Erschienen in: Neurochemistry International ; 59 (2011), 6. - S. 837-846
https://dx.doi.org/10.1016/j.neuint.2011.08.012

Combined anti-inflammatory effects of β_2-adrenergic agonists and PDE4 inhibitors on astrocytes by upregulation of intracellular cAMP

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Keywords:
Astrocytes
Inflammation
cAMP
PDE4
β-Adrenoceptors

A B S T R A C T

Inflammation is an important hallmark of all neurodegenerative diseases and activation of different glial populations may be involved in the progression of some of these disorders. Especially, the activation of astroglia can lead to long-term detrimental morphological changes, such as scar formation. Therefore, improved strategies to modulate inflammation in these cells are currently being investigated. We investigated the interaction of phosphodiesterase (PDE) 4 inhibitors, such as rolipram, with other agents raising cellular cAMP levels. When used alone, none of the PDE4 inhibitors increased cAMP levels. The adenylate cyclase activator forskolin, the β_2-adrenergic agonist clenbuterol and the mixed β_1/β_2-adrenergic agonist isoproterenol increased intracellular cAMP levels of cortical murine astrocytes. This increase was synergistically elevated by rolipram or the PDE4 inhibitor RO-201724, but not by inhibition of PDE3. Inflammatory stimulation of the cells with the cytokines TNF-α, IL-1β and IFN-γ strongly induced PDE4B and augmented overall PDE4 activity, while PDE3 activity was low. Clenbuterol and forskolin caused downregulation of cytokines and chemokines such as IL-6 and MCP-1. This effect was further enhanced by rolipram, but not by the PDE3 inhibitor milrinone. The cAMP-raising drug combinations attenuated the upregulation of TNF-α and IL-6 mRNA and the secretion of IL-6, but did not affect initial NF-κB signalling triggered by the stimulating cytokines. These results indicate that PDE4 may be a valuable anti-inflammatory target in brain diseases, especially under conditions associated with stimulation of cAMP-augmenting astrocyte receptors as is observed by clenbuterol treatment.

1. Introduction

Inflammation is associated with many disorders affecting the central nervous system (CNS) (Saijo et al., 2010), and some studies suggest that anti-inflammatory treatment may slow disease progressions (Hartmann et al., 2003; Villoslada et al., 2008; Fletcher et al., 2010). Although they are not regarded as traditional immune cells, astrocytes are capable of mounting a pronounced inflammatory response when they are triggered by endogenous inflammatory mediators, or by ligands of certain toll-like receptors (Chung and Benveniste, 1990; Choi et al., 1999; Falsig et al., 2004; Bsibsi et al., 2002). The spectrum of factors secreted and upregulated after stimulation with a cytokine mix is remarkably similar to that of activated microglia and astrocytes are considered a second important line of host defence for the CNS (Minagar et al., 2002; Falsig et al., 2006, 2008).

Several studies have shown the regulation of glial inflammatory mediators by noradrenaline (Feinstein et al., 2002; Dello et al., 2004; Simonini et al., 2010). Some of these effects may be related to the increase in intracellular cAMP levels caused by the selective stimulation of β-adrenoceptors (Szabo et al., 1997). Accordingly, specific β-adrenergic agonists have been shown to modulate inflammation by inhibiting TNF-α production and by up-regulating IL-10 expression (Szabo et al., 1997; Farmer and Pugin, 2000). Especially the agonist clenbuterol appears interesting as it shows neuroprotective effects in a mouse and rat model of cerebral ischemia, and closer investigation has shown that it not only blocks astrocytosis in this model, but also reduces the upregulation of the inflammatory marker GFAP in cultured astrocytes (Culmsee et al., 1999, 2007). The role of intracellular cAMP levels in inflammation is further supported by multiple studies showing that inhibition of phosphodiesterase (PDE) 3 and 4 is immunomodulatory (Schudt et al., 1999; Mendes et al., 2009; Pages et al., 2009). For

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instance, rolipram, a widely used inhibitor of PDE4, reduces inflammation and has been shown to suppress the clinical manifestations of an animal model of multiple sclerosis (Sommer et al., 1997; Block et al., 2004; de Visser et al., 2008). However, only little information is available on the effects of PDE4/PDE3 inhibition on astrogliosis in disease models.

The most prominently expressed PDE subtypes in immune cells are PDE3 and PDE4 (Lugnier, 2006). In some cell types a combined inhibition of both enzyme activities is required to obtain maximal increases of cAMP and attenuation of inflammation (Gantner et al., 1997, 1999). In other cells PDE inhibitors have no effect unless they are combined with a cAMP-increasing stimulus (Selige et al., 2010a). For instance, β-adrenergic agonists and PDE inhibitors may show synergy (Bruss et al., 2008).

More information is required on the PDE spectrum of resting and activated murine astrocytes, and on how inhibition of PDE3 or PDE4 would affect the anti-inflammatory activity of β_2-adrenergic agonists. Here, we examined these questions by using the adenylate cyclase activator forskolin, the β_2-adrenergic agonist clenbuterol and the mixed β_1/β_2-adrenergic agonist isoproterenol together with specific PDE3 and PDE4 inhibitors. In murine primary cortical astrocytes, stimulated with a complete cytokine mix consisting of TNF-α, IL-1β and IFNγ, we examined how the PDE4 inhibitor rolipram, in combination with other drugs, would affect mRNA levels and secretion of mediators such as IL-6.

2. Materials and methods

2.1. Materials

The β-adrenergic agonists, PDE inhibitors, forskolin, and the constituents of the complete cytokine mix (CCM), i.e. 10 ng/ml murine Il-1β, 10 ng/ml murine TNF-α and 5 U/ml murine IFN-γ, were from Sigma–Aldrich (Copenhagen, DK). All media for cell culturing were from Invitrogen Life technologies (Taastrup, DK) while plates, dishes and flasks were purchased from NUNC (Roskilde, DK).

2.2. Primary astrocyte cultures

Pregnant C57BL/6JolaHsd mice were purchased from Harlan (Horst, The Netherlands). All experimental procedures were carried out in accordance with the directives of the Danish National Committee on Animal Research Ethics and the European Communities Council Directive #86/609 for care of laboratory animals.

Primary cortical astrocytes were prepared as described earlier in detail (Falsig et al., 2004). In brief, cortices from 1 to 2 day old mice were dissected and the hippocampi and meninges were removed. The cortices were digested in 1 ml phosphate-buffered saline (PBS) pH 7.4, containing 2 g/l glucose and 0.001% (w/v) phenol red), containing 10 mg/ml trypsin TRL (Worthington Biochemical, Lakewood, NJ), 1 mg/ml DNase 1 (Worthington Biochemical) and 5 mg/ml $MgSO_4$ (37 °C, 3 min). The tissue was triturated in 0.5 mg/ml DNase and filtered through a nylon mesh. The filtered cells were centrifuged for 10 min on a percoll gradient and the astrocytes were recovered from the interface. The cells were seeded in 185 cm^2 culture flasks at a density of 11.000 cells/cm^2 and left to incubate at 37 °C, 5% CO_2 and 95% humidity. Growth medium was changed twice a week for 14 days at which time the cells were trypsinized and re-seeded in 96-well plates (10.000 cells/well) or in dishes for qRT-PCR (500.000 cells/dish). Cells were always used 1 week ± 1 day after replating at which time they had reached confluence. The combination of percoll gradient centrifugation and replating on day 14 increased the purity of astrocyte monocultures compared to other protocols. This was assessed by IB4 microglial staining showing <1% positive cells and the observation that LPS, a powerful microglial activator, induced neither TNF-α nor NO responses (Falsig et al., 2004).

