Lysophosphatidic acid (LPA) is a growth factor that exerts a number of well characterized biological actions on fibroblasts and other cells. In the present study, we investigated the possibility that LPA activates the transcription factor NF-κB. NF-κB is a target of cytokines, but its activation by other classes of agonists has raised considerable interest in the control of processes such as inflammation and wound healing through varied mechanisms. We find that LPA causes a marked activation of NF-κB in Swiss 3T3 fibroblasts as determined by the degradation of IκB-α in the cytosol and the emergence of κB binding activity in nuclear extracts. The EC\textsubscript{50} for activation of NF-κB is 1–5 μM, a range similar to that reported for reinitiation of DNA synthesis and activation of the serum response element. Activation of NF-κB is attenuated by pertussis toxin and inhibitors of protein kinase C, and it is completely blocked by the Ca\textsuperscript{2+} chelator BAPTA-AM. The combination of phorbol ester and thapsigargin promotes an activation comparable with that of LPA. Activation by LPA is additionally inhibited by tyrphostin A25 but not genistein or AG1478, with that of LPA. Activation by LPA is additionally inhibited by tricyclodecan-9-yl-xanthogenate (D609), implying a requirement for hydrolysis of phosphatidylcholine. The activation is also inhibited by reactive oxygen species. The activation also is inhibited by lysophosphatidic acid (LPA1; 1-acyl-2-lyso-sn-glycerol-3-phosphate) is a naturally occurring, water-soluble glycerophospholipid that exhibits striking hormone- and growth factor-like activities (1, 2). Synthesized and released by platelets, LPA represents a major bioactive constituent of serum, and its actions on fibroblasts, endothelial cells, and smooth muscle cells in particular suggest roles in wound healing among other events. Indeed, LPA acts on a large number of cells to achieve a broad range of immediate and long lasting effects. Specific responses to LPA include changes in cell shape and tension, chemotaxis, proliferation, and differentiation.

The molecular actions of LPA have been characterized best in rodent fibroblasts, where at low concentrations (i.e. 10–100 nm) LPA stimulates phosphoinositide hydrolysis (3) and promotes the Rho-dependent formation of stress fibers and focal adhesions (4). The stimulation of phosphoinositide hydrolysis is thought to occur through the GTP-binding regulatory protein (G protein) G\textsubscript{i}. The formation of stress fibers and focal adhesions is consistent with activation of Rho through G\textsubscript{12} or G\textsubscript{13} (5). One or a combination of these G proteins is also responsible for the protein tyrosine phosphorylation elicited by LPA (6–8). LPA additionally inhibits adenyl cyclase, an action achieved through the pertussis toxin (PTX)-sensitive G protein G\textsubscript{i} (9). LPA uses G\textsubscript{i}, moreover, to activate Ras, Raf, and the extracellular signal-regulated kinases (ERKs) ERK1 and ERK2 (10, 11). The activation of ERKs (11), and presumably the inhibition of adenyl cyclase (3), occurs at concentrations of LPA comparable with those stimulating phosphoinositide hydrolysis and cytoskeletal changes. At higher concentrations (i.e. 5–70 μM), LPA promotes reinitiation of DNA synthesis in quiescent fibroblasts (9). Whether G proteins are sufficient for this action is unclear, but the sensitivity of the phenomenon to PTX implies that G\textsubscript{i} represents at least one necessary input. The need for high concentrations of LPA in this context may relate to a requirement for more persistent signaling and/or engagement of other receptors or pathways. Micromolar concentrations of LPA also promote arachidonic acid formation, a second phase of inositol phosphate accumulation (PTX-insensitive), and activation of serum response factor (9, 12, 13). Receptors that recognize LPA are poorly characterized; however, several have been identified that conform to the seven-transmembrane domain motif characteristic of G protein-coupled receptors (GPCRs) (14–17).

NF-κB (nuclear factor-κB) is the prototype of a family of dimers whose constituents are members of the Rel family of transcription factors (18). In most types of cells, NF-κB is present as a heterodimer comprising p50 (NF-κB1) and p65 (RelA). NF-κB is normally retained in the cytosol in an inactive form through interaction with IκB inhibitory proteins. Release of NF-κB for translocation to the nucleus and interaction with cognate DNA sequences is accomplished through a signal-induced phosphorylation and subsequent degradation of IκB. Originally described as a necessary element for expression of the immunoglobulin κ gene in mature B cells, NF-κB is now recognized to be an important transcriptional regulatory protein in a variety of cell types (19).

