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Rolipram impairs NF- κ B activity and MMP-9 expression in experimental autoimmune encephalomyelitis

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Abstract

Rolipram suppresses experimental autoimmune encephalomyelitis (EAE) and diminishes cell infiltration of the central nervous system (CNS). In Lewis rats with EAE, rolipram induced a reduction of matrix metalloproteinases-9 (MMP-9) gene expression in lymph node cells (LNCs) and spinal cord, decreased basal levels of nuclear (p50/p65) NF- κ B in LNCs from treated rats, and impaired CD3 mediated NF- κ B translocation. Rolipram also reduced the luciferase activity directed by the NF- κ B binding site of the MMP-9 gene and diminished the ability of a myelin basic protein (MBP) specific cell line to migrate across artificial basement membranes. IL-2 induced MMP-9 proteolytic activity was only slightly reduced indicating that additional factors contribute to inhibit cell migration mediated by rolipram.

Keywords: Experimental autoimmune encephalomyelitis; Rolipram; Nuclear factor kappa B; Matrix metalloproteinase-9

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a neuroinflammatory disease induced in genetically susceptible animals by active immunization against antigens of the central nervous system or by adoptive transfer of activated CD4+ Th1 cells specific for these antigens (Khoruts et al., 1995). It serves as a model of the human demyelinating disease multiple sclerosis (MS). Increased levels of enzymes such as matrix metalloproteinases (MMPs) contribute to the pathogenesis of the disease. Matrix metalloproteinases are a gene family of zinc-dependent endopeptidases, with more than 20 members, including gelatinases, matrilysins, stromelysins, metalloelastases, membrane-type metalloproteinases and interstitial collagenases. They are involved in a number of physiologic and pathologic functions that require proteolytic modelling of the extracellular matrix. In disease, these proteases not only play a major role in malignant tumors (Curran and Murray, 1999), but they are also deeply involved in the pathogenesis of several autoimmune disorders of the peripheral and central nervous systems (Hartung and Kieseier, 2000). One of the MMPs, the gelatinase-B (MMP-9), efficiently degrades basement membranes, cleaves myelin basic protein and processes cytokines and chemokines, regulating the extravasation of immune cells and the tissue destruction during inflammation (Opdenakker et al., 2001). Type 4 phosphodiesterase (PDE4) is considered a major PDE isoenzyme responsible for catabolism of cyclic AMP (cAMP) and regulation of the inflammatory function in many cells, including monocytes and lymphocytes (Ichimura and Kase,

1993; Livi et al., 1990). Previous results from our laboratory demonstrated that rolipram (a PDE4 inhibitor) improves EAE by inducing a delay in the entry of inflammatory cells into the central nervous system (CNS) (Martinez et al., 1999). Although the therapeutic effect of rolipram has been related to inhibition of proinflammatory cytokines (Sommer et al., 1995), the precise mechanisms of this drug remain elusive. *In vitro*, it has been shown that rolipram inhibits the nuclear factor kappa B (NF- κ B) (Jimenez et al., 2001; Navarro et al., 1998). This nuclear factor has a key role in the regulation of inflammatory processes and is involved in the expression of many genes whose activity has been related to EAE and MS, including MMP-9 (Kieseier et al., 1998). Although the transcriptional expression of MMP-9 is modulated by other transcription factors (Sato and Seiki, 1993), a pivotal role has been proposed for NF- κ B activity in the inducible expression of MMP-9 (Bond et al., 1998).

To investigate possible molecular mechanisms by which rolipram treatment might diminish the CNS infiltration of inflammatory cells, we studied the expression of MMP-9 both in lymphocytes and CNS from rolipram treated EAE rats. In those animals, we found a lower expression of MMP-9 in the CNS and the periphery. This observation correlated with down-regulation *in vivo* of NF- κ B activity in lymphocytes, after treatment of EAE with rolipram and with inhibition of *in vitro* transcriptional activity directed by the NF- κ B site (located at position -600) of the MMP-9 promoter. Furthermore, we found that, *in vitro* treatment of an MBP-specific cell line with rolipram inhibited the MMP-9 activity and the migration

ability across artificial basement membranes.

