Immunopharmacological Potential of Selective Phosphodiesterase Inhibition. II. Evidence for the Involvement of an Inhibitory-κB/Nuclear Factor-κB-Sensitive Pathway in Alveolar Epithelial Cells

JOHN J. HADDAD, STEPHEN C. LAND, WILLIAM O. TARNOW-MORDI, MAREK ZEMBALA, DANUTA KOWALCZYK, and RYSZARD LAUTERBACH

Neuroscience Research Laboratory, Department of Anesthesia and Perioperative Care, University of California Medical Center, San Francisco, California (J.J.H.); Oxygen Signaling Group, Center for Research into Human Development, Tayside Institute of Child Health, Faculty of Medicine, Ninewells Hospital and Medical School, University of Dundee, Dundee, Scotland, United Kingdom (S.C.L.); Department of Neonatal Medicine, Westmead Hospital and New Children’s Hospital Neonatal Service, University of Sydney, New South Wales, Sydney, Australia (W.O.T.-M.); and Departments of Clinical Immunology and Microbiology and Neonatology, Jagiellonian University Medical College, Cracow, Poland (M.Z., D.K., R.L.)

Received July 27, 2001; accepted October 19, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

In this report we investigated the immunopharmacological role of selective and nonselective phosphodiesterase (PDE) inhibition in regulating the inhibitory-κB (IκB-α)/nuclear factor-κB (NF-κB) signaling transduction pathway. In fetal alveolar type II epithelial cells, PDE blockade at the level of the diverging cAMP/cGMP pathways differentially regulated the phosphorylation and degradation of IκB-α, the major cytosolic inhibitor of NF-κB. Whereas selective inhibition of PDEs 1, 3, and 4, by the action of 8-methoxymethyl-3-isobutyl-1-methylxanthine, amrinone, and rolipram, respectively, exhibited a tendency to augment the translocation of NF-κB, (p50, RelA (p65), RelB (p68), and c-Rel (p75), selective blockade of PDE 5, 6, and 9, by the action of 4-[[3',4'-[methylenedioxy]benzyl]amino]-6-methoxyquinazoline and zaprinast, attenuated lipopolysaccharide-endotoxin (LPS)-mediated NF-κB translocation. Pentoxifylline, a nonspecific PDE inhibitor, reversed the excitatory effect of LPS on NF-κB subunit nuclear localization, in a dose-dependent manner. Furthermore, analysis of NF-κB activation under the same conditions revealed a biphasic effect mediated by LPS. PDEs 1, 3, and 4 inhibition was associated with up-regulating NF-κB transcriptional activity. In contrast, blockading the activity of PDEs 5, 6, and 9 negatively attenuated LPS-mediated NF-κB activation, similar to the effect of 3,7-dihydro-3,7-dimethyl-1-(5-oxohexyl)-1H-purine-2,6-dione (pentoxifylline). These results indicate that selective and nonselective interference with the control of the dynamic equilibrium of cyclic nucleotides via PDE isoenzyme regulation represents an immunoregulatory mechanism that requires the differential, biphasic targeting of the IκB-α/NF-κB pathway.

Although the transcription factor nuclear factor-κB (NF-κB) has been originally recognized in regulating gene expression in B-cell lymphocytes (Sen and Baltimore, 1986), subsequent investigations have demonstrated that it is one member of a ubiquitously expressed family of Rel-related transcription factors that serve as critical regulators of many genes, including those of proinflammatory cytokines (Siebenlist et al., 1994; Baldwin, 1996, 2001; Haddad et al., 2001b). The translocation and activation of NF-κB in response to various stimuli are sequentially organized at the molecular level. In its inactive state, the heterodimeric NF-κB, which is mainly composed of two subunits, p50 (NF-κB1) and p65 (RelA), is present in the cytoplasm associated with its inhibitory protein, IκB (Siebenlist et al., 1994; Baldwin, 1996). Upon stimulation, such as with cytokines and lipopolysaccharide-endotoxin (LPS), derived from the cell wall of Gram-negative bacteria, IκB-α, the major cytosolic inhibitor of NF-κB (Baldwin, 1996; Haddad et al., 2001b), undergoes phosphorylation on serine/threonine residues, ubiquitina-

ABBREVIATIONS: NF-κB, nuclear factor-κB; IκB, inhibitory-κB; LPS, lipopolysaccharide-endotoxin; NLS, nuclear localization sequence; PDE, phosphodiesterase; PKAc, catalytic subunit of protein kinase A; PDEI, phosphodiesterase inhibitor; MBMQ, 4-[[3',4'-[methylenedioxy]benzyl]amino]-6-methoxyquinazoline; 8-methoxymethyl-IBMX, 8-methoxymethyl-3-isobutyl-1-methylxanthine; IκK, IκB kinase; NFAT, nuclear factor of activated T cells.
tion, and subsequent proteolytic degradation, thereby un-
masking the nuclear localization signal (NLS) on p65 and
allowing nuclear translocation of the complex. This sequen-
tial propagation of signaling ultimately results in the release
of NF-κB subunits from IκB-α inhibitor, allowing transloca-
tion and promotion of gene transcription.

