Inhibitors of Protein Kinase C (PKC) Prevent Activated Transcription

ROLE OF EVENTS DOWNSTREAM OF NF-κB DNA BINDING

Matthew C. Catley, Lisa M. Cambridge, Yasuyuki Nasuhara, Kazuhiro Ito, Joanna E. Chivers, Andrew Beaton, Neil S. Holden, Martin W. Bergmann, Peter J. Barnes, and Robert Newton

From the Department of Thoracic Medicine, National Heart and Lung Institute, Imperial College, Dovehouse Street, London SW3 6LY, United Kingdom, and the BioMedical Research Institute, Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

In pulmonary A549 cells, the protein kinase C (PKC) inhibitor, Ro 31-8220, and the phosphotidylcholine-specific phospholipase C inhibitor, D609, prevent NF-κB-dependent transcription, yet NF-κB DNA binding is unaffected (Bergmann, M., Hart, L., Lindsay, M., Barnes, P. J., and Newton, R. (1998) J. Biol. Chem. 273, 6607–6610). We now show that this effect also occurs in BEAS-2B bronchial epithelial cells as well as with other PKC inhibitors (Gö 6976, GF10920X, and calphostin C) in A549 cells. Similarly, phorbol ester, a diacylglycerol mimetic, activates NF-κB-dependent transcription and potentiates tumor necrosis factor α (TNFα)-induced NF-κB-dependent transcription, yet unlike TNFα, poorly activates IκB kinase (IKK) activity, IκBα degradation, or NF-κB DNA binding in both A549 and BEAS-2B cells. As phorbol ester-induced NF-κB-dependent transcription was relatively insensitive to the proteasome inhibitor, MG-132, PKC may affect NF-κB-dependent transcription via mechanisms other than the core IKK-IκB pathway. This is supported by Gal4 one hybrid activation of p65/RelA transactivation, which was potentiated by TNFα and phorbol ester and was inhibited by Ro 31-8220 and D609. Additionally, a number of PKC isoforms, particularly the novel isoform PKCe, induced p65/RelA transactivation. Phosphorylation of p65/RelA and cAMP-responsive element-binding protein (CREB)-binding protein (CBP) was increased by TNFα treatment and, in the case of CBP, was prevented by Ro 31-8220 or D609. However, p65/RelA-CBP interactions were unaffected by either compound. As this effect was not limited to NF-κB, but was a more general feature of inducible gene transcription, we suggest PKC isoforms may provide a point of intervention in diseases such as inflammation, or cancer, where activated gene expression is prominent.

In inflammation, the binding of proinflammatory cytokines, such as TNFα or IL-1β, to their respective receptors results in the rapid activation of the transcription factor nuclear factor-κB (NF-κB). This process involves various signaling molecules and leads to the activation of the IκB kinase (IKK) complex, which consists of two closely related kinases, IKKα and IKKβ, and the structural protein IKKy (1). Phosphorylation and activation of this complex, particularly IKKα, leads to the phosphorylation, ubiquitination and subsequent degradation of the NF-κB inhibitor protein, IκBα. Loss of IκBα releases NF-κB, typically heterodimers of p50 (NFκxB1) and p65 (RelA), which can then translocate to the nucleus and activate transcription. However, there is considerable data to suggest that this process is not sufficient for transcriptional action (2). For example, we have previously reported that the protein kinase C inhibitor, Ro 31-8220, and the phosphotidylcholine-specific phospholipase C (PC-PLC) inhibitor, D609, had no effect on the induction of NF-κB DNA binding by TNFα or IL-1β yet totally ablated NF-κB-dependent transcription (3). A number of similar observations have also been made using inhibitors of the p38 mitogen-activated protein kinase (4–6), phosphatidylinositol 3-kinase (7), protein kinase A (8), tyrosine kinases (9), and others (see Ref. 2). Taken together these data suggest that a number of additional signal transduction pathways are required, which impact on events post-DNA binding, but are nevertheless necessary for NF-κB-dependent transcription.

