^{CIP/WAF}$ mRNA levels in keratinocytes stimulated by FA

Treatment of keratinocytes with FA at the concentration 1 μM for 72 h caused significant increase of p21 and p27 mRNA and protein levels in 3PC cells; however, no effect in the papilloma cell line MT1/2 and only a slight increase for p27 protein level in the carcinoma cells Ca3/7 were observed (Fig. 2). Combination of both PDE4 inhibitors and FA exerted completely different effects on non-transformed (3PC) and transformed keratinocytes (MT1/2 and Ca3/7). Co-administration

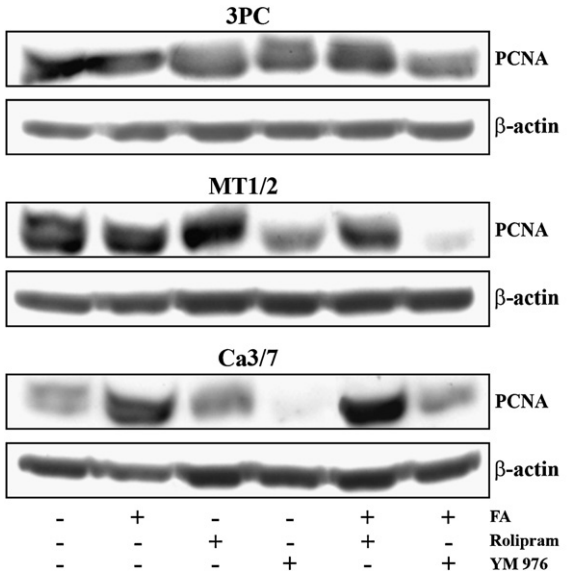

Fig. 4. Effect of FA and PDE4 inhibitors on the PCNA level. Cells were incubated in the presence of ethanol (EtOH), FA (1 μM), rolipram (10 μM), YM976 (10 μM) or with a combination of FA (1 μM) and rolipram (10 μM) or YM976 (10 μM) for 72 h. The PCNA protein was detected by Western blot analysis. For normalization of differences in the amounts of protein loaded onto gel, the procedure was repeated with β-actin antibodies.

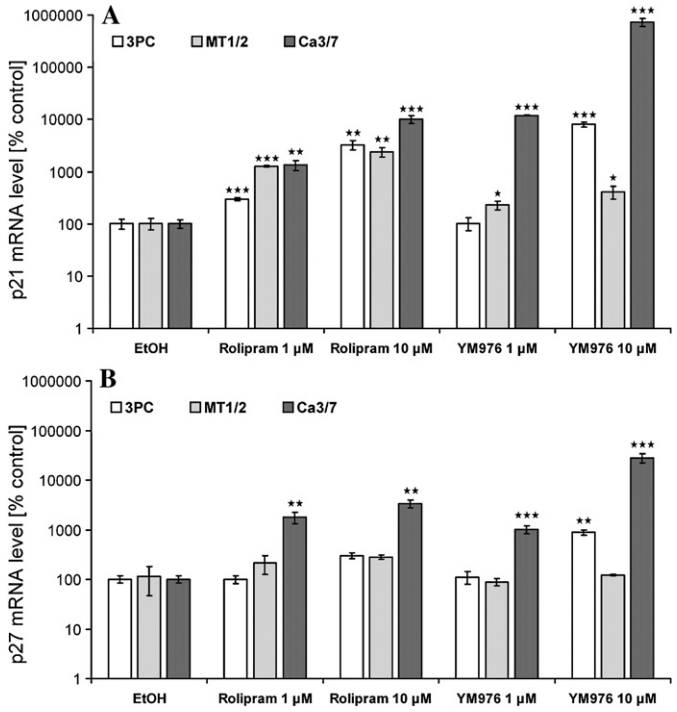

Fig. 5. Changes in $p21^{CIP/WAF}$ (A) and $p27^{KIP1}$ (B) mRNA level in 3PC, MT1/2 and Ca3/7 cell lines after treatment with rolipram and YM976. Cells were grown in 6 well plates for 72 h in medium containing rolipram (1 and 10 μM) or YM976 (1 and 10 μM), then were harvested and in isolated total RNAs, p21 and p27 mRNA levels were measured by RT-PCR assay. Each point with vertical bar represents the average (±S.D.) from four to six repetitions. *P* value<0.05 (*), <0.01 (**) or <0.001(***) vs. control, ethanol-treated group (EtOH).

