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7-Benzylamino-6-chloro-2-piperazino-4-pyrrolidino-pteridine, a potent inhibitor of cAMP-specific phosphodiesterase, enhancing nuclear protein binding to the CRE consensus sequence in human tumour cells

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Abstract

The cAMP-specific phosphodiesterase isoenzyme family PDE4 represents the highest cAMP-hydrolysing activity in many human cancer cell lines including the human large cell lung carcinoma cell line LXFL529L. Treatment of LXFL529L cells with the potent PDE4 inhibitor 7-benzylamino-6-chloro-2-piperazino-4-pyrrolidino-pteridine (DC-TA-46) induces dose-dependent growth inhibition. Cells are arrested in the G_1-phase of the cell cycle and the induction of apoptosis is observed. In this study, we investigated the effect of DC-TA-46 on downstream elements of the cAMP-pathway. DC-TA-46 mediated inhibition of PDE4 activity in LXFL529L cells resulted in an increase of the intracellular cAMP level and significant induction of the activity of protein kinase A (PKA). The regulatory PKA subunit RIα was predominantly expressed in LXFL529L cells. In contrast to effects induced by cAMP analogues like 8-Cl-cAMP, the expression of the regulatory subunits of PKA remained unaffected by DC-TA-46. Treatment of LXFL529L cells with DC-TA-46 enhanced the binding of nuclear proteins to the cAMP-responsive element (CRE) consensus sequence TGACGTCA in a time- and dose-dependent manner, indicating the activation of transcription factors by PKA phosphorylation.

Keywords: cAMP; DC-TA-46; Phosphodiesterase; cAMP responsive element; Nuclear protein binding; PKA

1. Introduction

Cyclic AMP is an important intracellular second messenger mediating the transcriptional regulation of many genes [1]. It plays a central role in response to external signals for cell proliferation and differentiation [2,3]. The hydrolysis of cyclic adenosine monophosphate (cAMP) is accomplished by 3′,5′-cyclic nucleotide phosphodiesterases (PDE), a superfamily of isoenzymes, consisting of at least 11 isoenzyme families differing in substrate specificity, kinetic characteristics and sensitivity to isoenzyme specific modulators [4–11]. Amongst the 11 isoenzyme families known so far, the cAMP-specific rolipram-sensitive isoenzyme family PDE4 has been found to play an important role in the regulation of cAMP homeostasis in tumour cells. In B16 murine melanoma and MCF-7 human mammary carcinoma cells PDE4 represents the major cAMP-hydrolysing activity [12]. In murine keratinocytes, representing different stages of malignant transformation, cAMP-hydrolysing activity was mainly attributable to PDE4 and was found to be enhanced with increasing malignancy, primary cells representing the lowest activity. Inhibition of PDE4 with the pteridine derivative DC-TA-46 induces an increase of intracellular cAMP and a dose-dependent inhibition of tumour cell growth. After 24 hr treatment, CarB cells are arrested in the G_1/G_0-phase of the cell cycle. At concentrations >2 μM, the onset of apoptotic cell death is observed [13].

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Abbreviations: cAMP, cyclic adenosine monophosphate; CRE, cAMP-responsive element; CS, fetal calf serum; IBMX, isobutylmethylxanthine; PDE, phosphodiesterase.

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An increase in the level of the second messenger cAMP affects the function of the tetrameric PKA holoenzyme. Binding of cAMP to PKA leads to the release of the catalytic subunits, which subsequently phosphorylate target proteins. In the nucleus, PKA-mediated phosphorylation influences the transcriptional regulation of various genes through cAMP-responsive elements in the promoter regions [14,15].

In the present study, we addressed the question which downstream effectors of the cAMP-pathway are modulated as a result of PDE4 inhibition and might therefore contribute to the growth inhibitory effects of DC-TA-46. First of all, we investigated the effect on the activity of PKA and, with emphasis to cAMP-analogues like 8-chloro-cAMP, also effects on the expression of the different PKA isoforms. The mitogen-activated protein kinase (MAPK) cascade, one of the central pathways in the regulation of cell proliferation, can be blocked as a result of deactivating phosphorylation of Raf-1 by PKA [16,17]. We previously showed that in the human vulva carcinoma cell line A431, the MAP kinase cascade is effectively shut down by DC-TA-46 [18]. In the present study, we focussed on the question whether also nuclear targets of the PKA are affected as a result of effective PDE4 inhibition, with emphasis on the modulation of protein binding to selected CRE sites. Established effectors of this pathway like forskolin, isobutylmethylxanthine (IBMX) and dibutyryl-cAMP (db-cAMP) were used for comparison.

2. Materials and methods

2.1. Chemicals

[^{3}H]cAMP (specific activity: 39 Ci/mmol) and [γ-^{32}P]-ATP (500 μCi/mmol) was obtained from Amersham Pharmacia Biotech (Freiburg, Germany). Forskolin and IBMX were purchased from ICN Biomedicals GmbH (Eschwege, Germany) and dibutyryl-cAMP from Sigma–Aldrich Chemie GmbH (Deisenhofen, Germany). Rolipram (4-[3-cyclopentyloxy-4-methoxy-4-methoxyphenyl]-2-pyrrolidone) was kindly provided by Schering AG (Berlin, Germany). DC-TA-46 was synthesised as described previously [19].

2.2. Cell culture

LXFL529L cells were kindly provided by Prof. Dr. H.H. Fiebig, University of Freiburg, Germany [20]. LXFL529L cells were grown at 37° in RPMI 1640 (Gibco, BRL; Life Technologies GmbH, Eggenstein, Germany), supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), in a humidified atmosphere of 5% CO_2. The cells were tested routinely for absence of mycoplasm contamination. In most experiments, cells were seeded in 10 cm^2 Petri dishes or 25 cm^2 tissue culture flasks (10^6 cells). Cells were allowed to grow for at least 24 hr. The plates were used for the experiments at a density of approximately 70% (logarithmic growth).