The binding of agonists to certain GPCRs promotes activation of NF-κB. Agonists include serotonin (working through the 5-HT\textsubscript{1A} receptor) (20), platelet-activating factor (21), thrombin
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(22), and bradykinin (23). That GPCRs are linked to NF-κB is particularly significant, since these receptors are widely distributed, the actions of NF-κB are notable, and the coincident activation of NF-κB and other GPCR-regulated transcription factors can provide unique forms of transcriptional regulation. Because LPA exerts a wide range of actions in part or entirely through GPCRs, and because NF-κB is especially relevant to inflammation and wound healing, we instituted efforts here to understand whether LPA promotes the activation of NF-κB.

We explored the possible relationship between LPA and NF-κB in fibroblasts and the mechanisms by which this relationship is established.

**EXPERIMENTAL PROCEDURES**

**Reagents—** α-L-lysophosphatidic acid (C18:1, cis-9), cycloheximide, ascorbic acid, pyridinedithiocarbamate, and dimethyl sulfoxide were obtained from Sigma. Phorbol-12-myristate-13-acetate (PMA), calphostin C, Ro 31-8220, bisindolylmaleimide I, tyrphostin A25 and AG1478, 1,2-bis(o-aminophenoxy)ethane-N,N′,N″-tetracetic acid (tetra-oxymethyl) ester (BAPTA-AM), thapsigargin, N-acetylcysteine, and diphenylethiodione were obtained from Calbiochem. Dithiothreitol was obtained from Boehringer Mannheim. Tricyclodecan-9-yl xanthate (D609) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA) or Sigma. TNFα was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Potassium ethylxanthate was obtained from Aldrich. The double-stranded oligonucleotide conforming to 5′-AGTTGAGGGACTTTC-CAGGC-3′ was obtained from Promega Corp. (Madison, WI), and those conforming to 5′-AGTTGAGGGACTTTC-CAGGC-3′ and 5′-ATTCGATCGGGGCGGGGCGAGC-3′ and the antibody toward p65 (RelA) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [32P]ATP was obtained from NEN Life Science Products. Electrophoretic reagents were obtained from Bio-Rad.

**Cell Culture—** Swiss 3T3 mouse embryonic fibroblasts (a gift from Dr. E. Rozengurt, Imperial Cancer Research Fund, London, UK) were maintained at 37 °C under a humidified atmosphere of 10% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum, and the cells were incubated for an additional 18 h. LPA (prepared as a stock of 1 mg/ml in phosphate-buffered saline containing 10 mg/ml essentially fatty acid-free bovine serum albumin (Sigma)) and/or other reagents or vehicles were added to achieve the concentrations specified.

**Nuclear Extract Preparation—** Nuclear extracts were prepared by the method of Dignam et al. (24) with minor modifications. Following incubation with LPA or other reagents, cells were washed twice in ice-cold phosphate-buffered saline, harvested, and resuspended in 400 μl of hypotonic buffer (10 mM HEPES (pH 7.9), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After 10 min on ice, 30 μl of Nondenat P-40 (10% (v/v)) was added with mixing for 2 s. The nuclei were pelleted by centrifugation at 20,000 × g for 10 s. The supernatant was removed, and the nuclei were resuspended in hypotonic buffer (20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride) and shaken for 45 min at 4 °C. The samples were centrifuged at 20,000 × g for 30 s, and the supernatants (nuclear extracts) were saved. Protein concentrations were determined using the Bradford method (Bio-Rad).