2. Materials and methods

2.1. Induction of EAE, treatment protocols and clinical evaluation

EAE was induced in Lewis rats (Charles River, France) as described elsewhere (Puerta et al., 2000). Briefly, an inoculum containing 50 µg of guinea pig myelin basic protein (gp-MBP) (Sigma Chemical Co. St. Louis, Mo) and 500 µg of *M. tuberculosis* (strain H37 Ra, Difco, Detroit, MI) in incomplete Freund's adjuvant (Difco) was injected subcutaneously into the hind footpads. Rats injected with complete Freund's adjuvant (CFA) were inoculated with *M. tuberculosis* and adjuvant in the same way. EAE rats were treated with the racemate of rolipram suspended in physiological saline with the dispersing agent Tween 80 (Sigma) 2:25 v/v; a single dose of 5 mg/kg body weight per day was administered subcutaneously, as reported elsewhere (Martinez et al., 1999). Sham-treated controls were injected with saline / Tween 80 in the same way. Rats were examined daily for the presence of neurological signs, using the following scale: 0 = no EAE; 1 = partial loss of tail tonicity; 2 = loss of tail tonicity; 3 = unsteady gait and mild paraparesis; 4 = hind-limb paralysis; and 5 = death. Grades 3 and 4 were often accompanied by urinary and fecal incontinence. All experiments were carried out following the Spanish regulations for experimental work with animals.

2.2. Cell isolation and culture

Rat popliteal and inguinal lymph nodes were removed, passed through a 200-µm stainless steel mesh sieve and the lymph node cells (LNCs) suspension was washed and resuspended in Click's medium (RPMI 1640, 2 mM glutamine, 1 mM sodium pyruvate, essential and non-essential amino acids, 10 mg/ml streptomycin and 100 U/ml penicillin and 0.1 M 2-mercaptoethanol (Gibco)) supplemented with 10% heat-inactivated fetal calf serum (FCS). Before extraction of proteins, cells were stimulated by plate-bound anti-rat CD3 (BD Pharmingen, San Diego, CA) (1 µg/ml) during the incubation periods indicated in the figures.

To establish a rat MBP-specific T cell line, popliteal and inguinal lymph nodes from gp-MBP immunized rats were harvested, and the LNCs (2×10^6 cells/ml) cultured with gp-MBP (20 µg/ml) in Click's medium supplemented with 10% heat-inactivated FCS for 3 days at 37°C and 5% CO₂. The cells were expanded in the presence of recombinant rat IL-2 (1.6 ng/ml) (R&D systems, Abingdon, UK) for 10 days and restimulated with mitomycin C-treated spleen cells from Lewis rats at 0.5×10^6 cells/ml, in the presence of 15 µg/ml gp-MBP. The culture was maintained through additional rounds of stimulation and expansion. On repeated experiments, these cells exhibited a high encephalitogenic capacity when injected intravenously to naïve Lewis rats (data not shown).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) and semiquantitative analysis

Total RNA was extracted from frozen segments of the spinal cords and fresh LNCs using TriPure™ (Boehringer Mannheim, Heidelberg, Germany) according to the manufacturer's instructions. The RT and PCR reactions were performed as described elsewhere (Puerta et al., 2000). Briefly, one µg RNA was reverse transcribed, using AMV reverse transcriptase and

oligo (dT)₁₅ primers (Promega). cDNA (undiluted or diluted 1/10th for spinal cord and LNCs respectively) was amplified using the following primers: 5'-ACATCACCTATTGGATCCAAAG-3' and 5'-AGCCATACAGCTTATCCGGTC-3' for MMP-9 and 5'-TTGTAACCAACTGGGACGATATGG-3' and 5'-GATCTTGATCTTCATGGTGCTAGG-3' for β-actin. All primers used span introns.

