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Cyclic AMP enhances resolution of allergic pleurisy by promoting inflammatory cell apoptosis via inhibition of PI3K/Akt and NF-κB

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ABSTRACT

Selective and timely induction of apoptosis is an effective means of resolving inflammation. The effects and putative mechanisms by which cyclic AMP (cAMP) modulates leukocyte apoptosis *in vivo* are still unclear. The present study aims at identifying intracellular pathways underlying the ability of cAMP elevating agents to resolve eosinophilic inflammation in a model of allergic pleurisy in mice. Ovalbumin (OVA) challenge of immunized mice induced eosinophil recruitment that peaked at 24 h and persisted till 48 h. Treatment with the PDE4 inhibitor rolipram, cAMP mimetic db-cAMP or adenylate cyclase activator forskolin, at 24 h after antigen-challenge resulted in profound resolution of eosinophilic inflammation, without a decrease of mononuclear cell numbers. There was a concomitant increase in number of apoptotic cells in the pleural cavity. The effects of rolipram and db-cAMP were inhibited by the PKA inhibitor H89. Inhibition of PI3K/Akt or NF-κB induced resolution of inflammation that was associated with increased apoptosis. OVA-challenge resulted in a time-dependent activation of Akt and NF-κB, which was blocked by treatment with rolipram or PI3K/Akt pathway inhibitors. Thus, cAMP elevating agents resolve established eosinophilic inflammation by inducing leukocyte apoptosis. Mechanistically, the actions of cAMP are dependent on PKA and target a PI3K/Akt-dependent NF-κB survival pathway.

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1. Introduction

Eosinophils are effectors cells that play an important role in the pathophysiology of allergic diseases [1,2]. In allergic diseases, such as asthma, eosinophils are a crucial source of cytotoxic proteins, lipid mediators, oxygen metabolites, and cytokines, which may contribute to the severity of disease [3]. The accumulation of eosinophils in tissue depends not only on the number of cells being recruited at any particular time, but also on the number of cells that are cleared or leave the tissue [4]. Thus, defective removal of these cells may play an important role in the initiation and propagation of chronic inflammatory diseases. There are two main mechanisms that underlie the clearance of inflammatory cells from tissues, namely apoptosis followed by their subsequent removal by phagocytes and necrosis. Whereas the latter is undoubtedly associated with enhanced inflammation and tissue injury, the former is accompanied by shut down of cellular activity and inhibition of the inflammatory response [4]. Apoptosis is characterized by specific morphologic and biochemical events including cell shrinkage, cytoplasmic vacuolation, membrane blebbing, chromatin condensation and nuclear fragmentation associated with endonucleolytic DNA cleavage [5].

More recently, there has been great interest in understanding of the signal transduction pathways relevant for induction of the apoptosis or survival of leukocytes *in vivo* [6–8]. Cyclic adenosine 3′,5′ monophosphate (cAMP) is an important intracellular second messenger produced after adenylate cyclase activation that regulates different cellular processes by cAMP effectors [9,10]. Phosphodiesterases (PDEs) controls the intracellular cAMP levels by catalyzing its hydrolysis and inactivating these second messengers [10]. PDE isoenzymes have been classified into eleven distinct families [10]. Of these, PDE3, PDE4 and PDE7 are the most

Abbreviations: cAMP, cyclic adenosine monophosphate; db-cAMP, dibutyryl-cAMP; i.pl., intrapleural; PDE4, phosphodiesterase 4; PI3K, phosphatidylinositol 3-kinase; NF-κB, nuclear factor kappa B; Akt/PKB, protein kinase B; EMSA, electrophoretic mobility shift assay.

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important for the regulation of cAMP in various types of cells. In neutrophils, eosinophils, mast cell and basophils, PDE4 isoenzymes appear to play a more important function in the regulation of cAMP in leukocyte [10]. Indeed, PDE4 inhibitors induce an increase in the intracellular levels of cAMP in leukocytes and have potent anti-inflammatory activity [11–14]. Intracellular actions of cAMP can be mimicked by administration of the cell permeable analogue dibutyryl-cAMP (db-cAMP) or the adenylate cyclase activator forskolin. Similarly to rolipram, these compounds also inhibit leukocyte function and possess significant anti-inflammatory effects *in vivo* [15]. *In vitro*, inhibition of PDE4 enzymes and increase of intracellular levels of cyclic AMP may modify the survival of eosinophils. Indeed, cAMP elevating agents may enhance or prevent apoptosis of eosinophils depending on their activation status [16]. The effects of cAMP elevating/mimetics on leukocyte apoptosis and survival *in vivo* are not well-established.

The PI3K/Akt pathway has been also shown to mediate survival in many cell types [17]. Recently, we have demonstrated [8] that the PI3K/Akt pathway was important for the survival of eosinophils *in vivo*. It has been reported that there is a cross-talk between the cAMP-dependent and phosphatidylinositol 3-kinase (PI3K) pathways, but the effects of cAMP on PI3K/Akt activity are quite varied [18,19] and cAMP can either stimulate or inhibit Akt activity. For example, cAMP activates PI3K/Akt in thyroid cells and hepatocytes [20,21], whereas inhibition of PI3K/Akt pathway by cAMP has been reported in fibroblast and leukemia cells [22,23]. The transcription factor nuclear factor kappa B (NF-κB) is a key regulator of several cellular functions, including leukocyte activation and survival [24–26]. The pro-survival/anti-apoptotic effects of Akt can be mediated by NF-κB. For example, Akt may phosphorylate IκB kinase (IKK) leading to NF-κB activation [27,28]. It is not known whether the pro-survival effect of the PI3K/Akt pathway during allergic inflammation is mediated via modification of NF-κB function. Hence, it is of interest to examine whether any resolving effect of cAMP on allergic inflammation is mediated by prevention of the function of PI3K/Akt and consequent change in NF-κB function.

In the present study, we examined the ability of the PDE4 inhibitor rolipram and of cAMP-inducers/mimetics, forskolin and db-cAMP, to resolve eosinophilic inflammation in a model of allergic pleurisy in mice [1,8,29,30]. We show that rolipram, db-cAMP and forskolin resolve established eosinophilic inflammation by promoting apoptosis of inflammatory cells and by inhibiting a PI3K/Akt-dependent NF-κB survival pathway.

2. Materials and methods

2.1. Animals

All procedures described here had prior approval from the Animal Ethics Committee of Universidade Federal de Minas Gerais. Male C57/BL6 mice (8–10 weeks) obtained from the Bioscience Unit of Instituto de Ciências Biológicas were housed under standard conditions and had free access to commercial chow and water.

2.2. Drugs, reagents and antibodies

Rolipram (Biomol®, Plymouth Meeting, PA), forskolin and Akt inhibitor-IV (both from Calbiochem, San Diego, CA), gliotoxin (Glio) (Fluka Biochemika, Switzerland), LY294002 (Alamone labs, Jerusalem, Israel), and pyrrolidine dithiocarbamate (PDTC, Sigma–Aldrich, St Louis, MO) were diluted in DMSO and further in PBS. Dibutyryl-cAMP was from Sigma and was diluted in PBS. Annexin-V Detection Kit was from Caltag Laboratories (Burlingame, CA). Rabbit anti-P-Akt (Ser 473), anti-Akt, anti cleaved caspase-3 and mouse anti-phospho-IκB-α were from Cell Signaling Technology (Beverly MA, USA). Rabbit anti-IκB-α (C-21/sc-371), anti p65/*Rel*A (C-20/sc-372), anti p50/NF-κB1 (H-119/sc-7178) and anti Bax (P-19/sc-526) or secondary anti-rabbit peroxidase conjugate antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, EUA). Anti β-actin and anti-mouse peroxidase conjugate antibodies were from Sigma.