Candidate protein targets for these additional activation pathways include components of the transcriptional apparatus (5), co-activator molecules (8), as well as NF-κB itself (2). In this context, p65 is widely reported to exist as a phosphopro-
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tein, and a number of studies have documented its signal-
induced phosphorylation (10–13). Similarly, p50 may also be
phosphorylated (11). While the exact role (or roles) of NF-κB
phosphorylation is currently equivocal, an initial report that
phorbol ester-induced phosphorylation of p65, within the C-
terminal activation domain, correlated with increased transac-
tivation potential has set the prevailing theme (10). Likewise,
inhibitors of the p38 mitogen-activated protein kinase and the
extracellular regulated kinase pathway were able to reduce p65-
dependent transactivation (14), while the P3K/Akt (protein
kinase B) pathway was implicated in p65 phosphorylation and
p65-dependent transactivation (7, 15, 16). Analysis and map-
ping of phosphorylated residues in p65 has variably revealed
series 529 and 536, within the transactivation domain as
being phosphorylated following TNF-α treatment (17, 18). Phos-
phorylation of these residues, at least in some studies, is be-
lieved to occur via the IKKs and appears to enhance transcriptional
activation (17–19). This contrasts with reports that
implicate serine 276 in phosphorylation and transcriptional activation by protein kinase A (PKA) via a mechanism that
involves enhanced association with the transcriptional co-acti-
vator CREB-binding protein (CBP) (8, 20).

In the present manuscript, we have extended our previous
observations by further exploring the mechanism of inhibition of
NF-κB-dependent transcription by the PKC inhibitor, Ro
31-8220, and the PC-PLC inhibitor, D609 (3). We provide evi-
dence that implicates serine 276 in phosphorylation and trans-
criptional activation by protein kinase A (PKA) via a mechanism that
involves enhanced association with the transcriptional co-acti-
vator CREB-binding protein (CBP).
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Inhibitors of Protein Kinase C Prevent NF-κB-dependent Transcription but Not p65 Translocation—In our previous studies (3), we showed that the induction of NF-κB-dependent transcription by TNFa was completely blocked by D609, a reported PC-PLC inhibitor, and Ro 31-8220, a PKC inhibitor, yet the activation of NF-κB DNA binding or IκBα degradation was unaltered. To further explore this effect, we have also tested the known inhibitors of PKC, Go6976 and D609, as well as the structurally unrelated inhibitor, calphostin C. In each case, there was no effect on DNA binding as determined by EMSA, whereas robust inhibition of NF-κB-dependent transcription was observed (Fig. 1, A and B). This result was confirmed by Western blotting of nuclear extracts, which revealed no effect of either Ro 31-8220 or Go6976 on nuclear translocation of p65 (Supplemental Fig. S-1). Similarly, confocal microscopy revealed that neither the Go6976 nor D609 had any inhibitory effect on the nuclear translocation of p65 following TNFa stimulation of A549 cells (Supplemental Fig. S-2).

Ro 31-8220 and D609 Prevent NF-κB-dependent Transcription in BEAS-2B Cells—To examine the possibility that these effect were due to a peculiarity of the A549 cells system, the bronchial epithelial cell line, BEAS-2B, was also treated with TNFa in the presence or absence of both Ro 31-8220 or D609. In neither case was there any effect on the induction of NF-κB DNA binding activity (Fig. 1C), yet activation of the NF-κB-dependent reporter, 6xBtk, was substantially repressed (Fig. 1D) suggesting that our data are more generally applicable.

Phorbol Ester Activates NF-κB-dependent Transcription by a Mechanistically Distinct Process—To further substantiate the role of PKC, 6xBtk A549 cells were stimulated with the DAG mimetic, PMA, which is a potent activator of PKC. This markedly induced NF-κB-dependent transcription and was also pre-
vent by inhibitors of PKC (Fig. 1B). Analysis of the concentration-response characteristics for PMA revealed that a maximal response was achieved at $10^{-7}$ M (Fig. 2A), a value that is consistent with other PKC-mediated events. A combination of a maximally effective dose of TNFα (10 ng/ml) with a maximally effective dose of PMA ($10^{-7}$ M) resulted in a strongly enhanced luciferase response suggesting that these two treatments act via independent pathways to activate transcription (Fig. 2A). Similarly, PDBu ($10^{-7}$ M) also induced NF-κB-dependent transcription, whereas the inactive analog 4α-PMA ($10^{-7}$ M) was without effect suggesting that these effects are indeed specific (data not shown). Analysis of NF-κB DNA binding was consistent with our previous findings in showing a strong increase following TNFα or IL-1β treatment and only a very weak induction following PMA treatment (Fig. 2B) (28, 29). To our initial surprise the combination of TNFα plus PMA resulted in an apparent decrease in DNA binding activity with respect to TNFα alone. However, this result could be explained by the increased NF-κB-dependent transcription following combined TNFα + PMA treatment resulting in elevated expression of the NF-κB-dependent IκBα gene (30). This in turn would accelerate feedback inhibition of NF-κB DNA binding.

