Ras is a guanine nucleotide-binding protein that acts as a molecular switch controlling cell growth. The Ras GTPase-activating proteins (GAPs) p120-GAP and neurofibromin are candidates as Ras effectors. The GTPase-activating activity of both proteins is inhibited by mitogenic lipids, such as arachidonic acid and phosphatidic acid, and differential inhibition of the two GAPs led to the hypothesis that both were effectors in a Ras-controlled mitogenic pathway (Bollag, G., and McCormick, F. (1991) Nature 351, 576–579). We have studied the mechanism of inhibition by arachidonic acid in three ways: first, by measurements of catalytic activity under multiple turnover conditions; second, using p-(6-phenyl)-1,3,5-hexatrienyl)benzoic acid as a fluorescent probe for ligands binding to GAPs; and third, by using a scintillation proximity assay to measure direct binding of Ras to neurofibromin. We found no significant differential inhibition between p120-GAP and neurofibromin by arachidonic acid. The inhibition by arachidonic acid included a major component that is competitive with Ras-GTP. These data suggest that in some cases the arachidonic acid effects of lipids are mediated via inhibition of GAPs, GAPs are not Ras effector proteins. Additionally, lipids can exert a non-competitive type effect, consistent with a protein denaturing activity, making difficult extrapolations from in vitro data to the situation within cells, and possibly explaining the variability of literature data on inhibition by lipids.

The ras genes encode guanine nucleotide-binding proteins that act as molecular switches for signal transduction pathways controlling cell growth and differentiation (1–5). In the GTP-bound form, Ras is active and interacts with effector proteins to propagate a signal from the outside of the cell to the nucleus or cytoskeleton (6,7). A region on Ras has been mapped out through mutagenesis and structural studies as the effector binding region (7). Ras has a low intrinsic GTPase activity, which is accelerated by the GTPase-activating proteins (GAPs)1 p120-GAP and NF1 (6, 8). GAPs can thus act as negative regulators by converting Ras to the inactive GDP form. Activation of Ras to the GTP form occurs by nucleotide exchange, catalyzed by exchange factors (6).

Several candidate effector proteins have been proposed. Thus, very convincing evidence has emerged for the role of the serine-threonine kinase, c-Raf, as an effector controlling the activation pathway for MAP kinase (9–13). Phosphatidylinositol 3-OH kinase interacts with the effector binding region of Ras and might well be an effector for a Ras signaling pathway controlling phosphoinositide metabolism (14). Rap-GDP dissociation stimulator also binds to the effector binding region of Ras, but it is not known to have a biological activity associated with an effector function (15). Both p120-GAP and NF1 have many properties expected from a Ras effector, in that they bind preferentially to Ras-GTP rather than Ras-GDP and interact with Ras at the effector binding region (6–8). Evidence both for and against such a role has been presented. There is much experimental data to support a role of p120-GAP in signaling, other than just to down-regulate Ras, whereas with NF1 most data are consistent purely with a negative regulatory role (8). However, an effector role for both p120-GAP and NF1 was suggested by Bollag and McCormick (16) based on their data on the differential inhibition of p120-GAP and NF1 activity by lipids.

An early response to mitogenic stimulation is a rapid alteration in levels of various lipids such as diacylglycerol, phosphatidic acid, arachidonic acid, and metabolites of phosphatidylinositol (17–21). Phosphatidic acid itself acts as a mitogen in specific cells (22). Microinjection of a neutralizing anti-Ras antibody blocked the mitogenic activity of phosphatidic acid showing that its activity is completely Ras-dependent (22). Among other lipids, phosphatidic acid and arachidonic acid inhibit p120-GAP and NF1 in vitro (16, 19, 23–25). This suggested the possibility that the mitogenic effects of these lipids might be mediated by inhibition of p120-GAP, leading to an increase in Ras-GTP, and hence an activation of Ras. Bollag and McCormick (16) reported that phosphatidic acid inhibited NF1 catalytic activity but did not block binding of Ras to NF1. This led to an hypothesis in which NF1 was a Ras effector, modulated by lipid inhibition (16).