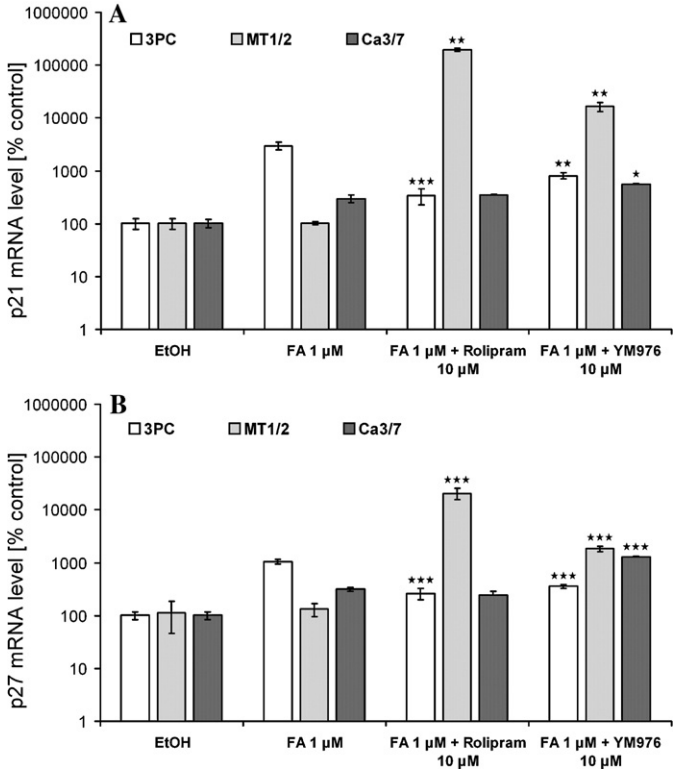

Fig. 6. Effect of PDE4 inhibitors on and $p21^{CIP/WAF}$ (A) and $p27^{KIP1}$ (B) mRNA level in keratinocytes stimulated by FA. Cells were grown in the presence of FA (1 μM) or in combination of FA (1 μM) with rolipram (10 μM) or YM976 (10 μM) for 72 h. Cells were harvested and in isolated total RNAs, p21 and p27 mRNA levels were measured by RT-PCR analysis. Each point with vertical bar represents the average (±S.D.) from four to six repetitions. *P* value<0.05 (*), <0.01 (**) or <0.001(***) vs. FA treated group (FA 1 μM).

of rolipram or YM976 with FA lowered FA-induced, mRNA and protein levels of both cell cycle inhibitors in 3PC cells, while in MT1/2 cells it caused a significant increase (Figs. 2 and 6). For p27 protein, similar effect was observed in Ca3/7 cell line treated with FA and YM976 (Fig. 2). Co-treatment with rolipram and FA did not have a significant influence on p21 and p27 expression in Ca3/7 cells. This finding seems to correlate with the results obtained from the proliferation study.

When administered together with FA, they increased proliferation rate of 3PC cells, decreasing percentage of cells in G1 phase (where p21 and p27 protein levels are going down) and decreased the growth of MT1/2 and Ca3/7 (increasing cell population in G1 phase) cells where both inhibitors of the cell cycle are upregulated (Fig. 2).