To examine the ability of TNFα and PMA to activate the primary IKK-IκBα pathway involved in NF-κB activation, the IKK complex was immunoprecipitated and kinase activity assessed. As is normally observed (31, 32), TNFα produced a profound activation of IKK activity that was maximal within 5 min of treatment (Fig. 2C). This activity was mirrored in the rapid phosphorylation (mobility shift) of IκBα observed at 5-min post-stimulation and the complete degradation within 15 min of treatment (Fig. 2D). In marked contrast, PMA failed to induce IKK kinase activity to any great extent and did not result in substantial loss of IκBα (Fig. 2, C and D).

Analysis of the 6xBtk BEAS-2B cells also revealed maximally effective concentrations of $10^{-7}$ M (EC50 = 1.43 $10^{-8}$ M) and 10 ng/ml (EC50 = 0.16 ng/ml) for PMA and TNFα, respectively (data not shown), and these when added together resulted in a slight (but nonsignificant) enhancement of reporter activity. Consistent with the A549 study, PMA produced a very minor increase in NF-κB DNA binding over basal levels and failed to induce both IκBα degradation or the appearance of S-32 phosphorylated IκBα (Fig. 2, F and G). Conversely, TNFα strongly activated both NF-κB DNA binding, phosphorylation of IκBα, and subsequent loss of IκBα (Fig. 2, F and G). Therefore the data from these two cell line models demonstrate that, whereas TNFα robustly activates the IKK-IκBα pathway to result in NF-κB nuclear translocation and transcriptional activation, this is not primarily the mechanism that accounts for transcriptional activation by phorbol esters.

To test the requirement for proteasome activity in the re-
response to PMA, A549 cells and 6xBtk A549 cells were treated with TNFα or PMA in the presence or absence of the proteasome inhibitor, MG-132 (33). This has previously shown to be effective in A549 cells against TNFα-induced activation of NF-κB and IL-8 expression (34). Western blot analysis of IκBα degradation revealed a marked inhibition by MG-132 (Fig. 3A). No effect of MG-132 was observed on IκBα following PMA treatment due to the lack of obvious degradation by PMA alone. Likewise, MG-132 was an effective inhibitor of TNFα-induced NF-κB-dependent transcription at concentrations (30 μM) that also inhibited IκBα degradation (Fig. 3B). Little or no effect was observed on PMA-induced NF-κB-dependent transcription, suggesting that such proteasome degradation does not play a role in this pathway.

Role of Protein Kinase A and Phosphatidylinositol 3-Kinase in NF-κB-Dependent Transcription—As both the PKA and PI3K pathways have been implicated in the activation of NF-κB-dependent transcription (see Ref. 2 and references therein), we tested the effect of H-89, a potent inhibitor of PKA, and wortmannin and LY294002, both potent inhibitors of PI3K. Whereas, the PKC inhibitor, Ro 31-8220, was effective at inhibiting either TNFα- or PMA-mediated NF-κB-dependent transcription between 10^-7 and 10^-6 M (EC50 values 1.53 x 10^-7 M and 4.63 x 10^-7 M, respectively), H-89 was at least 10-100-fold less effective with the respective EC50 values lying between 10^-4 and 10^-5 M (Fig. 3C). As the K value for H-89 on PKA is around 48 nM, and this drug is known to inhibit PKC isoforms at 31.7 μM (Calbiochem data sheet), it is likely that these data point again to the involvement of PKC rather than PKA. This conclusion is supported by the additional finding that forskolin, an activator of PKA, or inhibitors of phosphodiesterase 4, which raise intracellular cAMP and would be expected to activate PKA, either show little effect or down-regulate activated NF-κB-dependent transcription (data not shown). Likewise adenosine overexpression of protein kinase inhibitor, a potent endogenous inhibitor of PKA (35), also showed no effect on NF-κB-dependent transcription in A549 cells (data not shown).