The optimal number of cycles was determined in preliminary experiments intended to identify a linear range of amplification for each gene. Cycles used were: 32 for MMP-9 and 23 for β-actin, in the spinal cord samples; and 30 for MMP-9 and 23 for β-actin, in the LNCs samples. PCR reactions for each gene were performed simultaneously to minimize variability. Negative controls (samples lacking cDNA) were included in each assay. PCR products were separated by electrophoresis in 1% agarose gels (0.5 x TBE), blotted on nylon membranes (Boehringer Mannheim) by capillary action under alkaline conditions (Ausubel, 1992), and hybridized with specific probes containing digoxigenin. The chemoluminescent detection was applied according to a standard protocol (DIG Luminescent Detection Kit, Boehringer Mannheim). The density of the band was quantitated using a digital imaging system. Band intensity was normalized to the β-actin signal to account for any differences in total RNA content for each sample. Values were expressed as relative densitometric units. The units were calculated as a fraction of the maximum sample density for each blot. In order to compare data from treated and untreated animals, all RT-PCR products of each gene were measured from the same blot.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins extraction and generation of ³²P end-labeled probe were performed as reported previously (Dorado et al., 1998). Nuclear proteins (2 µg) were incubated for 10 min in a final volume of 10 µl with 200 ng poly (dIdC) (Amersham-Pharmacia) before addition of 0.4 ng of 5'-end labelled ds-oligonucleotide κB, which contains a NF-κB binding site (5'-CAAGGAAAGGGGATTCTCCTGCCGCGGC-3') (Arima et al., 1992). Binding reactions were performed during 30 minutes at room temperature and electrophoresed in a 5% polyacrylamide gel in TBE (0.5x). DNA-protein complexes were detected by X-ray film exposition. For supershift assays, the binding mixtures were preincubated for 3 h with the indicated antibody before adding the end-labelled probe. Competition assays were carried out in the presence of a 50-fold molar excess of cold oligonucleotide 10 min before the addition of the probe.

2.5. Plasmid construction, transfection and luciferase assays

To construct κB-pGL3-prom an hexamer of the the sequence from -600 to -590 of the rat MMP-9 promoter (Eberhardt et al., 2000) was inserted at the XhoI site of pGL3-prom (Promega Corporation, Madison, WI). Jurkat cells (2×10^6 cells/ml) were electroporated at 270 V and 960 µF in 0.4-cm cuvettes using a Bio-Rad Gene Pulser with 5 µg of κB-pGL3-prom or the empty vector. Each sample was transfected with 1 µg of pRL-TK plasmid, wich contains the cDNA encoding renilla luciferase (Promega). After 20 h, the cells were divided for the different culture conditions indicated. Transfections were stimulated with 20 ng/ml phorbol-miristate acetate (PMA) for 3 h and protein extraction and luciferase determinations were carried out by the dual-luciferase assay system of Promega. Each value of the firefly luciferase activity codified by pGL3-

prom or κ B-pGL3-prom plasmids was normalized to the renilla luciferase activity.

2.6. Gelatin zymography

Nonreduced samples were subjected to SDS-PAGE zymography as described (Xie et al., 1994). Briefly, 20 μ l of the cell culture medium were applied to a 8% polyacrylamide gel containing 2 mg/ml of gelatin; after electrophoresis, the gel was washed for 1 h in 2.5% Triton X-100 and then incubated overnight at 37°C in 40 mM Tris-HCl, pH 7.5, 10 mM CaCl₂. The gel was stained with a solution of 0.25% Coomassie brilliant blue R-250 and destained in 7% acetic acid and 10% methanol. Gelatinolytic activity was observed as a clear band. The density of the bands was quantitated using a digital imaging system.

2.7. Migration assay

Twenty-four-well BD BioCoat Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA) were used, following the manufacturer's protocol. Briefly, 5 x 10⁵ MBP-specific lymphocytes were seeded into the upper chamber with 0.5 ml RPMI 1640 serum-free medium. Cells were preincubated with rolipram for 90 min at different concentrations. The bottom chamber contained 0.75 ml RPMI 1640 serum-free medium. After incubation at 37°C with 5% CO₂ for 20 h, the content of the lower chambers was collected, resuspended and then counted by trypan blue staining using a hemacytometer chamber. The equivalent of dimethyl sulfoxide (DMSO) present in samples with the highest concentration of rolipram did not affect migration.

3. Results

3.1. Rolipram reduces the MMP-9 gene expression in LNCs and CNS from EAE animals

As the extravasation of lymphocytes and cleavage of cytokines and chemokines in EAE is tightly regulated by the expression of matrix metalloproteinases, and one of them, MMP-9, is up-regulated in EAE and MS (Hartung and Kieseier, 2000), we analyzed MMP-9 gene expression in LNCs and spinal cord of rolipram treated and untreated EAE rats. On day 10 post-inoculation (PI), when cells are activated in the draining lymph nodes without neurological signs, rolipram treated rats had decreased levels of this metalloproteinase compared to EAE rats (Fig. 1, left panel); on day 13 PI, when clinical signs reach their highest severity in the untreated group and rolipram treated rats show a mild clinical score (Martinez et al., 1999), the MMP-9 gene expression in the CNS was significantly reduced in rolipram treated EAE rats, compared to the untreated control (Fig. 1, right panel).