2.3. Induction of pleurisy

Animals were immunized with OVA (Sigma) adsorbed to aluminium hydroxide gel as described [1,8,29,30]. Briefly, mice were injected s.c. (subcutaneous) on days 1 and 8 with 0.2 ml of a solution containing 100 μg of OVA and 70 μg of aluminium hydroxide (Reheiss, Dublin, Ireland). Sensitized mice were challenged by i.pl. (intrapleural) administration of antigen (OVA) or PBS. The cells present in the pleural cavity were harvested at different times after antigen-challenge by washing the cavity with 2 ml of PBS and total cell counts performed in a modified Neubauer chamber using Turk's stain. For the experiments evaluating leukocyte apoptosis, infiltrating leukocytes were examined 2 h (annexin-V, DNA fragmentation assays, caspase-3 cleavage) and 24 h (morphologic apoptosis) after drug treatment. Differential cell counts were performed on cyto-centrifuge preparations (Shandon III) stained with May–Grunwald–Giemsa using standard morphological criteria to identify cell types. The results are presented as the number of cells per cavity.

2.4. Treatment with drugs

The role of cAMP on eosinophil accumulation into pleural cavity was investigated by using rolipram (a specific PDE4 inhibitor), forskolin (an adenylate cyclase activator), and db-cAMP (a cell permeable cAMP analogue). Rolipram was administered systemically (i.p.) at dose of 150 μg/mouse (6.0 mg/kg), 24 h after i.pl. OVA-challenge. This dose was shown to be effective in other experimental system [14]. Forskolin 10 μg/mouse (0.4 mg/kg), Db-cAMP 100 μg/mouse (4 mg/kg), LY294002 (30 μg/mouse, 1.0 mg/kg), AKT inhibitor-IV 10 μg/mouse (0.4 mg/kg) and gliotoxin 20 μg/mouse (0.8 mg/kg) were gived i.pl. at a volume of the 100 μl, 24 h after OVA-challenge. PDTC was administered systemically (i.p.) at a dose of 100 mg/kg, 24 h after the i.pl. administration of OVA. As a positive control for anti-inflammatory activity, we used the synthetic glucocorticoid dexamethasone at dose of 2.0 mg/kg in PBS buffer. Glucocorticoids have been shown to induce eosinophil apoptosis and to enhance macrophage phagocytosis of apoptotic bodies [31]. Drugs were dissolved in DMSO and further diluted in PBS. Control mice received drug vehicle only.

2.5. Assessment of leukocyte apoptosis

2.5.1. Morphology

Apoptosis was assessed as previously described by us [8,24]. Briefly, cells (5×10^4) collected 48 h after antigen-challenge were cyto-centrifuged, fixed and stained with May–Grunwald–Giemsa and counted using oil immersion microscopy (×100 objective) to determine the proportion of cells with distinctive apoptotic morphology (cells presented chromatin condensation, nuclear fragmentation and formation of apoptotic bodies out or inside macrophages). Twenty-five fields were counted per slide and results are expressed as the mean ± S.E.M of number of apoptotic cells in 25 fields.

2.5.2. Annexin-V binding and propidium staining

Assessment of apoptosis was also performed by flow cytometry using FITC-labeled annexin-V (Caltag laboratories), which binds to phosphatidylserine exposed on the surface of apoptotic cells, and propidium iodide, as an index of loss of cell membrane integrity.

Annexin-V was added to 100 μl of 2.5×10^5 cells collected 2 h and 6 h after drugs treatment, in binding buffer. Following 20 min incubation at room temperature, these samples were treated with 5 μl of propidium iodide (50 μg/ml) and analyzed using a Becton Dickenson FACScan (San Jose, CA) and FlowJo 7.2.2 software (Tree Star Company). Results are expressed as cells undergoing the early stage of apoptosis quantified by staining with annexin-V but not propidium iodide. The cells were selected based on size and granularity, allowing separate analysis of granulocyte population. At the time point evaluated (26 h after OVA-challenge), morphological analysis showed that granulocytes were eosinophils (>95%, data not shown).

2.6. Lysate preparation and Western blot analysis

Inflammatory cells harvested from the pleural cavity were washed with PBS and total cell extracts or nuclear and cytoplasmic cell extracts were prepared, as described [32,33]. Protein amounts were quantified with the Bradford assay reagent from Bio-Rad (Bio-Rad, USA). Total cell extracts (40 μg), Nuclear (15 μg) and cytoplasmic (40 μg) extracts were separated by electrophoresis on a denaturing 10–15% polyacrylamide-SDS gel and transferred onto nitrocellulose membranes, as described [32]. Membranes were blocked overnight at 4 °C with PBS containing 5% (w/v) nonfat dry milk and 0.1% Tween-20, washed three times with PBS containing 0.1% Tween-20 and then incubated with specifics antibodies (1:1000) in phosphate-buffered saline containing 5% (w/v) BSA and 0.1% Tween-20. After washing, membranes were incubated with appropriated horseradish peroxidase-conjugated secondary antibody (1:3000). Immunoreactive bands were visualized by using ECL detection system, as described by the manufacturer (GE Healthcare, Piscataway, NJ).

2.7. Electrophoretic mobility shift assay (EMSA)

Band shift assay was carried out of 10 μg nuclear extracts essentially as described [32,33], using a 5′ [^{32}P]-end-labeled double-stranded probe (only one strand is shown) corresponding to the consensus-binding site of NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′). Heterologous competition assays were performed with a 100-fold molar excess of cold oligonucleotide corresponding to *c-fos* SRE.

2.8. Statistical analysis

All results are presented as the mean ± S.E.M. Normalized data were analyzed by one-way ANOVA, and differences between groups were assessed using the Student–Newman–Keuls post-test. A *P*-value