Phosphodiesterases (PDEs), a family of isoenzymes in-
volved in regulating the dynamic equilibrium of cyclic nu-
cleotides (cAMP/cGMP) (Pagani et al., 1992; Bolger et al., 1993;
Tsuboi et al., 1996; Ekholm et al., 1997; Ferry and Higgs,
1998; Essayan, 1999), have been recently implicated in regu-
ulating the IκB-α/NF-κB signaling pathway and other tran-
scription factors (Montmény, 1997). For instance, it was re-
ported that the transcriptional activity of NF-κB was regu-
lated by the IκB-α-associated catalytic subunit of protein
kinase A (PKAc) in a cAMP-independent mechanism (Zhong et al.,
1997). Furthermore, Wang et al. (1997) observed that c-Rel (p75), one of the members of the Rel family, formed a
selective target of pentoxifylline, a nonspecific PDE inhibitor,
in mediating the inhibition of T-lymphocyte activation. In
addition, pentoxifylline blocked reactive oxygen species-
mediated regulation of NF-κB independently of the phospho-
diesterase inhibitory activity (Lee et al., 1997). Relatively
recently, a correlation of note was observed between the
suppression of proinflammatory cytokine production and the
inhibition of NF-κB/NFAT signaling pathway mediated by
PDE type 4 isozymes (Navarro et al., 1998). Moreover, To-
mita et al. (1999) reported a novel role for dexamethasone
and theophylline, another nonspecific inhibitor of PDE, in
regulating NF-κB translocation/activation and cytokine ex-
pression. In addition, selective inhibition of PDE 3 atten-
uated the activation of NF-κB and subsequently blocked the
downstream cytokine signaling pathway (Matsumori et al.,
2000). However, the immunopharmacological role that selec-
tive and nonselective inhibition of PDE isoenzymes plays in
regulating the nuclear translocation and activation of NF-κB
is not well characterized and thereby remains to be identified
in the alveolar epithelium.

Therefore, the aim of the present investigation targeted a
dual analytical assessment of PDE inhibition. First, a deter-
mination was made of the interference of selective phospho-
diesterase isoenzymes in regulating the phosphorylation,
degradation, and accumulation of IκB-α within the cytosolic
compartment; and second, an evaluation was made of the
role that those isoenzymes play in determining the nuclear
translocation of selective NF-κB Rel subunits, thereby interfer-
ing with the activation of NF-κB, a transcriptional activity
involved in regulating genes encoding cytokines and the pro-
gression and evolution of inflammation.

Materials and Methods

All experimental procedures involving the use of live animals were
reviewed and approved under the Animals Act legislation, 1986
(United Kingdom). Unless indicated otherwise, chemicals/ reagents of
the highest analytical grade were obtained from Sigma-Aldrich
(Dorset, England) and Calbiochem (Nottingham, UK).

Primary Cultures of Alveolar Epithelia. Fetal alveolar type II
epithelial cells were isolated from lungs of rat fetuses on gestation
day 19, essentially as described elsewhere (Haddad and Land,
2000a,b; Haddad et al., 2000, 2001a,b,c).

LPS Exposure and Assessment of the Effect of Phosphodi-
esterase Inhibitors (PDEIs) on NF-κB Translocation/Activation.

The signaling mechanism mediating the effect of selective and nonse-
lective inhibition of PDEs in regulating NF-κB translocation and ac-
activation in the alveolar epithelium is not well characterized. Accordingly,
we designed a series of experiments to span NF-κB translocation/acti-
vation in response to LPS and PDEI treatment. Cells were challenged
with LPS (10 μg/ml) independently or in the presence of various PDEIs.
Subcellular cytosolic/nuclear extracts (24 h) were subsequently
prepared, followed by Western analysis and electrophoretic mobility gel
shift assay, essentially as described previously (Haddad and Land,
2000a; Haddad et al., 2000). Briefly, cytosolic/nuclear extracts were
prepared from monolayer filters washed twice in 5 ml of ice-cold, pre-
equilibrated phosphate-buffered saline and cells (1–2 × 10^6) were col-
centrifuged at 420g for 5 min at 4°C. Nuclei were released by re-
-suspending the pellet in 250 μl of buffer A containing 10 mM Tris-
HCl, pH 7.8, 10 mM KCl, 2.5 mM NaH_2PO_4, 1.5 mM MgCl_2, 1 mM
Na_2VO_4, 0.5 mM dithiothreitol, 0.4 mM 4-2-aminoethylbenzenesulfo-
nyl fluoride-HCl, and 2 μg/ml each of leupeptin, pepstatin A, and
aprotinin. The suspension was left in ice for 10 min following by a 45-s
homogenization at a moderate speed. Nuclei were collected by cen-
trifuging the slurry at 4500g for 5 min at 4°C and resuspending in 100 μl
of buffer B [buffer A adjusted to 20 mM Tris-HCl, pH 7.8, 420 mM KCl,
20% (v/v) glycerol]. The supernatants thus obtained were termed cyto-
solic extracts. The nuclei were then lysed at 4°C for 30 min with gentle
agitation, the debris cleared by centrifugation at 10,000g for an addi-
tional 30 min at 4°C, and the supernatants, termed nuclear extracts,
were frozen in liquid nitrogen and stored at −70°C until used. In all
cases, protein contents were determined by the Bradford method by
using bovine serum albumin as a standard (Haddad and Land,
2000a).