**Electrophoretic Mobility Shift Assay—** Electrophoretic mobility shift assays were performed using a double-stranded oligonucleotide containing a consensus κB binding site (5′-AGTTGAGGGACTTTC-CAGGC-3′), the underlined sequence represents the consensus κB region, which was end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. Nuclear extracts (2.5 μg of protein) were incubated in 10 μl Tris-HCl (pH 7.9), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 0.15 mg/ml poly(dI-dC), and 20–30 fmol of 32P-labeled oligonucleotide (50,000–100,000 cpm) in a total volume of 15 μl at room temperature for 10 min. The reaction mixture was then subjected to electrophoresis in a 5% polyacrylamide slab gel containing 50 μM Tris, 380 mM glycine, and 2 mM EDTA, pH 8. The gels were dried under vacuum for analysis by autoradiography (overnight exposure) or PhosphorImager analysis. For competition studies, nuclear extracts were incubated prior to the addition of labeled oligonucleotide for 10 min at room temperature with unlabeled oligonucleotide, unlabeled oligonucleotide containing a G→C substitution in the κB binding motif (5′-ATTCGATCGGGGCGGGGCGAGC-3′), or an unlabeled oligonucleotide containing the consensus binding site for Sp1 (5′-ATTCGATCGGGGCGGGGCGAGC-3′). For supershift analysis, nuclear extracts were incubated with approximately 2 μg of antibodies specific for p65 (RelA) or nonimmune goat IgG for 30 min at 4 °C in the presence of radiolabeled oligonucleotide prior to electrophoresis. The results shown in all figures are representative of at least three experiments.

**Western Blot Analysis—** Following exposure to LPA with or without cycloheximide as specified, cells were washed with ice-cold phosphate-buffered saline and lysed in 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 mM Na₃VO₄, 0.5% Triton X-100, 10 mg/ml leupeptin, 10 mg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 20,000 × g for 15 min at 4 °C. Supernatants were collected and subjected to SDS-polyacrylamide gel electrophoresis (11% acrylamide). Protein was transferred to nitrocellulose membrane and probed with polyclonal rabbit anti-human IκB-α (0.4 mg/ml) detected subsequently by chemiluminescence using a donkey anti-rabbit IgG conjugated with horseradish peroxidase and luminol as recommended by the manufacturer (ECL Western; Amersham Pharmacia Biotech).

**RESULTS**

The possibility that LPA activates the transcription factor NF-κB was investigated in Swiss 3T3 fibroblasts, which have been used extensively in studies of agonists, including LPA, linked to changes in cell morphology and reinitiation of DNA synthesis (4, 25, 26). We examined first the extent to which LPA promotes degradation of IκB-α, an inhibitory protein whose proteolysis would precede the translocation of NF-κB to the nucleus. As shown in Fig. 1 (left panel), LPA caused a transient degradation of this protein. Levels of IκB-α decreased slowly following introduction of LPA, reaching a minimum at 40–60 min, and increased thereafter to near control values. The time-dependent resynthesis of IκB-α is a common finding in cytokine action (27) and appears to reflect activation of the IκB-α gene by NF-κB as part of a feedback loop (28). To circumvent resynthesis of IκB-α, we also evaluated degradation of this protein in the presence of cycloheximide. As expected, degradation of IκB-α under this condition, where protein synthesis is blocked, was complete.

A more direct evaluation of NF-κB activation was carried out by electrophoretic mobility shift assays. The data in Fig. 2 demonstrate that LPA promotes a time- and concentration-dependent appearance of a factor(s) within nuclear extracts that binds to an oligonucleotide probe containing an NF-κB binding site. The proinflammatory cytokine TNFα also promotes the appearance of this factor. The relevant protein-DNA complex was evident as a band of radioactivity (denoted by an arrow) positioned above two less prominent bands. This band, but not the other two, was supershifted with a p65 (RelA)-directed antibody (Fig. 3, top panel), confirming the identity of the induced factor as NF-κB. The nature of the protein-DNA interaction was evaluated further in competition experiments (Fig. 3, bottom panel), where the 32P-labeled oligonucleotide was found to be displaced by unlabeled oligonucleotide. The same unlabeled oligonucleotide, but containing a mutation in the κB site, and an altogether unrelated oligonucleotide (containing an
by the arrow fibroblasts were incubated with 40 \( \mu \)M LPA, or LPA or TNF\( \alpha \) at the indicated concentrations for 40 min (lower panel). Nuclear extracts were then prepared for electrophoretic mobility shift assays. No or 0 LPA corresponds to vehicle alone (0.2 mg/ml BSA). The LPA-induced complex of \( ^{32}P \)-labeled oligonucleotide and NF-\( \kappa \)B is designated by the arrow.

Sp1 binding site) did not displace the \( ^{32}P \)-labeled oligonucleotide. The EC\(_{50}\) for LPA based on the intensity of the shifted band was 1–5 \( \mu \)M, and the time required for full development of the response was 40–60 min (Fig. 2). The response was transient, as the level of shifted oligonucleotide began to decrease by 3 h (not shown).