3.5. Effect of PDE4 inhibitors on FA stimulated expression of MT-1 gene

To determine whether PDE4 inhibitors can affect glucocorticoid receptor function in tumorigenic cell lines, the role of PDE4 inhibitors

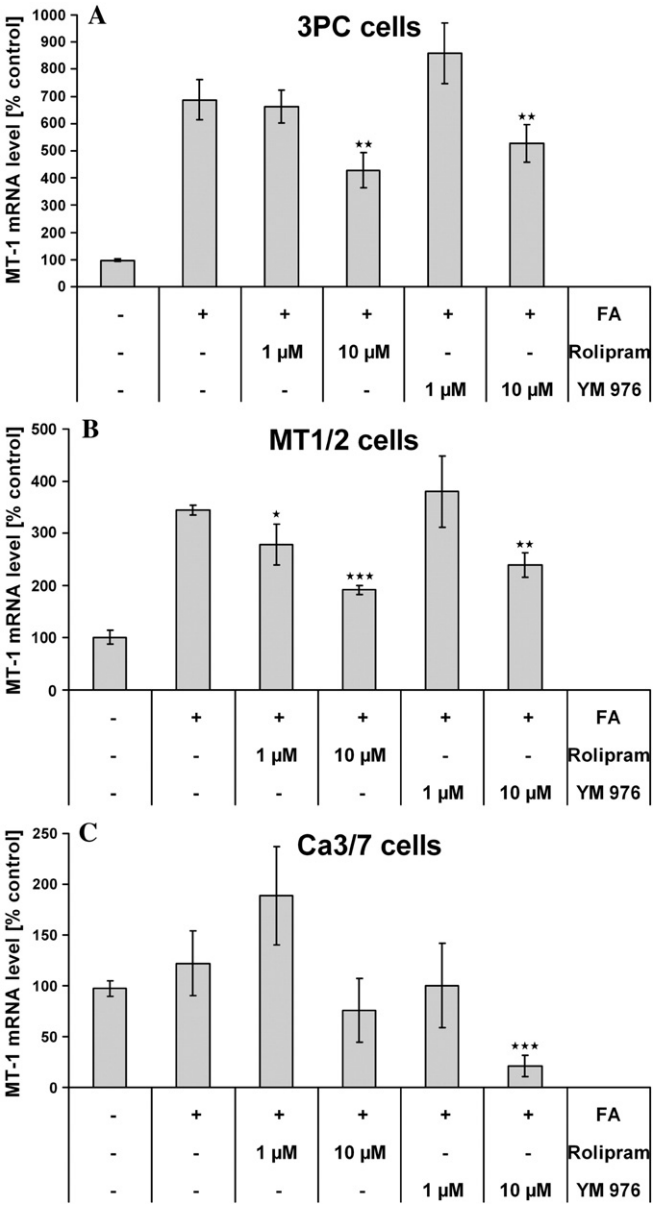

Fig. 7. Expression of MT-1 gene in 3PC (A), MT1/2 (B) and Ca3/7 (C) cells treated with FA, rolipram and YM976; mRNA levels were measured by RT-PCR analysis. Cells were exposed for 72 h in medium containing indicated concentration of tested compounds. Each point with vertical bar represents the average (±S.D.) from four to six repetitions. *P* value<0.05 (*), <0.01 (**) or <0.001(***) vs. FA treated group.