Similarly, the PI3K inhibitor, wortmannin, which shows an IC50 value in the low nanomolar (5 nM) and is physiologically effective at 100 nM (Calbiochem data sheet), had little effect at up to 10^-3 M (Fig. 3C). Likewise, LY294008 (IC50 1.4 μM for PI3K, Calbiochem data sheet) also showed a similar lack of effect (data not shown) leading us to conclude that the PI3K pathway does not play a key role in the activation of NF-κB-dependent transcription in this experimental system.

p65 Transactivation Is Inhibited by D609 and Ro 31-8220 and Potentiated by PKC—The above data indicate that D609 and Ro 31-8220 act at a point downstream of NF-κB translocation and DNA binding. To examine the effect on transactivation by p65, we established a Gal4 one-hybrid assay for p65 transactivation in A549 cells (10, 36). In this system the transactivation potential of p65 was increased by both TNFα and PMA, and in each case both D609 and Ro 31-8220 substantially inhibited transactivation (Fig. 4, A and B). To determine whether PKC isoforms were actually able to increase p65-dependent transactivation, constitutively active versions of PKCa, -β1, -δ, -ε, -η, and -ζ were overexpressed (25). In all cases, apart from PKCe, some degree of potentiation was observed (Fig. 4C). This effect was most apparent for the novel isoforms with PKCe giving rise to the greatest overall levels of activation. These data therefore support the idea that one or more PKC isoforms can act on or downstream of p65 to potentiate NF-κB transcriptional potential.

Analysis of Potential Downstream Targets of Phosphorylation—To examine whether p65 itself was a downstream kinase target, cells were stimulated with TNFα in the presence of 32P-labeled inorganic phosphate, and p65 was immunoprecipitated (Fig. 5A). This experiment clearly shows that the phosphorylation status of p65 is increased rapidly (around 3-fold) following TNFα stimulation. This effect was maximal within 15 min, and by 1 h levels of p65 phosphorylation were falling (Fig. 5A and data not shown). However, as neither D609 nor Ro 31-8220 affected this increase (Fig. 5B), we conclude that the effect of these compounds must lie downstream of not only p65 translocation and DNA binding but also of p65 phosphorylation.

A potential candidate molecule that is known to interact with p65 and could be targeted by phosphorylation is CBP (37, 38). This co-activator protein is believed to act both as a physical link with the basal transcriptional machinery and to facilitate transcriptional activation by virtue of its associated HAT activity (38). Following 32P metabolic labeling of proteins, CBP was immunoprecipitated and subject to autoradiography. This revealed a striking increase in radioactivity associated with CBP indicating increased phosphorylation (Fig. 6A). Unlike the rapid increase observed for p65, CBP phosphorylation increased gradually over 1 h until reaching a maximum 1–2 h post-TNFα stimulation. In each case, prior treatment with D609 or Ro 31-8220 markedly repressed this increase indicating that the event was downstream of processes that were targeted by these compounds (Fig. 6B). Effect of D609 and Ro 31-8220 on CBP-associated HAT and Interaction with p65—To examine the possible mechanistic significance of CBP phosphorylation, the HAT activity associ-
Fig. 4. One-hybrid assay of p65 transactivation. A, A549 cells, transfected with the Gal4-p65 expression plasmid and the Gal4-luc reporter, were treated with TNFα (10 ng/ml) or PMA (0.1 μM) for 6 h before harvesting for luciferase assay and protein determination. Luciferase activity was normalized to protein concentration and expressed as fold induction. Data (n = 6) are shown as means ± S.E. B, cells were transfected as in A and then treated with D609 (50 μM), Ro 31-8220 (0.1 μM), before stimulation with PMA (0.1 μM) or TNFα (10 ng/ml), and harvested after 6 h for luciferase assay and protein determination. Luciferase activity was normalized to protein concentration and expressed as fold induction. Data (n = 4) are shown as means ± S.E. C, cells were transfected with Gal-p65 expression plasmid and Gal-luc as in A along with various quantities (μg) of PKC overexpression plasmids. Following transfection, cells were incubated for 24 h before harvesting for luciferase determination. Data (n = 4–8) are plotted as fold induction as means ± S.E.