The extent to which the G protein \( G_i \) might contribute to the activation of NF-\( \kappa \)B was assessed with PTX, which suppresses activation of \( G_i \) by GPCRs. Pretreatment of cells with PTX attenuated LPA-induced activation of NF-\( \kappa \)B by approximately 60% (Fig. 4, top panel). Efforts to enhance the attenuation by manipulating pretreatment time and concentration of PTX were unsuccessful. That the actions of LPA typically assigned to \( G_i \), are not completely suppressed by PTX, for example ERK activation and reinitiation of DNA synthesis, is not without precedent (9–11).

A downstream target for both \( G_i \) and \( G_q \) is the phosphoinositide-specific phospholipase C-\( \beta \), whose activation results in recruitment of PKC and mobilization of Ca\(^{2+}\). Overnight treatment of cells with a high concentration of PMA (1 \( \mu \)M) to induce down-regulation of classical and novel forms of PKC suppressed activation of NF-\( \kappa \)B by 70–80% (not shown), as did Ro-31-8220 (Fig. 4, middle panel), which inhibits all forms of PKC. Other inhibitors of PKC, including bisindolylmaleimide I (not shown) and calphostin C, inhibited activation of NF-\( \kappa \)B nearly as well. Activation of NF-\( \kappa \)B was completely suppressed by pretreatment of cells with the cell-permeable Ca\(^{2+}\) chelator BAPTA-AM (Fig. 4, bottom panel).

Given the apparent requirements for PKC and intracellular Ca\(^{2+}\), we tested whether the activation of PKC and/or mobilization of Ca\(^{2+}\) might be sufficient to activate NF-\( \kappa \)B. Some degree of activation was achieved with PMA, but not the extent observed with LPA (Fig. 5). Only a small degree of activation was achieved with thapsigargin, moreover, an inhibitor of the endoplasmic reticular Ca\(^{2+}\)-ATPase that causes a time-dependent increase in cytosolic Ca\(^{2+}\). However, the combination of thapsigargin and PMA activated NF-\( \kappa \)B ultimately to an extent somewhat greater than that achieved with LPA.

Because considerable attention has been devoted to the utilization of the EGF receptor and other tyrosine kinases by GPCRs, including the one or more receptors that mediate the actions of LPA (29, 30), we tested inhibitors of different tyrosine kinases for their effect on LPA-induced activation of NF-\( \kappa \)B. Genistein, which inhibits a wide range of tyrosine kinases, was without effect (Fig. 6). Tyrphostin AG1478, a relatively specific inhibitor of the EGF receptor tyrosine kinase, caused some degree of inhibition but only at very high concentrations (16 \( \mu \)M is shown; concentrations normally used to inhibit the EGF receptor are 0.125–1 \( \mu \)M (29, 31)). At concentrations less than 5 \( \mu \)M, AG1478 had no effect. In contrast, tyrphostin A25, like genistein regarded as a general inhibitor of tyrosine ki-
nuclear extracts were prepared for electrophoretic mobility shift assays.

Swiss 3T3 fibroblasts were pretreated with or without genistein, tyrphostin A25 or thapsigargin for 1 h with AG1478 for 2 h at the indicated concentrations and then with 40 μM LPA (or vehicle) for 40 min. Nuclear extracts were prepared for electrophoretic mobility shift assays.

Activation of NF-κB by thapsigargin and phorbol ester.

Swiss 3T3 fibroblasts were incubated with 40 μM LPA (or vehicle), 250 nM PMA, and/or 100 nM thapsigargin for the indicated times, and nuclear extracts were prepared for electrophoretic mobility shift assays.

Inhibition of NF-κB by pyrrolidinedithiocarbamate.

As shown in Fig. 7, N-acetylcysteine completely inhibited LPA-induced activation of NF-κB. Pyrrolidinedithiocarbamate was similarly effective. Dimethyl sulfoxide achieved a less extensive, but still notable, inhibition. Ascorbic acid and dithiothreitol were without effect. The activation of NF-κB was also highly sensitive to diphenyleneiodonium, an inhibitor of flavanoid-containing enzymes such as NADPH oxidase.