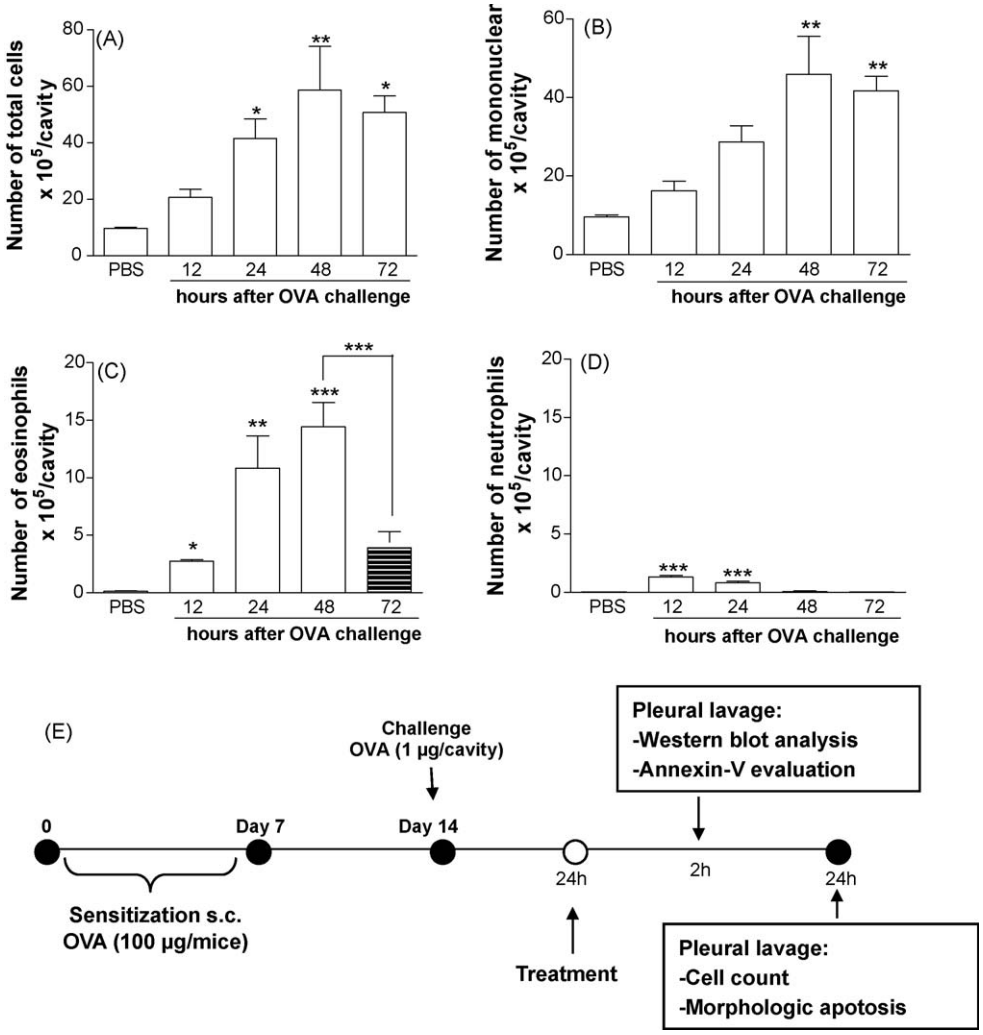

Fig. 1. Kinetics of leukocyte influx in allergic inflammation. Immunized mice were challenged with an i.pl. injection of OVA (1 μg/cavity) or PBS. The cells that migrated into the pleural cavity were collected at indicated times and processed for counting of leukocytes (A), mononuclear cells (B), eosinophils (C), and neutrophils (D). Results are expressed as the means ± S.E.M of five mice in each group. $^*P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared with PBS-injected mice. (E), schematic representation of the protocol of the induction of pleurisy and of the treatments used in this paper.

<0.05 was considered significant. Calculations were performed using the prism 4.0 software program for Windows (GraphPad software, San Diego, CA).

3. Results

3.1. Cyclic AMP elevating agents promote resolution of allergic pleurisy by inducing leukocyte apoptosis

The model of allergic pleurisy used in the present experiment is a well-established model of acute eosinophilic inflammation previously described by our group [1,8,29,30] and by others [13]. Injection of 1 μg of OVA into the pleural cavity of sensitized mice induced a time-dependent influx of leukocytes. As shown in Fig. 1A–D, there was an increase in the total number of leukocytes, eosinophils, mononuclear cells and neutrophils in OVA-challenged mice. Total leukocyte influx reached a maximum at 48 h and decreased at 72 h as compared with PBS-treated mice (Fig. 1A). Eosinophil influx was first detectable at 12 h, reached maximal at 24–48 h and dropped thereafter (Fig. 1C). The time-course of mononuclear infiltrate mirrored the total leukocyte influx (Fig. 1B). Antigen-challenge of sensitized mice also induced an early