2.3. PDE assay

After DC-TA-46 treatment (15 min, 12 hr), cells were harvested by removing the medium and washing with 5 mL of ice-cold PBS. Harvesting and preparation of the cytosol and membrane fraction was performed at 4°. Cells were harvested by scraping in homogenisation buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 4 mM benzamidine hydrochloride, 0.5 mM trypsin inhibitor from soybeans, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol, 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 1 μM pepstatin, 1 μM leupeptin) and cracked two times in liquid nitrogen. After centrifugation (10,000 *g*, 10 min), the supernatant (soluble fraction) was carefully removed and subjected to the PDE assay. The pellet was resuspended in homogenisation buffer, containing 0.5% Triton X-100. Lysis of the membranes was assisted by ultra-sound. After centrifugation (10,000 *g*, 15 min) the supernatant (soluble membrane fraction) was analysed for PDE activity as described previously [12].

2.4. HPLC/MS/MS

Cells were seeded in 175 cm^2 cell culture plates (6×10^6 for 15 min incubation, 5×10^6 for 12 hr incubation) and allowed to grow for 24 hr. The cells were incubated with 100 μM rolipram (0.1% DMSO) for the respective time. Thereafter, the cells were washed twice with PBS (5 mL) and harvested by scraping into 700 μL Tris–buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA). The cells were cracked by four cycles of freezing and thawing in liquid nitrogen. Separation of cytosolic and particulate fraction was achieved by centrifugation at 100,000 *g* for 1 hr. The remaining pellet was carefully resuspended. The proteins in the cytosolic and particulate fractions were precipitated by methanol (−20°) overnight. The samples were centrifuged at 10,000 *g* for 15 min. The supernatants were submitted to the analysis of the rolipram content by HPLC/MS/MS.

The HPLC system consisted of two Series-200 LC micropumps and a Series-200 autosampler (Sciex, Concord, Canada). The chromatographic separation was performed on a RP-18e LiChrospher® 100, 125-4 (Merck) together with a mixture of 10 mM ammonium acetate–MeOH with a linear gradient starting at 85:15 rising up to 5:95 for 19 min with a flow-rate of 1 mL/min. The mobile phase was degassed in an ultrasonic bath prior to analyses. The injection volume was 200 μL. Mass spectrometry was performed using a PE Sciex API-2000 tandem triple–quadrupole equipped with a turbo ion spray source in the positive ion ionisation mode. Ultra-high purity nitrogen was used as nebulisation and courtan gas. Calibration of the mass analyser was performed by infusion (10 μL/min)

of a commercial mixture of PPGs (polypropylene glycols, Applied Biosystems, St. Quentin-en-Yvelines, France) using a syringe pump. The selected reaction monitoring (SRM) mode was chosen for quantification. The electrospray capillary was set at 5000 V and the declustering potential to 51 V. The ion source temperature was set at 350° and the courtain gas at 25 psi. The pressure of the nitrogen used for the collision dissociation (CAD) was 2 psi, the nebuliser gas at 15 psi and the courtain gas flow at 5 psi. The collision energy was set to 40 V, focusing potential to 70 V and the entrance potential to −12 V. The ions monitored were $m/z = 276.3$ and 131.0 daughter ion of rolipram. Quantification of rolipram was performed by external standard method.

2.5. Intracellular cAMP

The intracellular cAMP-level was determined in duplicate, using a [^{3}H]cAMP assay kit from Amersham Pharmacia Biotech (Freiburg, Germany). Results were expressed as picomoles cAMP per milligram protein. The samples were prepared according to the manufacturers instructions.

2.6. Protein kinase A assay

Cells were incubated as described above. At respective time points, cells were washed twice with PBS buffer and subsequently harvested by scraping in a small volume of extraction buffer (50 mM Tris–HCl, pH 7.5, 5 mM Na_2EDTA, 100 μg/mL trypsin inhibitor, 1 μM leupeptin, 1 μM pepstatin, 0.5 mM phenylmethylsulfonylfluoride). Cells were cracked in liquid nitrogen and centrifuged at 14,000 *g* for 6 min. The supernatant was applied to the assay, according to the instructions of the manufacturer (Gibco, Karlsruhe, Germany).

2.7. Nuclear extracts

Nuclear extracts of LXFL529L cells were prepared according to the modified method of Huang *et al.* [21]. After removing the medium, cells were washed twice with cold phosphate-buffered saline (PBS) and harvested by scraping in ice-cold PBS. After centrifugation (200 *g*, 4°, 5 min) the cell pellet was suspended in 0.5 mL NP-40 buffer (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 0.5 mM phenylmethylsulfonylfluoride, 0.5 mM dithiotreitol) and incubated for 30 min on ice. For complete lysis, cells were passed through a 20-gauge needle and the nuclei separated by centrifugation (200 *g*, 4°, 10 min). Cell nuclei were resuspended in 50 μL NE/NaCl buffer (20 mM Tris–HCl, pH 7.9, 20% glycerol, 350 mM NaCl, 0.2 mM Na_2EDTA, 0.5 mM phenylmethylsulfonylfluorid, 0.5 mM dithiotreitol) and lysed using a micro stirring bar in an Eppendorf tube. After centrifugation at 14,000 *g* for 6 min at 4°, the crude nuclear extract was removed from the pellet fraction.

2.8. Oligonucleotides

For DNA/protein-binding assay, double-stranded oligonucleotides of the following sequence were used: CRE_{SOM} 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′, CRE_{TAT} 5′-AGCTTCTGCGTCAGCGCCAG-3′, TRE 5′-CGCTTGATGAGTCAGCCGGAA-3′. The corresponding binding sites are underlined. The oligonucleotides were purchased from Promega (Mannheim, Germany) and Amersham Pharmacia Biotech (Freiburg, Germany).