Cytosolic and nuclear proteins (20–25 μg) were resolved over
sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5%)
gels at room temperature, blotted onto nitrocellulose membrane, and
nonspecific binding sites were subsequently blocked. Mouse mono-
clonal IgG anti-IκB-α (H-4), IgG_2a anti-p1IκB-α (B-9), rabbit poly-
clonal IgG anti-p50 (NLS), anti-p52 (K-27), anti-p65 (RelA; A), anti-
p68 (RelB; C-19), and anti-p75 (c-Rel; N) (Santa Cruz Biotechnology,
Wiltshire, UK) antibodies were used for primary detection. Anti-
rabbit IgG-biotinylated antibody (Amersham plc, Little Chalfont,
Buckinghamshire, UK) was used for secondary detection followed by
the addition of streptavidin-horseradish peroxidase conjugate and
visualized on film by chemiluminescence. β-Actin standard was used
as an internal reference for semiquantitative loading in parallel
lanes for each variable. Western blots were scanned by NIH Magi-
ScanII and subsequently quantitated by UN-Scan-IT automated digi-
tizing system (version 5.1; 32-bit), and the ratio of the density of the
band to that of β-actin was subsequently performed.

Custom deoxyoligonucleotide probe sequences were purchased
from Genosys (Cambridge, UK): NF-κB, 5′-AGTGGAGGGGACTT-
TCCCAGGCG-3′ (binding sequence underlined). Gel-purified double-
stranded DNA was end-labeled with [γ-32P]ATP (PerkinElmer Life
Sciences Ltd., Cambridge, UK). Identical amounts of radioactive
probe (1–2 × 10^4 counts/min) were added to binding reactions con-
taining 1 to 5 μg of fetal alveolar type II nuclear extracts in a final
volume of 40 μl in DNA binding buffer (20 mM HEPEs, pH 7.9; 1 mM
MgCl_2; 4% Ficoll)). Reaction mixtures were incubated for 30 min at
25°C before separating on nondenaturing 4% polyacrylamide gels at
room temperature and subjected to electrophoresis with 1:10
Tris-borate-EDTA buffer. A nonspecific competitive polyoxydi-
osinic-deoxycytidylic acid (poly(dI-dC)) (Amersham plc) was added
to reaction mixtures after addition of labeled probe. Gels were
transferred to ion-exchange chromatography paper, vacuum dried, and
then electronically visualized on a Packard Instant PhosphorImager
(Packard BioScience Ltd., Berkshire, UK). Specific quantitation of the
corresponding DNA gel shift bands was performed with phos-
phorimaging (Haddad and Land, 2000a,b; Haddad et al., 2000,
2001a,b).

Statistical Analysis and Data Presentation. Data are the means
and the error bars the S.E.M. of at least three independent
cell cultures. Statistical evaluation was performed by one-way anal-

**Results**

**Involvement of Selective Phosphodiesterase Isoenzymes in Regulating IκB-α Signaling.** In a previous study, we have reported a detailed account of IκB-α signaling in response to exogenous LPS, derived from *Escherichia coli* (Haddad et al., 2001b). As shown in Fig. 1A, LPS (10 μg/ml; 24 h) mediated the degradation of IκB-α within the cytosolic compartment. Coincubation of cells with LPS and PDEIs differentially regulated LPS-mediated degradation of IκB-α (Fig. 1). The effect of 8-methoxymethyl-IBMX (PDEI1) on IκB-α abundance is shown in Fig. 1A. 8-Methoxymethyl-IBMX had no effect on LPS-dependent degradation of IκB-α at 1 and 10 μM, but reversed the effect of LPS at 100 μM, thereby allowing its cytosolic accumulation (Fig. 1A). The effect of 5-amino-(3,4-dihydro-5-(2-propoxyphenyl)-7H-1,2,3-triazolo[4,5-d]pyrimidine-7-one (zaprinast) (PDEI5/6/9) in regulating LPS-mediated phosphorylation/degradation was shown in Fig. 1B. MBMQ did not affect or reverse the effect of LPS at all doses (Fig. 1B). The role of pentoxifylline (nonselective PDEI) on IκB-α abundance is displayed in Fig. 1B. Pentoxifylline partially restored IκB-α abundance, especially at 1 and 10 μM, but not at 100 μM (Fig. 1B). Histogram analysis of the effect of selective PDE inhibition on IκB-α abundance is shown in Fig. 2A. Analysis of the half-maximal (50%) excitatory concentration (EC₅₀) of selective and nonselective phosphodiesterase inhibitors on IκB-α abundance/degradation regulated by LPS (10 μg/ml; 24 h) is given in Table 1.

