Adenovirus-mediated Delivery and Expression of a cAMP-dependent Protein Kinase Inhibitor Gene to BEAS-2B Epithelial Cells Abolishes the Anti-inflammatory Effects of Rolipram, Salbutamol and Prostaglandin E₂: A Comparison with H-89

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**Running title:** Adenovirus-mediated expression of PKIα in BEAS-2B cells

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**Abbreviations:** BSA, bovine serum albumin; cAMP, cyclic adenosine-3',5'-monophosphate; GM-CSF, granulocyte/macrophage colony-stimulating factor; 8-Br-cAMP, 8-Bromo-cyclic adenosine-3',5'-monophosphate; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PDE, phosphodiesterase; COPD, chronic obstructive pulmonary disease; GEFs, guanine nucleotide exchange factors; GFP, green fluorescent protein; PKI, heat-stable inhibitor of PKA; Cα, α-isoform of the catalytic subunit of PKA; CRE, cAMP response element, CREB, cAMP response element binding protein; CREM, cAMP response element modulator; AA, arachidonic acid, MCP, monocyte chemotactic peptide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; HBSS, Hanks’ balanced salt solution; FCS, fetal calf serum; DMEM, Dulbecco’s-modified Eagles medium; HEK, human embryonic kidney; CMV, cytomegalovirus; EGF, epidermal growth factor; DAPI, 4',6-diamidino-2-phenylindole; CNS, central nervous system.

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Abstract

cAMP-Elevating drugs are thought to mediate their biological effects by activating the cAMP/cAMP-dependent protein kinase (PKA) cascade. However, this hypothesis is difficult to confirm due to a lack of selective inhibitors. Here, we have probed the role of PKA in mediating inhibitory effects of several cAMP-elevating drugs in BEAS-2B epithelial cells using an adenovirus vector encoding a PKA inhibitor protein (PKIα), and have compared it to H-89, a commonly used small molecule PKA inhibitor. Initial studies established efficient gene transfer and confirmed functionality of PKIα 48 h after virus infection. All cAMP-elevating drugs tested promoted the phosphorylation of CREB, activated a CRE-driven luciferase reporter gene and suppressed both GM-CSF generation and [3H]AA release in response to interleukin-1β and monocyte chemotactic protein (MCP)-1 respectively. These effects were abolished by PKIα. In contrast, H-89 behaved unpredictably under the same conditions. Thus, while CREB phosphorylation evoked by a range of cAMP-elevating drugs was abolished by H-89, neither activation of the CRE-dependent luciferase reporter gene construct nor the inhibition of GM-CSF generation by was inhibited. Paradoxically, H-89 antagonised MCP-1-induced [3H]AA release and enhanced the inhibitory effect of submaximal concentrations of rolipram and 8-Br-cAMP. We suggest that expression of PKIα in susceptible cells provides a simple and unambiguous way to assess the role of PKA in cAMP signalling and to probe the mechanism of action of other drugs and cAMP-dependent responses where the participation of PKA is equivocal. Furthermore, these data suggest that H-89 is not a selective inhibitor of PKA and should be avoided.
Through highly coordinated changes in the rate of synthesis and degradation, cAMP mediates the effect of a large number of hormones, autacoids and neurotransmitters. Current dogma holds that agonism of Gs-coupled receptors augments the basal activity of one or more isoforms of adenylyl cyclase. The cAMP signal then is propagated and amplified through the activation of PKA ultimately to effect a change in cell function (see Beavo and Brunton, 2002). In the inactive state PKA is tetramer composed of two catalytic and two regulatory subunits (Francis and Corbin, 1999). cAMP, when elevated, binds to the regulatory subunits resulting in the dissociation of the inactive holoenzyme and the release of two catalytically-active subunits, which then phosphorylate various target proteins (Francis and Corbin, 1999). Conversely, re-association of catalytic and regulatory subunits to form an inactive holoenzyme occurs when the cAMP concentration falls below a certain threshold. Typically, this is achieved enzymatically following the conversion of cAMP to 5′-adenosine monophosphate by one or more PDEs of which multiple distinct families have been described (Beavo, 1995, Soderling and Beavo, 2000). Although a wealth of data support the idea that cAMP-elevating drugs mediate their effects by activating PKA, there are many reports that are inconsistent with this hypothesis (Martin et al., 2001, Staples et al., 2001). Indeed, cAMP is now known to interact with, and/or signal through, multiple intermediates including PDE2, PKG, GEFs and certain ion channels (see Beavo and Brunton, 2002). Thus, cAMP-induced responses are not invariably mediated by a common mechanism involving PKA.

A significant factor that has hampered the unequivocal assignation of PKA to cAMP-induced responses is a lack of selective pharmacological tools. Many reported compounds marketed as PKA inhibitors (e.g. H-7, H-8, H-89) are isoquinolinesulfonamides, which, in cell-free systems at least, behave remarkably non-selectively (Davies et al., 2000). Another limitation is that H-89 is a β-adrenoceptor antagonist with an affinity similar to that required for the inhibition of PKA (Penn et al., 1999). More recently, certain Rp-cAMPS analogues have been shown to selectively inhibit PKA (Dostmann et al., 1990). However, these compounds must also be used with prudence given their potential to interact with other cAMP-binding proteins (Enserink et al., 2002, Ingram and Williams, 1996, Rangarajan et al., 2003).
The most selective inhibitors of PKA currently defined belong to a family of small heat-stable proteins that compete with substrate by acting at the catalytic site of the enzyme (Collins and Uhler, 1997, Olsen and Uhler, 1991b, Scarpetta and Uhler, 1993). In humans three genes (PKIA, PKIB, PKIG) encode the α, β and γ families of PKIs respectively (Zeng et al., 2000). These proteins have a cell type-specific distribution (Collins and Uhler, 1997, Olsen and Uhler, 1991b, Van Patten et al., 1992; Zeng et al., 2000) although in many of cells including leukocytes and lung little if any PKI is expressed (Collins and Uhler, 1997). In addition to acting as pseudosubstrate inhibitors, each PKI has a nuclear export signal and is able to form a complex with PKA catalytic subunits at the nucleus and export them to the cytosol where an inactive PKA holoenzyme can reform (Fantozzi et al., 1992, 1994). Despite their high selectivity for PKA over other kinases, PKIs and inhibitory peptides derived from these proteins are poorly cell permeant and are limited to studies in permeabilized cells (Ensenat-Waser et al., 2002, Harris et al., 1997). Improvements in peptide permeation can be achieved by N-myristoylation (Ensenat-Waser et al., 2002, Harris et al., 1997), but this approach is compromised by incomplete uptake into the cell and the selective partitioning of N-myristoylated-peptides into the plasma membrane (Harris et al., 1997). One method to circumvent these problems is to transfect cells with plasmids encoding active peptides derived from PKIα (Grove et al., 1987, Olsen and Uhler, 1991a). However, while effective, this approach is restricted to the relatively few cell types that can be transfected efficiently, and is not germane to primary cells.