2.9. DNA/protein-binding assay

The oligonucleotides were labelled with digoxigenin using a 3′-end labelling kit (Promega, Mannheim, Germany). The crude nuclear extract was incubated with the labelled oligonucleotide-mixture in gel retardation buffer (20% (v/v) glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiotreitol, 250 mM NaCl, 0.25 mg/mL poly[d(I-C)], 50 mM Tris–HCl, pH 7.5). After incubation (90 min on ice) gel loading solution (250 mM Tris–HCl, pH 7.5, 0.2% (w/v) bromphenolblue, 0.2% (w/v) xylene cyanol, 40% (v/v) glycerol) was added and subjected to electrophoresis on a 5% native polyacrylamide gel. Specificity of the DNA/protein-binding was established by preincubating the crude nuclear extract with a 75-fold molar excess of unlabelled oligonucleotide (10 min at RT) prior to adding the labelled oligonucleotide and incubating for 1 hr followed by native PAGE as described above. After electrophoresis, the proteins were electrotransferred to a nylon membrane, fixed on the membrane by UV light (254 nm, 4 min) and detected by chemoluminescence.

2.10. Immunoprecipitation of RIα, RIIα and RIIβ cAMP receptor proteins after photoaffinity labelling with 8-N_3-[^{32}P]cAMP

Cells were harvested and washed two times with cold PBS. After centrifugation, cell pellets were suspended in 0.5 mL buffer Ten [22] (20 mM Tris–HCl, pH 7.4, 0.1 M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$, 0.1 mM pepstatin, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 0.4 mg/mL aprotinin, and 0.5 mg/mL soybean trypsin inhibitor, filtered through a 0.45 μM pore size membrane), passed through a 20-gauge needle five times using a 1-mL syringe, allowed to sit at 4° for 15 min, and centrifuged for 5 min in an Eppendorf microfuge at 4°. The supernatant was used as lysate. Protein concentration was determined by the method of Lowry as modified by Smith *et al.* [23] with BSA as standard. Photoaffinity labelling and immunoprecipitation of cAMP receptor proteins were carried out as described previously [22]. Cellular proteins were subjected to SDS–polyacrylamide gel electrophoresis, and the resolved proteins were electrotransferred to nitrocellulose sheets.

Radioactivity was detected by autoradiography using Kodak X-Omat film.

3. Results

3.1. Inhibition of intracellular PDE activity

In LXFL529L cells, out of the various cAMP-elevating agents tested, DC-TA-46 was the only to potently inhibit PDE activity in both, the cytosol and the membrane compartment (Fig. 1A and B). Dose dependence was observed for the cytosol, but in that concentration range not for the particulate associated PDE activity. Incubation with 1 μM DC-TA-46 reduced the membrane bound PDE activity to 37% already after 15 min incubation, an effect that was not significantly intensified by higher concentration or longer incubation (up to 12 hr). No significant effect on cytosolic and membrane-bound PDE activity was observed by treatment with the selective PDE4 inhibitor rolipram (100 μM). Also forskolin (50 μM), an activator of adenylate cyclase, induced no effect on the cytosolic PDE