The tricycloedan xanthogenate D609 inhibits the hydrolysis of phosphatidicholine at the level of a phosphatidicholine-specific phospholipase C-like enzyme and/or phospholipase D (34–36), among perhaps other actions (37), and has been used to explore signaling pathways utilized by growth factors, TNF, and GTPase-deficient G protein subunits. Schütze et al. (35), for example, found that D609 inhibits the activation of NF-κB by TNFα, and Wadsworth et al. (38) demonstrated that this compound inhibits Na+/H+ exchange stimulated by α12. We found here that D609 inhibits quite effectively the activation of NF-κB by LPA (Fig. 8). The EC50 was about 3 μg/ml, and the maximum degree of inhibition was greater than 90%. Potassium ethylxanthate had no effect. D609 was not nearly as potent an inhibitor of TNFα’s activation of NF-κB as it was of LPA’s. Inhibition in the case of TNFα occurred only at concentrations of D609 exceeding 50 μg/ml. The activation of NF-κB by LPA was thus selectively inhibited by D609.

DISCUSSION

That proinflammatory cytokines such as interleukin-1 and TNFα activate NF-κB has long been appreciated, and the sequence of events by which the activation occurs is now emerging (39, 40). Of perhaps equal significance, however, is the fact that agonists working through GPCRs can also activate NF-κB (20–23). In the work described here, we have focused on LPA. LPA has a rich and important biology; it induces a number of cells to proliferate and others to differentiate and, at a molecular level, works through one or more G proteins to stimulate MAP kinases, phospholipid metabolism, and cytoskeletal rearrangement. Activation of NF-κB clearly constitutes an additional action of LPA, and one to be reconciled in any setting of transcription relevant to this agonist.

LPA triggers a pronounced activation of NF-κB, as ascertained by the degradation of IκB-α and the emergence of κB binding activity in nuclear extracts. Based on supershift experiments, the activated form of NF-κB contains the p65 (RelA) subunit. The other component may be p50 (NF-κB1), given the widespread occurrence of NF-κB as a p50-p65 heterodimer, but this remains to be determined. Dimeric complexes of NF-κB that contain p65 function as strong activators of gene expression (41). The transience of NF-κB activation suggests that IκB-α alone of the inhibitory proteins is the target of LPA’s action (28).

The concentration of LPA supporting activation of NF-κB (EC50 = 1–5 μM) is higher than that reported to inhibit adenyl cyclase (3), activate ERKs (10), elevate IP3 and Ca2+ (3), and stimulate formation of stress fibers and focal adhesions (4) but is similar to that needed for activation of serum response factor (12), initiation of a “second” phase of inositol phosphate production (9), and reinitiation of DNA synthesis (9). It is tempting to speculate that activation of NFF-κB by LPA, like that of serum response factor, may function as a counterpart to proliferative signaling, for example through activation of genes immediately relevant to DNA synthesis.

Because the roles of G proteins in the phenomena induced by LPA are not completely established, a basic question is whether LPA utilizes G proteins to achieve its activation of NF-κB. That PTX attenuates activation of NF-κB by oncogenic Ras is required for progress toward cell transformation and, in particular, that NF-κB prevents a Ras-induced apoptosis that would otherwise abrogate transformation. It is not implausible that mitogens such as LPA, as they drive replication, similarly use NF-κB to foil any tendency of the cell to enter into an apoptotic program. Alternatively, the activation of NF-κB may play a more direct role in proliferative signaling, for example through activation of genes immediately relevant to DNA synthesis.

The widespread occurrence of NF-κB in carcinomas and leukemias may reflect a role for Gi. The participation of this G protein would not be surprising; NF-κB has long been appreciated, and the se-

Discussion
Fig. 7. Activation of NF-κB by LPA is sensitive to certain antioxidants. Swiss 3T3 fibroblasts were pretreated with or without the antioxidants ascorbic acid (90 min), dithiothreitol (DTT) (60 min), dimethyl sulfoxide (DMSO) (30 min), N-acetylcysteine (90 min), or pyrroldinedithiocarbamate (PDTC) (60 min) (left panel) or with diphenyleneiodonium (DPI) (30 min) (right panel) at the indicated concentrations prior to treatment with 40 μM LPA (or vehicle) for 40 min. Nuclear extracts were prepared for electrophoretic mobility shift assays.

Fig. 8. Inhibition of NF-κB activation by D609. Swiss 3T3 fibroblasts were preincubated as indicated for 30 min with D609 or the inactive analog potassium ethylxanthate (PEX) and then exposed to 40 μM LPA (or vehicle) or 30 ng/ml TNFα for 40 min. Nuclear extracts were prepared for electrophoretic mobility shift assays.