Here we report the validation and utility of an alternative method to inhibit PKA by using an adenovirus vector to deliver and express PKIα. Given the controversy that surrounds the mechanism of action of cAMP-elevating in many pro-inflammatory and immune cells (e.g. see Takayama et al., 2002, Pang and Knox, 2001, Staples et al., 2001) we have tested the hypothesis that cAMP-elevating drugs evoke responses in BEAS-2B epithelial cells through activation of PKA. This technique provides a simple and unambiguous way to assess the biological role of PKA in many cells and may aid the identification of novel mechanisms of signalling that could be exploited to therapeutic advantage.
Materials and Methods

Generation of recombinant adenoviruses. A recombinant adenovirus containing a 251-bp DNA fragment encoding the complete amino acid sequence of rabbit skeletal muscle PKIα (Day et al., 1989) was prepared as outlined in figure 1 (Lum et al., 1999). The DNA was cloned into the shuttle vector pACCMVpLPa (Becker et al., 1994), which is derived from the plasmid pAC, producing pACCMV-PKIα (Gomez-Foix et al., 1992). In this vector the E1a and part of E1b sequences (required for replication) were replaced between map units 1.3 and 9.1 of the Ad5 genome with the CMV immediate early promoter. The pUC19 single cloning cassette was inserted immediately downstream from the CMV promoter followed, in order, by a fragment of the SV40 genome, including the small t antigen intron, and polyadenylation signal. Equimolar amounts of pACCMV-PKIα (0.2 µg) and the plasmid pJM17 (0.8 µg) were co-transfected in to HEK 293 cells (Graham et al., 1977) using cationic liposomes (Lum et al., 1999). The plasmid pJM17 contains the full-length Ad5 DNA molecule (with a non-functional E3 region) and a 4.3 kb pBRX bacterial insert (providing ampicillin and tetracyclin resistance sequences) in the E1 region at 3.7 map units, thereby exceeding the packaging limit of the Ad5 capsid (McGrory et al., 1988). Homologous recombination between the two plasmids generated an E1′, E3′ Ad genome that could replicate and be packaged into virions only in HEK 293 cells in which E1 function is supplied in trans by integrated, constitutively expressed Ad E1 sequences. A single clone of recombinant virus was isolated by serial dilution using a plaque assay and expanded in HEK 293 cells. The genome of the Ad5.CMV.PKIα vector was confirmed by PCR amplification of contiguous Ad/expression cassette sequences, purified by double caesium chloride ultra-centrifugation and exhaustively dialyzed against buffer A (10 mM Tris - pH 7.4, 10 mM MgCl2, 10% v/v glycerol). Stocks of virus containing ~10^{11} pfu/ml were prepared in buffer A and stored at -80°C. Under these conditions there was no precipitation of virus particles or loss of virus infectivity due to inactivation or aggregation (Nyberg-Hoffman & Aguilar-Cordova, 1999).

To control for the biological effect of the virus per se, the vector, Ad5.CMV.Null, expressing no transgene, was constructed in a similar manner but without sub-cloned gene sequences between the CMV promoter and the polyadenylation signal. In preliminary experiments the efficiency of infection
was assessed using an Ad5 vector encoding GFP (Ad5.CMV.GFP), which is available commercially (Qbiogene-Alexis Ltd, Nottingham, UK).

**Infection of BEAS-2B cells with Ad5 vectors.** BEAS-2B cells (passage number < 50) were seeded in 6- or 24-well plastic plates containing keratinocyte serum-free medium supplemented with 5 ng/ml EGF, 50 µg/ml bovine pituitary extract, 100 mg/ml penicillin and 100 µ/ml streptomycin. The cells were cultured until 50% confluent and then infected with either Ad5.CMV.PKIα or Ad5.CMV.Null (MOI = 10 to 150 as indicated), or left untreated (naïve) for 48 h at 37°C. Cells were growth arrested for 12 h in serum-free medium and processed for biochemical and functional measurements as described below. The efficiency of transgene expression was determined by calculating the number of GFP-expressing cells as a percentage of total number of cells that were counterstained with the nuclear marker DAPI.

**Measurement of PKA activity.** Naïve and Ad5-infected BEAS-2B cells were treated at 37°C with 8-Br-cAMP (1 mM; 30 min), rolipram (30 µM; 30 min), salbutamol (10 µM; 5 min), PGE₂ (10 µM; 5 min) or vehicle as indicated in the text. Cells were removed from culture plates, collected in 15 ml of ice-cold HBSS and centrifuged (15000 x g; 5 min). Each pellet was resuspended in 200 µl of ice-cold buffer B (20 mM MOPS – pH 7.2; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 150 mM NaCl), left for 15 min and sonicated with a probe attachment for 10 s. PKA activity in the resulting supernatants was determined by measuring the phosphorylation of Kemptide as described previously (Giembycz and Diamond, 1990). One unit of activity is defined as that amount of PKA that catalyzed the incorporation of 1 pmol phosphate from ATP into Kemptide per minute per mg protein at 30°C and pH 7.2. The activation state of PKA is expressed as an activity ratio. This index is defined as the specific activity of Kemptide kinase obtained in the absence of cAMP divided by the specific activity of Kemptide kinase obtained in the presence of a concentration of exogenous cAMP required to maximally activate PKA. Kemptide phosphorylation that was insensitive to PKIα (i.e. residual kinase activity in cells expressing the Ad5.CMV.PKIα transgene) represents a kinase other than PKA and was subtracted from the total specific activity in naïve and Ad5.CMV.Null-treated cells before activity ratios were calculated.
Measurement of PKIα inhibitory activity. Naïve and Ad5-infected BEAS-2B cells (multiplicity of infection [MOI] = 100; 48 h) were removed from the culture plates, suspended in 200 µl ice-cold buffer C (20 mM MOPS – pH 7.2, 5 mM DTT, 1 mM EDTA, 1 mM EGTA) containing PMSF (50 µg/ml), aprotinin (25 µg/ml) leupeptin (10 µg/ml) and pepstatin (10 µg/ml) and sonicated with a probe attachment for 10 s. Cell lysates were centrifuged (1200 × g; 15 min) and the protein concentration in the resulting supernatant was adjusted to 1µg/µl with buffer C containing proteinases inhibitors. After heating (95°C; 10 min), the inhibitory activity of the cytosolic extract from naïve and Ad5-infected cells against recombinant Cα and Type Iα PKG (both 100 u/tube) was determined. In these experiments heat-inactivated cytosol was added to the PKA assay cocktail, incubated for 30 min at 30°C and processed as before (Giembycz and Diamond, 1990). Protein kinase G was measured in the same way as PKA except that the assay was conducted at 4°C a reaction mix supplemented with cGMP (5 µM) and with Glasstide (100 µM) as substrate.