Large differences in potency and specificity of inhibition of GAPs by lipids have been observed by the various researchers in this field. For example, Bollag and McCormick (16) reported that arachidonic acid inhibits p120-GAP with an IC50 of ~200 μM, and the catalytic domain of NF1 (NF1-GRD) with an IC50 of ~30 μM, whereas Golubic et al. (23) reported that the catalytic domains of NF1 and of p120-GAP were both inhibited by arachidonic acid with IC50 values between 8 and 16 μM. With
phosphatidic acid, Bollag and McCormick (16) found no inhibition of p120-GAP, but an \( I_{50} \) of \(-10 \mu M \) with NF1-GRD. In contrast, Golubic et al. (23) found little or no inhibition by phosphatidic acid of the catalytic domains of either NF1 or p120-GAP. This diversity appears to be a reflection of different experimental procedures, conditions, and components utilized. Thus, there are reports showing that the inhibitory potency differs dependent upon whether full-length or catalytic domains are expressed (23, 24, 26). The assay conditions and the procedure by which the lipids are solubilized also appear to be important factors. Lipids have been introduced either as pure micelles or as mixed micelles with apparently differing results (19, 24). Furthermore, some assays have been performed in the presence of detergents (16, 24), whereas others have not (16, 23). In this study, we decided to keep the experimental system as simple as possible by performing experiments with arachidonic acid in the absence of detergents.

We report here an investigation into the mechanism of arachidonic acid inhibition of both p120-GAP and NF1 to see if it was different from that reported for phosphatidic acid. We show that there is a strong competitive element in the mechanism of arachidonic acid inhibition, such that arachidonic acid can block binding of NF1 to Ras. This has important implications for any hypothesis in which arachidonic acid, through interaction with GAPs, acts as a modulator of Ras signaling.

**EXPERIMENTAL PROCEDURES**

**Proteins**

NF1-334 was purified as described by Eccleston et al. (27) and GST-NF1-334 and [Leu\(^{38}\)]Harvey-Ras (residues 1-166) as described by Skinner et al. (28). Normal Harvey-Ras (residues 1-166) was expressed in Escherichia coli and purified by procedures similar to those in Ref. 28. GAP-344 was purified as described by Skinner et al. (29).

**Arachidonic Acid**

Arachidonic acid (Sigma) was dissolved in ethanol (Spectrograde grade, BDH) to form a stock solution at 40 mM. Appropriate dilutions were made in ethanol, and aliquots of these added to the experiments such that the final concentration of ethanol was not more than 2%, and in general was chosen to be 1%.

**Nucleotide Complexes**

RasGTP or Ras\(^{[3H]}\)GTP complexes were prepared by nucleotide exchange in the presence of a GTP regenerating system (28). Ras-mantGTP complexes were prepared as described by Eccleston et al. (27).

**CMC Determination**

CMC was determined by two independent procedures, one based on light scattering and the other based on fluorescence changes of the probe DPH. In both cases the experiments were performed in 20 mM Tris/HCl, pH 7.5, 1 mM MgCl\(_2\), 0.1 mM dithiothreitol. In the former procedure, fatty acids were dissolved in ethanol and titrated into buffer such that a maximum of 2% ethanol was present. Light scattering was monitored in a fluorimeter at 500 nm. The sample was taken to be the concentration of lipid at which a sharp discontinuity occurred in the light scattering versus concentration graph. Alternatively, CMC was determined by titrating the lipid into a solution of DPH and was taken to be the concentration of lipid at which a discontinuity occurred in the graph of fluorescence intensity versus concentration of lipid added.

**Steady-state Fluorescence**

Measurements were performed on a Perkin Elmer LS-50B spectrophuorometer thermostatted at 30 °C. Experiments were performed in 20 mM Tris/HCl, pH 7.5, 1 mM MgCl\(_2\), 0.1 mM dithiothreitol.