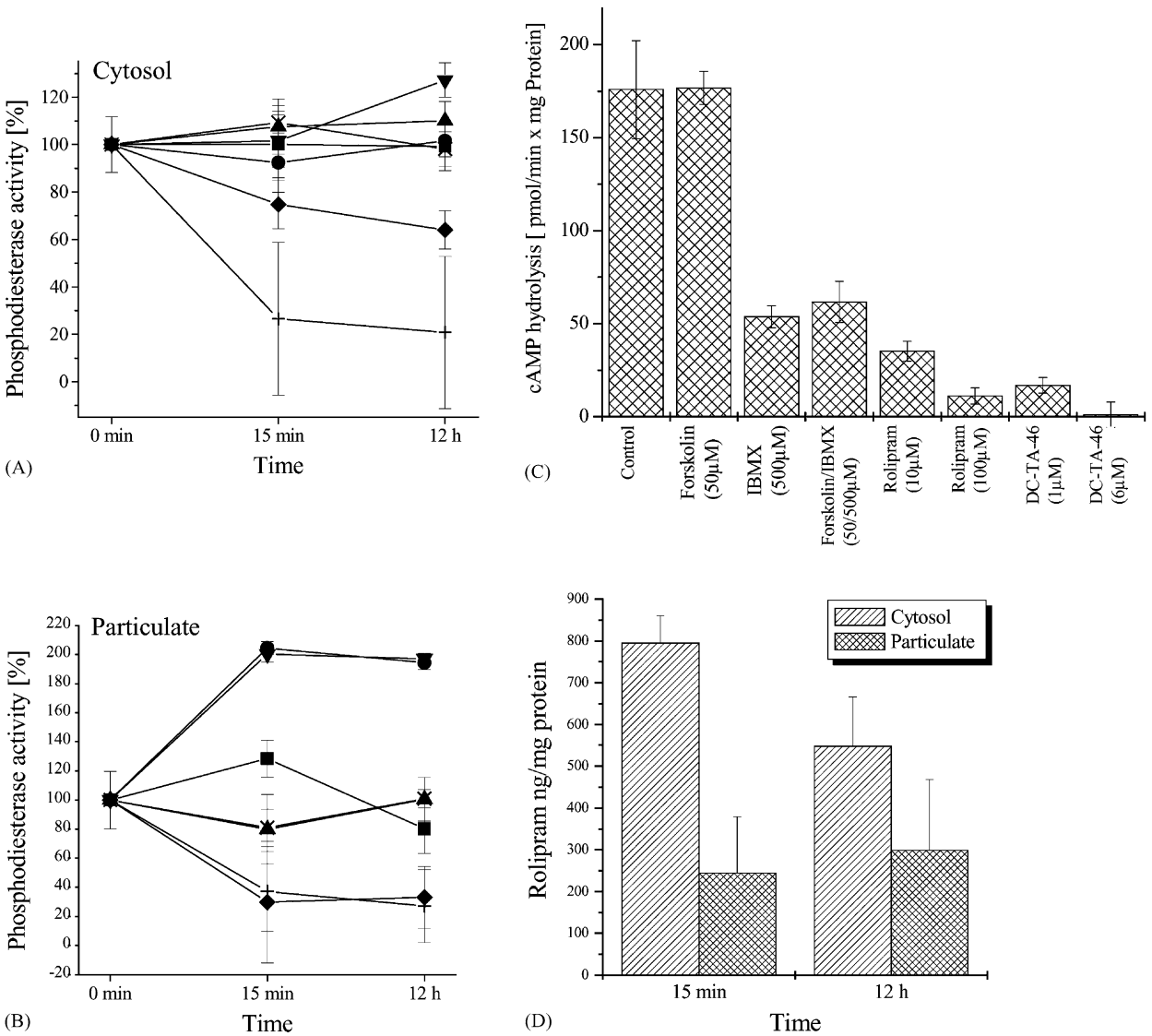

Fig. 1. (A) Influence of DC-TA-46, forskolin and IBMX on the PDE activity in cytosol and (B) membranes of LXFL529L cells. Cells were incubated with forskolin, 50 μM (●); IBMX, 500 μM (▲); forskolin/IBMX, 50/500 μM (▼); DC-TA-46, 1 μM (◆); DC-TA-46, 6 μM (+); rolipram, 100 μM (×). Control cells (■) were treated with DMSO only. All incubations were performed in serum-free medium. 15 min and 12 hr after drug addition, cells were harvested in homogenisation buffer and lysed. After centrifugation, the supernatant (cytosolic fraction) was removed and subjected to the PDE assay ($n = 3$). The pellet was resuspended in homogenisation buffer, containing 0.5% Triton X-100, lysed and centrifuged again. The supernatant (soluble membrane fraction) was also subjected to the assay. The whole experiment was performed four times (values are presented as mean ± SD). (C) Inhibition of cytosolic PDE activity of LXFL529L cells when the compounds are directly applied to the enzyme preparation in the PDE assay. The data presented are the mean ± SD of three independent experiments, each done in triplicate. (D) Uptake of rolipram by LXFL529L cells. Cells were incubated with 100 μM rolipram for 15 min or 12 hr, respectively. The content of rolipram in the cytosol and the cellular membranes was determined by HPLC/MS/MS. The data presented are the mean ± SD of three independent experiments.

activity. However, in combination with the unspecific PDE inhibitor IBMX (500 μM), a slight but significant induction of cytosolic cAMP hydrolysing activity was observed after 12 hr of incubation (Fig. 1A). In contrast to the cytosol, membrane-associated PDE activity was strongly enhanced by forskolin alone or in combination with IBMX already after 15 min, remaining at that level until the end of the incubation time (12 hr) (Fig. 1B).

The lack of efficacy of rolipram and IBMX was only observed when the intact cells were treated with the respective compounds. Both compounds effectively inhibited the cAMP-hydrolysis when directly applied to a cytosolic preparation of LXFL529L cells in the PDE assay (Fig. 1C). Under that experimental conditions already 10 μM of rolipram inhibited the major proportion of the cytosolic PDE activity. DC-TA-46 showed the highest inhibitory potency.

Due to the lack of efficacy to inhibit PDE activity in intact LXFL529L cells, the uptake and subcellular localisation of rolipram was determined by HPLC/MS/MS (Fig. 1D). Rolipram was found to be predominantly localised in the cytosol. The compound is taken up from LXFL529L cells only to a minor extent, reaching about 0.8 μg/mg protein after 15 min incubation. After 12 hr of incubation, the content in the cytosol was even lower with a concomitant slight but not significant increase of the rolipram content in cellular membranes.

3.2. cAMP level and PKA-activity

Treatment of LXFL529L cells with 10 μM DC-TA-46 resulted in a time-dependent increase of the intracellular cAMP level, followed by an increase in PKA activity (Fig. 2A). Treatment with db-cAMP (1 mM) induced a strong and rapid increase within the first 30 min of incubation, reaching a peak activity about three times the PKA activity induced by DC-TA-46 at that time point. However, whereas DC-TA-46 induced PKA activation continued to increase, db-cAMP induced activation rapidly declined beyond 30 min (Fig. 2B).