2.3. Cyclic AMP measurements

In order to assess cellular cAMP levels a commercially available [^{125}I]-cAMP flashplate assay was used (PerkinElmer, Hvidovre, DK). All assay reagents were obtained and used according to the manufactures instructions. In brief, experiments were stopped by removing the supernatant from the astrocytes followed by lysis of the cells with 100 μl/well 0.1 M HCl at 4 °C. One hour post-lysis the samples were neutralized with 68 μl/well 0.15 M NaOH + 50 mM sodium acetate. For the analysis, 40 μl assay buffer (50 mM sodium acetate, pH 6.2) was added to each well on a flashplate (96-well scintillation plates, precoated with anti-cAMP antibodies) together with 60 μl of sample or 60 μl cAMP standard. Then, 100 μl [^{125}I]-cAMP tracer in carrier buffer (50 mM acetate sodium, 0.1% sodium azide, 12 mM $CaCl_2$, 1% bovine serum albumin, pH 6.2) was added to each well. The flashplates were sealed and left to incubate at 4 °C for 20 h. Luminescence of flashplates was measured on a Wallac Microbeta® system, and data were calculated according to the cAMP calibration curve and standardized to the cell number.

2.4. Quantitative RT-PCR

Cells were lysed and RNA extracted using Trizol reagent according to the manufactures instructions. Purified RNA was treated with "DNA-Free" (Ambion, Huntingdon, UK). Total RNA (1 μg) was reverse-transcribed using TaqMan RT-mastermix (Applied Biosystems, Naerum, DK) using random hexamers in a 100 μl reaction on a PTC-200 DNA engine Thermal Cycler (VWR International, Albertslund, DK). Relative quantification was performed as described earlier (Falsig et al., 2004; Lund et al., 2005) In brief, 2.5 μl of cDNA was mixed with 10 μl SYBR mastermix (Biorad, Grenaa, DK), 4.3 μl water, 0.2 μl HK-UNG 0.1 U/μl and 750 nM of forward and reverse primers and run using a program of 2 min at 50 °C, 3 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Collected data was analyzed and a threshold cycle (tc) for each sample was determined. Relative quantification was achieved by comparing each sample to in-plate reference genes using the delta-delta method. Water controls and genomic DNA controls were run simultaneously with the samples on all plates and did not differ significantly from background. Reference gene selection was made using four different housekeeping genes tested against each other. Primers used were GAPDH sense: 5′-TGC ACC ACC AAC TGC TTA G-3′, antisense: 5′-GGA TGC AGG GAT GAT GTT C-3′; HPRT sense: 5′-AGC TAC TGT AAT GAT CAG TCA ACG-3′, antisense: 5′-AGA GGT CCT TTT CAC CAG CA-3′; RNA polymerase II sense: 5′-TGC GCA CCA CGT CCA ATG ATA-3′, antisense: 5′-GGA GCG CCA AAT GCC GAT AA-3′; Beta-actin sense: 5′-GAT GCT CCC CGG GCT GTA TTC-3′, antisense: 5′-CTC TTG CTC TGG GCC TCG TCA C-3′; TNF-α sense: 5′-CTA TGG CCC AGA CCC TCA CAC TCA-3′, antisense: 5′-CAC TCC AGC TGC TCC ACT TG-3 CTC-3′; IL-6 sense: 5′-GGA GCC CAC CAA GAA CGA TAG TCA-3′, antisense: 5′-GAA GTA GGG AAG GCC GTG GTT GTC-3′; MCP-1 sense 5′-CAT GCT TCT GGG CCT GCT GTT C-3′, antisense: 5′-CCT GCT GCT GGT GAT CCT CTT GTA G-3′; RANTES sense 5′-GCA GCT GCC CTC ACC ATC ATC-3′, antisense: 5′-GAG GCA GCG CGA GGG AGA G-3′. The efficiency of all primers was between 90% and 110%.

The gene expression of murine PDE4 subtypes were measured and presented as described earlier (Selige et al., 2010a). The following endogenous 18S rRNA control primers and probe were used: sense 5′-CGG CTA CCA CAT CCA AGG AA-3′, antisense 5′-GCT GGA ATT ACC GCG GCT-3′, probe 5′-VIC-TGC TGG CAC CAG ACT TGC

CCT C-TAMRA-3′. For analysing the mRNA expression of PDE4 subtypes we used the following TaqMan® gene expression assays from Applied Biosystems (Darmstadt, Germany): Mm00480071_m1 (PDE4A), Mm00480166_m1 (PDE4B), Mm01343237_m1 (PDE4C) and Mm00456878_m1 (PDE4D).

2.5. Measurements of PDE isoenzyme activities and preparation of cellular extracts

Cells (1–3 × 10^6) were counted and washed twice in PBS and resuspended in 1 ml homogenization buffer (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM, HEPES 10 mM, EGTA 1 mM, $MgCl_2$ 1 mM, β-mercaptoethanol 1 mM, pepstatin A 5 mM, leupeptin 10 mM, phenylmethylsulfonyl fluoride 50 mM, soybean trypsin inhibitor, benzamidine 2 mM, pH 8.2). Thereafter, cells were disrupted by sonification, and PDE activity was assessed as described by Thompson et al. (1979) with some modifications (Bauer and Schwabe, 1980). The assay mixture (final volume 200 μl) contained: cellular lysates, Tris–HCl 30 mM; pH 7.4, $MgCl_2$ 5 mM, EGTA 0.1 mM and 0.5 mM of [^{3}H]cAMP as substrate. Reactions were performed for 30 min at 37 °C in 96-well plates and terminated by addition of 50 μl 0.2 M HCl per well. Assays were left on ice for 10 min and then 25 μg 5′-nucleotidase (Crotalus atrox) was added. Following incubation for 10 min at 37 °C assay mixtures were loaded onto QAE-Sephadex A25 columns to bind adenosine and cAMP and subsequently eluted with 30 mM ammonium formiate (pH 6.0). Thereafter, radioactivity (phosphate) in the eluate was measured. Results were corrected for blank values (measured after heat denaturation of samples) that were below 2% of total radioactivity.