The role of PDE inhibition in regulating the phosphorylation of IκB-α is shown in Fig. 1A. In comparison with either PDEI1 and 3, rolipram partially reversed the effect of LPS on IκB-α abundance, allowing its accumulation but to a lesser extent than the effect of amrinone. In contrast to PDEI1, amrinone reversed the degradation effect of LPS at all doses used in this study, thereby allowing the accumulation of IκB-α in the cytosol (Fig. 1A). In comparison with either PDEI1 and 3, 4-[3-(cyclopentolox)-4-methoxyphenyl]-2-pyrrrolidinone (rolipram) (PDEI4) partially reversed the effect of LPS on IκB-α abundance, allowing its accumulation but to a lesser extent than the effect of amrinone (Fig. 1A). The effect of MBMQ (PDEI5) on IκB-α abundance is displayed in Fig. 1B. MBMQ did not affect or reverse the effect of LPS at all doses (Fig. 1B). 8-Methoxymethyl-IBMX up-regulated LPS-mediated phosphorylation of IκB-α at 1 μM, but not at higher concentrations. Amrinone up-regulated LPS-dependent phosphorylation of IκB-α at the highest dose used (100 μM), but not at lower doses. Rolipram suppressed LPS-dependent phosphorylation of IκB-α in a dose-independent manner.}

**Fig. 1.** Role of selective phosphodiesterase inhibition in regulating IκB-α phosphorylation/degradation. A, effect of 8-methoxymethyl-IBMX (PDEI1), amrinone (PDEI3), and rolipram (PDEI4) on LPS (10 μg/ml; 24 h)-mediated degradation of IκB-α within the cytosolic compartment. 8-Methoxymethyl-IBMX has no effect on LPS-dependent degradation of IκB-α at 1 and 10 μM, but reversed the effect of LPS at 100 μM, thereby allowing its cytosolic accumulation. Amrinone reversed the degradation effect of LPS at all doses, thereby allowing the accumulation but to a lesser extent than the effect of amrinone. B, effect of MBMQ (PDEI5), zaprinast (PDEI5/6/9), and pentoxifylline (nonspecific PDEI) on IκB-α abundance. MBMQ did not affect the effect of LPS at all doses. Zaprinast partially reduced LPS-induced IκB-α degradation at 100 μM. Pentoxifylline partially restored IκB-α abundance, especially at 1 and 10 μM, but not at 100 μM. C, effect of 8-methoxymethyl-IBMX, amrinone, and rolipram on LPS-mediated IκB-α phosphorylation. 8-Methoxymethyl-IBMX up-regulated LPS-mediated phosphorylation of IκB-α at 1 μM, but not at higher concentrations. Amrinone up-regulated LPS-dependent phosphorylation of IκB-α at the highest dose used (100 μM), but not at lower doses. Rolipram suppressed LPS-dependent phosphorylation of IκB-α in a dose-independent manner. D, effect of MBMQ, zaprinast, and pentoxifylline LPS-mediated IκB-α phosphorylation. MBMQ up-regulated IκB-α phosphorylation at 100 μM, with similar effects to LPS at the lower range of the dose-response curve. Zaprinast up-regulated LPS-dependent phosphorylation of IκB-α at all doses. Pentoxifylline (PTX) up-regulated LPS-mediated phosphorylation of IκB-α at the lowest dose (1 μM), but suppressed this effect at higher doses. The housekeeping gene protein product β-actin was used as an internal reference for semiquantitative loading per lane. n = 3 to 4, representing the number of independent experiments.
tion of IκB-α, the major cytosolic inhibitor of NF-κB (Baldwin, 2001; Haddad et al., 2001b), is not well characterized in the alveolar epithelium. Subsequently, after having determined IκB-α abundance within the cytosolic compartment, we aimed at investigating the role that these isoenzymes play in regulating IκB-α phosphorylation. As shown in Fig. 1C, 8-methoxymethyl-IBMX up-regulated LPS-mediated phosphorylation of IκB-α at 1 μM, but not at higher concentrations, whose effects were similar to that of LPS alone (Fig. 1C). The effect of amrinone on IκB-α phosphorylation is displayed in Fig. 1C. Amrinone up-regulated LPS-dependent phosphorylation of IκB-α at the highest dose used (100 μM), but not at lower doses (Fig. 1C). Rolipram suppressed LPS-dependent phosphorylation of IκB-α in a dose-independent manner (Fig. 1C). The effect of MBMQ on IκB-α phosphorylation is shown in Fig. 1D. MBMQ up-regulated IκB-α phosphorylation at 100 μM, with similar effects to LPS at the lower range of the dose-response curve (Fig. 1D). The role of zaprinast in regulating LPS-mediated IκB-α phosphorylation is displayed in Fig. 1D. Zaprinast up-regulated LPS-dependent phosphorylation of IκB-α at all doses (Fig. 1D). The effect of pentoxifylline on IκB-α phosphorylation is shown in Fig. 1D. Pentoxifylline up-regulated LPS-mediated phosphorylation of IκB-α at the lowest dose (1 μM), but suppressed this effect at higher doses (Fig. 1D). Histogram analysis of the effect of selective PDE inhibition on IκB-α phosphorylation is shown in Fig. 2B. Analysis of the half-maximal (50%) excitatory and inhibitory concentrations (EC50/IC50) of selective and nonselective phosphodiesterase inhibitors on IκB-α phosphorylation regulated by LPS (10 μg/ml; 24 h) is given in Table 1.