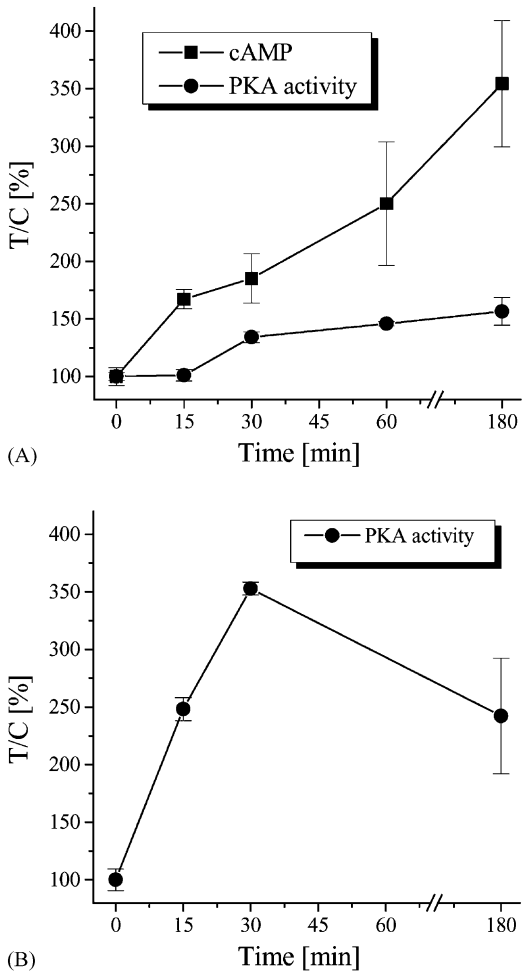

Fig. 2. Total cAMP-level and PKA-activity after incubation of LXFL529L cells with (A) 10 μM DC-TA-46, (B) 1 mM db-cAMP. At the respective time points, cells were harvested and prepared for both experiments. For the cAMP-assay the medium was removed, the cells washed with Tris–EDTA buffer and subsequently harvested by scraping in ethanol. After centrifugation the supernatant was removed. The precipitate was resuspended and centrifuged again. The supernatants were united and lyophilised. The resulting residue was dissolved and directly applied to the assay. For PKA assay, cells were washed twice with ice-cold PBS, harvested in extraction buffer and cracked in liquid nitrogen. After centrifugation, the supernatant was applied to the PKA assay. Data are expressed as percent of the control. Each point represents the mean ± SD of six to nine samples from three separate experiments.

3.3. Expression of PKA-isoforms

In LXFL529L cells, the regulatory subunit RIα was found to represent the predominantly expressed subunit of PKA as detected by photoaffinity labelling followed by immunoprecipitation (Fig. 3). In addition to RIα, low levels of RIIα were expressed. After incubation of LXFL529L cells with DC-TA-46 (up to 3 μM) for 3 (Fig. 3) and 5 days (data not shown), this ratio of regulatory subunit isoform expression remained unchanged.

3.4. Modulation of nuclear protein binding to cAMP-responsive elements

Nuclear extracts of LXFL529L cells, treated with DC-TA-46, were incubated with a digoxigenin end labelled 27-mer oligonucleotide. The selected oligonucleotide sequence (CRE_{SOM}) originates from the somatostatin gene, that contains the CRE consensus sequence TGACGTCA. Incubates were subjected to electrophoresis on a native gel (5%). After transfer onto a nylon membrane, bands were detected by chemoluminescence.

Incubation of LXFL529L cells with DC-TA-46 was found to increase the binding of nuclear proteins to the CRE consensus sequence, detected as retardation of the oligonucleotide CRE_{SOM} (Fig. 4A). Compared to untreated cells, nuclear extracts of DC-TA-46 treated cells exhibited a different pattern of retardation as a consequence of nuclear protein binding to the CRE_{SOM}-oligonucleotide. Intensities of the most strongly retarded band (band 1) and

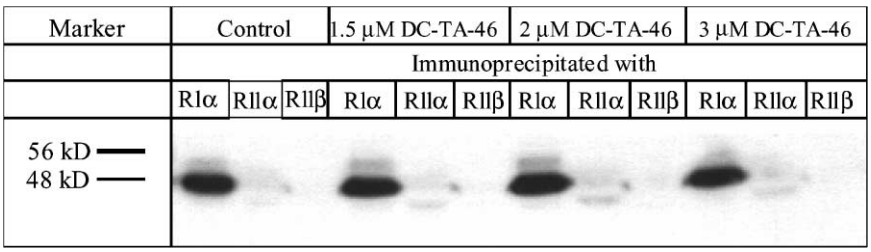

Fig. 3. Effect of DC-TA-46 on the expression of RIα, RIIα and RIIβ cAMP receptor proteins in LXFL529L-cells. Cells were treated with various concentrations of DC-TA-46 for 3 days, then harvested and extracted. The cAMP receptor proteins present in the extract were labelled by photoactivated incorporation of 8-N_3-[^{32}P]cAMP and immunoprecipitated using polyclonal antibodies against RIα, RIIα or RIIβ. The samples were applied to a SDS/PAGE gel and the PKA subunits were separated by electrophoresis. Detection was performed by autoradiography. The blot shown is representative for two independent experiments.