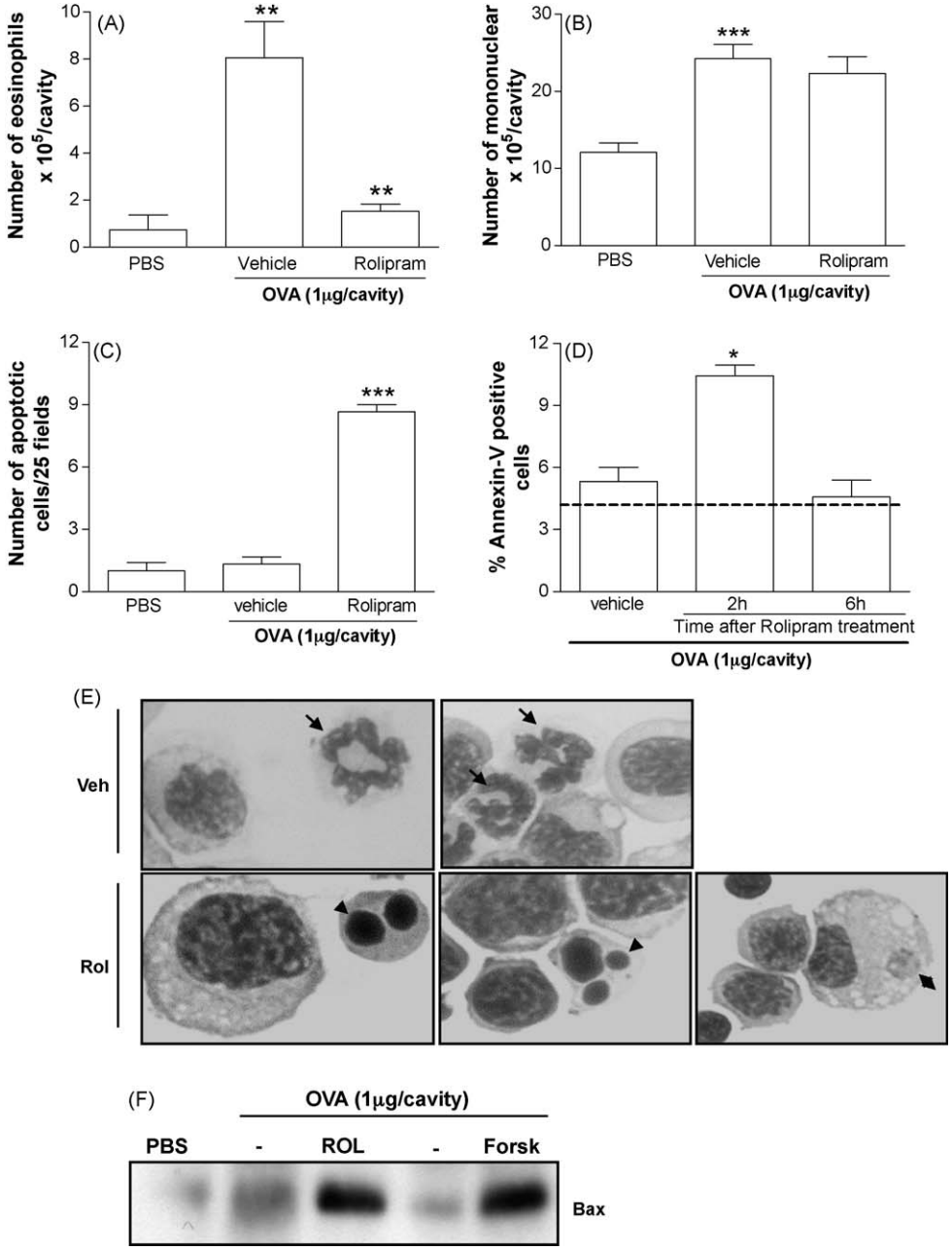

Fig. 2. Delayed treatment with rolipram resolves eosinophilic inflammation by promoting leukocyte apoptosis. Immunized mice were challenged with OVA or PBS and 24 h later, received a systemic (s.c) injection of rolipram (6 mg/kg, i.p.) or drug vehicle. The number of eosinophils (A), mononuclear cells (B) and cells with distinctive apoptotic morphology (C) was assed 48 h after antigen-challenge as described in Section 2. Results are expressed as the number of cells per cavity, or number of cells with distinctive apoptotic morphology in 25 fields, and are shown as the mean ± S.E.M of five mice in each group. $^{**}P < 0.01$ or $^{***}P < 0.001$ when compared with PBS-injected mice or with vehicle treated OVA-challenged mice. In (D) 2.5×10^5 cells were collected 2 h and 6 h after drug or vehicle treatment and incubated with FITC-labeled annexin-V and propidium iodide. Results are expressed as the means ± S.E.M of the percentage of cells staining with annexin-V-FITC alone. There were at least five mice in each group. $^{*}P < 0.05$, when compared with vehicle treated OVA-challenged mice. (E) Representative figures of apoptotic and non-apoptotic eosinophils. Vehicle (upper panels) and rolipram-treated (lower panels) animals are shown. Arrows indicate intact eosinophils, arrowheads indicate apoptotic eosinophils and double arrow indicates an apoptotic body inside a macrophage. In (F), total extracts obtained of inflammatory cells harvested from the pleural cavity 2 h after drug treatments (i.e. 26 h after OVA-challenge) were subjected to Western blot to analysis Bax accumulation as described in Section 2.

recruitment of neutrophil peaking at 4 h [29] and dropping rapidly to background levels by 24 h (Fig. 1D).

The next experiments were designed to investigate whether agents that promote increase of cAMP levels could interfere with eosinophil accumulation in the pleural cavity. We initially used rolipram, a selective PDE4 inhibitor [12]. Eosinophil influx was maximal at 24–48 h, with minor neutrophil contamination in the exudates at these times (compare Fig. 1C and D). Hence, we treated mice with rolipram 24 h after OVA-challenge, when inflammatory cell influx was already established, and performed the pleural lavage 24 h after rolipram treatment (i.e. 48 h after OVA-challenge) (See schematic representation in Fig. 1E).

Mice that were treated with rolipram showed a significant reduction in the accumulation of eosinophils in the pleural cavity at 48 h after challenge (Fig. 2A), without change in the number of mononuclear cells (Fig. 2B). The reduction of eosinophils was associated with an increase in the number of apoptotic cells at the pleural cavity, as demonstrated by morphologic criteria (Fig. 2C). The morphologic features of leukocytes at 24 h after treatment with rolipram are show in Fig. 2E. In agreement with the morphological assessment, there was a rapid increase in annexin-V^+ cells 2 h after treatment with rolipram, when compared with vehicle treated mice (Fig. 2D). Treatment with rolipram also induced the expression of the pro-apoptotic protein Bax (Fig. 2F).

PDE4 inhibitors enhance intracellular levels of cAMP by inhibiting its degradation [12]. To investigate whether increases in cAMP by other means affected eosinophil apoptosis, we studied the effects of forskolin, an adenylate cyclase activator, and db-cAMP, a cell permeable cAMP analogue. The administration of forskolin or db-cAMP in the pleural cavity, when the inflammatory process was established, decreased eosinophil accumulation (Fig. 3A) and increased the number of apoptotic cells (Fig. 3B). Treatment with forskolin also enhanced Bax expression (Fig. 2F). A PKA inhibitor H89 prevented the resolution of eosinophilic inflammation caused by rolipram and db-AMP (Fig. 3C), implicating PKA as the cAMP effector in this resolving process.

3.2. Resolution of OVA-induced pleurisy by rolipram is associated with inhibition of PI3K/Akt

The PI3K/Akt pathway has been shown to mediate survival in many cell types [17]. Recently, we have demonstrated [8] that the PI3K/Akt pathway was important for the survival of eosinophils *in vivo*. With this in mind, we examined the levels of Akt phosphorylation after antigen-challenge and showed that there was a time-dependent increase of Akt phosphorylation in the inflammatory cells recovered from pleural cavity (Fig. 4A). The time-course of Akt phosphorylation mirrored the eosinophil influx into the pleural cavity (compare Fig. 4A and 1C). Treatment with rolipram 24 h after antigen-challenge (24 h of OVA + 2 h of rolipram) rapidly inhibited Akt phosphorylation to baseline levels (Fig. 4B). Similarly, treatment with db-cAMP or forskolin reduced Akt phosphorylation (data not shown). As a positive control, treatment with the PI3K inhibitor LY294002 also prevented Akt phosphorylation (Fig. 4B).

To explore the importance of the PI3K/Akt pathway for eosinophil recruitment/survival to the pleural cavity after antigen-challenge of immunized mice, we used the PI3K inhibitor LY294002 and the Akt inhibitor-IV. Treatment with the LY294002 or Akt inhibitor-IV reduced the number of eosinophils in the pleural cavity induced by antigen-challenge and increased the number of apoptotic cells (Fig. 4C and F). Altogether, these experiments show that inhibition of PDE4 or administration of cAMP mimetic induces clearance of eosinophils by preventing the phosphorylation of Akt, an important signal for eosinophil survival in the system.

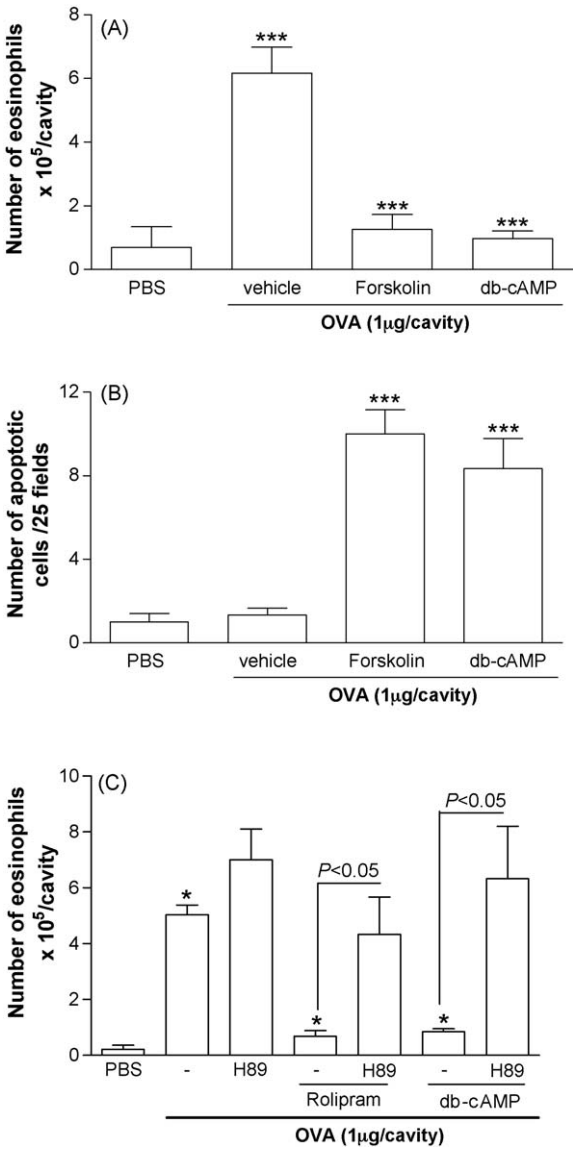

Fig. 3. Delayed treatment with cAMP elevating agents resolves eosinophilic inflammation in a PKA-dependent manner. Immunized mice were challenged with an intrapleural (i.pl.) injection of OVA (1 μg/cavity) or PBS and 24 h later were treated with a local (i.pl.) injection of forskolin (10 μg/mouse), db-cAMP (100 μg/mouse) or drug vehicle. In (C), mice were pre-treated by 30 min with H89 (60 μg/mouse, i.pl.) before receive rolipram or db-cAMP treatment. (A and C) Eosinophil recruitment and (B) number of apoptotic cells were evaluated 24 h after drug treatment, i.e. 48 h after antigen-challenge. Results are expressed as the number of eosinophils per cavity or as number of apoptotic cells in 25 fields and are shown as the mean ± SEM of at least five mice in each group. $^*P < 0.05$ or $^{***}P < 0.001$, when compared with PBS-injected mice or with vehicle treated OVA-challenged mice.