Role of Phosphodiesterase Isoenzyme Inhibition in Regulating Nuclear Translocation of Selective NF-κB Rel Subunits. Although LPS up-regulated the nuclear translocation of NF-κB1 (p50), RelA (p65), RelB (p68), and c-Rel (p75), it had no apparent effect on NF-κB2 (p52). 8-Methoxymethyl-IBMX had no inhibitory effect on LPS-mediated translocation of p50, p65, p68, and p75, as shown in Fig. 3A. Similar to the effect of 8-methoxymethyl-IBMX, amrinone did not suppress LPS-mediated NF-κB subunit translocation (Fig. 3B). As shown in Fig. 3C, rolipram did not inhibit the translocation of NF-κB subunits. The housekeeping gene protein product β-actin was used as an internal reference for semiquantitative loading per lane (Fig. 3). The effect of MBMQ on NF-κB subunit translocation is displayed in Fig. 4A, where there was an inhibitory effect at doses ≥1 μM (p50), ≥10 μM (p65), ≥1 μM (p68), and ≥10 μM (p75). As shown in Fig. 4B, zaprinast blocked LPS-mediated NF-κB translocation of p50 (≥10 μM), p65 (≥1 μM), p68 (≥1 μM), and p75 (≥10 μM). Pentoxifylline reduced the nuclear localization of p50 (≥1 μM), p65 (≥1 μM), p68 (≥1 μM), and p75 (≥1 μM), as shown in Fig. 4C. The housekeeping gene protein product β-actin was used as an internal reference for semiquantitative loading per lane (Fig. 4). Analysis of the half-maximal (50%) excitatory and inhibitory concentrations (EC50/IC50) of selective and nonselective phosphodiesterase inhibitors on NF-κB subunit nuclear abundance regulated by LPS (10 μg/ml; 24 h) is given in Table 2.

Effect of LPS on NF-κB DNA-Binding Activity: Time-Response Analysis. As shown in Fig. 5A, incubation of epithelial cells with LPS (10 μg/ml) induced, in a time-dependent manner, NF-κB activation. The nuclear activity of NF-κB in response to LPS emerged significantly as early as

---

**TABLE 1**

Analysis of the EC50/IC50 of selective and nonselective phosphodiesterase inhibitors on IκB-α abundance/degradation and phosphorylation regulated by LPS (10 μg/ml; 24 h)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IκB-α Abundance</th>
<th>IκB-α Phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (μM)</td>
<td>IC50 (μM)</td>
</tr>
<tr>
<td>8-Methoxymethyl-IBMX</td>
<td>27.31 ± 1.25</td>
<td>2.01 ± 0.18</td>
</tr>
<tr>
<td>Amrinone</td>
<td>49.10 ± 2.58</td>
<td>46.44 ± 3.25</td>
</tr>
<tr>
<td>Rolipram</td>
<td>&gt;100</td>
<td>93.15 ± 5.07</td>
</tr>
<tr>
<td>MBMQ</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Zaprinast</td>
<td>&gt;100</td>
<td>76.01 ± 3.62</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td>&gt;100</td>
<td></td>
</tr>
</tbody>
</table>

---

**Fig. 2.** Analysis of the effect of selective phosphodiesterase inhibition on LPS-mediated IκB-α phosphorylation/degradation within the cytosolic compartment. A, histogram analysis of PDE inhibition on IκB-α abundance. B, histogram analysis of PDE inhibition on IκB-α phosphorylation. *P < 0.05, **P < 0.01, ***P < 0.001, as compared with control (LPS alone); †P < 0.05, lowered compared with control. n = 3 to 4, representing the number of independent experiments.
2 h postaddition to monolayers, and continued to increase in an exponential manner to maximize at 16- to 24-h time point (Fig. 5A). LPS-mediated activity of NF-κB thereafter subsided beyond the 24-h time point, although it remained significantly different from control (no LPS) until 72 h, when it became insignificant at 96 h (Fig. 5A). Histogram analysis of the corresponding gel-shifted bands is given in Fig. 5B.