**Scintillation Proximity Assay**

This was performed basically as described by Skinner et al. (28), except that the buffer was 20 mM Tris/HCl, pH 7.5. The assay was performed by adding 80 μl of a solution of 0.2 μM NF1-GST in 20 mM-Tris/HCl, pH 7.5, 2 mM dithiothreitol to each well, followed by 120 μl of a mixture of 0.07 μM Ras\(^{[3H]}\)GTP, 0.031 mg/ml of anti-glutathione S-transferase antibody and 4.2 mg/ml of Protein A polyvinyltoluene scintillation proximity assay beads (Amersham) suspended in 20 mM Tris/HCl, pH 7.5, 2 mM dithiothreitol, 2 mM MgCl\(_2\). Lipids were either added to 80 μl of the solution of NF1-GST, followed by addition of 120 μl of a mixture containing scintillation proximity assay beads, anti-GST, and radiolabeled Ras, or were added directly to the complete mixture of 200 μl of these two components.

**HPLC Analysis**

\(^{[3H]}\)GTP and \(^{[3H]}\)GDP were quantified by HPLC separation using ion-pair chromatography on a Lichrosorb RP18 (5 μm particle size; 250 x 4 mm) eluting isocratically at 1 mL/min \(^{-1}\) with 12% acetonitrile, 88% tetraethyleneammonium hydroxide (2.25 mM) dissolved in 56 mM potassium phosphate buffer, pH 5.3, essentially as described by Pingoud et al. (30). Radiolabeled compounds were detected and quantified by in-line mixing with scintillant, using a Berthold radiochemical detector.

**Catalytic Activity Measurements**

**RasGTP Hydrolysis**—The rates of GAP-344- or NF1-334-stimulated RasGTPase were measured by incubating a stoichiometric normal Ras\(^{[3H]}\)GTP complex with the appropriate GAP, in the presence of phosphatidic acid and in the absence of detergents. Incubations were performed in 20 mM Tris/HCl, pH 7.5, 1 mM MgCl\(_2\), 0.1 mM dithiothreitol at 25 °C for 10 min. The concentration of Ras was about 7 μM. Final concentrations of GAP-344 and NF1-334 were 0.040 μM and 0.014 μM, respectively. The concentrations of GAPs were chosen so that in the absence of inhibitor the maximum extent of Ras-GTP hydrolysis was 80% and generally was less and so gave a reasonable estimate of the true initial rate. The extent of hydrolysis in the absence of GAP-344 or NF1-334 was negligible as compared to that in its presence. At the end of the incubation, samples were transferred to ice and immediately quenched by addition of an equal volume of HPLC running buffer. The samples were stored at -70 °C prior to HPLC analysis to determine the extent of conversion of Ras\(^{[3H]}\)GTP to Ras\(^{[3H]}\)GDP. Fatty acids were added from ethanolic stocks such that the final concentration of ethanol was less than 2%. Control experiments showed that 2% ethanol had no effect on the GTP hydrolysis reaction.

**Ras-mantGTP Hydrolysis**—Hydrolysis was measured under multiple turnover conditions in which catalytic amounts of NF1-334 (=10% the molar concentration of Ras) were mixed with Ras-mantGTP. Experiments were performed either on the home-built stopped flow fluorimeter (27) or on a Hi-Tech SF-61 instrument with the excitation monochromator set at 365 nm and emission monitored through a Wratten 47B filter. In these experiments, stock solutions of Ras-mantGTP were diluted in buffer to which arachidonic acid was also added when required. This Ras solution was mixed in the instrument with an equal volume of a solution of NF1-334 in buffer, again containing arachidonic acid if required. All concentrations subsequently quoted are after mixing.

The initial rate of fluorescence change was measured directly from the photomultiplier output versus time plot in units of volts \(^{-1}\). To compensate for changes made in the applied photomultiplier voltage to allow measurements over a wide range of concentration of Ras-mantGTP, it was necessary to establish the relationship between volts and molar concentration of Ras-mantGTP. This was done in parallel experiments in which the Ras-mantGTP solution of known concentration was mixed in the stopped flow fluorimeter with a solution consisting of 500 μM GDP, 40 mM EDTA, and 400 mM ammonium sulfate. This caused complete displacement of bound mantGTP from the Ras protein (cf. Fig. 2, trace c), and the amplitude of the photomultiplier fluorescence decrease was measured. This allowed the relationship between total Ras-mantGTP and photomultiplier output to be established. The conversion of Ras-mantGTP to Ras-mantGDP results in about a 10% decrease in fluorescence (31). Although this was not precisely determined in our experiments, we used a figure of 10% to allow us to calculate rates in units of molar concentration.