Fig. 5. Analysis of p65 phosphorylation. A, A549 cells were treated with TNFα (10 ng/ml) for the times indicated in the presence of [32P]orthophosphate. Metabolically labeled p65 was immunoprecipitated and subjected to SDS-PAGE. Gels were dried down and autoradiography (AR) performed. Western blotting (WB) for p65 (RelA) was performed to confirm the presence of p65 in immunoprecipitates. Representative blots are shown, and data (n = 2) were normalized to p65 protein and are expressed as fold induction ± S.E. BP = immunoprecipitation performed in the presence of blocking peptide. Blots representative of three such experiments are shown. B, cells were pretreated with either Ro 31-8220 (10 μM) or D609 (50 μM) in the presence of [32P]orthophosphate and then treated with TNFα (10 ng/ml) for 15 min. As in A, representative blots are shown, and data (n = 3) were normalized to p65 protein, expressed as a percentage of TNFα treated and plotted and means ± S.E.

Fig. 6. Effect of Ro 31-8220 and D609 on CBP phosphorylation, associated HAT activity, and association with p65. A, A549 cells were treated with TNFα (10 ng/ml) for the times indicated in the presence of [32P]orthophosphate. Metabolically labeled CBP was immunoprecipitated and subject to SDS-PAGE. Gels were dried down and autoradiography (AR) performed to reveal metabolically labeled proteins. A blot representative of two such experiments is shown. B, cells were pretreated with either Ro 31-8220 (10 μM) or D609 (50 μM) in the presence of [32P]orthophosphate and then treated with TNFα (10 ng/ml) for 1 h prior to harvesting and immunoprecipitation of CBP. Autoradiography (AR) and Western blotting (WB) for CBP was performed. BP = immunoprecipitation performed in the presence of blocking peptide. Blots representative of three such experiments are shown. C, A549 cells were treated as in (B) as indicated and harvested after 1 h. CBP was immunoprecipitated, and the associated HAT activity was assayed by measuring the transfer of [3H]acetate from [3H]acetyl-CoA to total histone proteins in vitro. Data (n = 5) are plotted as means ± S.E. D, cells were treated with drugs and TNFα as in B. After 1 h cells were harvested, and p65 was immunoprecipitated prior to Western blot analysis of both CBP and p65. E, cell were treated and harvested as in D. CBP was immunoprecipitated and Western blot analysis for p65 was performed.
were harvested for luciferase assay, and the data (n) of NF-

and in each case this was almost totally prevented by the prior
treatment with these compounds, indicating PKC involvement. In each case this was consistent with more recent reports documenting repression of NF-κB by PKC (see Ref. 2). However, many of these reported events are readily distinguishable from the pathway presented here. Thus, contrary to models suggested by other experimental systems (7, 8, 15), the analysis of a number of kinase inhibitors in the present study excludes a role for PI3K and PKA, while a role for PKC is reinforced. The lack of a role for PKA is further strengthened by our finding that adenoviral overexpression of protein kinase inhibitor, an endogenous and potent inhibitor of PKA (39), or the PKA activator, forskolin, were without effect on NF-κB-dependent transcription (data not shown). This is consistent with more recent reports documenting repression of NF-κB transactivation by PKA (40). Similarly, the finding that the phorbol esters, PMA and PDBu, but not the inactive analog 4α-PMA, are potent activators of NF-κB-dependent transcription suggests the existence of a DAG-dependent effect and again raises the possibility of PKC involvement. In each case the induction of NF-κB-dependent transcription was prevented by a number of inhibitors of PKC further supporting the involvement of this kinase. However, many of the reports implicating PKC in signaling to NF-κB suggest a role upstream of NF-κB DNA binding (2, 41). For example the novel isoform, PKCδ, can mediate IκB degradation and NF-κB DNA binding (42), while the atypical isozymes, PKCγ, which may be activated by serine and PKCζ, have been shown to phosphorylate and activate IκBα. Clearly this process is mechanistically distinct from the one observed in the present study as the inhibitors of PKC or the PC-PLC inhibitor, D609, have no effect on induction of NF-κB nuclear transloca-

obtained in the converse experiment in which CBP was immuno-

precipitated and the presence of p65 was assayed (Fig. 6E). These data support the existence of a signal-induced interaction between p65 and CBP and further suggest that this inter-

action is not disrupted by the prior treatment with these inhibitors.