**Effect of Phosphodiesterase Isoenzyme Inhibition on Nuclear Activation of NF-κB.** In association with the differential regulation of selective PDE inhibition on NF-κB subunit translocation, PDEI revealed a novel role in regulating LPS-dependent NF-κB activation by interfering with the binding to specific κB moieties. As shown in Fig. 6A, 8-methoxymethyl-IBMX augmented LPS-induced NF-κB activation at 1 and 10 μM, with no apparent effect at 100 μM, the effect of which was similar to LPS alone. Histogram analysis of the corresponding gel-shifted bands with 8-methoxymethyl-IBMX is given in Fig. 8A. Although amrinone at doses 1 and 10 μM behaved in a similar manner to LPS, it up-regulated the effect of LPS at 100 μM (Fig. 6B). Histogram analysis of the corresponding gel-shifted bands with amrinone is given in Fig. 8B. Rolipram up-regulated LPS-mediated activation of NF-κB in a dose-dependent manner (Fig. 6C). Histogram analysis of the corresponding gel-shifted bands with rolipram is given in Fig. 8C. The effect of MBMQ on NF-κB activation is displayed in Fig. 7A, where its inhibitory effects are evident at doses ≥10 μM. Histogram analysis of the corresponding gel-shifted bands with MBMQ is
TABLE 2

Analysis of the EC_{50}/IC_{50} of selective and nonselective phosphodiesterase inhibitors on NF-κB subunit translocation and activation regulated by LPS (10 μg/ml; 24 h)

<table>
<thead>
<tr>
<th>Phosphodiesterase Inhibitor</th>
<th>NF-κB Subunit Nuclear Abundance</th>
<th>NF-κB Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Methoxymethyl-IBMX</td>
<td>p50 &gt;100, p52 &gt;100, p65 &gt;100, p68 &gt;100, p75 &gt;100</td>
<td>1.25±0.17</td>
</tr>
<tr>
<td>Amrinone</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Rolipram</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>IC_{50} (μM)</td>
<td>MBMQ</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Zaprinast</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

![LPS (10 μg/ml)](A)

![NF-κB Subunit Nuclear Abundance](B)

**Discussion**

To accommodate an ever-changing microenvironment, cells adjust the pattern of gene expression by adaptive regulation of a host of transcription factors, which bind their respective cognate sites in the regulatory elements of targeted genes (Makarov, 2000; Baldwin, 2001; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). NF-κB comprises the Rel family of inducible transcription factors that are key mediators in regulating the progression of the inflammatory process (Yamamoto and Gaynor, 2001). Therefore, activation/regulation of the NF-κB/Rel transcription family, via nuclear translocation of cytoplasmic entities and complexes, plays a central role in the evolution of inflammation through regulation of genes essentially involved in encoding proinflammatory cytokines and other inflammatory mediators (Baldwin, 1996, 2001; Tak and Firestein, 2001). The NF-κB/Rel family includes five members: NF-κB_{1} (p50/p105 {p50 precursor}), NF-κB_{2} [p52/p100 (p52 precursor)], RelA (p65), RelB (p68) and c-Rel (p75) (Tak and Firestein, 2001). Despite the ability of most Rel members (with the exception of p68) to homodimerize, as well as form heterodimers, with each other, the most prevalent activated form of NF-κB is the heterodimer p50-p65, which possesses the transactivity domains necessary for gene regulation (Baldwin, 1996; Makarov, 2000; Baldwin, 2001). The NF-κB members contain a Rel homology domain, which is responsible for dimer formation, nuclear translocation, sequence-specific consensus DNA recognition, and interaction with IκB, the cytosolic inhibitors of NF-κB (Baldwin 2001; Tak and Firestein, 2001). In unstimulated cells, NF-κB resides in the cytoplasm as an inactive NF-κB/IκB complex, a mechanism that hinders the recognition of the NLS by the nuclear import machinery, thereby retaining the NF-κB complex within the cytosol (Baldwin, 1996, 2001).
Signals emanating from membrane receptors, such as those for interleukin-1 and tumor necrosis factor-α, activate members of the mitogen-activated protein kinase kinase kinase-related family, including NF-κB inducing kinase and mitogen-activated protein kinase kinase kinase kinases, both of which are involved in the activation of IκB kinases, IKK₁ and IKK₂, components of the IKK signalosome (Mercurio and Manning 1999; Zandi et al., 1997; Makarov, 2000). These kinases phosphorylate members of the IκB family, including IκB-α, the major cytosolic inhibitor of NF-κB (Baldwin, 2001; Haddad et al., 2001b), at specific serines within their amino termini, thereby leading to site-specific ubiquitination and degradation by the proteasome. This sequential trajectory culminating in the inducible degradation of IκB, which occurs through consecutive steps of phosphorylation and ubiquitination, allows freeing of the NF-κB complex, which translates to the nucleus to bind specific κB moieties and initiate gene transcription (Makarov, 2000; Baldwin, 2001).
munoregulatory potential aimed at targeting the NF-κB signaling pathway, therefore, remains of particular interest. Because NF-κB regulates host inflammatory and immune responses by increasing the expression of specific genes and enzymes whose products contribute to the pathogenesis of the inflammatory process (Makarov, 2000; Yamamoto and Gaynor, 2001), selective modulation of this transcription factor bears a typical therapeutic approach for the control and regulation of inflammatory-associated diseases. Unfortunately, due to convergence of more than one mechanism upon the onset and progression of the inflammatory process, which regulates NF-κB signaling, it has been extremely difficult to solely target this pathway without affecting other cellular functions. Within this context, the anti-inflammatory immunoregulatory role that phosphodiesterase inhibition plays in regulating this pathway is not well understood or characterized. Therefore, the major aim of the present investigation was to shed light on the role that selective phosphodiesterases play in regulating IkB-α/NF-κB signaling, thereby showing for the first time that PDE inhibition differentially and dually regulates this transduction pathway, bearing consequences for the therapeutic treatment of inflammatory disease involving NF-κB and regulated by the respiratory epithelium (Makarov, 2000; Perkins, 2000; Haddad et al., 2001c,d; Yamamoto and Gaynor, 2001).