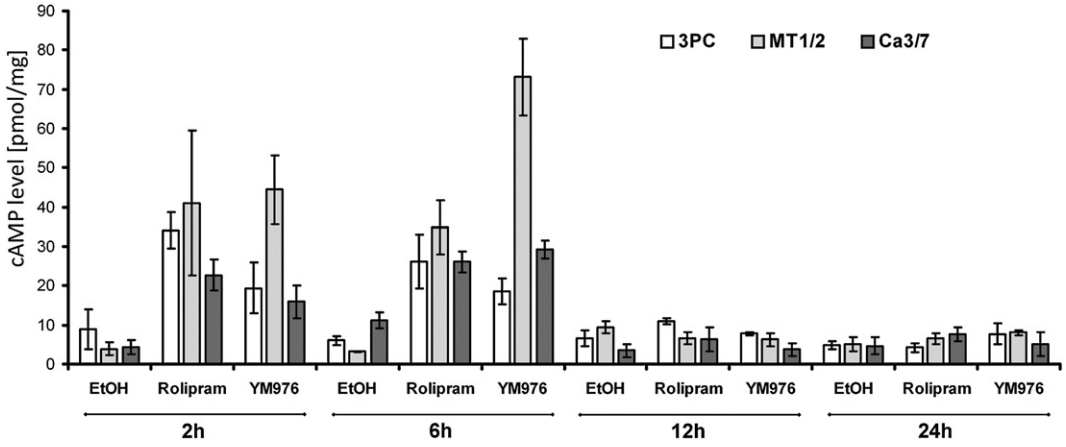

Fig. 8. Time-course of cAMP concentration in different keratinocyte cell lines exposed to YM976 (10 μM) and rolipram (10 μM). Each point with vertical bar represents the average (±S.D.) from three to five repetitions. Statistical significance of the data was observed only after 2 and 6 h of incubation.

on the FA stimulated expression of an endogenous glucocorticoid-responsive gene was studied. We have examined mRNA level of metallothionein 1 (MT-1), a small metal-binding protein expressed in mouse epidermis. Expression of the MT-1 gene is positively regulated by glucocorticoids in different tissues, and a sequence homologous to the GRE in the promoter region of this gene was described. Real time PCR analysis of RNA isolated from keratinocytes treated for 72 h with FA (1 μM), both PDE4 inhibitors (1 μM and 10 μM) or with their combinations showed a dramatic increase in the MT-1 mRNA level in the glucocorticoids sensitive non-tumorigenic 3PC cell line treated with FA only (Fig. 7). As expected, in MT1/2 cells, FA had a smaller effect on the MT-1 gene expression and did not significantly affect the MT-1 expression in Ca3/7 cells. Both PDE4 inhibitors slightly stimulated the MT-1 expression in MT1/2 cells (data not shown) but rolipram and YM976, combined with 1 μM FA caused considerable and statistically significant decreases in the MT-1 mRNA level in 3PC and MT1/2 cell lines at a concentration of 10 μM.

3.6. The effect of YM976 and rolipram on cAMP accumulation

Shown in Fig. 8 are time-courses for YM976 (10 μM) – and rolipram (10 μM)-induced accumulation of cAMP in the keratinocyte cell lines representing different stages of tumorigenesis. Both inhibitors caused great increase in cAMP level during first 6 h in all cell lines. The highest increase in cAMP level was observed in the papilloma cell line MT1/2. A dramatic increase in cAMP level was cause by YM976 in MT1/2 cells after 6 h of treatment. The prolonged incubation for 12, 24, 48, and 72 h showed cAMP levels typical to the control ethanol-treated cells.

4. Discussion

Glucocorticoids due to their anti-inflammatory and anti-proliferative properties, are widely used as drugs to treat different inflammatory, autoimmune and hyperproliferative diseases such as arthritis, asthma, dermatitis, psoriasis and many others. Glucocorticoids are also considered as anticancer agents and are used for the treatment of different hematological malignancies including leukemias, lymphomas and multiple myelomas. Even though, the role of glucocorticoids as anticancer agents in epithelial tumors has not been elucidated, it is well known that glucocorticoids prevent tumor promoter-induced inflammation and skin hyperplasia (Schwarz et al., 1977; Slaga, 1984); they are also very potent inhibitors of mouse skin tumor formation (Verma et al., 1983; DiGiovanni et al., 1988). On the other hand, transformed keratinocytes become resistant to the growth inhibitions by glucocorticoids (Budunova et al., 1997).