Role of Global Histone Acetylation—Analysis of [3H]Acetyl-

cate incorporation into total histone revealed an increase following 

TNFα treatment (Fig. 7A). As with CBP-associated HAT activity, this was reduced to near basal levels by prior treatment with D609 but not Ro 31-8220. Western blot analysis of acety-

lated histone H4 also confirmed this result (Fig. 7B).

To examine the link between histone acetylation and NF-κB-
dependent transcription, cells were treated with the histone deacetylase inhibitor, TSA. As expected this resulted in in-

creased levels of acetylated histone H4 (Fig. 7B). Analysis of 

NF-κB-dependent transcription revealed no effect of TSA on unstimulated transcription but resulted in a concentration-de-

pendent enhancement of TNFα-stimulated transcription (Fig.

7C). This confirms that increased overall HAT activity does promote NF-κB transcriptional responses. By contrast the in-

duction of NF-κB-dependent transcription by PMA was only weakly potentiated by TSA, possibly because this pathway is already activated by PMA.

Effect on Various Transcriptional Responses—To examine the possibility that these compounds were merely causing non-

specific effects on transcription, the effects of D609 and Ro 31-8220 were tested on both low level constitutive (TATA) and high level constitutive (SV40) transcription (Fig. 8A). In each case these reporters revealed no responsiveness to a range of treatments including TNFα (10 ng/ml), IL-1β (1 ng/ml), PMA (10⁻⁷ M), or dexamethasone (10⁻⁶ M), and this situation was unchanged by the presence or absence of D609 or Ro 31-8220. By contrast A549 cells containing CRE-, TRE-(AP-1), or GRE-

responsive reporters revealed responsiveness to stimulation and in each case this was almost totally prevented by the prior

addition of D609 or Ro 31-8220. As A549 cells respond rather poorly to cAMP elevating agents and the CRE reporter appears to act more like an AP-1/ATF-driven reporter (Fig. 8A and data not shown), the effect of these drugs was also analyzed on the CRE reporter in BEAS-2B cells. In these cells the CRE reporter responded robustly to a variety of cAMP-elevating agents, including salbutamol, prostaglandin E2, and the phosphodiester-

ase 4 inhibitor, rolipram, as well as to the cAMP analog 8-bromo-cAMP and the adenylyl cyclase activator, forskolin (data not shown). In present study, CRE-dependent transcription was dramatically enhanced by a combination of forskolin + 
rolipram and was substantially reduced by both D609 and Ro 31-8220 (Fig. 8A).

To test whether these observations are also observed on bona fides genes, A549 cells were treated with IL-1β, TNFα, or PMA in the presence or absence of D609 or Ro 31-8220. Northern blot analysis revealed increased mRNA expression of c-fos, c-jun, and IκBα at 1 h, and in each case expression was almost totally prevented by Ro 31-8220 (Fig. 8B). However, D609 resulted in potentiation of c-fos expression by IL-1β and TNFα, little effect on c-jun expression, and repression of IκBα expression. Simi-

larly analysis of cyclooxygenase-2, IL-8 and p50/p105, revealed an up-regulation by each stimulus and a profound repression by Ro 31-8220 (Fig. 8B). D609 resulted in a further increase in COX-2 and IL-8 expression and showed little effect on p50/p105 expression.

DISCUSSION

In a previous study (3), the PKC inhibitor, Ro 31-8220 and the PC-PLC inhibitor, D609, were shown to profoundly inhibit NF-κB-dependent transcription, yet no effect on loss of IκBα or on induction of NF-κB DNA binding was observed. This led to a hypothesis whereby activation pathways in addition to the core IKK-IκB pathways are necessary for transcriptional activation of NF-κB (3). In the current manuscript, we now confirm these findings in bronchial epithelial BEAS-2B cells and sug-

gest that this effect is more generally applicable to such cells.