Western immunoblot detection of PKIα, pCREB/pATF-1 and β-actin. Naïve and Ad5-infected BEAS-2B cells were treated (30 min) with 8-Br-cAMP (1 mM), rolipram (30 µM) or vehicle as indicated in the text, and harvested in buffer D (50 mM Tris HCl, 150 mM NaCl, 0.1 mM EDTA, 1% v/v Triton X-100, 0.1 v/v SDS, 0.5% w/v deoxycholate) containing proteinases inhibitors. After denaturation, protein (~ 25 µg/lane) was electrophoresed on 8% (for PKIα) or 10% (for β-actin and pCREB/pATF-1) SDS-polyacrylamide gels and transferred to nitrocellulose for 2 h at 1A in buffer E (183 mM glycine, 25 mM Tris-base and 20% v/v methanol). The nitrocellulose was incubated for 1 h in buffer F (25 mM Tris-base - pH 7.4, 150 mM NaCl, 0.1% v/v Tween 20) containing 5% (w/v) non-fat dry milk and incubated overnight in the same buffer supplemented with 5 mg/ml BSA containing either anti-PKIα, anti-pCREB/pATF-1 or anti-β-actin (diluted 1:500, 1:1000 and 1:500 respectively). Membranes were washed with buffer F and incubated with horseradish peroxide-linked anti-rabbit IgG (diluted 1:4000 in buffer F-containing 5% w/v non-fat dry milk) for 1 h at room temperature. The nitrocellulose was washed again and developed using ECL™ western blotting detection reagents on Kodak X-OMAT-S film. Relevant bands were quantified by laser-scanning densitometry.
Staining and visualization of PKIα and Cα. Naïve and Ad5-infected BEAS-2B cells were grown on glass coverslips placed in six-well tissue culture plates containing 2 ml keratinocyte serum-free medium supplemented with 5 ng/ml EGF, 50 µg/ml bovine pituitary extract, 100 mg/ml penicillin and 100 u/ml streptomycin. Cells were kept at 37°C until semi-confluent, exposed to 8-Br-cAMP (1 mM; 30 min), rolipram (30 µM; 30 min) or vehicles, washed free of drug and fixed in formaldehyde (4% v/v in PBS). The fixative was decanted and cells were permeabilized with NP-40 (0.5% v/v in PBS). Following rehydration in glycine (100 mM in PBS), BEAS-2B cells were “blocked” by immersion in buffer G (0.5% v/v BSA, 0.1% w/v gelatin in PBS) followed by overnight incubation at 4°C with rabbit, anti-human antibodies directed against PKIα or Cα (both diluted 1:250). Unbound antibody was washed away and a biotinylated swine, anti-rabbit IgG secondary antibody was added for 60 min at room temperature. Cells were then incubated (60 min) with fluorescein-conjugated streptavidin (diluted 1:100 in buffer G) and counter-stained with the nuclear marker, DAPI (1 µg/ml; 5 min). The subcellular localization of Cα and PKIα was visualized by laser-scanning confocal microscopy.

Measurement of GM-CSF. Naïve and Ad5-infected BEAS-2B cells were treated (30 min) with 8-Br-cAMP, rolipram, salbutamol, Org 9935 (a PDE3 inhibitor), PGE2 or vehicle at the concentrations indicated in the text prior to stimulation with IL-1β (1 ng/ml). The amount of GM-CSF released in to the culture supernatant was measured 24 later using a commercially available ELISA (R & D Systems, Europe Ltd, Abingdon, Oxon, UK) according to the manufacturer’s instructions. The detection limit of this assay is 8 pg/ml.

Measurement of AA release. Confluent BEAS-2B epithelial cells in 24-well plates were incubated overnight in 0.5 ml serum-free medium containing 0.125 µCi [3H]AA. Cells were washed twice in fresh medium and once in medium supplemented with 2 mg/ml fatty acid-free BSA to absorb AA metabolites. Cells were treated (30 min) with 8-Br-cAMP or rolipram at the concentrations indicated in the text, prior to the addition of the CCR2 chemokine, MCP-1 (1 ng/ml; ~EC50). At 24 h the supernatants and cells were harvested in 1% SDS for liquid scintillation counting. Thin layer chromatography was not used in the present study to determine the proportion of radiolabel released by
MCP-1 that was authentic [\textsuperscript{3}H]AA. Accordingly, data are presented as “agonist-induced tritium released” and expressed as a percentage of the total radioactivity incorporated at 24h.

**Generation of a CRE-dependent luciferase reporter construct.** The plasmid pADneo2-C6-BGL contains 6 tandemly repeated CREs upstream of a minimal β-globin promoter driving a luciferase gene (Himmler et al., 1993). BEAS-2B cells at ~ 50% confluence in T-75 flasks were transfected with 8 µg of plasmid DNA using Tfx 50. After overnight incubation, cells were passaged and cultured in T-162 flasks in the presence of 75 µg/ml G-418. Medium was changed every 2 - 3 days until foci of stable transfectants appeared, usually after 2 - 3 weeks of culture. These were then harvested to create a heterogenous population of cells in which the sites of plasmid integration were randomised.

**Measurement of luciferase.** Naïve and Ad5-infected BEAS-2B cells that has been transiently transfected with pADneo-C6-BGL were treated (30 min) with 8-Br-cAMP, rolipram, salbutamol, PGE\textsubscript{2}, forskolin or their respective vehicles at the concentrations indicated in the text and figure legends. Six hours later the cells were harvested in Reporter lysis buffer and the luciferase assay was performed according to the manufacturer’s instructions. All data are normalized to total protein.

**Assessment of cell viability.** The viability of virus-infected BEAS-2B cells was determined colorimetrically by measuring the reduction of the tetrazolium salt, MTT, to formazan by mitochondrial dehydrogenases.

**Protein determination.** The protein concentration was determined according to the method of Bradford.

**Drugs and analytical reagents.** HBSS and keratinocyte serum-free medium were from Flow Laboratories (Rickmansworth, Hertfordshire, UK), Tfx 50, G-418, Reporter lysis buffer and the luciferase assay were from Promega (Southampton, UK), and [\gamma\textsuperscript{32}P]-ATP (20-40 Ci/mmol) and [5,6,8,9,11,12,14,15-\textsuperscript{3}H] AA (74 Ci/mmol) were obtained from Amersham International (Amersham, Buckinghamshire, UK). Primary antibodies, recombinant proteins and enzyme inhibitors were obtained from the following sources: murine C\alpha (code # 539481), bovine Type I\alpha PKG (code # 370650), H-89 and rolipram were from CN Biosciences (Nottingham, UK); goat anti-human PKI\alpha (code sc#1944) and
goat anti-human actin (code sc# 1615) were from Autogen Bioclear (Calne, Wiltshire, UK); and pCREB/pATF-1 (code 9191S) was purchased from New England Biolabs (Hitchin, Hertfordshire, UK). MCP-1 was from R & D Systems, (Europe Ltd, Abingdon, Oxon, UK) and FCS, MTT, 8-Br-cAMP, Kemptide and all other reagents were from Sigma-Aldrich (Poole, Dorset, UK). Org 9935 was from Organon Laboratories (Lanarkshire, Scotland, UK).

**Statistical Analyses.** Data points, and values in the text and figure legends, represent the mean ± s.e.mean of ‘n’ independent determinations. Concentration-response curves were analysed by least-squares, non-linear iterative regression with the ‘PRISM’ curve fitting program (GraphPad software, San Diego) and EC50 and IC50 values were subsequently interpolated from curves of best-fit. Where appropriate data were analysed statistically using Student’s paired t-test or by one-way ANOVA/Newman-Keuls multiple comparison test. The null hypothesis was rejected when P < 0.05.
Results

Ad5-mediated gene transfer. The efficiency of gene expression was determined using an Ad5 vector containing a cDNA encoding the amino acid sequence of GFP and monitored by fluorescence microscopy. Treatment of BEAS-2B cells with Ad5.CMV.GFP resulted in the expression of the transgene as a function of the MOI. When expressed as a percentage of the total number of cells counterstaining for the nuclear marker DAPI, 24.3 ± 2.7%, 49.7 ± 2.3%, 92 ± 1.2% and 91.7 ± 1.9% (n = 3) of those cells were GFP+ at an MOI of 1, 10, 20 and 50 respectively. The viability of GFP-expressing cells was assessed in parallel by monitoring the reduction of MTT to formazan and was not adversely affected by the virus at an MOI ≤ 30 (data not shown).