Cyclic AMP degradation did not exceed 25% of the amount of substrate/tracer added. The final DMSO concentration was 0.3% (v/v) in all assays. Selective inhibitors and activators of PDE isoenzymes were used to determine activities of PDE families as described previously (Rabe et al., 1993). At the concentrations used in the assay piclamilast (1 μM) and motapizone (10 μM) completely blocked PDE4 and PDE3 activities without interfering with activities from other PDE families. PDE4 activity was calculated as the difference of total cAMP-specific PDE activity in the presence or absence of 1 μM piclamilast. The difference between piclamilast-inhibited cyclic AMP hydrolysis in the presence and absence of 10 μM motapizone was defined as PDE3 activity. PDE1/2 were measured after addition of their respective allosteric activators to the cell lysates. PDE1 was defined as the increment of cyclic AMP hydrolysis (in the presence of 1 mM piclamilast and 10 mM motapizone) induced by 1 mM Ca^{2+} and 100 nM calmodulin. The increase of cyclic AMP degrading activity (in the presence of 1 mM piclamilast and 10 mM motapizone) induced by 5 mM cyclic GMP represented PDE2. The PDE2 inhibitor, 9-(6-phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)purin-6-one (100 nM) completely inhibited this cyclic GMP-induced activity increment further verifying that this activity is PDE2.

2.6. Transcription factor NF-κB nuclear translocation

The nuclear translocation of NF-κB was measured on the Cellomics Arrayscan II system as described elsewhere (Falsig et al., 2004; Lund et al., 2006; Henn et al., 2009). Briefly, cells were preincubated for 5 h with forskolin, clenbuterol and rolipram followed by stimulation with CCM for 30 min. Stimulation was ended by aspiration of the medium and the cells were fixed for 10 min in 4% paraformaldehyde. The NF-κB p65 subunit was immunostained, the nucleus was identified by H-33342 staining and cells were imaged. The p65 intensity difference between the nucleus and cytoplasm was measured. A total of 20 fields/well were imaged at 20× magnification for each sample. All assay reagents were from the Cellomics HitKit™ series and were used according to the manufactures instructions.

2.7. Enzyme-linked immunoassays

ELISA kits were used to measure IL-6 (eBioscience, UK) and MCP-1 (BDbioscience, UK). All assay reagents were obtained and used according to the manufactures instructions.

2.8. Statistics

Data were analyzed as appropriate using Student's *t*-test, one-way ANOVA or two-way ANOVA followed by Bonferroni post-hoc test. Data are presented as mean values ± SEM. *P*-values below 0.05 were considered statistically significant. Data are given for replicates of one representative biological experiment. Experiments were performed 2-4 times. Only results that were reconfirmed are displayed.

3. Results

3.1. Upregulation of cAMP levels by forskolin and β-adrenergic agonists under conditions of PDE4 inhibition

Astrocytes were treated with the adenylate cyclase activators forskolin, clenbuterol and isoproterenol at relatively high, but frequently used, pharmacological concentrations. The effect on average cellular cAMP levels was measured at different time points ranging from 1 h to 24 h. The total cellular cAMP levels of forskolin-/or clenbuterol-stimulated cells (10–20 pmoles/10^6 cells) were not affected, whereas the treatment with isoproterenol (1 μM) alone increased the level of cAMP significantly.

In a parallel experiment the stimulators were combined with the PDE4 inhibitor rolipram. This agent was used at a concentration of 10 μM, which is known to cause nearly complete inhibition of its target (Suttorp et al., 1993). The combination of the direct adenylate cyclase activator forskolin with rolipram led to a 5-fold increase in cAMP concentration after 4 h. Elevated cAMP levels were also observed at 8 h and 24 h (Fig. 1A). The combination of the β_2-adrenergic agonist clenbuterol and rolipram led to about 7-fold increased cellular cAMP concentrations in the time period 4–24 h after the start of the exposure (Fig. 1B). The most pronounced and rapid effect was observed when the mixed β_1/β_2-adrenergic agonist isoproterenol was combined with rolipram. Already 1 h after the stimulation cAMP was increased >15-fold remaining increased for further 23 h (Fig. 1C).

To obtain more information on the concentration-response relationship of the cAMP increase triggered by the adenylate cyclase activators measurements were performed at a fixed time point of 5 h after stimulation. At high concentrations (10 μM) forskolin alone triggered a significant increase. This was further increased by rolipram (Fig. 2A). Clenbuterol alone led to a small (non-significant in our experiments) elevation of cAMP at concentrations of 1–10 μM. However, in the presence of rolipram, it increased cAMP concentration significantly with an EC_{50} of 25 nM (Fig. 2B). Also isoproterenol induced cAMP levels significantly more in the presence of rolipram than when administered alone. In both cases EC_{50} values for the β-agonist were 230–250 nM, but the effect size was 3-fold larger when the PDE4 inhibitor was present (Fig. 2C). In summary, these findings show that PDE inhibition can synergistically augment cAMP levels of astrocytes exposed to different adenylate cyclase activators, while effects of rolipram on average cellular concentrations of the second messenger are negligible in unstimulated astrocytes.

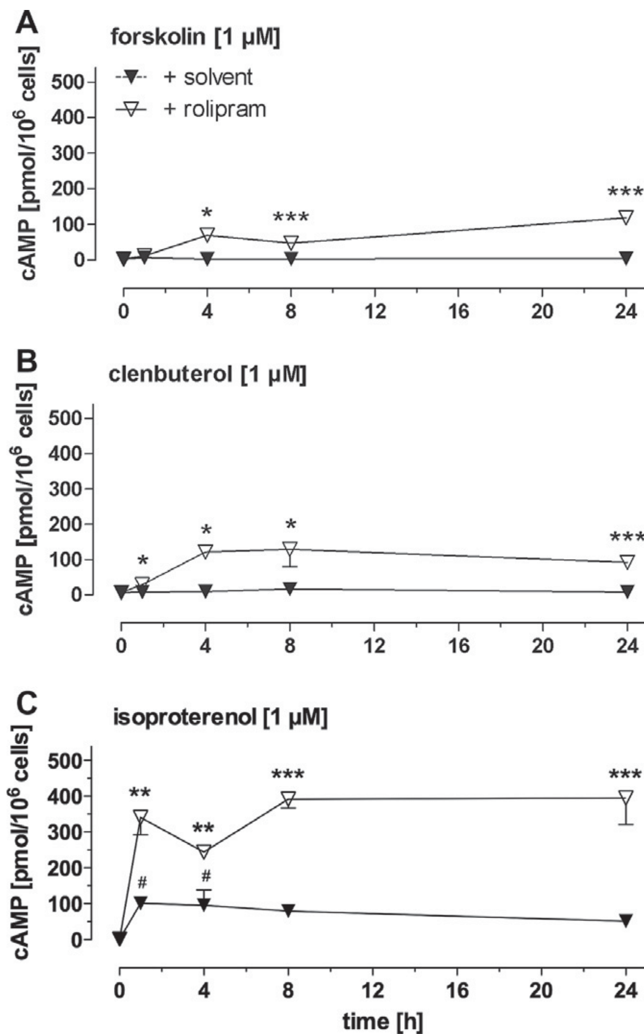

Fig. 1. Synergistic effects of rolipram and β-adrenergic agonists on astrocyte cAMP. Cells were treated with (A) forskolin, (B) clenbuterol or (C) isoprenaline alone (+ solvent) or together with 10 μM rolipram and intracellular cAMP concentrations were determined after the times indicated. Data are means ± SEM of triplicate determinations. Two-way ANOVA showed a significant time and treatment effect ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. no rolipram). Time series with no rolipram were analysed by one-way ANOVA with appropriate post-hoc tests ($^{\#}p < 0.05$ vs. 0 h).