Although the inflammatory signals mediated by LPS are recognized in other systems and cell models, the role of LPS-mediated signaling and its modulation by PDE isoenzymes in the alveolar epithelium is not well characterized. Administration of LPS differentially regulated NF-κB nuclear subunit translocation. Despite the observation that LPS has no influence on the unit composition of p52, its stimulatory effect on p50, p65, p68, and p75 is evidently prominent. Besides, the promoters of genes encoding cytokines contain multiple cis-acting motifs, including those that bind specific subunits (i.e., p50-p65) of such transcription factors as NF-κB (Makarov, 2000; Baldwin, 2001; Tak and Firestein, 2001). Furthermore, the release of free NF-κB upon extracellular stimulation due to IkB phosphorylation and degradation, leads to DNA binding to initiate transcription of related genes, including immunoreceptors, cytokines, and, interestingly, its own inhibitor, IkB (Mercurio et al., 1997; Baldwin, 2001; Haddad et al., 2001b). Two unique features of the NF-κB/IkB complex system are deduced from its feedback regulation. The transcriptional activation of NF-κB triggers the synthesis of IkB, and NF-κB-activated transcription is maintained by continuous degradation of IkB, which is sustained by an extracellular stimulus (Perkins, 2000; Baldwin, 2001; Haddad et al., 2001b). Thus, the expression of IkB parallels both NF-κB activity and the duration of the activating extracellular stimulation, suggesting that this temporal parallelism between IkB accumulation/degradation and an effective external stimulation is a mechanism allowing dual, biphasic, regulation of NF-κB within the alveolar space.

The novel interference of specific PDE isoenzyme inhibition in regulating IkB-α phosphorylation/degradation, translocation of selective NF-κB subunits, and the activation of this transcription factor remains of particular interest. Phosphodiesterase regulation of IkB-α/NF-κB signaling pathway, however, is not well understood and remains to be elucidated. Coward et al. (1998), for example, reported that nonselective PDE inhibition possesses an anti-inflammatory activity via suppression of NF-κB, bearing consequences for the treatment of asthmatic patients. Furthermore, pentoxifylline, a nonspecific methylxanthine-derived PDE inhibitor, selectively targeted the c-rel (p75) NF-κB subunit, with variable and inconsistent effect on RelA (p65), in the treatment of T-cell-dependent diseases (Wang et al., 1997), an observation correlating with another investigation (Lee et al., 1997). Alternatively, it was demonstrated that the PKAc, but not the protein kinase A regulatory subunit, binds IkB proteins and is associated with the NF-κB-IkB complex (Zhong et al., 1997). The authors concluded that PKAc interacts with IkB-α and IkB-β through sequences from the N terminus of the protein, and that this interaction inhibits the catalytic activity of PKAc. Of note, the observation that stimulation of cells with inducers of NF-κB activity, such as LPS, agents that do not elevate intracellular cAMP, led to degradation of IkB proteins and consequent activation of IkB-bound PKAc (Zhong et al., 1997). To the best of our knowledge, this is the

![Fig. 8. Analysis of the effect of selective phosphodiesterase inhibition on LPS-mediated activation of NF-κB within the nuclear compartment.](image-url)
Phosphodiesterase Regulation of IxB-α/NF-κB Signaling

first report that has given a detailed account of the role of selective and nonselective PDE interference in regulating IxB-α/NF-κB signaling. The differential regulation of IxB-α phosphorylation, in particular, implicated a PDE-sensitive upstream kinase. However, from the present data alone it cannot be concluded which of the upstream kinases are directly regulated by selective regulation of PDE isoenzymes. Nevertheless, because the IKK signalosome (Mercurio et al., 1997; Zandi et al., 1997; Makarov, 2000) is involved in regulating IxB-α phosphorylation in response to various stimuli, including LPS, it remains tempting to suggest that this complex is likely to form a target of the selective regulation by PDEs. Furthermore, whether this differential regulation of IxB-α phosphorylation/degradation is cAMP/cGMP-dependent cannot be confirmed based on the aforementioned observations alone; however, because PDE inhibitors are involved in regulating the dynamic equilibrium of these cyclic nucleotides, the possibility that LPS-mediated IxB-α phosphorylation is cAMP/cGMP-sensitive cannot be excluded, an observation correlating with the transcriptional activity of either nucleotide (Montminy, 1997; Zhong et al., 1997; Ma et al., 1999).