Expression of the PKIα transgene. Western blotting was used to detect the expression of PKIα after infection of BEAS-2B cells with Ad5.CMV.PKIα. In uninfected cells PKIα was not detected in any experiment. However, 48 h after virus infection a single peptide was labelled by the anti-PKIα antibody that migrated as a 12 kDa band on SDS polyacrylamide gels and increased in intensity as a function of the MOI relative to the house-keeping protein, β-actin (Fig. 2a). Fluorescence microscopy confirmed that 48 h after virus infection at a MOI of 20 the number of PKIα+ cells expressed as a percentage of DAPI+ cells was comparable to the data obtained with Ad5.CMV.GFP (Fig. 2b). Unless stated otherwise, all other experiments using Ad5 vectors were conducted at a MOI of 20.

Functionality of the PKIα transgene. A comprehensive set of experiments was performed to confirm the functionality of PKIα expressed in BEAS-2B cells.

Inhibitory activity of PKIα. A cytosolic extract was prepared from uninfected (naïve) cells and from cells exposed to Ad5.CMV.PKIα and Ad5.CMV.Null (empty virus) for 48 h at a MOI of 100. After denaturing proteins (95°C; 5 min) the ability of heat-stable PKIα to inhibit the phosphorylation of Kemptide by the α-isoform of PKA catalytic subunits (Cα) was assessed. As shown in figure 3a, heat-inactivated cytosol from Ad5.CMV.PKIα-infected cells inhibited Cα activity in a concentration-dependent manner with an IC50 = 495.6 ± 41.1 ng protein. Cytosol prepared under identical conditions
from Ad5.CMV.Null and naïve cells was inactive at concentrations up to 10 µg protein (Fig. 3a). PKIα did not inhibit the activity of Type Iα PKG at any protein concentration examined (Fig. 3b).

**PKA activity ratio.** Naïve and virus-infected BEAS-2B cells (MOI = 20; 48 h) were treated with 8-Br-cAMP (1 mM; 30 min), PGE₂ (10 µM; 5 min), salbutamol (10 µM; 5 min) or the PDE4 inhibitor rolipram (30 µM; 30 min), which is the major cAMP-hydrolysing activity in essentially all pro-inflammatory cells, and the PKA activity ratio determined by *in vitro* kinase assay. Virus infection *per se* had no effect on total (+ 10 µM cAMP) PKA activity (in pmol/min/mg protein: naïve: 868.5 ± 64.7; Ad5.CMV.Null: 873.3 ± 65.1, n = 17, P > 0.05). After correcting for PKA-independent activity (see below), the basal PKA activity ratio in naïve and Ad5.CMV.Null-infected cells was approximately 28% (Table 1). This level of activation increased to approximately 100%, 46%, 47% and 52% after treatment of BEAS-2B cells with 8-Br-cAMP, rolipram, PGE₂ and salbutamol respectively (Table 1). In untreated cells expressing the PKIα transgene, basal PKA activity in the absence of exogenous cAMP was significantly lower (~ 58%) than in naïve and Ad5-CMV.Null-infected cells, and was not activated by exogenous cAMP (10 µM; Table 1) or further inhibited by 10 µM PKI(4-22), an active peptide derived from full length PKIα (data not shown). Identical results were obtained in Ad5.CMV.PKIα-infected cells that were treated with 8-Br-cAMP, rolipram, PGE₂ and salbutamol (Table 1).

**CREB/ATF-1 phosphorylation.** Naïve and virus-infected BEAS-2B cells (20 MOI; 48 h) were treated (30 min) with 8-Br-cAMP (1 mM) or rolipram (30 µM) and the phosphorylation status of CREB and ATF-1 was determined by western blotting using a phosphospecific antibody. As shown in figure 4a, both proteins and a third species that is probably a CREM variant, were detected in a phosphorylated state in unstimulated naïve cells and this level of basal expression was not significantly altered in cells infected with either adenovirus vector. 8-Br-cAMP and rolipram routinely increased the levels of pCREB and pATF-1 by approximately 300% in both naïve and Ad5.CMV.Null-infected cells, whereas phosphorylation over baseline expression was significantly attenuated (90- 95%) in cells expressing PKIα (Figs. 4a & c). Similar experiments were conducted in naïve cells pre-treated (30 min) with the purported PKA inhibitor, H-89 (10 µM), which abolished the phosphorylation, above baseline, of
CREB/ATF-1 effected by 8-Br-cAMP and rolipram (Fig. 4b). Salbutamol could not be used in this or any other experiment as H-89 is a β-adrenoceptor antagonist at concentrations that inhibit PKA (Penn et al., 1999).

**Nuclear export of Cα.** The ability of virally expressed PKIα to export catalytic subunits of PKA from the nucleus to the cytosol was assessed by immunconfocal microscopy using polyclonal antibodies that recognise unique epitopes in human Cα and PKIα, together with DAPI, a nuclear stain that selectively binds to dsDNA. Relative to the IgG control, which shows a very low level of non-specific labelling (Fig. 5a,(i)), the expression of PKIα (red) in untreated cells was uniformly distributed throughout the cell (Fig. 5a (ii)). After exposure of BEAS-2B cells to 8-Br-cAMP (1 mM; 30 min) and rolipram (30 µM) the distribution of PKIα was changed with more intense immunoreactivity detected in the cytosol relative to the nucleus (Fig. 5a (iii) & (iv)).

Relative to the IgG controls (Fig. 5b (i) & (v)), the labelling of Cα (green) in naïve cells (and cells infected with Ad5.CMV.Null - data not shown) was confined predominantly to the cytoplasm whereas nuclei were counter-stained blue with DAPI (Fig. 5b (ii) & (vi)). The localisation of Cα under these conditions is consistent with the majority of this catalytic subunit existing as part of an inactive R2Cα2 holoenzyme, which is retained in the cytosol. Treatment of BEAS-2B cells with 8-Br-cAMP (1 mM; 30 min) resulted in a marked translocation of Cα to the nucleus presumably due to dissociation of the PKA holoenzyme (Fig. 5b (iii) & (vii)). In contrast, Cα was found exclusively in the cytoplasm of Ad5.CMV.PKIα-infected cells treated with 8-Br-cAMP (Fig. 5b (iv) & (viii)), which is consistent with the elegant demonstration by Fantozzi et al., (1992, 1994) that PKIα can promote the nuclear export of Cα. Similar data were obtained with rolipram (30 µM; 30 min; data not shown).