3.2. Treatment of active EAE with rolipram inhibits the nuclear NF- κ B levels in lymphocytes

The translocation of NF- κ B to the nucleus is an early step for the expression of proinflammatory genes, and rolipram impairs NF- κ B activation *in vitro* (Jimenez et al., 2001; Navarro et al., 1998). As PDE4 inhibition in EAE leads to a decreased expression of proinflammatory molecules (Martinez et al., 1999), and to a reduced MMP-9 gene expression, both in the CNS and the periphery, we wanted to know whether *in vivo* the molecular mechanism of PDE4 inhibition involved the same modification of NF- κ B activation as *in vitro*. For that purpose,

we examined by EMSA the levels of nuclear NF- κ B in lymphocytes from the lymph nodes of rolipram treated EAE rats. Nuclear NF- κ B levels were analyzed in freshly obtained cells or upon *in vitro* CD3 engagement. As shown in Fig. 2A, nuclear translocation of NF- κ B was seen in cells from CFA rats activated with anti-CD3 antibody. The bands detected were specific for the sequence employed, as shown by competition assay, and composed by homodimers p50/p50 and heterodimers p50/p65, as revealed by supershift reactions with antibodies against p50 and p65 subunits. The inactive form of NF- κ B (p50/p50) was observed in LNCs from CFA animals, whereas the active form (p50/p65) was induced only upon CD3 engagement (Fig. 2B). Nevertheless, the active heterodimer was detected in cells obtained from EAE rats either in the presence or in the absence of the stimulus. *In vivo*, treatment with rolipram had two main effects: impairment of (p50/p65) induction upon CD3 engagement (CFA vs CFA+ROL) and inhibition of the cell activation state in the absence of stimulus (EAE vs EAE+ROL).

These data show that the observed amelioration of EAE, diminished MMP-9 (Fig. 1) and proinflammatory gene expression (Martinez et al., 1999) in rolipram treated animals correlates with an inhibitory effect on NF- κ B activation.

To further confirm the inhibitory effect of rolipram on the nuclear translocation of NF- κ B in lymphocytes, an encephalitogenic MBP-specific T-cell line was cultured for 6 hours with or without stimulus (10 ng/ml PMA and 1 μ M ionomycin). Rolipram (10 μ M) was added to the cultures. Cell viability, measured by Trypan blue dye exclusion, was unaffected by any of the concentrations used of this drug. Control cultures without rolipram were treated with a DMSO concentration equivalent of that used in rolipram treated cultures. As shown in Fig. 3, rolipram impaired the induction of the active form of NF- κ B (p50/p65) in stimulated cells.

3.3. Rolipram decreases promoter activity in a construct containing the NF- κ B binding sequence of the MMP-9 gene

To examine whether the inhibition of NF- κ B translocation detected by EMSA was associated with a reduction in the transcriptional activity mediated by the κ B site present in the MMP-9 promoter, we transfected Jurkat cells with the κ B-pGL3-prom plasmid, containing an hexamer of the NF- κ B binding site of the MMP-9 gene. Transfections were stimulated with phorbol myristate acetate in the presence of different concentrations of rolipram. As shown in Fig. 4, PMA promoted about 10-fold more transcriptional activity in the κ B-pGL3-prom than in the empty vector transfections, as assessed by luciferase activity. Treatment of stimulated κ B-pGL3-prom transfections with rolipram reduced luciferase activity in a dose-dependent manner. Under the same experimental conditions, pGL3-prom transfections showed no significant changes in luciferase activity, indicating that the effect of rolipram on luciferase activity was exerted through the κ B sequence present in κ B-pGL3-prom. Taken together, these results demonstrate that rolipram *in vivo* impairs NF- κ B translocation, and may reduce *in vitro* the transcriptional activity driven by the NF- κ B site of the MMP-9 promoter.