3.3. Inhibition of NF-κB promotes resolution of established eosinophilic inflammation via induction of apoptosis

The transcription factor nuclear factor kappa B is a key regulator of several cellular functions, including leukocyte activation and survival [24–26]. The pro-survival/anti-apoptotic affects of Akt can be mediated by NF-κB. For example, Akt may phosphorylate IκB kinase leading to NF-κB activation [27,28]. To better characterize the involvement of NF-κB in allergic pleurisy, we determined the time-course and role of NF-κB activation in the model of OVA-induced pleurisy. As shown in Fig. 5, the kinetics of NF-κB activation in cells of pleural exudates, analyzed by NF-κB-DNA-binding activity (Fig. 5A), nuclear accumulation of the NF-κB p65 and p50 and IκB-α phosphorylation (Fig. 5B), paralleled the

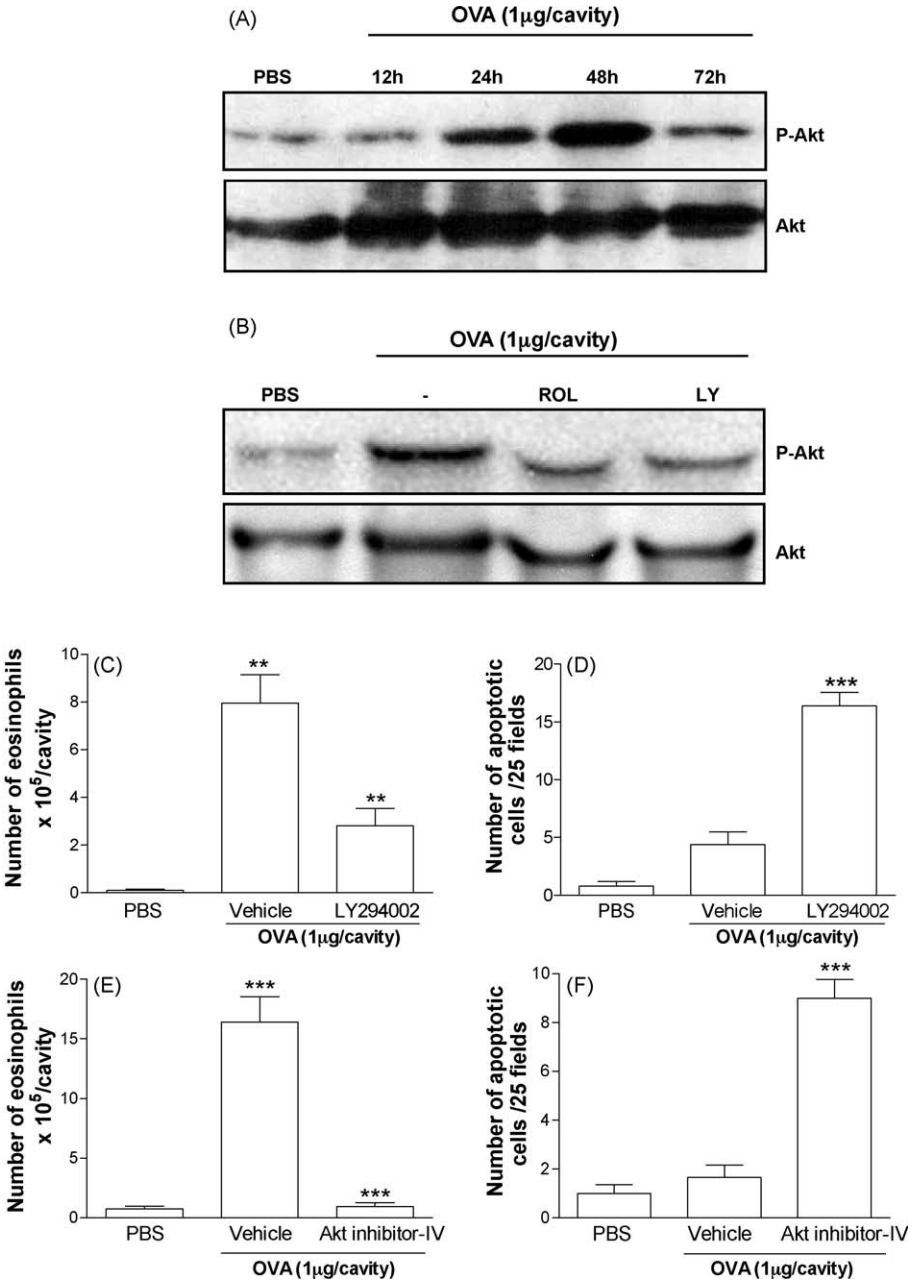

Fig. 4. Akt phosphorylation is decreased by rolipram and delayed treatment with inhibitors of the PI3K/Akt pathway resolve eosinophilic inflammation. Immunized mice were challenged with an intrapleural (i.pl.) injection of ovalbumin (OVA, 1 μg/cavity) or PBS at indicated times (A) or were treatment with rolipram (6 mg/kg, i.p.) or LY294002 (30 μg/mouse, i.pl.) 24 h after OVA-challenge (B). Akt phosphorylation was evaluated at indicated times (A) or after 26 h after antigen-challenge (24 h OVA-challenge + 2 h rolipram) (B) by Western blot analysis performed of total cell extracts obtained of pools of cells from at least five animals as shown in Section 2. Data shown are representative of three independent experiments. In (C–F), the PI3K inhibitor LY294002 (30 μg/mouse, i.pl), Akt inhibitor-IV (10 μg/mouse, i.pl) or drug vehicle were administered in 24 h-OVA-challenged mice and eosinophil recruitment (C and E) and number of apoptotic cells (D and F) were evaluated 24 h after drug treatment, i.e. 48 h after antigen-challenge. Results are expressed as the number of eosinophils per cavity or as number of apoptotic cells in 25 fields and are shown as the mean ± SEM of at least five mice in each group. $^{**}P < 0.01$ or $^{***}P < 0.001$, when compared with PBS-injected mice or with vehicle treated OVA-challenged mice.

kinetics of total inflammatory cell influx into the pleural cavity, i.e. NF-κB activation was first detectable at 12 h, peaked at 24–48 h of OVA-challenge and decreased thereafter (72 h).