**Effect of cAMP-elevating drugs on CRE-dependent luciferase gene expression in BEAS-2B cells infected with Ad5.CMV.PKIα or treated with H-89.** Exposure of BEAS-2B cells that were stably transfected with a CRE-driven luciferase reporter gene, to IL-1β (10 ng/ml; 6 h) failed to promote luciferase expression whereas 8-Br-cAMP and rolipram activated this construct in a concentration-dependent manner (Figs. 6a, b & c). Induction of the luciferase gene in cells expressing the PKIα transgene was suppressed in a graded fashion as a function of the MOI but was not affected in cells...
infected with the empty vector, Ad5.CMV.Null (Fig. 6a, b & c). In a separate series of experiments 8-Br-cAMP, (1 mM), rolipram (30 µM), PGE₂ (10 µM), salbutamol (1 µM) and forskolin (10 µM) induced the luciferase gene (Figs. 6d & e). This effect was abolished in Ad5.CMV.PKIα-infected cells but completely unaffected by the empty vector or after treatment (30 min) with H-89 at a concentration (10 µM) that abolished CREB/ATF-1 phosphorylation (Figs. 4b, 6d & e). Cell viability was not adversely affected by either Ad5 vector (data not shown).

**Effect of cAMP-elevating drugs on GM-CSF release from BEAS-2B cells infected with Ad5.CMV.PKIα.** Exposure of naïve cells to IL-1β (1 ng/ml; 24 h) resulted in the elaboration of GM-CSF (329.3 ± 22.9 pg/ml, n = 24) that was not significantly different (P > 0.05) from cells infected (MOI = 20) with Ad5.CMV.Null (353.6 ± 24.1 pg/ml, n = 24) or Ad5.CMV.PKIα (277.1 ± 21.6 pg/ml, n = 24). Pre-treatment (30 min) of naïve and Ad5-CMV.Null-infected cells with 8-Br-cAMP, rolipram, Org 9935 and salbutamol prior to IL-1β suppressed GM-CSF output in a concentration-dependent manner (Fig. 7a-d) whereas PGE₂ was inactive. The suppression of GM-CSF release was abolished in cells infected with Ad5.CMV.PKIα (Fig. 7a-d). Cell viability was not adversely affected by either Ad5 vector (data not shown).

**Effect of 8-Br-cAMP and rolipram on GM-CSF release from BEAS-2B cells treated with H-89.** Exposure of naïve cells to IL-1β (1 ng/ml; 24 h) resulted in the elaboration of GM-CSF (321.5 ± 28.8 pg/ml) that was not significantly different (P > 0.05) from cells infected (MOI = 20) with Ad5.CMV.PKIα (303.9 ± 12.2 pg/ml) or treated (30 min) with 3 or 10 µM of the isoquinolinesulfonyl, H-89 (249.5 ± 3.5 and 232.7 ± 14.9 pg/ml respectively; Fig. 8a). Pre-treatment (30 min) of Ad5.CMV.Null-infected cells with 8-Br-cAMP (1 mM) or rolipram (30 µM) prior to IL-1β suppressed GM-CSF output by > 80% (Fig. 8a). This effect was abolished in cells expressing the PKIα transgene, but completely unaffected in cells infected with Ad5.CMV.Null or treated with H-89 (Fig. 8a). Cell viability was not adversely affected by H-89 (data not shown).

**Effect of 8-Br-cAMP and rolipram on [3H]AA release.** The CCR2 chemokine, MCP-1 promoted the elaboration of tritium from naïve BEAS-2B cells labelled to equilibrium with [3H]AA. This effect was concentration-dependent with an EC₅₀ and maximum response of 99.7 ± 65.3 pg/ml and 3.35 ± 0.43%
of the total incorporated radiolabel respectively (n = 5; data not shown). The sensitivity of cells to MCP-1 and the amount of tritium released was not changed after infection with Ad5.CMV.Null (data not shown) or Ad5.CMV.PKIα (Figs. 9a, b & c). 8-Br-cAMP, rolipram and salbutamol inhibited MCP-1 (1 ng/ml)-induced tritium release in a concentration-dependent manner with IC₅₀ values of 433.3 ± 148.1 µM, 7.03 ± 2.8 µM and ~ 3 µM respectively (Fig. 9a, b & c). This effect was not altered in cells infected with Ad5.CMV.Null, but prevented following expression of the PKIα transgene. In contrast, H-89 (10 µM; 30 min) reduced by 72% MCP-1 (1 ng/ml)-induced tritium release. Moreover, the suppression of tritium release evoked by 300 µM 8-Br-cAMP and 10 µM rolipram (but paradoxically not higher concentrations of these agents) was significantly enhanced by H-89 (Fig. 9d).
Discussion

Many drugs and endogenous ligands increase, directly or indirectly, the concentration of cAMP inside cells and evoke responses that historically were believed to involve exclusively activation of PKA. Over the last twenty years this dogma has been discredited with the discovery of multiple, functionally-distinct cAMP-binding proteins (Beavo and Brunton, 2002). However, the major problem that continues to hinder the simple and unambiguous assignation of PKA to cAMP-driven responses is a lack of selective inhibitors. Many compounds exemplified by H-89 (Chijiwa et al., 1990) are isoquinolinesulfonamides that, in cell-free systems, are non-selective (Davies et al., 2000) presumably because they block a conserved ATP-binding site found among many protein kinases (Engh et al., 1996). The potential limitations of H-89 and related compounds prompted us to evaluate an alternative method to inhibit PKA in intact cells with extremely high selectivity. To this end, SV40-transformed human bronchial epithelial cells (BEAS-2B) were infected with an Ad5 vector encoding the complete amino acid sequence of PKI\(\alpha\), an endogenous and highly selective inhibitor of PKA. BEAS-2B cells were selected for the validation as they are simple to culture and manipulate, express the necessary adhesion molecules to facilitate Ad5 attachment and internalization and do not express detectable endogenous PKI\(\alpha\). In most experiments cells infected with Ad5.CMV.PKI\(\alpha\) were compared to those treated with concentrations of H-89 (3 and 10 µM) that are routinely employed to “inhibit” PKA in intact cells.

Properties of the PKI\(\alpha\) transgene

An exhaustive series of experiments was performed to establish the optimal conditions of infection and, subsequently, to confirm the functionality of the PKI\(\alpha\) transgene. The vector, Ad5.CMV.PKI\(\alpha\), at a MOI of 20 led to the expression after 48 h of PKI\(\alpha\) in >90% of exposed cells at a level that did not adversely affect cell viability or responsiveness to stimuli. Like native PKI\(\alpha\), the expressed protein was heat-stable (Walsh et al., 1971), and potently inhibited PKA in a cell free system. In contrast, PKI\(\alpha\) did not inhibit Type I\(\alpha\) PKG, a highly homologous enzyme to which PKA is most closely related (Takio et al., 1984). These results are entirely consistent with previous findings where PKI\(\alpha\) at a concentration \(\sim 10^6\)-times higher than its affinity for PKA (\(K_i = 50\) to 100 pM) fails to inhibit PKG and, in fact, may not bind to this enzyme at all (Glass et al., 1986). Expression of PKI\(\alpha\) also prevented the activation of
PKA in intact BEAS-2B cells (measured by *in vitro* kinase assay) stimulated with a range of agents known to elevate cAMP. This observation suggests that the binding of PKIα to free catalytic subunits released upon dissociation of the PKA holoenzyme is preserved during cell lysis and throughout the duration of the *in vitro* kinase assay presumably because of its extremely high affinity for the enzyme (Collins and Uhler, 1997, Glass et al., 1986). A consistent finding was that the phosphorylation of Kemptide was not abolished in PKIα-expressing cells and neither increasing the MOI nor adding PKI(4-22) to the assay cocktail affected this residual “PKA-independent” activity. Thus, although Kemptide is a relatively specific substrate for PKA (Giembycz and Diamond, 1990, Walsh et al., 1971) other kinases will phosphorylate this peptide, and their activities need to be subtracted when calculating PKA activity ratios (Table 1). Expression of an active PKIα transgene was also confirmed in intact cells where the ability of 8-Br-cAMP, rolipram and PGE₂ ordinarily to promote the phosphorylation of CREB and ATF-1 was inhibited. However, as shown in figure 3, CREB, ATF-1 and, in some experiments, a third species that may be a CREM variant, were detected in a phosphorylated state in unstimulated cells. This basal level of expression was not altered in cells expressing the PKIα transgene, consistent with the *in vitro* kinase data, indicating that the phosphorylation of CREB and related transcription factors in unstimulated cells is maintained by a kinase(s) other than PKA.