3.4 Rolipram reduces IL-2 induced MMP-9 enzymatic activity in MBP-specific rat lymphocytes

To test if rolipram was able to modulate the overall MMP-9 proteolytic activity in activated T lymphocytes, we established a rat cell line reactive to MBP, treated the cells with increasing doses of the drug, and assayed MMP-9 activity by gelatin zymography in the supernatant of cultured cells. Fig. 5 shows

that MMP-9 activity was almost undetectable in the absence of a stimulus and was induced in the presence of the proinflammatory molecule IL-2. Rolipram treatment of IL-2 activated lymphocytes reduced MMP-9 activity in a dose-dependent way, without affecting cell viability as measured by trypan-blue exclusion. Treatment of unstimulated cells with rolipram induced no changes in the enzyme activity (data not shown).

3.5. Rolipram reduces transmigration of MBP-specific T lymphocytes

To assess whether this drug could modify the migratory ability of a rat T cell line specific for MBP, we cultured these lymphocytes in presence of different doses of rolipram for 90 min. Afterwards, cells were allowed to migrate across the matrigel membrane during 20 hours. Fig. 6 shows that the percentage of cells that could migrate across the matrigel membrane was significantly reduced by 50%, even at the lower dose used.

4. Discussion

Rolipram and other PDE4 inhibitors have been considered promising anti-inflammatory drugs for the treatment of several conditions, including EAE (Genain et al., 1995; Sommer et al., 1995). In EAE, we have previously shown that this drug down-regulates or delays the production of proinflammatory cytokines in the spinal cord of rats primed to develop EAE (Martinez et al., 1999). Other reports have confirmed these results (Agnello et al., 2000). In this work, we have analyzed the effects of rolipram on NF- κ B, a transcriptional factor involved in inflammatory processes (Burke, 2003). It was found to be induced in the spinal cord by EMSA (Pahan and Schmid, 2000) and immunocytochemistry (Kaltschmidt et al., 1994) in the peak of the clinical disease. Blocking the NF- κ B function can be an effective way to prevent EAE; mice deficient in the p50 subunit are resistant to EAE induction, and this is associated with an abnormal differentiation of antigen-specific T cells into either Th1 or Th2 effector cells (Hilliard et al., 1999). All these data have underlined a key role of NF- κ B in EAE development. The results presented here show that levels of nuclear NF- κ B are increased in LNCs from animals primed to develop EAE as compared with healthy controls. When rats primed to EAE were treated with rolipram, the high levels of NF- κ B activation in freshly obtained cells were prevented. Although *in vitro* CD3 stimulation of these LNCs allowed NF- κ B activation as efficiently as in healthy controls, *in vivo* NF- κ B activation through CD3 signaling was deeply impaired by treatment with rolipram in CFA animals. These results indicate that some of the EAE therapeutic effects mediated by rolipram can be related to an inhibition of the NF- κ B activity, whose translocation can be also blocked *in vitro* by other treatments improving EAE (Du et al., 2001b; Du et al., 2001a; Heneka et al., 2001).

The inhibition of NF- κ B activity mediated by rolipram could lead to a reduction of the expression levels of a number of genes regulated by this transcription factor and associated with EAE development, such as proinflammatory cytokines and factors involved in T cell migration and CNS infiltration. Among those genes, MMP-9 is one of the candidates to explain some of the rolipram therapeutic effects. Although other regulators such as AP-1 or Stat1 are involved in the gene

expression of MMP-9, NF- κ B action seems to be essential for the up-regulation of this metalloprotease (Eberhardt et al., 2000; Han et al., 2001; Munaut et al., 1999; Yan et al., 2004). Taking into account the critical role assigned to MMP-9 in the pathogenesis of both EAE and MS (Gijbels et al., 1992; Gijbels et al., 1994; Kieseier et al., 1998; Lee et al., 1999; Lichtinghagen et al., 1999; Waubant et al., 1999), we investigated possible effects of rolipram on MMP-9 regulation, and observed that, *in vivo*, treatment with rolipram diminished the MMP-9 mRNA production not only in cells from the draining lymph nodes on day 10 PI, a moment of maximum T cell activation, but also in the CNS on day 15 PI, when clinical signs were full blown in untreated rats. Thus, the improvement in EAE clinical signs mediated by rolipram is accompanied by a decrease in the NF- κ B DNA binding activity and in the MMP-9 gene expression. We could also demonstrate that inhibition of NF- κ B mediated by rolipram decreased the transcriptional activity directed by the κ B element present in the MMP-9 promoter.