In the present study, we have shown that two selective inhibitors of PDE 4 could potentiate the effect of glucocorticoids in transformed, keratinocytes which are resistant to glucocorticoids. The anti-proliferative properties of PDE4 inhibitors have been reported in a variety of cell models but mostly against the T and B cells in lymphoid leukemias. Elevation in cAMP levels caused by rolipram or forskolin induces cell cycle arrest and apoptosis (Ogawa et al., 2002); anti-proliferative effect has also been demonstrated against murine carcinoma cells (Marko et al., 1998). A large body of data describes the effect of elevation of cAMP level on glucocorticoid receptor signal transduction but the synergistic effect of cAMP signaling and glucocorticoid signaling has been studied mostly in lymphoid cells (Ogawa et al., 2002; Miller et al., 2002; Meyers et al., 2007). However, the effect of PDE4 inhibitors on cells proliferation and on interaction with glucocorticoid signaling using mouse keratinocytes which are resistant to growth inhibition by glucocorticoids has not been investigated.

To study the action of PDE4 inhibitors in transformed mouse keratinocytes, we have used a panel of keratinocyte cell lines representing different stages of mouse skin carcinogenesis. All keratinocyte cell lines were derived from SENCAR mice to maintain similar genetic backgrounds in our experiments. The 3PC cell line is an immortalized mouse keratinocyte cell line originally derived from SENCAR mouse keratinocytes exposed *in vitro*, for 24 h to 7,12-dimethylbenz[*a*] anthracene (DMBA) (Klann et al., 1989). The MT1/2, papilloma-producing cell line was derived from SENCAR mouse keratinocytes initiated *in vivo* with *N*-methyl-*N*′- nitro-*N*-nitrosoguanidine followed by treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Conti et al., 1988). The Ca3/7, carcinoma-producing cell line was obtained from squamous cell carcinomas induced in SENCAR mice by the standard two-stage DMBA-TPA protocol (Klann et al., 1989). All these cell lines are altered by treatment with tumor initiator and/or promoter: 3PC cells are immortalized but do not give rise to tumors, MT1/2 papilloma-producing cells are more advanced than 3PC, and Ca3/7 cells represent the highest stage of malignant transformation.

Transformed keratinocyte cell lines (MT1/2 and Ca3/7) are resistant to the growth inhibition by synthetic glucocorticoid FA even at a concentration of 100 μM while untransformed 3PC cell line is very sensitive to glucocorticoids, *i.e.*, FA (0.01 μM) caused a significant growth inhibition. The most common mechanism of cell growth inhibition by glucocorticoid hormones is G_0/G_1 cell cycle arrest by activation of genes coding a cyclin-dependent kinase (CDK) inhibitors, *Cdkn1a* (*p21Cip1*) and *Cdkn1b* (*p27Kip1*) (Rogatsky et al., 1999; Yemelyanov et al., 2007) that regulate cell cycle progression and are considered to be the negative modulators of cell cycle. We found that FA treatment caused an increase of PCNA level in Ca3/7 cell line, and a decrease in p21 and p27 whereas a significant increase in mRNA level

of p21 and p27 was found in glucocorticoids sensitive cell line (3PC). These results confirm that our Ca3/7cell line is still resistant to glucocorticoids and are consistent with our previous data on the resistance of those transformed mouse keratinocyte cell lines (Spiegelman et al., 1997) and skin papillomas to the inhibition of proliferation by the glucocorticoid hormone (Budunova et al., 1997).