A physiological role of PKIs is their ability to terminate cAMP-signalling. Each variant expresses a nuclear export signal and will bind PKA catalytic subunits at the nucleus and export them to the cytosol where an inactive PKA holoenzyme can reform (Fantozzi et al., 1992, 1994). This property was also shared by the PKIα transgene expressed in BEAS-2B cells. Thus, Cα was confined almost exclusively to the cytoplasm in untreated cells and to the nucleus after exposure to 8-Br-cAMP and rolipram. However, following expression of PKIα transgene, Cα was localized to the cytoplasm and did not accumulate at the nucleus regardless of whether cells were treated with 8-Br-cAMP or rolipram. Collectively, these results convincingly show that expression of PKIα in BEAS-2B cells following infection with Ad5.CMV.PKIα has the same properties at the native protein and can be used to establish the extent to which PKA mediates cAMP-dependent responses.
Application of PKIα to probe the role of PKA in cAMP-dependent responses

BEAS-2B cells were transfected with a CRE reporter construct and used as a positive control to monitor PKA-dependent transcription. Regardless of the means by which cAMP was mimicked or elevated, activation of the luciferase gene was routinely observed in naïve and Ad5.CMV.Null-infected cells. However, upon expression of the PKIα transgene cAMP-driven luciferase expression was abolished. Given the potency and remarkable specificity of PKIα (see above), these data clearly and unequivocally establish positive CRE-dependent regulation of the luciferase gene by PKA. An absolute dependence on PKA was also established for the inhibition of GM-CSF release and \[^3\text{H}\]AA mobilisation from IL-1β- and MCP-1-stimulated BEAS-2B cells respectively evoked by a diverse range of agents that elevate cAMP. Thus, we suggest that expression of PKIα in susceptible cells provides a simple and unambiguous means to establish the extent to which PKA participates in biological processes.

One observation that merits brief discussion is the discrepancy between the difference in the extent to which PKA is activated by rolipram (~46%) and 8-Br-cAMP (100%) and the equivalence with which these two agents promote CREB phosphorylation and suppress IL-1β- and MCP-1-induced GM-CSF and \[^3\text{H}\]AA release respectively. Perhaps the most likely explanation of these data is that rolipram only activates a sub-population of PKA, out of the total cellular pool, that is in close proximity to PDE4 (which could account for at least 46% of the total PKA), whereas 8-Br-cAMP would be expected to activate all PKA regardless of its sub-cellular localisation.

Studies with H-89

Treatment of BEAS-2B cells with H-89 abolished the ability of 8-Br-cAMP and rolipram to promote the phosphorylation of CREB and ATF-1 indicating that PKA was inhibited. These observations confirm the results of several previous studies where H-89 was shown to directly inhibit PKA in cell-based and cell-free systems (Chijiwa et al., 1990, Martin et al., 2001, Staples et al., 2001). However, H-89 failed to block the suppression of GM-CSF output or the activation of the CRE reporter construct elicited by range of cAMP-elevating agents. This latter finding was particularly unexpected as the luciferase gene is driven by six tandemly repeated CREs upstream of a minimal β-globin promoter and
is assumed to be exclusively dependent upon the phosphorylation of CREB by PKA. Indeed, this classical mode of regulation was strongly supported in the present study by the ability of PKIα to abolish CREB phosphorylation and transcription of the luciferase gene. If it is assumed that the suppression of cAMP-induced CREB phosphorylation by PKIα and H-89 reflects the inhibition of PKA then these data provide overwhelming evidence that H-89 exerts non-specific actions in intact cells and suggest that much of the published data with this compound are erroneous. Indeed, H-89 has also been shown to inhibit cAMP-dependent CREB phosphorylation in human monocyte-derived macrophages (Takayama et al., 2002), human T-lymphocytes (Staples et al., 2001) and human airway smooth muscle cells (Pang and Knox, 2001) yet the associated functional responses (inhibition of MIP-1β, IL-5 and eotaxin release respectively) were unaffected. In the present study H-89 was also found to significantly attenuate MCP-1-induced [3H]AA release yet enhance the inhibitory effect of submaximal concentrations of rolipram and 8-Br-cAMP indicating again a lack of specificity for PKA. Thus, taken together these results strongly support the finding that H-89 interacts with signalling intermediates other than PKA. While the identity of these additional proteins was not a subject of this investigation, it is known that isoquinolinesulfonamides can bind, in a cell-free system, to many enzymes with high affinity (Davies et al., 2000, Serizawa et al., 1993) and some of these can have a profound influence on gene expression (Dubois et al., 1994).

**Summary and general utility of Ad5.CMV.PKIα**

Persuasive evidence is presented herein that expression of PKIα in BEAS-2B cells provides a powerful and unambiguous means to determine the role of PKA in cAMP-dependent cell signalling. This technology holds several significant advantages over existing methods to inhibit PKA. In particular, PKIα has extremely high specificity for PKA. This unique property is not shared by small molecule inhibitors (e.g. H-89, Rp-cAMPS analogs) or active peptides derived from PKIα, which are less potent, poorly cell permeant and can inhibit PKG with micromolar potency (Glass et al., 1986). In addition, Ad5 vectors can express PKIα in many primary cell types that feature the coxsackie B virus receptor and αv integrins (essential for attachment and internalisation of Ad5 respectively (Nemerow, 2000)) including cardiac, skeletal and smooth muscle cells, epithelial and endothelial cells; fibroblasts, synoviocytes and osteoclasts. This is not the case when delivering peptides by plasmids (Grove et al.,
1987, Olsen and Uhler, 1991a), which are restricted to the relatively few cell types that can be transfected with high efficiency.

One important consideration that may have practical implications is whether PKIα can be effectively employed as a pharmacological tool in tissues that express high levels of endogenous PKIs such as the CNS, striated muscle and testis (Ashby & Walsh, 1972). The abundance of PKI varies widely between different cell types (Whitehouse & Walsh, 1983) and it has been calculated that even in the most highly expressing tissues PKA will be inhibited by no more than 20% (Whitehouse & Walsh, 1983). This is an important finding as most cells express considerably less PKI than the brain implying that the inhibitor plays, at most, a modest role in regulating PKA activity under normal conditions. Thus, the Ad5.CMV.PKIα vector can be used in a wide variety of cells and should be invaluable in probing the mechanism of action of drugs and cAMP-dependent responses where the role of PKA is uncertain.