We also evaluated whether the use of the NF-κB inhibitors given in the same way as cAMP elevating agents, i.e. at 24 h after antigen-challenge (Fig. 1E), could enhance resolution of eosinophilic inflammation. As seen in Fig. 6A, gliotoxin treatment given at 24 h after OVA-challenge drastically reduced the accumulation of eosinophils observed at 48 h but did not alter the number of mononuclear cells (PBS, $23.4 \pm 0.8 \times 10^5$ mononuclear per cavity; OVA + vehicle, $43 \pm 3.3 \times 10^5$ mononuclear per cavity; OVA + Glio, $47.7 \pm 2.7 \times 10^5$ mononuclear per cavity; $n = 5$, $P < 0.001$ when compare PBS × OVA). The reduction of eosinophil number at 48 h was also seen when another structurally distinct NF-κB inhibitor, PDTC, was given at 24 h (OVA + vehicle, $10.9 \pm 1.5 \times 10^5$ eosinophils per cavity; OVA + PDTC, $2.5 \pm 0.8 \times 10^5$ eosinophils per cavity; $n = 5$, $P < 0.001$). For comparison, treatment with dexamethasone (Dexa), a potent anti-inflammatory drug with various cellular targets [31], at 24 h after challenge diminished the accumulation of eosinophils in the pleural cavity (Fig. 6A).

Next, we evaluated the efficacy of the compounds at blocking NF-κB activity at 2 h after compound administration (i.e. 26 h after

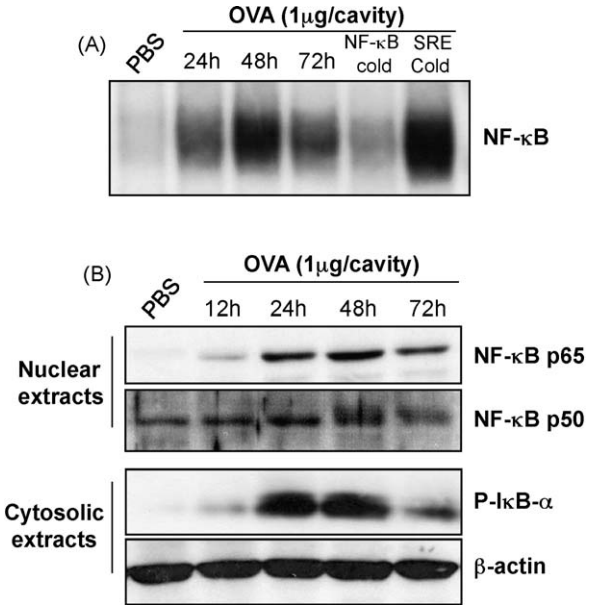

Fig. 5. Kinetics of NF-κB activation in allergic inflammation. Immunized mice were challenged with an i.pl. injection of OVA (1 μg/cavity) or PBS. The cells in the pleural cavity were colleted at indicated times and processed for protein extraction for EMSAs (A) and Western blot (B) analysis as described in Section 2. (A) EMSA was carried out of 10 μg of nuclear protein incubated with an end-labeled probe containing the consensus NF-κB site. Specificity of the interactions was confirmed by competition of the probe with 100-fold molar excess of the indicated cold oligodeoxynucleotide. (B) Nuclear extracts (15 μg) or cytoplasmic extracts (40 μg) were fractionated on 10% SDS-PAGE, transferred onto nitrocellulose membrane and then probed with anti p65, anti p50 or anti-phospho-IκB-α antibody. Re-probing of membrane with anti β-actin was used as control. Data are representative of three independent experiments in pools of cells from at least five animals.

antigen-challenge). As seen in Fig. 6B, treatment with gliotoxin inhibited OVA-induced NF-κB-DNA-binding activity and nuclear levels of p65.

The next experiments were performed in order to evaluate whether induction of apoptosis was involved in the ability of NF-κB inhibitors to resolve eosinophilic accumulation. To this end, apoptosis was assessed in several ways after the treatment with NF-κB inhibitors. The number of apoptotic cells, as accessed by morphologic criteria at 24 h after drug treatment, was markedly elevated in the pleural cavity of antigen-challenged mice treated with gliotoxin (Fig. 6C). Similarly, treatment with PDTC (OVA + vehicle, 0.5 ± 0.8 apoptotic cells/25 fields; OVA + PDTC, 6.0 ± 0.5 apoptotic cells/25 fields $n = 5$, $P < 0.01$) or dexamethasone (Fig. 6C) significantly increased the number of apoptotic events observed in the cavity of antigen-challenged mice. In agreement with the morphological assessment, there was a rapid increase in annexin-V positive cells 2 h after treatment with gliotoxin or dexamethasone when compared with vehicle treated mice (Fig. 6D and E). Chromatin fragmentation assay showed a similar result (data not shown). Caspase activation may be involved in gliotoxin-induced apoptosis in granulocytes [24]. Consistent with the latter possibility, treatment with gliotoxin or dexamethasone increased caspase-3 cleavage in cells of the pleural cavity of OVA-challenged mice, as analyzed 2 h after drug treatment (Fig. 6B). Altogether, the results suggest that inhibition of NF-κB induces inflammatory cell clearance from the pleural cavity of OVA-challenged mice by enhancing apoptosis of inflammatory cells.

3.4. Resolution of OVA-induced pleurisy by rolipram is associated with inhibition of NF-κB

Next, we evaluated whether NF-κB inhibition was associated with rolipram-induced resolution. NF-κB activation was evaluated by EMSA and Western blot analysis for IκB-α in cells recovered from the pleural cavity. Treatment with rolipram or LY294002 24 h after OVA-challenge greatly inhibited NF-κB-DNA-binding activity and prevented IκB-α degradation (Fig. 7A and B). Similarly, treatment with forskolin or db-cAMP also prevented the antigen-associated increased in IκB-α degradation (Fig. 7B).

4. Discussion

An understanding of the mechanisms involved in eosinophil recruitment, activation and survival in sites of allergic inflammation may be useful for the development of novel pharmacological therapies to control allergic diseases. In the present study, we demonstrate that increase of cAMP levels by means of PDE4 inhibition, adenylate cyclase activation or by mimicking cAMP action is effective at resolving eosinophilic inflammation after antigen-challenge of immunized mice. These agents induce the apoptosis of eosinophils resident in the pleural cavity in a PKA-dependent manner and by preventing signaling via the PI3K/Akt pathway and, consequent, NF-κB activation.

Treatment with the PDE4 inhibitor, forskolin or db-cAMP at peak of eosinophil accumulation greatly reduced the number of these cells. The reduction of eosinophil number was associated with an increase in the number of apoptotic events, as assessed by morphologic criteria, annexin-V binding and enhanced expression of Bax. Of note, eosinophil clearance was not associated with a decrease of mononuclear cells, suggesting that apoptotic cells were indeed eosinophils. Our findings that rolipram and forskolin treatment induced Bax accumulation is in agreement with previous findings showing that PDE4 inhibitors suppressed the expression of anti-apoptotic members of the Bcl-2 family and induced the pro-apoptotic protein Bax, thereby shifting the balance between pro- and anti-apoptotic members of the Bcl-2 family towards a pro-apoptotic direction in CLL cells [34,35]. In different cell lineages, cAMP-mediated signaling can be either anti-apoptotic or pro-apoptotic. There have been conflicting reports on the effects of cAMP elevating agents on eosinophil survival/apoptosis *in vitro*. In some experiments, cAMP has been shown to enhance survival and prevent apoptosis [36–38], whereas cAMP was shown to be involved in the induction of apoptosis in other experiments [39–41]. These discrepancies are probably due to differences in the source of eosinophils, dynamic abundance and distribution of intracellular cAMP effectors, previous priming of the cells and whether apoptosis-inducing agents were used or not. In our experiments, *in vivo* administration of compounds with different mechanism of action was clearly associated with resolution of eosinophilic inflammation. Hence, the net effect of cAMP elevation in the course of allergic inflammation is to resolve eosinophil, but not macrophage, accumulation.