In association with targeting the IxB-α signaling pathway, we observed differential regulation of NF-κB translocation and activation. Despite the observation that selective inhibition of PDEs 1, 3, and 4 exhibited no inhibitory effect on LPS-mediated translocation of NF-κB subunits, blockading the activity of 5, 6, and 9 differentially attenuated, and to relatively variable degree, LPS-dependent translocation of these subunits. Because the latter isoenzymes are directly involved in cGMP signaling, it is possible that cAMP-mediated signaling tends to up-regulate NF-κB nuclear accumulation, whereas the former pathway mediates an inhibitory effect, thereby retarding the nuclear localization of selective subunits. This discrepancy between the modes of action of either pathway suggested that there is a line of demarcation highlighting the divergence of these signaling mechanisms, especially on the bifurcation of interest that substantially resides within and/or above the IxB/NF-κB complex. Substantially working with effectiveness as competitive as selective inhibition of PDEs 5, 6, and 9, pentoxifylline reduced LPS-mediated NF-κB translocation, suggesting the involvement of a common signaling pathway converging on NF-κB.

Analysis of this differential regulation of NF-κB subunit translocation revealed the involvement of a novel biphasic pathway mediating the interference in NF-κB activation. Although selective inhibition of PDEs 1, 3, and 4 had a mild tendency to up-regulate the activation of this transcription factor, ostensibly due to accumulation of intracellular cAMP, selective inhibition of PDEs 5, 6, and 9 (↑ cGMP), along with pentoxifylline, negatively regulated LPS-mediated NF-κB activation. This phenomenon is rather reinforced by the observation that the molecular mechanism of inhibition by cAMP was found to correlate with NF-κB, in particular, the RelA component of the complex (Neuman et al., 1995). Based on their chemical structures, PDE inhibitors (1, 3, and 4 versus 5, 6, and 9) tend to elevate cAMP/cGMP, respectively; however, it may also be true that in some situations this may not be the only mode of action. In preference to this understanding, some of the effects of PDE inhibition, for example, may be implicated in regulating the process of cellular differentiation, an effect not mimicked by cAMP (Yang et al., 1995).

Furthermore, exogenous addition of TNF-α in the presence of rolipram, purported to elevate intracellular cAMP, restored NF-κB activation but not that of NFAT (Navarro et al., 1998). Of note, a possible relationship of pentoxifylline to Ca²⁺ mobilization has been declared because optimal c-Rel induction has been shown to require the dual signal of phorbol-12-myristate-13-acetate and ionomycin, where it was inferred that this nonselective PDE inhibitor would blockade c-Rel induction by attenuating a component of the calcium response (Yang et al., 1995). From the aforementioned mechanisms reported, it is not clear, however, whether PDE inhibition is involved in regulating selective NF-κB subunits in correlation with the suppression or augmentation of transcriptional activation. This investigation has created a basis for the hypothesis claiming that selective PDE blockade differentially regulate NF-κB translocation/activation, with detailed mechanics of action on certain subunits, where we have shown that not only c-Rel or RelA are targets for the mode of action of PDEs but also other components of the complex along with the machinery of IxB signaling that converge on regulating the NF-κB pathway. Despite the selective regulation of specific NF-κB subunit translocation/activation by PDE isoenzymes reported in this study, whether the mode of action is solely cAMP/cGMP-sensitive cannot be inferred. However, previous studies in our laboratory have demonstrated that cyclic nucleotides and their mimetics (forskolin, dibutyryl cAMP, and dibutyryl cGMP) have differentially regulated proinflammatory cytokine biosynthesis, a phenomenon shown to be correlated with the selective interference of NF-κB translocation and activation (J. J. Haddad, N. E. Saadé, B. Safieh-Garabedian, and S. C. Land, unpublished observations).

We herein report a novel immunopharmacological potential of selective and nonselective PDE inhibition in the process of regulating the IxB-α/NF-κB signaling transduction pathway. The results could be highlighted as follows: 1) PDE blockade at the level of the diverging cAMP/cGMP pathways differentially regulated the phosphorylation and degradation of IxB-α, the major cytosolic inhibitor of NF-κB; 2) inhibition of PDEs 1, 3, and 4 exhibited a tendency to augment the process of selective NF-κB subunit translocation, an effect associated with up-regulating transcriptional activity; and 3) blockading the activity of PDEs 5, 6, and 9 negatively attenuated LPS-mediated NF-κB translocation/activation. It is concluded that selective and nonselective interference with the control of the dynamic equilibrium of cyclic nucleotides via PDE isoenzyme regulation represents an immunopharmacological approach targeting the IxB-α/NF-κB complex and the downstream signaling pathway, thereby conferring a novel mode of action for targeting a transcriptional activity notoriously implicated in regulating the progression of the inflammatory process and its contraction in disease.

References


Address correspondence to: Dr. John J. Haddad, Neuroscience Research Laboratory, Department of Anesthesia and Perioperative Care, University of California Medical Center, San Francisco, CA 94143. E-mail: jhaddad@itsa.ucsf.edu