In contrast to PKIα, the results presented herein demonstrate that H-89 behaves unpredictably in intact BEAS-2B cells and cannot be used with confidence to implicate PKA in biological responses. However, there is a need to develop highly selective small molecule PKA inhibitors as the mechanism of action of PKIα and isoquinolinesulfonamides is distinct (see Introduction). Indeed, a comparison of the effect of PKIα and selective ATP-binding or catalytic site inhibitors may be instructive providing important new information regarding the role and regulation of PKA.
References


Dubois MF, Nguyen VT, Bellier S and Bensaude O (1994) Inhibitors of transcription such as 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole and isoquinoline sulfonamide derivatives (H-8 and H-7) promote dephosphorylation of the carboxyl-terminal domain of RNA polymerase II largest subunit. *J Biol Chem* **269**:13331-13336.


Footnotes

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Figure legends

Fig. 1. Strategy for the construction of the Ad5.CMV.PKIα recombinant adenovirus. The vector contains a transcription unit containing the CMV promoter/enhancer, a 251 bp cDNA encoding full length PKIα and a SV40 polyadenylation cassette. See Materials and Methods, Lum et al., (1999) and Gomez-Foix et al., (1992) for further details.

Fig. 2. Expression of Ad5-expressed PKIα in BEAS-2B cells. Cells were infected with Ad5.CMV.PKIα, Ad5.CMV.Null (MOI = 10 to 150 as indicated) or left untreated (naïve) for 48 h at 37°C. Cells were growth arrested in serum-free medium and processed for PKIα expression by western blotting (panel a) and confocal microscopy (panel b). Data are representative of three determinations. See Materials and Methods for further details.

Key: Blue – Nuclei stained with DAPI; Green - PKIα

Fig. 3. Effect of PKIα expressed in BEAS-2B cells on the in vitro activity of PKA and PKG. Cytosolic extracts were prepared from naïve and Ad5-infected cells (MOI = 100; 48h), denatured and tested for their ability to inhibit the phosphorylation of Kemptide by recombinant Cα (panel a). To confirm the selectivity of PKIα for PKA, cytosolic extracts from Ad5.CMV.PKIα-infected cells were also tested against recombinant Type Iα PKG in the presence of 10 μM cGMP and Glasstide as substrate (panel b). Data represent the mean ± s.e.m. of three independent determinations. See Materials and Methods for further details.
Fig. 4. Inhibition by PKI\(\alpha\) and H-89 of 8-Br-cAMP- and rolipram-induced CREB and ATF-1 phosphorylation. Naïve and Ad5-infected (MOI = 20) BEAS-2B cells were treated for 30 min with 8-Br-cAMP (1 mM), rolipram (30 \(\mu\)M) or vehicle and then probed for phospho-CREB/ATF-1 by western blotting. Panels \(a\) and \(b\) show the effect of the PKI\(\alpha\) transgene and H-89 (10 \(\mu\)M; 30 min) respectively on CREB/ATF-1 phosphorylation. Western blots are representative of three determinations. Panel (\(c\)) shows the mean result of PKI\(\alpha\) expression on CREB phosphorylation quantified by laser scanning densitometry. See Materials and Methods for further details.

Key: 1, vehicle; 2, 8-Br-cAMP; 3, rolipram.

* \(P < 0.05\), significant stimulation of CREB phosphorylation over basal level

** \(P < 0.05\), significant inhibition of 8-Br-cAMP- and rolipram-induced CREB phosphorylation

Fig. 5. Effect of 8-Br-cAMP and rolipram on the localization of PKI\(\alpha\) and C\(\alpha\). BEAS-2B cells were grown until 50% confluent, infected with virus vectors for 48 h and then exposed to 8-Br-cAMP (1 mM; 30 min), rolipram (30 \(\mu\)M; 30 min) or vehicle before fixing in formaldehyde. Cells were permeabilized, blocked and probed with rabbit, anti-human antibodies directed against PKI\(\alpha\) or C\(\alpha\) as described in Materials and Methods. The subcellular localization of C\(\alpha\) and PKI\(\alpha\) was then visualized by laser-scanning confocal microscopy. Panels \(a\) and \(b\) show the localization of PKI\(\alpha\) in Ad5-CMV.PKI\(\alpha\)-infected cells before and after drug treatment, and the effect of 8-Br-cAMP on distribution of C\(\alpha\) in naïve and Ad5.CMV.PKI\(\alpha\)-infected cells respectively.

Key: Red, PKI\(\alpha\); Green, C\(\alpha\); Blue, DAPI
**Fig. 6.** Effect of PKIα and H-89 on the induction by 8-Br-cAMP and several cAMP-elevating agents of a CRE-dependent luciferase reporter gene. BEAS-2B cells were infected with Ad5.CMV.PKIα, Ad5.CMV.Null (MOI = 20 or as indicated; 48 h) or treated with H-89 (10 µM; 30 min) before being exposed to 8-Br-cAMP (panels a & e), rolipram (panel b & e), salbutamol (panel d), PGE2 (panel d & e) or forskolin (panels d & e) as indicated. Six hours later the cells were harvested for luciferase activity. All data are normalized to total protein and represent the mean ± s.e.mean of three independent determinations. See Materials and Methods for further details.

* P < 0.05, significant inhibition of CRE-dependent transcription.

**Fig. 7.** Effect of expressing PKIα in BEAS-2B cells on the inhibitory effect of 8-Br-cAMP, rolipram, Org 9935 and salbutamol on GM-CSF output. Naïve and Ad5-infected cells (MOI = 20; 48 h) were treated for 30 min with 8-Br-cAMP (panel a), rolipram (panel b) or Org 9935 (panel c), or for 5 min with salbutamol (panel d). IL-1β (1 ng/ml) was then added and the cells were incubated at 37°C in a thermostatically-controlled incubator under a 5% CO2 atmosphere. At 24 h the amount of GM-CSF released into the culture supernatant was quantified by a sandwich ELISA. Each data point represents the mean ± s.e.mean of nine, four, three and seven independent determinations for 8-Br-cAMP, rolipram, Org 9935 and salbutamol respectively. The potency of the compounds tested in naïve and Ad5.CMV.Null-treated cells was not significantly different as follows:

- 8-Br-cAMP (IC50): naïve: 56.7 ± 15.2 µM; Ad5.CMV.Null: 85.6 ± 17.1 µM;
- Rolipram (IC50): naïve: 1.80 ± 0.5 µM; Ad5.CMV.Null: 2.6 ± 0.9 µM;
- Org 9935 (IC50): naïve: 19.0 ± 3.6 µM; Ad5.CMV.Null: 21.8 ± 5.2 µM;
- Salbutamol (EC50): naïve: 0.8 ± 0.3 µM; Ad5.CMV.Null: 1.1 ± 0.6 µM.
Fig. 7. Comparison of the effect of 8-Br-cAMP and rolipram on GM-CSF output from BEAS-2B cells treated with H-89 and infected with Ad5.CMV.PKIα. Naïve, Ad5-infected (MOI = 20; 48 h) and H-89 (10 µM; 30 min)-pretreated cells were exposed for 30 min to either 8-Br-cAMP (1 mM) or rolipram (30 µM). IL-1β (1 ng/ml) was then added and the cells were incubated at 37°C in a thermostatically-controlled incubator under a 5% CO₂ atmosphere. At 24 h the amount of GM-CSF released into the culture supernatant was quantified by a sandwich ELISA. Each data point represents the mean ± s.e.m. of nine and four independent determinations for 8-Br-cAMP and rolipram respectively. See Materials and Methods for further details.