Some production of inositol phosphates is observed at low concentrations of agonist, but the preponderance is achieved at micromolar concentrations. The latter (at least) is insensitive to PTX (9). It is conceivable that low concentrations of LPA activate G1 and that higher concentrations activate G12 and G13. Together with the notion that G12 and G13 might even be sensitive. However, FMA and thapsigargin would exert more potent and long-lasting actions than those achieved by agonist-activated G12. With respect to the involvement of PKC, we have used both down-regulation and several pharmacological inhibitors to implicate a role for this enzyme(s). That Ro-31-8220 can activate JNK is a potentially confounding issue, but bisindolylmaleimide I lacks this attribute (47).

The possibility that Rho is engaged by G12 or G13 in the activation of NF-κB by LPA is most intriguing. LPA appears to activate Rho (4), and Rho when overexpressed as a wild-type or constitutively active protein activates NF-κB (48). The activation of Rho by LPA no doubt proceeds through G12 and/or G13. The GTPase-deficient forms of α12 and α13, for example, cause a Rho-dependent formation of stress fibers and focal adhesions, as does LPA (5). Labeling of G1 proteins with [32P]GTP azido- nitride, moreover, reveals activation of G12 and G13, together with G1 and G13, by LPA (31). Tyrophostin A25 has been demonstrated previously to prevent the induction of stress fiber and focal adhesion assembly by the GTPase-deficient form of α12 (31), as it does that by LPA at a step upstream from Rho (32). Of interest, we find here that A25 prevents LPA-induced activation of NF-κB. Thus, the activation of NF-κB by LPA may well involve an A25-sensitive activation of Rho. Rho has recently been demonstrated to be required in the activation of NF-κB by bradykinin in human epithelial cells (49). How a Rho-dependent pathway integrates with those pathways sensitive to inhibitors of PKC and intracellular Ca2+ fluxes, D609, and PTX remains to be determined. In a departure from the results presented for cytoskeletal changes (31), we do not have firm evidence for the utilization of the EGF receptor by LPA. AG1478, an inhibitor of EGF receptor autophosphorylation, had only a modest impact at high concentrations on the activation of NF-κB. Genistein, which would block phosphorylation of the receptor by Src among other kinases, had no effect.

The relevance of reactive oxygen intermediates to the activation of MAP kinases and NF-κB has generated considerable interest (33, 50–52). N-Acetylcysteine has been used extensively in this regard and was found here to effectively inhibit the activation of NF-κB by LPA, as did pyrroldinedithiocarbamate and dimethyl sulfoxide. The nature and source of the reactive oxygen species is unclear, although the inhibition achieved with diphenyleneiodonium might suggest the involvement of NADPH oxidase. Of interest, several reports find reactive oxygen species to be involved in activating components of the ERK activation cascade, ostensibly through phosphorylation of the EGF receptor (50, 53). For reasons outlined above, we suspect that the EGF receptor is not a participant in the activation of NF-κB, and we know that ERK is activated at concentrations of LPA well below those required for activation of NF-κB. It is nevertheless intriguing that the reactive oxygen species-dependent phosphorylation of the EGF receptor reported in HeLa cells is implied to occur at only higher (i.e. micromolar) concentrations of LPA (53). Perhaps, then, the formation of reactive oxygen species will emerge as the most closely correlated requirement for NF-κB activation, SRF activation, and reinitiation of DNA synthesis.

The activation of NF-κB by LPA is exceptionally sensitive to inhibition by the tricyclodecan xanthogenate D609. Sensitivity to this compound has been used in other contexts to suggest the relevance of a phosphatidylcholine-specific phospholipase C (34, 35). More recent reports suggest that phospholipase D, too, can be inhibited by D609 (36, 54), perhaps at higher concentrations. Our use of D609 was prompted by the observation that D609 inhibits the activation of NF-κB by TNFα (35), as it does that of sphingomyelinase and of Raf by oncogenic Ras (34). We found that D609 indeed inhibited the activation of NF-κB by TNFα but only at high concentrations (>50 μg/ml), consistent with the observation of Schütte et al. (35). In contrast, the amount of D609 required to inhibit activation of NF-κB by LPA was considerably lower; almost complete suppression could be achieved at concentrations less than 10 μg/ml. D609 at these lower concentrations has been argued to inhibit a phosphatidylcholine-specific phospholipase C.
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