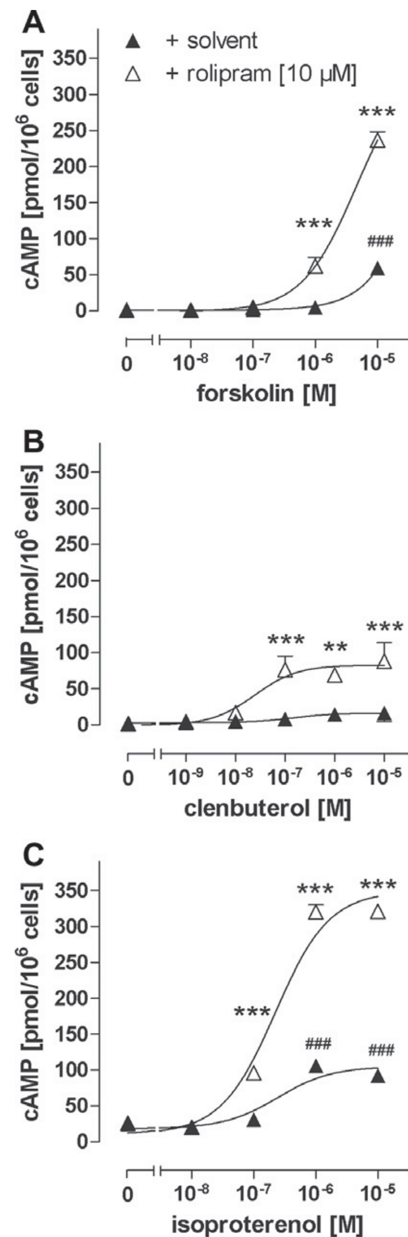

Fig. 2. Modification of cAMP response of forskolin and β-adrenergic agonists by rolipram. Primary astrocytes were stimulated for 5 h with different concentrations of (A) forskolin, (B) clenbuterol or (C) isoproterenol alone or together with 10 μM rolipram, and then intracellular cAMP concentrations were determined. Data are means ± SEM of triplicate determinations. Data were analysed by one-way and two-way ANOVA with appropriate post-hoc tests ($^{**}p < 0.01$, $^{***}p < 0.001$ vs. no rolipram; $^{\#\#\#}p < 0.001$ vs. control of non-stimulated).

3.2. Activity of PDE isoenzymes and gene expression of murine PDE4 subtypes in resting primary astrocytes

Besides PDE4, cAMP levels may be controlled by other phosphodiesterases. Therefore, more information was sought on such enzyme activities in astrocytes, and the enzymatic activities were determined in cell lysates. The cAMP turnover by PDE4 accounted for more than 50% of the total cAMP hydrolyzing enzymatic activity found in control astrocyte lysates. PDE3 activity was low and PDE2 was nearly absent. Notably, the enzymatic activities of PDE1 and PDE2 are regulated in cells by the actual concentrations of allosteric modulators. For determinations in cell lysates only theoretical maximum activities could be measured. These activities do not necessarily reflect the situation in the cell. They only indicate the amount of enzyme present (Fig. 3A). The four different isoenzymes accounting for PDE4 activity cannot be distinguished pharmacologically. Therefore, we obtained an estimate of their relative mRNA expression by quantitative PCR. The most prominent isoform was PDE4B. PDE4C was below the detection limit and the isoforms A and D were detected at low levels (Fig. 3A). As PDE4 is often directly associated with adrenergic receptors the above data suggest that this phosphodiesterase, and predominantly its isoform PDE4B, indeed take a major role in regulating the cellular cAMP levels in β-adrenergic agonist stimulated astrocytes.

3.3. Inhibition of PDE4, but not of PDE3 activity, affects cAMP in astrocytes

In several peripheral inflammatory cells PDE3 can significantly contribute to the modification of signals using cAMP as second messenger. Therefore, we tested here potential additional effects of PDE3 inhibition by milrinone, a drug that specifically targets this

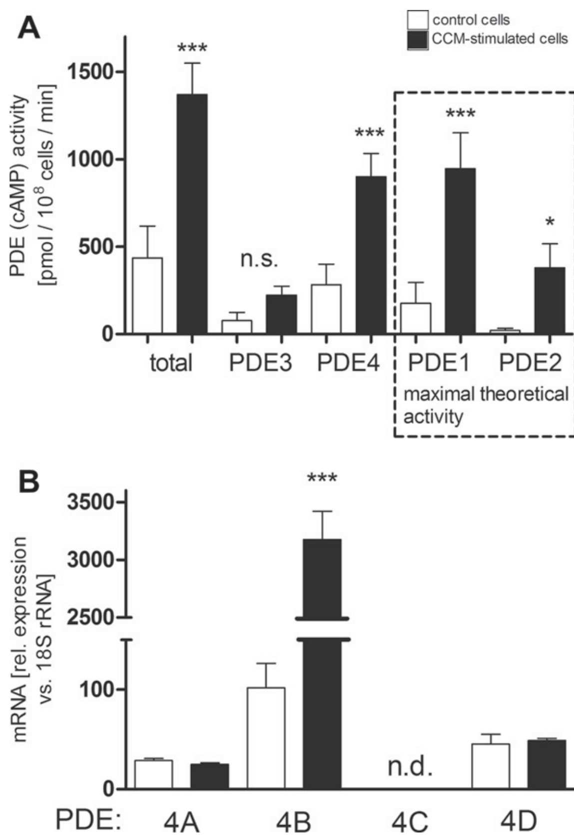

Fig. 3. Enzymatic activities of different PDEs and mRNA expression of PDE4 subtypes in resting and activated astrocytes. Cells were stimulated with a complete cytokine mix (CCM) consisting of 10 ng/ml murine Il-1β, 10 ng/ml murine TNF-α and 5 U/ml murine IFN-γ) for 24 h or were left unstimulated. Then cell lysates were prepared for the determination of PDE enzymatic activities and for mRNA extraction. (A) The total cAMP hydrolyzing activity was determined in addition to activities of different cAMP specific PDEs. Note that PDE1 and PDE2 activity is not comprised in the total activity, as the determination of their activities requires addition of allosteric stimulators. For PDE1 and PDE2, the maximal theoretical activity of the enzyme in the cell lysate was determined after addition of Ca2+/calmodulin (PDE1) or cGMP (PDE2). (B) Gene expression levels of PDE4 subtypes were determined by quantitative RT-PCR and are expressed relative to the levels of 18S rRNA. Data of PDE enzymatic activities are means ± SD from 2 different cell preparations for unstimulated and 4 preparations for CCM-stimulated astrocytes. Data from qRT-PCR are means ± SD from triplicate determinations (different cell preparations), and were analyzed by one-way ANOVA (controlled vs. stimulated; **p* < 0.05; ****p* < 0.001; n.s.: not significant; n.d.: not detectable).

isoenzyme (Howell et al., 1995; Horn et al., 2006). Forskolin, clenbuterol and isoproterenol were used at fixed concentrations, triggering small cAMP increases on their own. Rolipram was used over a wide range of concentrations to identify any potential role of PDE3 at different cellular PDE4 activities. Milrinone alone had no effect on cAMP levels. Also combinations of rolipram and milrinone did not increase the intracellular messenger. Milrinone did not further increase the cAMP levels when given together with an adenylate cyclase activator. Finally, addition of the PDE3 inhibitor did not significantly augment the cAMP response triggered by a combination of rolipram with forskolin or β-adrenergic agonists (Fig. 4).