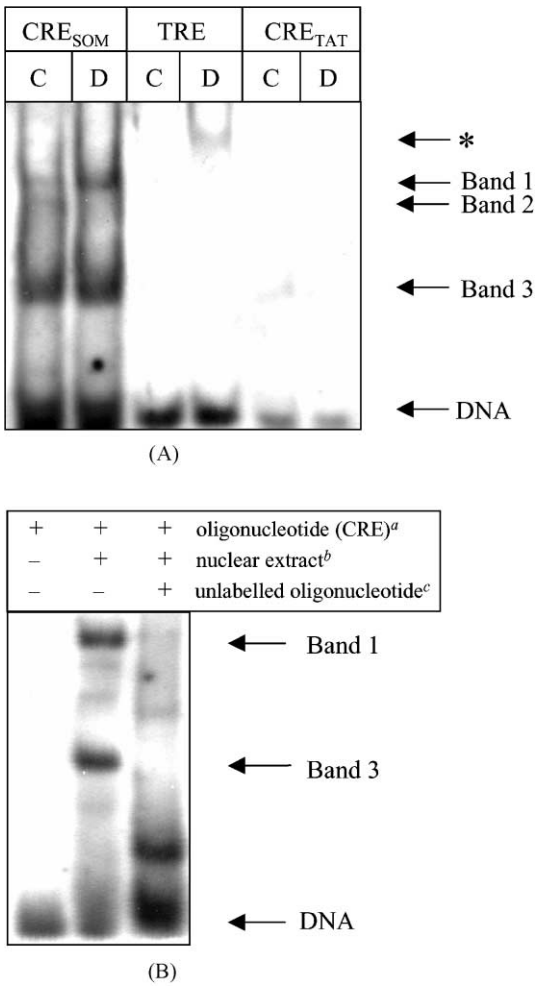

Fig. 4. (A) Gel mobility shift assays were performed using digoxigenin labelled oligonucleotides and LXFL529L nuclear extracts. The extracts were prepared upon incubation of the cells with 10 μM DC-TA-46 (D) or DMSO as a control (C) for 3 hr. The nuclear extract of the cells was incubated with digoxigenin labelled oligonucleotides (CRE$_{SOM}$, TRE and CRE$_{TAT}$). (B) Specificity of nuclear binding. In the oligonucleotide competition experiment, nuclear extracts of LXFL529L cells were preincubated with a 75-fold molar excess of unlabelled oligonucleotide CRE$_{SOM}$ for 10 min at RT, followed by the addition of the labelled oligonucleotide. Subsequently, the samples were submitted to electrophoresis on a native 5% polyacrylamide gel. For detection the proteins were electrotransferred to a nylon membrane and visualised by chemoluminescence. The blots show typical results done at least three times.

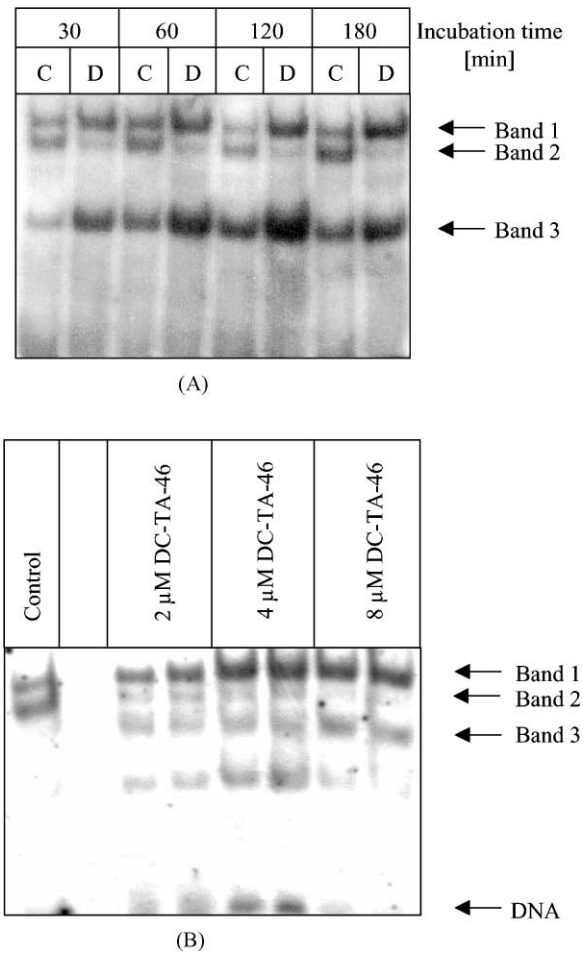

Fig. 5. Dependence of the DNA/protein-binding on (A) the concentration of DC-TA-46 and (B) the incubation time. (A) LXFL529L cells were incubated for 3 hr with 2, 4 and 8 μM DC-TA-46. (B) Treatment with 10 μM DC-TA-46 (D) or DMSO (C) for 30, 60, 120 and 180 min. In controls, cells were incubated with the solvent DMSO. The nuclear extract was incubated with the labelled oligonucleotide CRE$_{SOM}$ and submitted to an electrophoresis on a native 5% polyacrylamide gel. For detection the proteins were electrotransferred to a nylon membrane and visualised by chemoluminescence. The blot represents a typical experiment, performed two times.

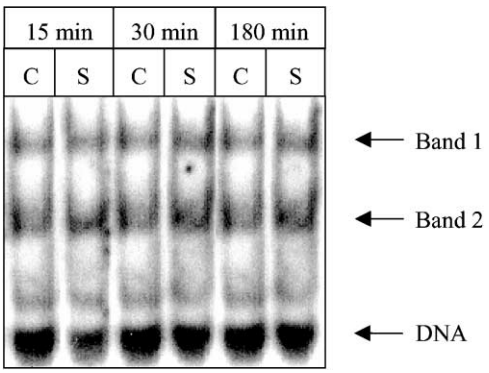

Fig. 6. Gel mobility shift assay with nuclear extracts from LXFL529L cells treated with 1 mM dibutyryl-cAMP (S) for 15, 30 and 180 min. Untreated cells were taken as control (C). The nuclear extract was incubated with the labelled oligonucleotide CRE_{SOM} and submitted to an electrophoresis on a native 5% polyacrylamide gel. For detection the proteins were electrotransferred to a nylon membrane and visualised by chemoluminescence. The blot represents one of three experiments that all gave similar results.

of band 3, were markedly enhanced in DC-TA-46 treated samples, whereas band 2 was diminished. In contrast, DC-TA-46 did not increase the binding affinity of nuclear proteins to an oligonucleotide containing the truncated CRE sequence CGTCA of the tyrosine aminotransferase gene (CRE_{TAT}). Binding of nuclear factors to an oligonucleotide containing the TPA responsive element (TRE) consensus sequence TGAGTCA was only very weakly influenced (Fig. 4A). The specific nature of the binding of nuclear proteins to CRE_{SOM} was confirmed by competitive preincubation with an excess of unlabelled CRE_{SOM} oligonucleotide (75-fold). This lead to complete disappearence of bandshift signals representing complexes with labelled oligonucleotide (Fig. 4B). Distinct time and concentration dependence of the increase in binding affinity is evident from Fig. 5A and B.