Drugs that elevate cAMP may inhibit several eosinophil functions, including respiratory burst, degranulation, aggregation and lipid mediator production [12,42]. Because the agents were given to the whole animal and may have had access to several cell types in addition to the eosinophil, it is difficult to pin-point their major site of action. Known eosinophil survival factors such as GM-CSF and IL-5 peak at 6 h after antigen-challenge [8,29,30], hence much earlier than the schedule of administration (given at 24 h after challenge) of the compounds tested here. Moreover, treatment with anti-IL-5 or anti-GM-CSF at 24 h after challenge did not clear the eosinophils from the cavity (our unpublished observations). Of note, pre-treatment of mice with similar doses of these antibodies blocked OVA-induced eosinophil recruitment in the pleural cavity (our unpublished observations) suggesting that they act by mechanisms other than promoting survival in the system. Thus, administration of PDE4 inhibitors or other cAMP elevating agents may resolve eosinophilic inflammation by acting

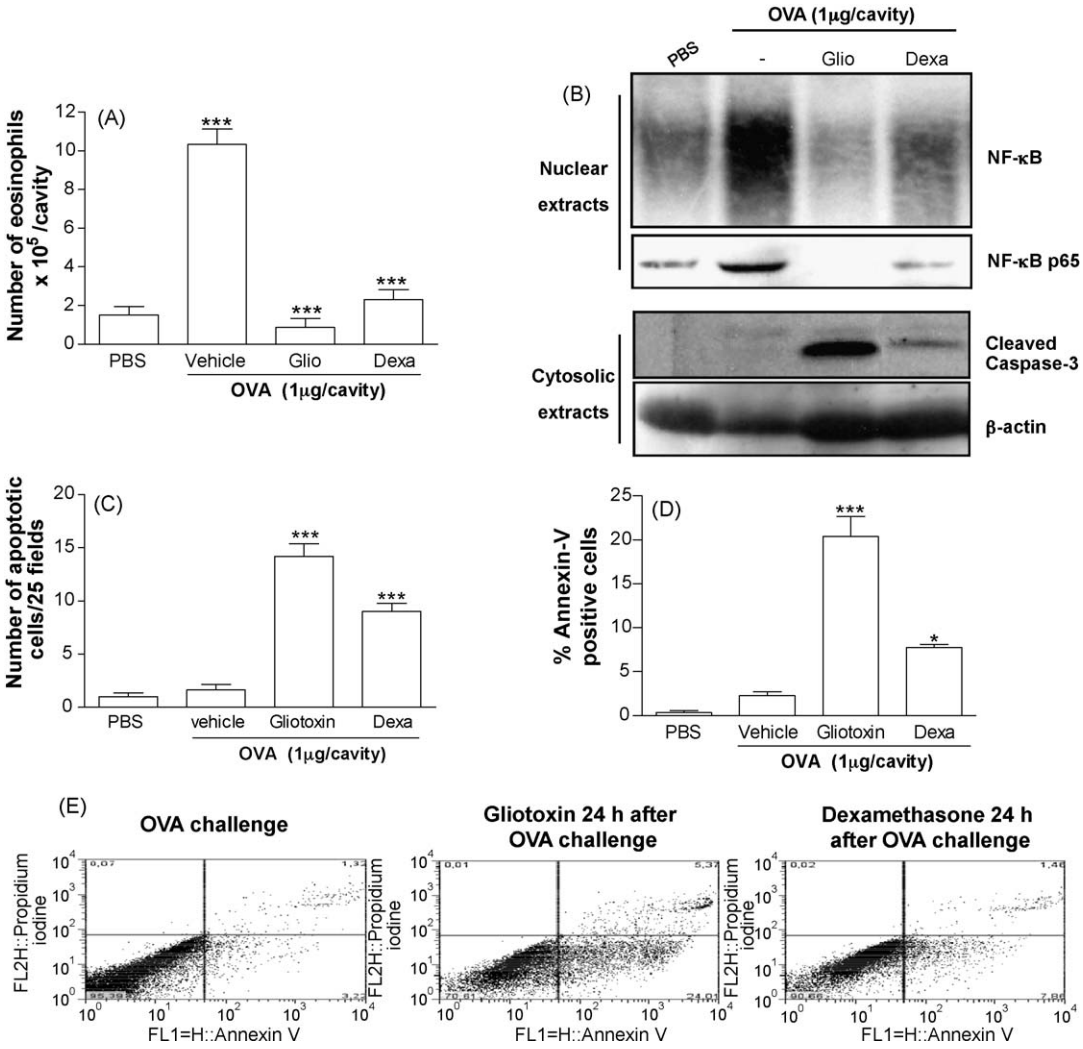

Fig. 6. Delayed treatment with NF-κB inhibitors reduces eosinophilic inflammation accumulation and induces apoptosis. Immunized mice were challenged with OVA or PBS and 24 h later, received a local (i.pl.) injection of gliotoxin (Glio) at dose of 20 μg/mouse (0.8 mg/kg) or systemic (s.c) injection of dexamethasone (Dexa) at dose of 2.0 mg/kg, or drug vehicle. In (A and C), cells were collected of the pleural cavity 48 h after OVA-challenge and were cyto-centrifuged, fixed, and stained with May–Grunwald–Giemsa. The proportion of cells with distinctive apoptotic morphology was evaluated in 25 fields. Results are expressed as the number of eosinophils per cavity or as number of apoptotic cells in 25 fields and are shown as the mean ± SEM of at least five mice in each group. In (B), cytosolic and nuclear extracts were obtained of inflammatory cells recovered of pleural cavity 2 h after drugs treatments e subjected to EMSA and Western blot analysis as described in Section 2. In (D and E), apoptosis was accessed by flow cytometry. Cells (2.5×10^5) were collected 2 h after drugs or vehicle treatments and incubated with FITC-labeled annexin-V and propidium iodide. Results are expressed as the means ± SEM of the percentage of cells staining with annexin-V-FITC alone. There were at least five mice in each group. $^*P < 0.05$ or $^{***}P < 0.001$, when compared with PBS-injected mice or with vehicle treated OVA-challenged mice.

on eosinophils themselves or by regulating the secretion of survival factors other than GM-CSF and IL-5.

The binding of cAMP to proteins such as PKA and Epac (exchange protein directly activated by cAMP) explain most of its functional activities but there are additional, less well-characterized effector proteins [9,18]. Although nonspecific effects of H89 may exist [43], this is a widely used tool to assess the role of PKA in *in vitro* and *in vivo* systems. In our model system, PKA inhibition by H89 limited cAMP-mediated eosinophil clearance, suggesting that PKA may be the cAMP effector.