**RESULTS**

Inhibition of GAP- and NF1-activated RasGTPase by Arachidonic Acid—Arachidonic acid was added to standard GAP activity assays from an ethanolic stock. Under these conditions, arachidonic acid inhibited GAP-344 and NF1-334 activities with \( I_{50} \) values of 10 and 5 μM, respectively (Fig. 1). The inhibitory potency was much higher than that reported by Bollag and McCormick (16). We noted one experimental difference, which was that these workers did not use pure lipid but
incorporated the detergent Nonidet P40 in all assays so that presumably mixed micelles were formed. We assayed the inhibitory potency of arachidonic acid on p120-GAP in the presence of 0.1% Nonidet P40, and found only 18% inhibition at 30 °C. Both proteins were in 20 mM Tris/HCl, pH 7.5, 1 mM MgCl2, 0.1 mM dithiothreitol. Fluorescence recordings are shown for experiments in the absence of arachidonic acid (trace a) and with 20 μM arachidonic acid added to both the NF1–334 and Ras-mGTP solutions (trace b). To convert fluorescence changes into molar concentrations, in separate experiments 1 μM Ras-mGTP was mixed with 500 μM GDP dissolved in 20 mM Tris/HCl, pH 7.5, 1 mM MgCl2, 0.1 mM dithiothreitol, containing additionally 40 mM EDTA and 400 mM ammonium sulfate to promote rapid nucleotide exchange (trace c) (see "Experimental Procedures"). The inset shows the initial phases of traces a and b on a larger scale.

The inhibitory potency of arachidonic acid was influenced by ionic strength. With increasing concentrations of NaCl up to 300 mM NaCl, an increase in inhibition by arachidonic acid was seen, but further increases in NaCl concentration resulted in a decrease in the level of inhibition (data not shown). Thus, 20 μM arachidonic acid inhibited by 45% with no added NaCl, 80% with 300 mM added NaCl and 41% with 1000 mM NaCl. At 150 mM NaCl, arachidonic acid inhibited NF1-stimulated Ras-mGTPase with IC50 ~20 μM. The biphasic effect on inhibition with increasing ionic strength is likely to be caused by an
Arachidonic Acid Inhibition of Ras GAPs

Fig. 3. Steady-state kinetic analysis of the inhibition of NF1-334-catalyzed Ras-mantGTPase by arachidonic acid. Initial rates ($k_{\text{act}}$) of NF1-334 catalyzed Ras-mantGTP hydrolysis were measured in experiments similar to those shown in Fig. 3. Experiments were performed with 0 µM (●), 12 µM (○), 18 µM (■), or 24 µM (▲) arachidonic acid. The data at each concentration of arachidonic acid were fitted by non-linear regression to the Michaelis-Menten equation to obtain values of $K_m$ and $K_{\text{act}}$. In A and B, the solid lines are based on these constants. $K_m$ at 0, 12, 18, and 24 µM arachidonic acid were 1.1, 1.3, 1.8, and 3.8 µM, respectively. $K_{\text{act}}$ at 0, 12, 18, and 24 µM arachidonic acid were 2.6, 2.3, 1.7, and 1.3 µM, respectively. In C, the ratio of $K_m$ to $K_{\text{act}}$ obtained from this curve-fitting has been plotted against the concentration of arachidonic acid used.

This value of $\alpha$ suggests that the mixed inhibition is predominantly of a competitive character, but the uncompetitive component is still significant. However, the data clearly do not fit this simple model in that the relationship between inhibition and the concentration of arachidonic acid at any fixed concentration of Ras is distinctly sigmoid and plots of either $K_m/K_{\text{act}}$ (Fig. 3C) or $1/K_{\text{cat}}$ (not shown) against arachidonic acid concentration are non-linear, parabolic upward.