To obtain evidence on the role of PDE4 independent of the use of rolipram we used another specific inhibitor, RO-201724, and compared its effects to those of rolipram in astrocytes exposed to adenylate cyclase activators (Fig. 5). RO-201724 elevated cAMP concentration-dependently, and was about 5–10 fold less potent than rolipram. These effects were consistent with the reported differences of cellular potency on eosinophils of about 6-fold (Dent et al., 1994), and the 10-fold difference in Ki with respect to PDE4B.

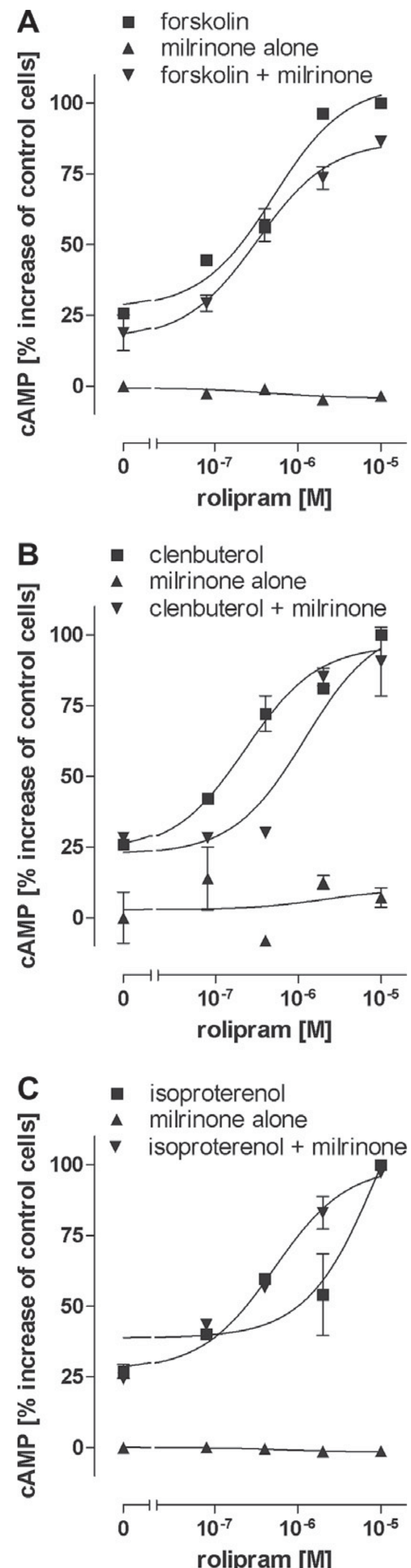

Fig. 4. Minor additional contribution of PDE3 inhibition to cAMP increase driven by PDE4 inhibition. Cells were stimulated for with (A) 2 μM forskolin and/or 10 μM milrinone, (B) 1 μM clenbuterol and/or 10 μM milrinone and (C) 0.5 μM isoproterenol and/or 10 μM milrinone. Rolipram was added with the other drugs at increasing concentrations and intracellular cAMP determined after 5 h. Data were normalized for the cell number and the maximal additional levels triggered by the agonists in the presence of 10 μM rolipram (see Fig. 2) in comparison to control cells. The 0% level indicates the cAMP content of control cells. Data are means ± SEM of triplicate determinations and were analysed by two-way ANOVA with appropriate post-hoc tests (**p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. milrinone).

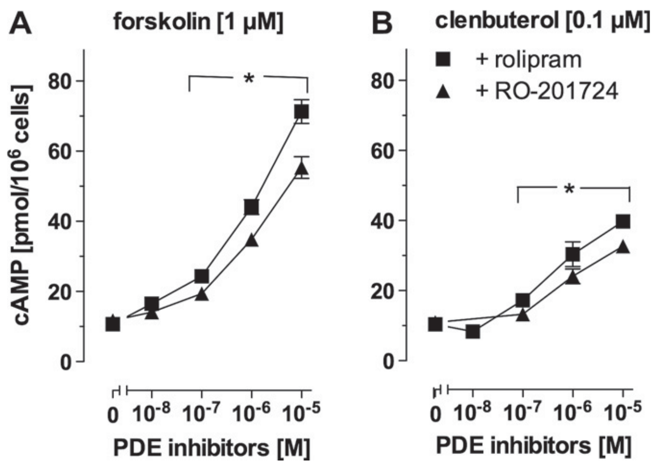

Fig. 5. PDE4 inhibiting properties of rolipram and RO-201724. Primary astrocytes were stimulated for 5 h with (A) 1 µM forskolin or (B) 0.1 µM clenbuterol in the presence of PDE inhibitors. Then intracellular cAMP concentrations were determined. Data are means ± SEM of triplicate determinations. Data were analysed by Student's *t*-test ($^{*}p < 0.05$)

Thus, different PDE4 inhibitors showed similar effects on astrocyte cAMP levels when corrected for their known potencies.

3.4. PDE isoenzymes and cAMP levels in inflammatory astrocytes

Exposure of astrocytes to additional mediators may affect their cAMP levels in experiments designed to investigate potential anti-inflammatory effects of PDE inhibitors. Therefore, cAMP levels were compared in resting astrocytes and in inflammatory astrocytes treated with a complete cytokine mix (CCM), a well-characterized astrocyte activator (Falsig et al., 2004; Henn et al., 2009, 2011), consisting of TNF-α, IL-1β and IFN-γ. In this regard, we used 13 different combinations of adenylate cyclase activators (different concentrations) with rolipram (10 µM). Under these conditions cAMP was measured after 5 h. The results showed consistently, and at all combinations, that the cAMP increases were attenuated by 30% in the presence of CCM (compared to resting cells; data not shown). Apart from this, all responses (e.g. concentration-dependence or potency range) were exactly as in non-inflammatory astrocytes (data not shown). These data suggest that combinations of PDE4 inhibitors and adenylate cyclase activators affect cAMP in activated astrocytes similarly as in resting astrocytes.

We also addressed the question of whether the PDE spectrum may change in astrocytes after exposure to inflammatory cytokines. The total cAMP hydrolyzing activity increased about 250% after astrocyte activation. Also, under these conditions PDE4 accounted for about 70% of the activity while PDE3 levels were relatively low. Notably, "potential PDE1 and PDE2 activities" were strongly upregulated under inflammatory conditions, indicating that these enzymes were induced and present in higher amounts in inflammatory cells. However, these enzymes do not contribute to cAMP turnover, unless cGMP and Ca^{2+}/calmodulin are increased simultaneously (Fig. 3A).