Indeed numerous other studies have also proposed various “additional” events that impact on transcriptional activation by NF-κB (3) (see Ref. 2). However, many of these reported events are readily distinguishable from the pathway presented here. Thus, contrary to models suggested by other experimental systems (7, 8, 15), the analysis of a number of kinase inhibitors in the present study excludes a role for PI3K and PKA, while a role for PKC is reinforced. The lack of a role for PKA is further strengthened by our finding that adenoviral overexpression of protein kinase inhibitor, an endogenous and potent inhibitor of PKA (39), or the PKA activator, forskolin, were without effect on NF-κB-dependent transcription (data not shown). This is consistent with more recent reports documenting repression of NF-κB transactivation by PKA (40). Similarly, the finding that the phorbol esters, PMA and PDBu, but not the inactive analog 4α-PMA, are potent activators of NF-κB-dependent transcription suggests the existence of a DAG-dependent effect and again raises the possibility of PKC involvement. In each case the induction of NF-κB-dependent transcription was prevented by a number of inhibitors of PKC further supporting the involvement of this kinase. However, many of the reports implicating PKC in signaling to NF-κB suggest a role upstream of NF-κB DNA binding (2, 41). For example the novel isoform, PKCδ, can mediate IκBα degradation and NF-κB DNA binding (42), while the atypical isozymes, PKCγ, which may be activated by ceramide, and PKCζ, have been shown to phosphorylate and activate IκBα. Clearly this process is mechanistically distinct from the one observed in the present study as the inhibitors of PKC or the PC-PLC inhibitor, D609, have no effect on induction of NF-κB nuclear transloca-
tion or DNA binding. In addition, we have previously shown that ceramide is incapable of activating or potentiating the transcriptional activity of NF-κB in A549 cells (48). Furthermore, despite proving to be effective at transcriptional induction, we found that PMA, which will only activate the DAG-sensitive classical and novel PKC isofoms, is in fact a very poor of inducer of NF-κB DNA binding and activation occurs gradually over a number of hours (28). Consistent with this, PMA failed to induce IKK activity and did not result in loss of IκBα in either A549 or BEAS-2B cells, whereas TNFα proved to be highly effective at each of these, suggesting that the induction of NF-κB transcriptional activity must occur via distinct cellular processes. This idea is strengthened by the observation that a maximally effect concentration of TNFα was further potentiated by PMA and the finding that this PMA-dependent pathway is relatively insensitive to proteasome inhibition. However, notwithstanding the above data, a low level of activation of NF-κB DNA binding is observed in both cell lines following PMA treatment and this raises the possibility that one or more PMA-activated PKC isofoms, possibly PKCα, may also way feed into the core IKK pathway (44).

An alternative explanation for PKC-dependent activation of NF-κB has previously been found at the level of p65/RelA phosphorylation (2), an event that may regulate the ability of p65/RelA to associate, when unphosphorylated, with the transcriptional repressor HDAC1 or when phosphorylated with PKCα (49). Thus phorbol ester-dependent phosphorylation of p65/RelA was previously shown to correlate with increased transactivation potential (10). More recently, PKCζ was implicated in p65/RelA phosphorylation, and increases in transactivation have now been correlated with phosphorylation of Ser311 of p65 and a subsequent increase in the association with CBP (50, 51). However, neither of these mechanisms can explain the effects in the present study as Ro 31-8220 failed to reverse TNFα-induced phosphorylation of p65/RelA and overexpression of a constitutively active PKCζ was without effect.

The fact that a number of non-PKC DAG-activated proteins have been identified led us to directly test whether PKC isofoms were actually able to activate NF-κB (52). However, the possibility that PKC isofoms may differentially input both onto NF-κB DNA binding and onto transcriptional potential makes the analysis of these separate events difficult (2). To functionally separate these entities, a Gal4 one-hybrid transactivation assay for p65 was established in the A549 cells (10). In this system the transactivation potential that is assayed derives only from DNA binding via the Gal4 DNA binding domain, which is constitutively nuclear, and not from signal-induced release of p65. Thus events that affect the release of p65 are not important, allowing us to show that both TNFα and PMA can enhance p65 transactivation, and importantly, both Ro 31-8220 and D609 are potent inhibitors of p65-dependent transactivation. Consistent with the suggested role for PKC in the transactivation of p65, the overexpression of a number of constitutively active PKC isofoms produced a robust activation of p65 transactivation. Interestingly, the atypically isofom, PKCζ, failed to elicit a response and this is consistent with the fact that phorbol esters, being diacylglycerol mimetics, will only activate classical and novel isofoms of PKC. The ability to elevate p65 transactivation was most apparent with the novel isofoms, particularly PKCε, suggesting that these kinases may be responsible for regulating transactivation. Similarly, PKCε has previously been shown to activate NF-κB-dependent transcription in RAW 264.7 cells providing support for a role of this kinase (53).