Treatment of LXFL529L cells with db-cAMP did not result in a significant change of the binding pattern to the CRE consensus sequence as compared to untreated control (Fig. 6). Again, competition with unlabelled CRE_{SOM} lead to disappearence of relevant bandshift signals (data not shown). Likewise, nuclear extracts of LXFL529L cells treated with forskolin did not exhibit altered complex formation, compared to untreated cells. This was also true for forskolin in combination with IBMX (data not shown).

4. Discussion

We have shown previously that in B16 murine melanoma cells, treatment with DC-TA-46 resulted in a dose-dependent increase of the intracellular cAMP-level and in efficient growth inhibition [12]. In the highly malignant murine spindle cell carcinoma cell line CarB DC-TA-46 likewise induced effective inhibition of the intracellular PDE activity and increased cAMP. Cells were arrested in the G_1-phase of the cell cycle and induction of apoptosis was observed [13]. Previously, we showed that the activity of the MAP kinase cascade, as a central signalling pathway in the regulation of proliferation, is effectively shut down as a consequence of DC-TA-46 treatment [18]. This might result from PKA-mediated deactivating phosphorylation of Raf-1 as it has been reported for other cAMP-elevating agents [16,17]. In the present study, we focussed on the question whether also downstream elements of the cAMP-pathway are modulated by treatment with DC-TA-46, which might contribute to the cellular effects of the compound. Especially effects on the PKA and subsequent potential nuclear targets were investigated.

The present study demonstrates that in LXFL529L cells DC-TA-46 induces a time-dependent cAMP increase, followed by a somewhat delayed, but sustained elevation of total PKA-activity. With respect to onset and peak level of cellular PKA activity, the response to treatment with the classical PKA activator db-cAMP was markedly different, compared to the DC-TA-46 induced effects, with a rapid and steep increase to peak activity at 30 min, followed by continuous activity decrease at later time points.

The two isoenzymes of cAMP-dependent protein kinase, PKAI and PKAII, differ in the regulatory subunit, but bind to identical catalytical subunits. Up to now, four isoforms of the regulatory subunit have been reported, RIα and RIβ for PKAI and RIIα and RIIβ for PKAII. The α-subunits (RIα and RIIα) are expressed in a variety of tissues at different ratio [24,25], whereas the occurrence of RIβ and RIIβ is more tissue specific [26,27]. Cho-Chung and Clair [28] found significantly higher expression of RI in neoplastic tissues and suggested that the balance between RI and RII might be pivotal, RI behaving more as a growth stimulating, potentially oncogenic factor and RII as a potentially tumour suppressing differentiation inducing factor [29,30]. Marked RI overexpression is also evident from our data in human LXFL529L cells. However, DC-TA-46 did not affect the expression of RI and RII.

In B 16 murine melanoma and MCF-7 human mammary carcinoma cells, cAMP-specific PDE4 isoenzymes have previously been found to account for the major cAMP-degrading activity. Treatment with the pteridine-based PDE4 inhibitor DC-TA-46 was found to induce an increase in intracellular cAMP, concomitant with dose-dependent growth inhibition [12]. Moreover, the human large cell lung tumour xenograft LXFL529 had been found to express very high PDE activity both, in solid tumour tissue grown in nude mice as well as in cell culture. About 86% of the overall PDE activity of the cell line LXFL529L could be ascribed to the PDE4 isoenzyme family [18]. In the present study, we demonstrated, that in LXFL529L cells DC-TA-46 inhibits cytosolic PDE activity in a dose-dependent manner (Fig. 1A). In cellular membranes, PDE activity is reduced within 15 min to a minimum already with 1 μM of the compound (Fig. 1B), whereas in the cytosol, a concentration of 6 μM DC-TA-46 is necessary to achieve

the same effect. This is in line with our findings that DC-TA-46 is rapidly taken up by cells to predominantly concentrate in cellular membranes [31]. About 88% of the cytosolic and 80% of the membrane-bound PDE activity isolated from LXFL529L cells is inhibited by 10 μM rolipram, a selective inhibitor of PDE4 isoenzymes [18]. This is approximately the proportion of the intracellular cAMP hydrolysing activity in the respective compartments which is inhibited by treatment with DC-TA-46. The remaining activity is probably due to other PDE isoenzymes than PDE4.

In contrast to the effect of DC-TA-46, treatment with forskolin alone or in combination with IBMX led to a rapid and steep increase of the PDE activity in cellular membranes, that persisted until the end of the observation period (Fig. 1B). In contrast, cytosolic PDE activity was much less affected, showing a slight but significant increase only at 12 hr (Fig. 1A). The short-time induction of PDE activity observed in the membrane compartment might result from PKA mediated phosphorylation as has been reported for PDE4D3 after treatment with db-cAMP, an effect already observed within 10 min incubation [32,33]. In LXFL529L cells, RT-PCR with PDE4 subtype-specific primers revealed among others the presence of mRNA for PDE4D3, D1 and D2 (unpublished data). Thus, whereas the almost instantaneous increase in particulate PDE activity is expected to result from phosphorylation predominantly of PDE4D3, the short-forms of PDE4D, 4D1 and 4D2, are upregulated by transcriptional activation, an effect that requires more time.