As in the resting astrocytes we also examined activated astrocytes exposed to CCM for 18 h for the mRNA levels of their PDE4 isoforms. The inflammatory activation increased the mRNA expression of PDE4B significantly (about 32-fold) whereas the expression of PDE4A and PDE4D was unaffected (Fig. 3B). Thus, PDE4B is the dominant isoform under inflammatory conditions, and may even be used as a biomarker of activation. The overall relative contribution of PDE4 to cAMP hydrolysis did not change in activated astrocytes. Thus, it is likely that the pharmacological actions of PDE4

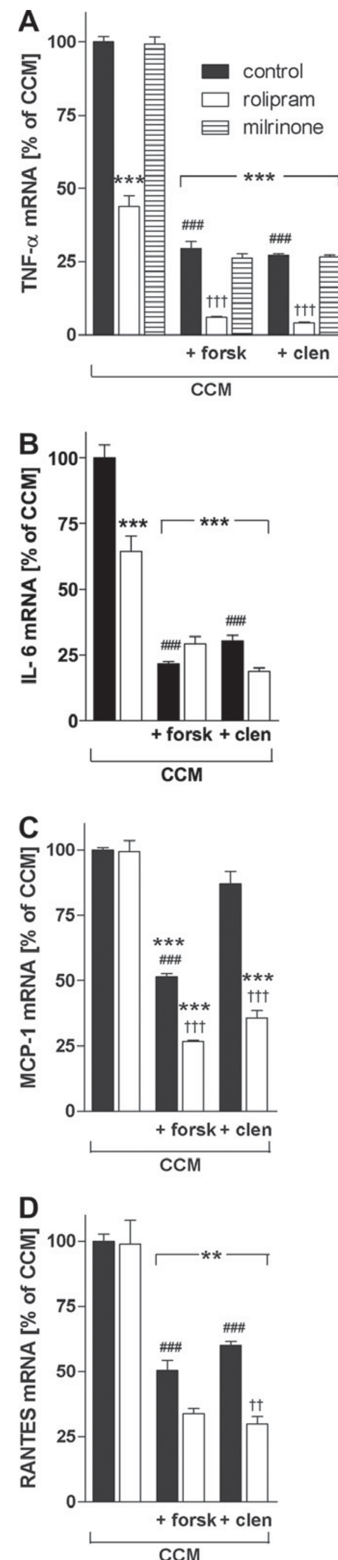

Fig. 6. Attenuated up regulation of mRNA for cytokines/chemokines by combined use of PDE inhibitors and β-adrenergic agonists in inflammatory astrocytes. Cells were preincubated for 5 hours with 1 µM forskolin (forsk), 0.1 µM clenbuterol (clen), 10 µM rolipram (rol) and 10 µM milrinone (mil) followed by 6 h of stimulation with CCM. The mRNA was prepared and analysed by quantitative RT-PCR. Values of cell exposed to CCM alone were set to 100% (positive control) and were always significantly increased compared to those from untreated astrocytes. Data are means ± SEM of triplicate determinations and are expressed as % change in mRNA expression compared to positive control. One-way ANOVA with appropriate post-hoc tests ($^{**}p < 0.01$, $^{***}p < 0.001$ vs. CCM; $^{\#\#\#}p < 0.001$ vs. rolipram; $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ vs. corresponding group without rolipram).

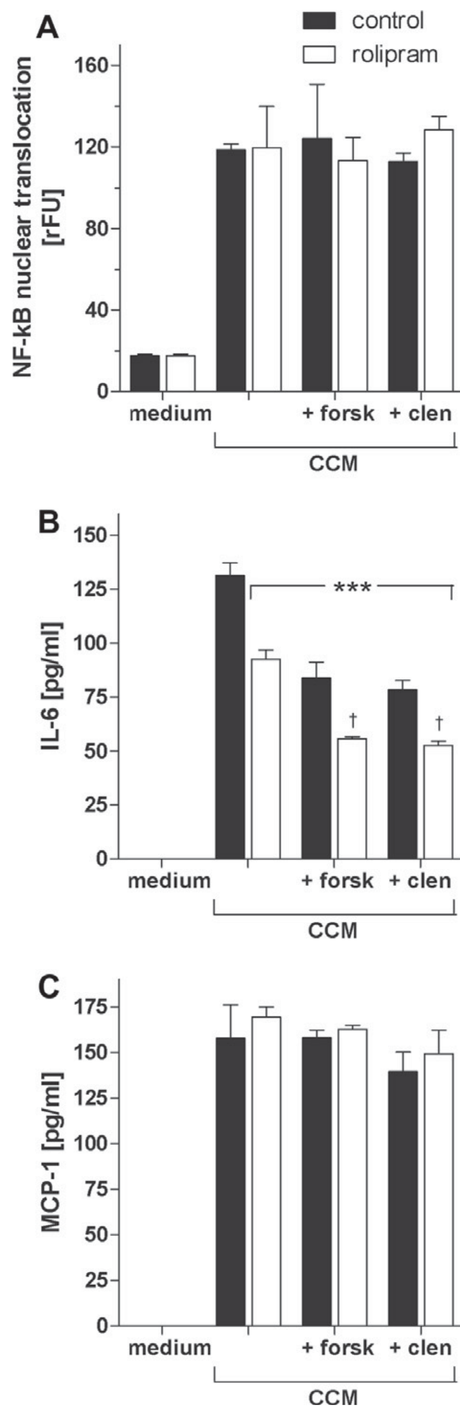

Fig. 7. Differential effects of PDE4 inhibition combined with β-adrenergic agonists on inflammatory mediators in cytokine-stimulated astrocytes. Cells were preincubated for 5 h with 1 μM forskolin, 0.1 μM clenbuterol and 10 μM rolipram followed by (A) 1 h and (B and C) 24 h of stimulation with CCM. (A) Cells were fixed and stained to visualize the nuclei and the distribution of the p65 NF-κB subunit. After microscopy imaging the staining intensities in the nucleus and the cytoplasm were quantified. Data are expressed as intensity difference between the nucleus and cytoplasm. (B–C) The supernatants were collected and analysed by ELISA to determine secreted polypeptides. Data are means ± SEM of triplicate determinations and were analysed by one-way ANOVA with appropriate post-hoc tests ($^{*}p < 0.05$, $^{***}p < 0.001$ vs. CCM; $^{\dagger}p < 0.05$ vs. corresponding group without rolipram).

inhibitors remained similar. The increased PDE activity in inflammatory astrocytes also correlated well with the slightly attenuated increase of cAMP in such cells when exposed to rolipram together with adenylate cyclase activators.

3.5. Anti-inflammatory effects of forskolin, clenbuterol and rolipram on mRNA regulation

Astrocytes were exposed to CCM for 6 h, which leads to a massive upregulation of inflammatory mediator mRNAs (Falsig et al., 2006). Preincubation with rolipram, but not milrinone attenuated the TNF-α response by about 50%. Also 1 μM forskolin and 0.1 μM clenbuterol attenuated the inflammatory effect. Only the combination of rolipram with the adenylate cyclase activators prevented CCM-induced TNF-α transcription nearly completely (Fig. 6A).