To explore the mechanistic basis of this effect, the co-activator protein, CBP, which is known to be instrumental in NF-κB
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transcriptional activation (37, 54), was immunoprecipitated and shown to be inducibly phosphorylated following TNFα stimulation as has been previously shown for IL-1β stimulation (26). That this phosphorylation was abolished by Ro 31-8220 and D609 presents a rational explanation for the observed transcriptional effects. As previous studies have shown that the phosphorylation of p65/RelA is critical in transcriptional activation and complex formation with CBP (20, 49) and that phosphorylation of CBP is also critical in transcriptional activation (55, 56), we speculated that reciprocal phosphorylation on CBP could also be important. In this context, p300 is implicated as a downstream target of PKC, although in that case the effect was to repress transcriptional output (57). In the present study, we show that following TNFα treatment the p65-CBP interaction is unaffected by either Ro 31-8220 or D609 in A549 cells, suggesting that the inhibition of CBP phosphorylation may play an alternative role. In this context, CBP-associated HAT activity was examined and found to be induced by TNFα but largely unaffected by the PKC inhibitor Ro 31-8220. By contrast, D609 caused a marked repression in CBP-associated HAT activity, and this was reflected in global changes in histone acetylation. To functionally link changes in histone acetylation with NF-κB-dependent transcription, cells were treated with the histone deacetylase inhibitor, trichostatin A. This increased both histone acetylation and potentiated NF-κB-dependent transcription demonstrating a positive link between these events. In terms of the data here, while these effects may explain the ability of D609 to inhibit NF-κB-dependent transcription, they do not appear to account for the repressive effect of PKC inhibitors.

Since CBP function is implicated in a variety of transcriptional responses in addition to NF-κB (38), we speculated that our finding may not be restricted to just NF-κB-dependent transcription and indeed analysis of AP-1, CRE, or GRE-dependent transcription responses confirms this and suggests a more general role in transcription. However, as low level, basal transcription, from just a TATA box or high level constitutive transcription, they do not appear to account for the repressive effect of PKC inhibitors.

As our studies have primarily focused on artificial reporter constructs, we have also examined the effect of Ro 31-8220 and D609 on the expression of acute phase genes following proinflammatory or mitogenic stimulation. Consistent with the above reporter analysis, known NF-κB-dependent genes, including IκBα (30), COX-2 (29), IL-8 (60), and p50/p105 (61), were substantially repressed by the PKC inhibitor, Ro 31-8220. This effect also extended to c-fos and c-jun, which are predominately regulated by Sis-inducible element, serum response element, and TRE (AP-1) sites (30, 62), but not the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, suggesting the validity of our reporter analyses. By contrast, D609 produced a highly variable response depending on the stimulus and the gene in question. Thus in many cases steady state mRNA levels were either unaffected or were potentiated. A similar effect was reported in respect of GM-CSF, expression of which was markedly increased by D609 via a mechanism that was at least partly due to a profound mRNA stabilization (63). This phenomenon, coupled with the global changes in histone acetylation observed following D609 treatment, have led us to treat these data with caution.

In conclusion, we present a body of data that support a role for additional regulatory signaling pathways in the transcriptional activation of NF-κB. On the basis of inhibitor data, known activators and overexpression of various PKC isoforms, we predict a role for one or more isoforms of this kinase, and we further indicate CBP as a downstream target of this pathway.

Analysis of additional luciferase reporters and real genes indicated that this effect is not limited to NF-κB but is in fact more generally applicable to activated transcriptional responses. The further exploration of this novel pathway will undoubtedly reveal additional insights into the regulation of activated transcriptional responses. Finally, as basal transcription was unaffected; this pathway could provide possible targets that may be exploited therapeutically to damp down the high levels of activated gene expression observed in many diseases including inflammation or cancer.

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