MMP-9 is one of the molecules associated with T cell migration across basement membranes *in vitro* (Leppert et al., 1995). The results presented here show that rolipram is also able to interfere significantly with migration of encephalitogenic T cells. Furthermore, *in vitro*, zymograms of supernatants from these T cells, cultured in presence of rolipram, showed a dose-dependent reduction of gelatinolytic activity. However, the fact that more than 60% of MMP-9 activity was maintained in cells treated with the highest rolipram concentration tested, suggests that additional factors contribute to inhibit the cell migration in these experiments. Lymphocyte migration to tissues involves a series of complex phenomena requiring activation, expression of adhesion molecules, polarization with the formation of a leading edge and an uropod, digestion of basement membranes and migration led by different chemoattractants. In addition, it has been recently shown that, in T cells, rolipram inhibits critical steps, such as adhesion to integrin ligands, aggregation, activation, polarization and transendothelial migration (Layseca-Espinosa et al., 2003). It is also able to reduce the chemokines MIP-1 α , MIP-2 and MCP-1 in the experimental autoimmune neuritis of Lewis rats (Abbas et al., 2000).

Rolipram may have various and complex effects on EAE, and our data indicate that the reduction of CNS infiltrating cells observed in previous experiments with this drug may be due in part to its inhibitory effect on T cell transmigration. The available evidence points towards NF- κ B inhibition as one of the key therapeutic mechanisms which would mediate down-regulation of genes codifying for proteins involved in cell migration. MMP-9 might be included among them, as suggested by the *in vivo* finding of a reduced gene expression in lymph nodes and CNS from EAE rats treated with rolipram, and the inhibition for its κ B regulating element mediated by the drug.

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Figure legends

Fig. 1. Effect of treatment with rolipram in EAE. Lewis rats were immunized with encephalitogen and divided into two groups. Rolipram was given from day 1 to 17 in a single daily dose of 5 mg/kg body weight s.c. Decreased MMP-9 gene expression in lymph node cells (LNCs) (left panel) and spinal cord (right panel) of rolipram treated EAE rats. LNCs and spinal cord from rolipram treated (EAE+ROL) and untreated rats (EAE) were obtained on days 10 and 13 PI respectively. Semi-quantitative RT-PCR was performed as described under materials and methods. Each point represents mean values of 4 rats; bars indicate SEM; asterisks denote statistically significant differences ($p < 0.05$) compared in the Student's *t*-test.

Fig. 2. Inhibition of NF- κ B translocation in LNCs from rolipram treated EAE rats. The presence of NF- κ B in nuclear proteins was detected by EMSA. (A): Competition and supershift assays. For competition, nuclear proteins from LNCs of CFA rats were incubated in the presence (+) or absence (-) of a 50-fold molar excess of cold oligonucleotide as competitor. For supershift, the same nuclear proteins were assayed in the presence or absence of the indicated antibody. A control without nuclear extract is shown. (B): Nuclear proteins were isolated from cells obtained from untreated (CFA) or EAE (EAE) rats, and rolipram treated non-immunized (CFA+ROL) or EAE (EAE+ROL) rats on day 10. The cells (2×10^6 cells/ml) were cultured in the presence (+) or absence (-) of plate-bound anti-rat CD3 (1 μ g/ml) for 1 h.

Fig. 3. Inhibition of NF- κ B activation on MBP-specific lymphocytes by rolipram. Cells were incubated with 10 mM rolipram for 6 hours in the presence (+) or absence (-) of stimulus: PMA (10 ng/ml) and ionomycin (1mM). Control cultures without rolipram were treated with a DMSO concentration equivalent of that used in rolipram treated cultures. Note the absence of induction of the active heterodimer (p50/p65) in stimulated cells cultured with rolipram.