We then examined IL-6, MCP-1, and RANTES as examples of mediator mRNAs that are particularly strongly upregulated in activated astrocytes (Falsig et al., 2006). The effects of rolipram alone were small or absent. The adenylate cyclase activators attenuated the response by about 50%, and in most cases this effect was significantly enhanced in the presence of rolipram (Fig. 6B–D). Thus, combinations of rolipram and clenbuterol showed an attenuating effect on astrocyte inflammatory mRNAs, but the extent of inhibition depended on the type of mediator.

3.6. Differential regulation of inflammatory mediators by forskolin, clenbuterol and PDE4 inhibition

We examined NF-κB activation as receptor-proximal signalling event in order to test whether the cAMP-elevating drugs would affect receptor availability and signalling. For this purpose, the translocation of the transcription factor to the nucleus was examined on the single cell level. After 30 min of stimulation with CCM an upregulation of nuclear NF-κB by 655% was observed. This nuclear translocation was unaffected by preincubation with forskolin, clenbuterol or combinations with rolipram (Fig. 7A). Thus, upstream immunological signalling in astrocytes was not disturbed by cAMP-elevating drugs.

Next we examined the release of inflammatory mediators following astrocyte activation. IL-6 and MCP-1 were chosen as examples for cytokine and chemokine secretion by activated astrocytes. TNF-α was not measured, as primary astrocytes do not secrete this cytokine (Falsig et al., 2004). Stimulation with CCM for 24 h increased IL-6 and MCP-1 secretion from below the detection limit (10 pg/ml) in the control cultures to 130 pg/ml and 160 pg/ml, respectively (Fig. 7B and C). IL-6 secretion was decreased by 30–40%, following preincubation with clenbuterol, forskolin and rolipram for 5 h (Fig. 7B). Co-administration of these drugs decreased the IL-6 secretion by 60%. No effect was observed by clenbuterol, forskolin and rolipram on MCP-1 secretion (Fig. 7C). Thus, the release of mediators was affected differentially by cAMP-elevating drug combinations.

4. Discussion

The present study showed that the combination of β-adrenergic agonists or the direct adenylate cyclase activator forskolin with PDE4 inhibitors increased the cAMP levels in cortical murine astrocytes. The enhancing effect of PDE4 inhibitors was consistent with PDE4 accounting for 50% of the cAMP hydrolyzing activity in resting or activated astrocytes. The altered cAMP levels after drug treatment had functional consequences as the combination of the β_2-adrenergic agonist clenbuterol with rolipram decreased mRNA levels of inflammatory mediators in activated astrocytes, and attenuated the release of IL-6. These findings may be of relevance *in vivo* as subpopulations of astrocytes are closely associated with brain noradrenergic terminals (Cohen et al., 1997). They have also been shown previously to express β-adrenoceptors (Sutin and Shao, 1992). Astrocyte β-adrenoceptor expression is dependent

on surrounding neuronal activity and may depend on the developmental stage. Accordingly, some studies found mainly β_1-adrenoceptor expression, while other studies suggest relatively higher β_1-adrenoceptor expression (Sutin and Shao, 1992; Cahoy et al., 2008; Hertz et al., 2010). The results of the present study indicate that the β_2-receptor, the target of clenbuterol, was expressed and functional. Moreover, as the effects of the mixed β_1/β_2-adrenergic agonist isoproterenol were more pronounced than those of the more selective β_2-adrenergic agonist clenbuterol, also β_1-adrenoceptors appeared to be functional. Further studies using selective β_1-adrenergic agonists and selective β-adrenoceptor antagonists would be required to unambiguously clarify the different expression levels. However, this was not the focus of the present study.

Our findings that the efficacy of forskolin, and of both β-adrenoceptor agonists, was augmented by rolipram indicate a high cAMP metabolism by PDE4 in astrocytes. The strong synergistic effects of β-adrenergic agonists and rolipram suggest that PDE4 activity played an important role in keeping average cellular cAMP levels at control levels even when adenylate cyclase was stimulated by clenbuterol or isoproterenol.

The immunomodulatory role of cAMP has been known for a long time, and pharmacological targets to modulate the second messenger have been investigated (Essayan, 2001). For instance, β_2-adrenoceptors and PDE4 inhibitors have been shown to increase intracellular levels of cAMP, and to have anti-inflammatory properties in non-neural cells (Szabo et al., 1997; Farmer and Pugin, 2000; Zhang et al., 2002; Pages et al., 2009). Furthermore, several studies demonstrated not only an additive effect of β_2-adrenoceptors and PDE4 inhibitors (in different cell types), but also provided evidence for the spatial proximity of these adenylate cyclase activators and cAMP-hydrolyzing enzymes (Xiang et al., 2005; Manning et al., 1996; Houslay et al., 2007).

To our knowledge, this is the first study examining the enzymatic activities of several cAMP-hydrolyzing PDE isoenzymes in astrocytes. The enzyme activity of PDE4 is strongly upregulated after cytokine stimulation. This indicates a critical role of the enzyme within the astrocytic inflammatory response. The underlying mechanism of the increased enzyme activities of PDE4 after CCM-stimulation in astrocytes remains uncertain. However, high concentrations of IL-1β as used in our study have been shown to increase the expression of PDE4 in human lung fibroblast by elevating cyclo-oxygenase-2 and prostaglandin E_2 (Selige et al., 2010b). The latter two factors are also known to be increased in activated astrocytes (Falsig et al., 2004).

Analyses of the mRNA expression of different PDE4 subtypes revealed a predominant role of the subtype PDE4B in this context. The strong upregulation of the PDE4B transcript is in line with the work of Borysiewicz et al., 2009, who showed an upregulation of PDE4B2 in activated astrocytes from newborn rat brains after toll-like receptor stimulation. PDE4B was also found upregulated in stimulated microglia (Lund et al., 2006). The significant upregulation of overall cAMP hydrolysing activity and of PDE4 activity in particular, in activated astrocytes may have different consequences. First, agonist-triggered rises of cAMP may be attenuated. This agrees with our findings of responses reduced by 30%. Second, inhibition of PDE4 may be pharmacological more effective in inflammatory cells than in resting cells. This effect may explain our observation that rolipram alone had an anti-inflammatory effect in activated cells, although it did not trigger measurable changes of cAMP in resting cells.