In general, cAMP provides a positive intracellular signal for cell proliferation in many differentiated cells (Dumont et al., 1989). However, in cancer cells cAMP could be a negative regulator of cell cycle progression. It has been reported that tumor cells exhibit a decreased intracellular cAMP level compared to normal cells (Epstein and Hachisu, 1984; Hickie et al., 1974). This might have resulted from elevated activation of PDEs enzymes in tumor cells (Drees et al., 1993). In keratinocytes representing different stages of carcinogenesis, the activity of PDE in primary keratinocytes was the same as in two papilloma cell lines but in the carcinoma cell line it was significantly higher. The intracellular level of cAMP was significantly lower in carcinoma cells than in primary keratinocytes and in papilloma cell lines (Marko et al., 1998). Despite these results, we did not find any differences in the basal cAMP level among the keratinocyte cell lines or in their phosphodiestarase activities (data not shown). Rolipram and YM976 are both PDE4 inhibitors but their structures are different. The structure of YM976 is lacking the 3-cyclopentyloxy-4-methoxyphenyl group, which is shared by rolipram and others PDE4 inhibitors. It has been reported that the inhibitory activity of YM976 was approximately 400-fold stronger than that of rolipram (IC_{50} values: YM976, 2.2 nM vs. rolipram, 820 nM) (Aoki et al., 2000). Despite that fact, the biological effect of the cAMP increase caused by rolipram is often more potent than that caused by YM976. When applied alone, both PDE4 inhibitors caused increases in the cAMP level in all tested cell lines in the first hour of treatment and after 72 h we could see only a slight inhibition of the cell growth of all cell lines, *i.e.*, by 40% at concentration of 100 μM in case of YM976; the effect of rolipram was less pronounced. The inhibitors also increased the mRNA level of p27 and/or p21 in a dose-dependent manner, but despite that, at concentration of 10 μM, both inhibitors did not cause any changes in cell cycle distribution in all cell lines. We also did not find any signs of caspase-3 and caspase-9 activation during the treatment of the PDE4 inhibitors up to 72 h (data not shown). Further results of our study revealed that both PDE4 inhibitors can increase the sensitivity of transformed cell lines to the growth inhibitory effect of FA. A combination of YM976 with FA caused a significant decrease in the cell numbers in MT1/2 and Ca3/7 cell lines, simultaneously raising the mRNA level of p21 and p27 (compared to FA treated and control cells), decreasing the PCNA protein level and increasing cell population in G1 phase. Rolipram administered together with FA caused a significant effect only in the papilloma cell line. On the other hand, the effect of PDE4 inhibitors applied together with FA in immortalized keratinocytes (3PC) was completely inversed. They weakened the inhibitory effect of FA on the cell growth rate and that was accompanied by the decrease in p21 and p27 mRNA levels, together with lowering percentage of cells in G1 (comparing to the FA treatment only). The effect of PDE4 inhibitors exerted on normal cells *versus* tumor cells appears to be completely different. It has been reported that PDE4 inhibitors up-regulate glucocorticoid receptor transcript levels in B-cell chronic lymphocytic leukemia (B-CLL) cells but not T-CLL cells or normal circulating T cells, B cells, monocytes, or neutrophils (Meyers et al., 2007). Rolipram was also able to synergize with glucocorticoids in inducing B-CLL but not T cell apoptosis (Tiwari et al., 2005).

To summarize, PDE4 inhibitors are able to sensitize tumorigenic cells to growth inhibitory effect of glucocorticoids. On the other hand, co-treatment with PDE4 inhibitors and FA caused an inhibition of the glucocorticoid-responsive gene (MT-1) expression in all cell lines (transformed or not transformed) in a dose-dependent manner, suggesting a negative effect of PDE4 inhibitors on glucocorticoid receptor. The FA action, in papilloma and carcinoma cells, in the presence of PDE4 inhibitors resembles the actions of dissociated glucocorticoids stimulating only a part of the glucocorticoid receptor response. Dissociated glucocorticoids are synthetic ligands of the glucocorticoid receptor and can activate only transrepression or transactivation activity of the glucocorticoid receptor (Schäcke et al., 2004). The transrepression activities of the glucocorticoid receptor were shown to be primary mechanism of the anti-inflammatory action of the glucocorticoids (Reichardt et al., 2001), while several side effects are thought to be predominantly mediated via transactivation. The action of glucocorticoids is limited by numerous side effects, and dissociated glucocorticoids were developed as compounds with the anti-inflammatory potency of standard glucocorticoids but with reduced side effects. Thus, PDE4 inhibitors could exert double effect on the glucocorticoid receptor; to restore the ability of the glucocorticoid receptor to inhibit the growth of cancer cells and to reduce side effects by inhibiting the glucocorticoid receptor transactivation. These findings warrant further, *in vivo* studies to examine if co-administration of PDE4 inhibitors with glucocorticoids is able to affect the growth of established papillomas or their conversion to carcinomas.

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