In addition to their central role in cell proliferation and migration, class I PI3K has also been implicated in the prevention of apoptotic cell death. For example, studies have demonstrated that the PI3K/Akt pathway is constitutively activated in the majority of human pancreatic cancer cell lines [44] and use of selective inhibitors of PI3K could inhibit growth and survival of tumors [45]. The PI3K pathway has also been shown to be an important factor of survival in monocytes [46], neutrophils [47], and eosinophils [8,48]. We have previously demonstrated that treatment with Wortmannin, a PI3K inhibitor, at the peak of eosinophilic inflammation decreased Akt phosphorylation and promoted eosinophil apoptosis [8]. Activation of Akt is a major mechanism by which PI3K provides survival signals [17]. Here, we find that antigen-challenge promoted Akt phosphorylation with a time-course that was parallel to the influx of eosinophils into the pleural cavity. The importance of the Akt pathway for eosinophil survival was evidenced by experiments using PI3K and Akt inhibitors. Moreover, treatment with rolipram inhibited antigen-induced Akt phosphorylation, suggesting that Akt is relevant for eosinophil survival *in vivo* and is a site for the action of cAMP elevating agents. Our results are consistent with studies which demonstrate a cross-talk between cAMP-dependent and PI3K pathways [22,23]. Particularly, the studies of Smith and colleagues [23] showed that cAMP-mediated apoptosis in diffuse large B-cell lymphoma was associated with marked inhibition of PI3K/Akt pathway. Although it is not clear how cAMP modifies Akt activity, a recent report suggests that cAMP-dependent inhibition of Akt in thyroid cells is mediated by phosphatase 2A involving both Epac and PKA cAMP

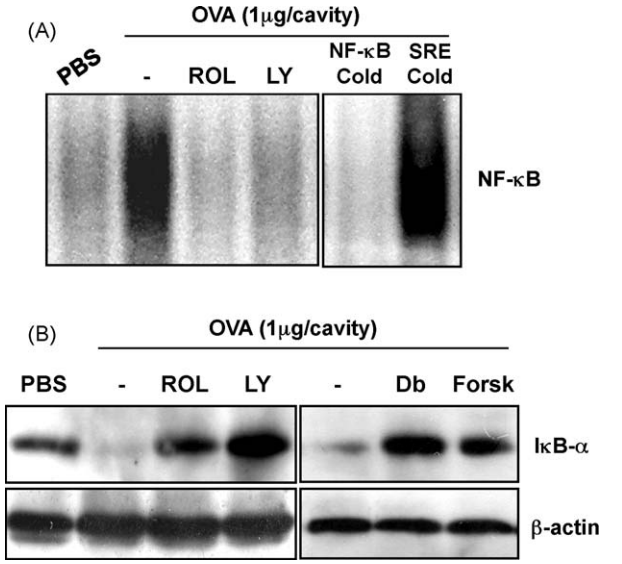

Fig. 7. Treatment with cAMP elevating agents and LY294002 prevents NF-κB activation in allergic inflammation. Immunized mice were challenged with an intrapleural (i.pl.) injection of OVA (1 μg/cavity) or PBS and 24 h later were treated with rolipram (6 mg/kg, i.p.), LY294002 (30 μg/mouse, i.pl.), forskolin (10 μg/mouse), db-cAMP (100 μg/mouse) or drug vehicle. Nuclear and cytoplasmic extracts obtained of inflammatory cells harvested from the pleural cavity 2 h after drug treatments (i.e. 26 h after OVA-challenge) were subjected to EMSA (A) and Western blot (B) analysis, respectively. (A) EMSAs were carried out of 10 μg of nuclear protein incubated with an end-labeled oligodeoxynucleotide probe containing the consensus NF-κB site. Specificity of the interactions was confirmed by competition of the probe with 100-fold molar excess of the indicated cold oligodeoxynucleotide. In (B) cytoplasmic extracts (40 μg) were fractionated on 10% SDS-PAGE, transferred onto nitrocellulose membrane and then probed with anti-IκB-α antibody (upper panel) or with β-actin antibody (under panel). Data are representative of two independent experiments in pools of cells from at least five animals.

effectors [19]. Thus, cAMP may mediate its survival/pro-apoptotic effects by modifying PI3K/Akt. Observations of opposing effects of Epac and PKA on Akt activation can provide a potential mechanism for the apparent cell type-specific effects of cAMP [18].

Akt/PKB has direct effects on the apoptosis pathway, for example by phosphorylating pro-apoptotic proteins such as caspase-9 and BAD. Akt also have effects in transcription factors,

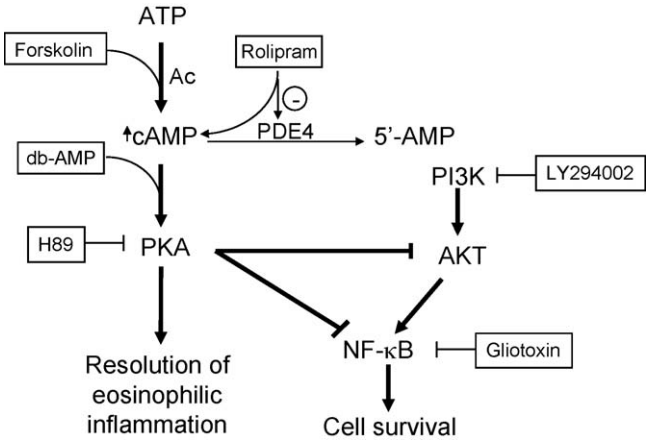

Fig. 8. Model for the resolution inducing effects of the delayed treatment with cAMP elevating agents in allergic eosinophilic inflammation. Increase in the intracellular levels of cAMP by means of the delayed treatment with an adenylate cyclase activator (forskolin), PDE4 inhibitor (rolipram) or db-cAMP promotes activation of a cAMP effector (PKA), which then inhibits signaling thought PI3K/Akt pathway. Down regulation of the Akt phosphorylation may itself lead to apoptosis or may prevent NF-κB activation, which is a relevant signal for eosinophil survival. Prevention of eosinophil survival leads to resolution of inflammation.

including the Forkhead transcription factor and NF-κB [17]. In this regard, Akt can induce cell survival by phosphorylating IκB kinase and, consequently, activating NF-κB [27–28]. The activated NF-κB may then control cell survival via induction of the expression of anti-apoptotic genes [24–26]. In our experiments, NF-κB activation, as evaluated by DNA-binding activity, p65/p50 nuclear accumulation and IκB-α phosphorylation correlated temporally with the infiltration of leukocytes in the pleural cavity of antigen-challenge mice. Treatment with gliotoxin, PDTC or dexamethasone at doses that inhibited NF-κB activation, induced resolution of eosinophilic inflammation and increased leukocyte apoptosis without decreasing number of mononuclear cells. Importantly, cAMP elevation or PI3K inhibitors decreased antigen-induced NF-κB activation by preventing IκB-α degradation and NF-κB-DNA-binding activity *in vivo*. Previous studies have shown that PDE4 inhibitors prevented NF-κB activation when given before or shortly after inflammatory stimulation [49–51], a finding consistent with the ability of PDE4 inhibitors to prevent leukocyte activation and recruitment [12]. However, our results are first to show the ability of delayed treatment with cAMP elevating agents to resolve eosinophilic inflammation and emphasize the importance of NF-κB for leukocyte survival *in vivo*. Moreover, our results are first to suggest that NF-κB activation is downstream of PI3K/Akt activation and resolution inducing effects *in vivo*.

Taken together, our data demonstrate that cAMP elevating agents or mimetics promote resolution of established eosinophilic inflammation in a PKA-dependent manner and by inhibiting Akt phosphorylation and consequent NF-κB activation (see Fig. 8). To our knowledge, this is the first observation that cAMP promotes apoptosis *in vivo* via inhibition of a PI3K/Akt/NF-κB pathway. Hence, we suggest that elevation of cAMP *in vivo* may represent a powerful anti-inflammatory strategy for the treatment of diseases in which eosinophil accumulation is thought to play a relevant role.

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