The increased "activity" of PDE2 in astrocytes after CCM-stimulation is interesting. PDE2 is allosterically activated by cGMP and nearly inactive at low levels of this cyclic nucleotide. Therefore, saturating amounts of cGMP were present in the assay buffer. Quiescent astrocytes had hardly any PDE2 enzyme activity. In contrast, after CCM-stimulation the "potential activity" of PDE2 was increased about 20-fold, and may thus play a major role in the immune response of astrocytes under conditions leading to elevated cGMP levels. Similar findings have been described for peritoneal macrophages or endothelial cells. In these systems, LPS- and TNF-α-stimulation have been reported to increase the activity of PDE2 (Witwicka et al., 2007; Seybold et al., 2005). CCM-stimulated astrocytes have an increased expression of the inducible NO-synthase (Falsig et al., 2004) and thus produce higher amounts of NO. This may lead to an increase of cGMP via activation of soluble guanylate cyclase and may finally contribute to an augmented activity of PDE2 (Gustafsson and Brunton, 2002). Further studies are required to determine the role of PDE2 in activated astrocytes under different conditions.

Stimulation of astrocytes with CCM induced nuclear translocation of NF-κB consistent with previous studies indicating involvement of this transcription factor in TNF/IL-1 signalling in astrocytes (Pawate et al., 2004; Brambilla et al., 2009; Henn et al., 2011). NF-κB translocation is triggered within minutes after receptor activation. The transcription factor then acts in the nucleus on the promoters of many inflammatory factors. Accordingly, raised cAMP levels can interact on many levels with NF-κB signalling and the eventual outcome depends on the cellular context and endpoint studied. For instance, in primary hepatocytes cAMP inhibits the IL-1β and IFN-γ induced NF-κB translocation (Hong et al., 2010). Also, in several other models PDE inhibitors attenuated NF-κB signalling (Kwak et al., 2005; Sánchez et al., 2005; Chi et al., 2004; Hervé et al., 2008). On the other hand, cellular models have been described in which cAMP or PDE inhibitors had no effect on NF-kB translocation or signalling activity (Ammit et al., 2000; Abdulla and Renton, 2005). It is even possible that PDE inhibitors do not affect initial NF-κB translocation in a given model, but they then attenuate its transcriptional activity (Gobejishvili et al., 2008). This complexity is due to multiple actions of cAMP, for instance dependent or independent of protein kinase A, and mediated or not via phosphorylation of CREB, NFAT or other relevant targets (Torgersen et al., 2002; Loza and Penn, 2010). Moreover, different promoters have several different binding sites for the transcription factors affected by cAMP. Thus, one cytokine may be downregulated while another one is upregulated, or not affected at all within the same cell (Kreckler et al., 2009; Spooren et al., 2010). In astrocytes, it appears as if the very initial translocation triggered by cytokines is not inhibited by raised cAMP, but later effects on NF-κB signalling that would be relevant for the release of inflammatory mediators cannot be excluded. These could include upregulation of IB expression. Such an effect was observed after noradrenaline treatment in astrocytes (Gavrilyuk et al., 2002) and could therefore also play a role in our experimental system.

The different mechanisms, types of effectors and pathways contributing to the effects of cAMP in cells may also explain the different effects of rolipram and clenbuterol on IL-6 secretion vs. chemokine release. For instance, the effects of forskolin or clenbuterol on IL-6 mRNA expression were not enhanced by rolipram, while the pharmacological treatments showed additive effects when IL-6 protein secretion was measured. This could have been due to different effects of cAMP on post-translational regulatory mechanisms, mRNA stability and transcription as discussed previously (Maier et al., 2009). The secretion of many inflammatory mediators is regulated post-transcriptionally, and this level of control may be affected by cellular cAMP pools in a different way, than transcription itself.

MCP-1 is another example of differential regulation that shows the importance of functional (protein) measurements in addition to the analysis of transcript levels. Rolipram alone did not affect MCP-1 mRNA levels. This might indicate a requirement for high concentrations of cAMP, or for an increased level of the second messenger throughout the cell for modulation of chemokine

expression. This is consistent with our findings, that only the combinations of forskolin or clenbuterol with rolipram decreased mRNA levels below 50%, and that the overall downregulation of MCP-1 mRNA was smaller than the one of IL-6. This smaller downregulation may not have been sufficient to affect the amount of protein secreted from the cells.

Besides their metabolic role, astrocytes possess both pro- and anti-inflammatory capabilities (Dong and Benveniste, 2001). We found here that clenbuterol inhibited the transcription of inflammatory mediators, although it had no effect on average cellular cAMP levels when administered alone. Rolipram alone was also able to down-regulate several inflammatory mediators without affecting overall cellular cAMP. This apparent inconsistency may be due to analytical reasons, as the cAMP-assay may not have picked up small changes of the low basal cAMP levels in astrocytes. An alternative explanation is the subcellular compartmentalisation of cAMP, and our inability to detect local increases within individual cells. It is well-established that different PDEs generate subcellular signalling compartments and nucleotide pools. Thereby, the increase of a PDE4 controlled cAMP-pool can exceed a required threshold to mediate the observed anti-inflammatory effects without elevating overall cellular cAMP (Houslay, 2010). Moreover, the temporal resolution of our assay did not allow for the detection of transient increases in cAMP levels. These may have occurred directly after receptor stimulation, or at later time points. Such changes may be involved in relatively fast neuronal signals (second – minute range), but they are unlikely to affect the inflammatory activation of astrocytes, which requires a continuous signal (hours range) to alter the transcriptional program.

Elevation of cAMP levels by PDE inhibition has been explored for many peripheral inflammatory cells, such as neutrophils, eosinophils, monocytes, macrophages, T cells and dendritic cells (Hatzelmann and Schudt, 2001), and also for microglia in the brain (Zhang et al., 2002). The present study now adds additional knowledge on the potential for the modulation of astrocyte inflammation by combinations of cAMP elevating agents. The combination of PDE inhibitors with clenbuterol appears interesting as the former compound can be used in humans, and has been shown to attenuate inflammation in rodent brain (Gleeson et al., 2010; Culmsee et al., 2007). In our study, both clenbuterol and forskolin showed significantly higher anti-inflammatory effects than rolipram. Co-administration of rolipram with either clenbuterol or forskolin led to a further attenuation of the inflammatory response of these agents, which fits well with the observed cAMP regulations. Although we are not aware of astrocyte studies showing such additive effects it has been indicated in a rodent depression model that this drug combination works in the brain (Zhang et al., 2005).

The absence of anti-inflammatory effects of a PDE3 inhibitor was in accordance with the observed lack of effect of PDE3 inhibition on cAMP levels, and with the low cellular PDE3 activity. Only few other cell types show such a PDE profile. Among these are neutrophils that, like astrocytes, are involved in the innate immune response. They respond maximally to PDE4 but not PDE3 inhibitors (Schudt et al., 1999).

In summary, the data of the present study indicate that combined β_2-adrenoceptor stimulation and PDE4 inhibition downregulates inflammation in cytokine-stimulated astrocytes. It thus seems that selective stimulation of astrocyte β_2-adrenoceptors in combination with inhibition of PDE4 could possible play a future therapeutic role in brain diseases aggravated by inflammation.

Acknowledgements

We are grateful to Heike Goebel from the Nycomed GmbH for the excellent technical support. Parts of the study were performed during the former employment of authors at H. Lundbeck A/S and supported by internal funds. Furthermore, the study was supported by the DFG, the Doerenkamp-Zbinden foundation and the University of Konstanz (ICRC for MemoStress).

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