Fig. 4. Rolipram inhibits PMA-induced transcriptional activity in a plasmid containing the NF- κ B binding site of the MMP-9 gene. Transfected Jurkat cells with the mock plasmid (pGL3-prom) or the construct containing an hexamer of the NF- κ B binding sequence (κ B-pGL3-prom) were stimulated or not with PMA for 3h. At the indicated concentration, rolipram was added simultaneously to the stimulus. Each value of the firefly luciferase activity was normalized to the renilla luciferase activity; results are represented as relative units. Each point is the mean of four independent determinations.

Fig. 5. Inhibition of IL-2 induced MMP-9 activity in the supernatant of MBP-specific lymphocytes by rolipram. Rat lymphocytes (2×10^6 cells/ml) were incubated in the presence (+) or absence (-) of recombinant rat IL-2 (50 ng/ml) at the indicated concentrations of rolipram (ROL) for 48 h. (A): culture supernatants were subjected to SDS-PAGE gelatin zymography as described under materials and methods. Zones of clearing represent gelatinolytic activity. (B): band intensity of zymograms was subjected to densitometric analysis, results are shown as units of optical density quantitated by PCBas software. Values represent the mean of optical density measurements obtained from three different experiments.

Fig. 6. Migration of MBP-specific lymphocytes across BioCoat Matrigel Invasion Chambers preincubated with 50 and 200 μ M rolipram for 90 min. Results are expressed as percentage of cells recovered from the lower compartment after 20 h, \pm SEM (A) where 100% corresponds to the value of the median in the control group of samples without rolipram. Individual values of controls and each rolipram condition were analyzed for *t*-test with Welch's correction by GraphPad Prism software. Differences between individual control values and each rolipram condition were statistically significant (***) with $p < 0.001$ (0.0004 and 0.0002 for 50 and 200 μ M of rolipram, respectively) (B).