* P < 0.05, significant inhibition of IL-1β-induced GM-CSF release.

Fig. 9. Effect of expressing PKIα in BEAS-2B cells on the inhibitory effect of 8-Br-cAMP, rolipram, and salbutamol on [3H]AA release. Confluent BEAS-2B epithelial cells (naïve and Ad5.CMV.PKIα-infected) were incubated overnight in 0.5 ml serum-free medium containing 0.125 µCi [3H]AA. Cells were washed and then treated (30 min) with 8-Br-cAMP, (panels a & d), rolipram (panel b & d) and salbutamol (panel c) at the concentrations indicated, prior to the addition of MCP-1 (1 ng/ml). At 24 h the supernatants and cells were harvested in 1% SDS for liquid scintillation counting. Tritiated species elaborated over the 24 h stimulation period are expressed as a percentage of the total [3H]AA incorporated. Data points represent the mean ± s.e.mean of five, five and three independent determinations for 8-Br-cAMP, rolipram and salbutamol respectively. See Materials and Methods for further details.

* P < 0.05, significant inhibition of MCP-induced tritium release.

** P < 0.05, significant inhibition of MCP-induced tritium release over the effect of 8-Br-cAMP and rolipram alone.
**TABLE 1**

Effect of 8-Br-cAMP and cAMP-elevating agents on PKA activity in naïve, Ad5.CMV.Null- and Ad5.CMV.PKI\(\alpha\)-treated BEAS-2B epithelial cells. Cells were treated with the agents indicated below and PKA activity was calculated as described in Material and Methods. One unit of activity is defined as that amount of PKA that catalyzed the incorporation of 1 pmol phosphate from ATP into Kemptide per minute per mg protein at 30°C and pH 7.2. The activation state of PKA is expressed as an activity ratio, which is the specific activity of Kemptide kinase obtained in the absence of cAMP divided by the specific activity of Kemptide kinase obtained in the presence of a concentration of exogenous cAMP required to maximally activate PKA. Kemptide phosphorylation that was not inhibited by PKI\(\alpha\) represents a kinase other than PKA and was subtracted from the total specific activity in naïve and Ad5.CMV.Null-treated cells before activity ratios were calculated.

<table>
<thead>
<tr>
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<th>Naive</th>
<th>Ad5.CMV.Null</th>
<th>Ad5.CMV.PKI(\alpha)</th>
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</thead>
<tbody>
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<td></td>
<td>Specific Activity (pmol/min/mg protein)</td>
<td>Specific Activity (pmol/min/mg protein)</td>
<td>Specific Activity (pmol/min/mg protein)</td>
</tr>
<tr>
<td>n</td>
<td>-cAMP</td>
<td>+cAMP</td>
<td>Ratio(^a)</td>
</tr>
<tr>
<td>Untreated</td>
<td>3</td>
<td>308 ± 094</td>
<td>0741 ± 133</td>
</tr>
<tr>
<td>8-Br-cAMP (1 mM)</td>
<td>4</td>
<td>819 ± 214</td>
<td>0817 ± 178</td>
</tr>
<tr>
<td>Rolipram (30 µM)</td>
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<td>0744 ± 061</td>
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<tr>
<td>Salbutamol (10 µM)</td>
<td>4</td>
<td>609 ± 104</td>
<td>0956 ± 092</td>
</tr>
<tr>
<td>PGE(_2) (10 µM)</td>
<td>3</td>
<td>586 ± 131</td>
<td>1072 ± 212</td>
</tr>
</tbody>
</table>

\(^a\) Ratios calculated after subtraction of PKI\(\alpha\)-insensitive activity

\(^b\) PKI\(\alpha\)-insensitive Kemptide phosphorylation

\(^*\) P < 0.05 - Significant increase over untreated cells in activity ratio

\(^**\) P < 0.05 - Specific activity in the absence of cAMP significantly lower than in naïve and Ad5.CMV.Null-infected cells
Recombination in HEK 293 Cells

Ad5.CMV.PKIα

PKIα

pUC19 cloning cassette

pAC.CMV.pL.pA
(8.8 kb)

pJM17
(40.3 kb)

0/100 1.3 3.7

pBRX

0 mu 1.3 mu 9.1 mu 100 mu

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JPET Fast Forward. Published on January 27, 2004 as DOI: 10.1124/jpet.103.060020
Figure 2

(a) MOI

MW B 10 20 30 50 60 80 100 150

43 kDa

35 kDa

12 kDa

Actin

PKIα

(b) Naive Ad5.CMV.Null (20 MOI) Ad5.CMV.PKIα (20 MOI)
Figure 3

(a) Protein kinase activity (% control) vs. heat-inactivated BEAS-2B cytosol (µg protein)

(b) Protein kinase activity (% control) vs. heat-inactivated BEAS-2B cytosol (µg protein)
Figure 4

(a) Naive Ad 5.CMV.Null Ad 5.CMV. PKI

<table>
<thead>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad5.CMV.PKIα</td>
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43 kDa

(b) Naive Naïve + H-89 Ad5.CMV.PKIα

<table>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naïve + H-89</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ad5.CMV.PKIα</td>
<td></td>
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</tbody>
</table>

43 kDa

(c) CREB Phosphorylation (O.D. Arbitrary units x 10^-3)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>O.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>8-Br-cAMP (1 mM)</td>
<td>18.0 ± 1.2 *</td>
</tr>
<tr>
<td>Rolipram (30 µM)</td>
<td>22.0 ± 1.5 **</td>
</tr>
</tbody>
</table>

* p < 0.05
** p < 0.01
Figure 5

(a) Control IgG Untreated 8-Br-cAMP (1 mM) Rolipram (30 μM)

PKIα

(i) (ii) (iii) (iv)

(b) Control IgG Untreated 8-Br-cAMP (1 mM) Ad5.CMV.PKIα + 8-Br-cAMP

Cα

(i) (ii) (iii) (iv)

Cα + DAPI

(v) (vi) (vii) (viii)
Figure 6

(a) (b) (c) (d) (e)
Figure 7

(a) GM-CSF Released (% Control) vs. log [8-Br-cAMP (M)]

(b) GM-CSF Released (% Control) vs. log [Rolipram (M)]

(c) GM-CSF Released (% Control) vs. log [Org 9935 (M)]

(d) GM-CSF Released (% Control) vs. log [Salbutamol (M)]
Figure 8
Figure 9

(a) Tritium Released (% Total Incorporated) as a function of log [8-Br-cAMP (M)]

(b) Tritium Released (% Total Incorporated) as a function of log [Rolipram (M)]

(c) Tritium Released (% Total Incorporated) as a function of log [Salbutamol (M)]

(d) Tritium Released (% Total Incorporated) in different conditions:
- Control
- + H-89 (10 µM)

Conditions:
- MCP-1 (1 ng/ml)
- 8-Br-cAMP (300 µM)
- 8-Br-cAMP (1 mM)
- Rolipram (10 µM)
- Rolipram (100 µM)