Characterization of the Interaction of Arachidonic Acid with NF1 and GAP Using the Fluorescent Probe DPH-carboxylic Acid—As the kinetic mechanism of inhibition of GAP activity by arachidonic acid is complex, we examined other methods to show whether arachidonic acid binds at the Ras-binding site, or at some other site, on GAPs. We postulated that p-(6-phenyl)-1,3,5-hexatrienyl)benzoic acid (DPH-carboxylic acid) might be a sufficiently close analogue of arachidonic acid that it would act as a fluorescent probe of the lipid-binding site. An increase in fluorescence of DPH-carboxylic acid caused by the inhibitor both decreases $k_{\text{cat}}$ and increases $K_m$. If the data are interpreted as fitting a mixed inhibition model (32), then it can be calculated from the intersection point of the lines that $K_m$ (or $K_s$ if the components are in rapid equilibrium) was around 1 µM and $\alpha K_m$ is -5, where $\alpha$ represents the ratio of $K_{iu}$ to $K_{ic}$ (Scheme 1).

Scheme I.

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Scheme I.
mixture with albumin, as expected if arachidonic acid competed with DPH-carboxylic acid for binding to albumin (Fig. 5). However, at concentrations of arachidonic acid up to 40 μM, there was no significant reduction in the fluorescence of DPH-carboxylic acid mixed with either NF1 (Fig. 5) or GAP. Indeed with GAP, an increase was seen. This suggested that with the latter two proteins the probe was binding at a site distinct from the arachidonic acid-binding site. However, we were still able to use DPH-carboxylic acid as a probe for ligands interacting with Gaps since the fluorescence of the protein-bound probe was sensitive to addition of ligands.

The fluorescence of a solution containing 1 μM DPH-carboxylic acid and 2 μM NF1–334 was dramatically reduced by addition of [Leu<sup>61</sup>]Harvey-RasGTP protein (Fig. 6). The reduction in fluorescence was nearly proportional to the concentration of added Ras, with half-maximal effect occurring at about 1 μM Ras-GTP. This is consistent with a relatively high affinity of interaction of Ras with NF1–334, as expected for the binding of NF1–334 to the [Leu<sup>61</sup>]Ras mutant. Since several species were present in this experiment, it was necessary to determine whether Ras-GTP was binding to NF1–334 or to the probe. Therefore, Ras-GTP was titrated into a mixture of 2 μM DPH-carboxylic acid and 1 μM NF1–334. In this experiment, the curve was shifted to the left with half-maximal reduction occurring at 0.5 μM Ras-GTP. These data are consistent with Ras binding to NF1–334 rather than to DPH-carboxylic acid. As a further control, normal Ras-GDP was titrated into the mixture of DPH-carboxylic acid and NF1–334, but only 20% reduction of fluorescence occurred at 4 μM Ras, consistent with the known weaker affinity of NF1 for Ras-GDP as compared with Ras-GTP.

These data suggested that the probe was able to monitor the interaction between Ras and NF1. Therefore, the effect of arachidonic acid on the fluorescence changes induced by Ras was examined (Fig. 6). The decrease in fluorescence caused by addition of Ras to the mixture of DPH-carboxylic acid and NF1–334 was largely abolished by 20 μM arachidonic acid, strongly suggesting competition between Ras and the lipid for binding to NF1. However, the dependence on concentration of arachidonic acid was not hyperbolic (Fig. 6B).