The differential response observed in cytosolic versus particulate compartments of LXFL529L cells towards treatment with the individual PDE inhibiting/cAMP elevating agents deserves specific consideration. First, although rolipram is widely considered and utilised as the reference PDE4 inhibitor, displaying IC_{50} values towards PDE4 isoenzyme preparations in the low to submicromolar range, it does not achieve significant inhibition of cytosolic or particulate PDE activity when whole cells are treated with concentrations up to even 100 μM (compare Fig. 1A and C). This might reflect a deficiency of rolipram to penetrate into the cell to a sufficient extent. In agreement with this, rolipram was not found to be growth inhibitory to human cancer cells (3 days incubation up to 100 μM, data not shown). The uptake of rolipram into LXFL529L cells was determined by HPLC/MS/MS (Fig. 1D). Indeed, rolipram was found to be taken up by the cells to only a minor extent, with about 0.8 μg/mg protein being present in the cytosol after 15 min incubation with 100 μM. In contrast, DC-TA-46 is effectively taken up by tumour cells such as LXFL529L [31]. After incubation with only 1 μM of DC-TA-46, 2.5 ± 0.07 μg/mg protein were detected in the cytosol after 30 min of incubation. The compounds also differ clearly with respect to their subcellular localisation. Rolipram was found to be predominantly localised in the cytosol (Fig. 1D), whereas the major proportion of DC-TA-46 is located in cellular membranes [31]. However, there is still enough cytosolic DC-TA-46 left to achieve a distinct dose-dependent inhibition of cytosolic PDE activity, approaching 80% inhibition already after 15 min with no significant induction at longer incubation time. Obviously this is a result of a very rapid equilibration of DC-TA-46 distribution between cytosolic and particulate compartments. Similar to rolipram, IBMX at a concentration as high as 500 μM neither significantly affects cytosolic PDE nor particulate PDE activity in intact cells, whereas the PDE activity in isolated cytosolic preparations are effectively inhibited (Fig. 1C). In addition, the potent adenylate cyclase activator forskolin also is of no influence towards cytosolic PDE (Fig. 1A and D). Why both compounds in combination induce the observed slight, yet significant long time induction of cytosolic PDE activity, that might be viewed as a result of transcriptional activation, is not clear at present. But the fact that forskolin alone or in combination with IBMX induces the observed rapid jump in particulate PDE activity argues for the relevance of both, cellular compartimentalisation of PDE activity, as well as distribution into cytosolic/particulate compartments of the substances utilised to influence intracellular cAMP levels by affecting adenylate cyclase and/or PDE activity. The preferential uptake of DC-TA-46 into the cellular membrane compartment obviously is responsible for the rather impressive and rapid PDE inhibitory effect in the particulate observed already at the lowest concentration used (Fig. 1B). After 24 hr incubation of LXFL529L cells with DC-TA-46, particulate PDE activity was inhibited with an IC_{50} value of 0.06 ± 0.02 μM [31]. However, when no such potent inhibitor is present in that compartment, cAMP enhancement by forskolin obviously entails posttranscriptional PDE activation in the particulate, most probably by PKA mediated phosphorylation. The outcome on downstream signalling elements might thus be expected to be dramatically different, depending on the cAMP modulating treatment applied. This is illustrated by the outcome of the gel mobility shift experiments. They clearly demonstrate that treatment with cAMP elevating/PKA activating agents alone, such as forskolin, forskolin/IBMX or db-cAMP does not result in enhanced protein binding to the CRE consensus sequence. The observed strong and rapid activation of particulate PDE activity by such treatments might be responsible for a rapid and efficient quenching of the cAMP signal before it can reach the nucleus. However, if a potent PDE inhibitor blocks the particulate as well as the cytosolic PDE activity, then the resulting enhanced cAMP levels can achieve PKA mediated activation of transcription factors, modulating their binding to the respective CREs.

Up to now, more than 10 CRE-binding factors have been described. Phosphorylation of a serine residue at position 133 of CREB, one of the first characterised CRE-binding proteins, is indispensable for its activation. However, the

influence of PKA-mediated phosphorylation on DNA binding of CREB is still not fully clarified. Experiments of Bullock and Habener [34] suggest, that phosphorylation of CREB alters the structure and charge characteristics of the CREB/DNA-complex resulting in an alteration of the binding affinity. It has also been reported that phosphorylation of CREB by PKA causes a modest increase in binding to high affinity CRE binding sites such as CRE_{SOM} and a stronger enhancement in binding to low affinity CRE binding sites like in the tyrosine aminotransferase gene such as CRE_{TAT} [35]. The consequence of enhanced nuclear factor binding to the CRE consensus sequence as a result of DC-TA-46 treatment depends on the respective transcription factors, if activators or inhibitors of transcription are bound. The characterisation of the respective transcription factors is currently under intensive investigation.

The modulation of nuclear factor binding as a consequence of DC-TA-46 treatment raises the question which genes are modulated in their expression. The clear arrest of cells in the G_1 phase of the cell cycle indicates that the expression of genes involved in cell cycle regulation might be influenced. Experiments on the expression of cyclins and cyclin-dependent kinases crucial for the G_1/S-transition showed a decrease of cyclin A protein in the cells after 24 hr treatment with DC-TA-46 (unpublished results). Cyclin A is known to possess a CRE in the promoter sequence. In contrast to the effect on cyclin A protein content, the level of the cyclin-dependent kinase inhibitors (CKI) p21 cip1 and p27 kip1, remained unchanged (data not shown).

Our results show that DC-TA-46 increases the binding affinity of nuclear proteins of LXFL529L cells to CRE_{SOM}. This effect is observed at a concentration range, where cellular effects like G_1-arrest and induction of apoptosis also are observed. Clearly, DC-TA-46 treatment results in the activation of transcription factors, modulating the transcription of CRE controlled genes. This is not the case for the other cAMP elevating/PKA activating treatments tested. Cellular compartimentalisation of cAMP modulating enzymes and of downstream signalling elements appears to be of great influence, as well as intracellular distribution kinetics of the test compounds. DC-TA-46 appears to be of particular promise since it is highly effective in inhibiting PDE in cytosolic as well as in particulate compartments, thus allowing long-term enhancement of cAMP levels. Although the chain of cellular events that finally lead to growth arrest and apoptosis has not been elucidated yet, it is quite obvious from our data that sustained PDE inhibition and cAMP elevation as achieved by DC-TA-46 is pivotal.

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