Figure 1

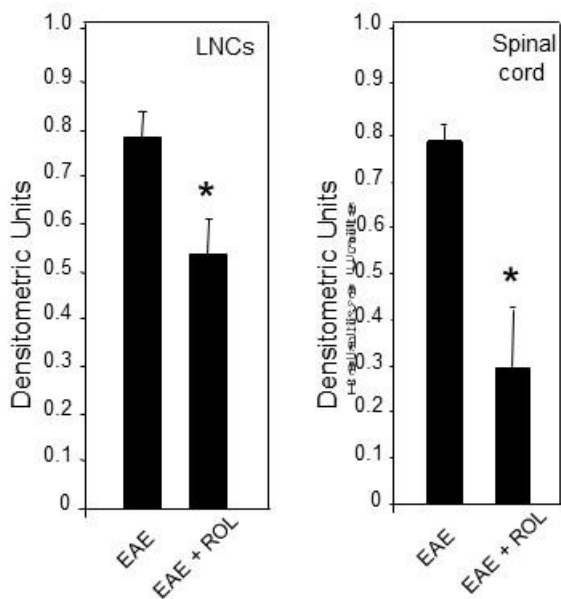


Figure 3

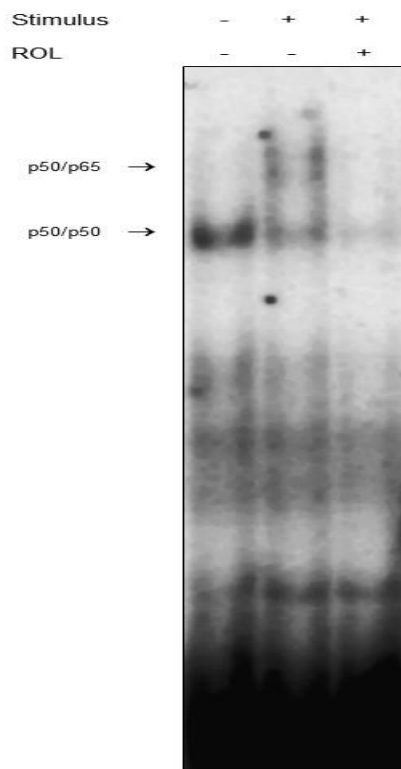


Figure 2

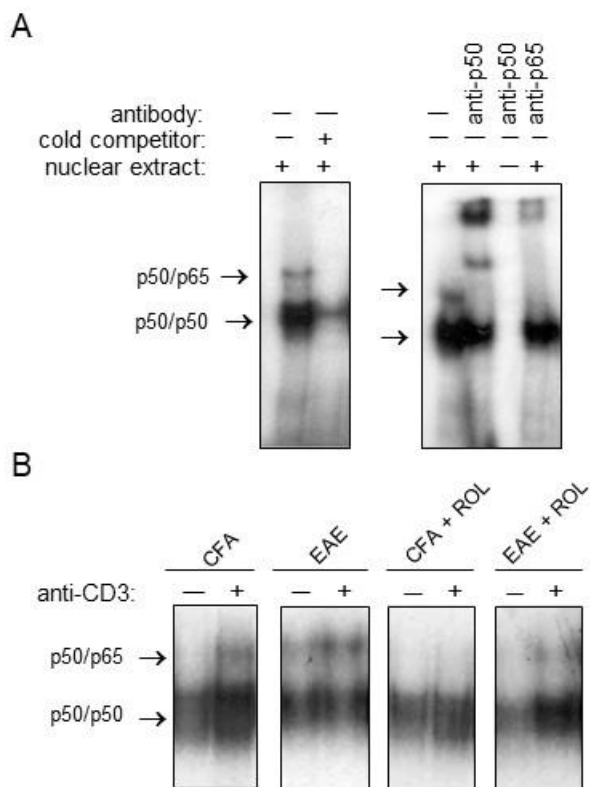


Figure 4

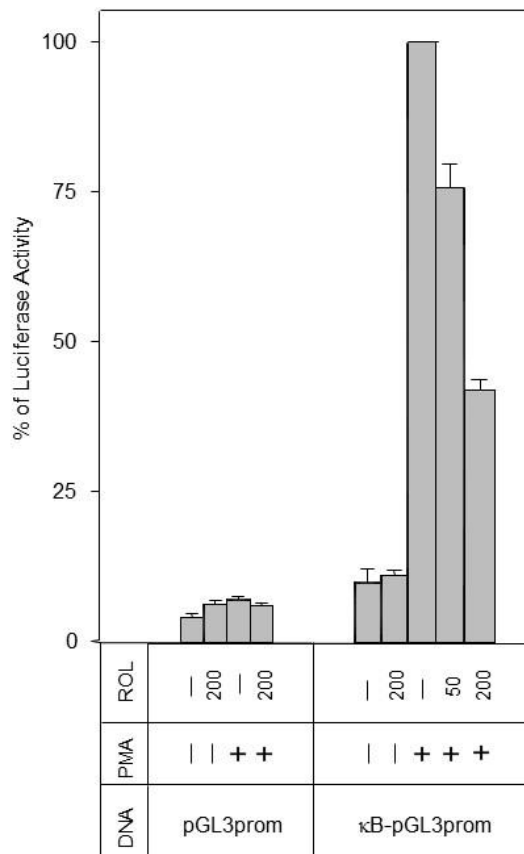


Figure 5

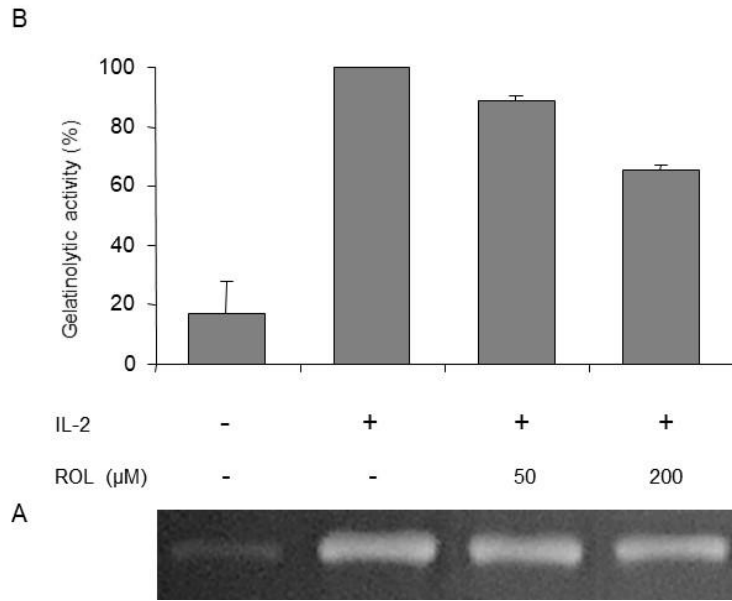


Figure 6