NF1/Ras Binding Scintillation Proximity Assay—The data from the kinetic characterization and use of the fluorescent probe suggested that arachidonic acid might, at least in part, be competing with Ras for binding to NF1 or GAP. We therefore tested whether arachidonic acid might block binding of Ras to NF1 by a more direct method. The recently described scintillation proximity assay procedure (28) was used to monitor the binding of Ras-GTP to NF1–334 and to test the effects of arachidonic acid on this interaction. In this assay, GST-NF1–334 fusion protein bound via an anti-GST antibody to protein A coated fluoromicrosphere beads interacts with a Ras<sup>[3H]GTP complex. When the Ras is in close proximity to the beads, i.e. when bound to NF1–334, scintillation occurs, whereas Ras in free solution does not cause any light emission. A key feature of the system is that it allows direct measurements of binding at
lipids might be artifactual. Other groups. These data suggested to us that some of the catalytic activity (data not shown), as Golubic et al. had shown by Golubic et al. (23) had demonstrated that arachidonic acid was not affecting binding of nucleotide to Ras, or binding between Protein A and antibody or between antibody and GST. In most experiments there was a non-hyperbolic dependence of inhibition on concentration of arachidonic acid, with a distinct lag at low inhibitor concentrations.

**DISCUSSION**

There are conflicting literature reports on the potency of arachidonic acid and phosphatidic acid, on their ability to differentially inhibit NF1 and p120-GAP activation of Ras-GTPase, and the mechanism by which this is achieved. We therefore investigated the effect of arachidonic acid on the interaction of Ras and GAPs using three different techniques in the simplest system possible.

Kinetic methods showed that in the absence of detergent, arachidonic acid inhibited the NF1- and p120-GAP activated activity of Ras-GTPase to similar extents with $I_{50}$ values of $5–15 \, \mu M$ (Fig. 1). These potencies are similar to those observed by Golubic et al. (23). In the presence of Nonidet detergent, the inhibitory potency of arachidonic acid on GAP activity was markedly reduced to levels reported by Bollag and McCormick (16) when they also used this detergent. We therefore performed all subsequent experiments in the absence of detergent. Under these conditions, we found phosphatidic acid to be only a weak reversible inhibitor of either GAP-334 or NF1-334 catalytic activity (data not shown), as Golubic et al. (23) had concluded, and in contrast to the more potent inhibition seen by other groups. These data suggested to us that some of the reported differential inhibition effects of NF1 and p120-GAP by lipids might be artifactual.

The mechanism of the inhibition of the NF1-activated Ras-GTPase was investigated in more detail by using the fluorescent analogue of GTP, mantGTP. The fluorescence decrease that occurs between RasmantGTP and RasmantGDP allowed the hydrolysis to be monitored continuously (Fig. 2). This has many advantages over the use of radiolabeled GTP, which requires analysis of many single time points. By varying the concentrations of both Ras-mantGTP and arachidonic acid, the effect of arachidonic acid on $K_{m}$ and $k_{cat}$ was established (Fig. 3). The inhibition was of a mixed character but predominantly competitive.

The second method to study the effect of arachidonic acid was to use the fluorescent probe DPH-carboxylic acid in equilibrium measurements of the interaction of Ras-GTP with NF1. Although DPH-carboxylic acid did not compete for the proposed arachidonic acid-binding site on NF1 (Fig. 5), as had been hoped, it still provided a probe on the interaction of NF1 with Ras-GTP, since the fluorescence of bound DPH-carboxylic acid was reduced on the binding of Ras-GTP (Fig. 6). Arachidonic acid largely abolished this effect (Fig. 6), strongly suggesting competition between the Ras and lipid for binding to NF1.

The third method to study the effect of arachidonic acid was to use a scintillation proximity assay (Fig. 7). Arachidonic acid completely abolished the binding interaction between [Leu$^{61}$]Ras-GTP and NF1, with 50% inhibition occurring at 5–10 $\mu M$.

All of the data above support the argument that arachidonic acid competitively inhibits the interaction of Ras-GTP with NF1. Furthermore, the inhibitory effects occur well below the CMC for arachidonic acid under the conditions used, showing that the effect is not caused by micelle formation, as had been suggested by Serth et al. (24).

Despite this evidence for competitive inhibition, our results suggest that arachidonic acid can exert other effects on the interaction. For example, the inhibition of the GAP-activated Ras-GTPase (Figs. 1 and 3) show a sigmoid or non-hyperbolic dose-response curve. In addition, the effect of arachidonic acid on the binding of Ras-GTP to NF1 as monitored by DPH-carboxylic acid (Fig. 6) or by the scintillation proximity assay (Fig. 7) is not hyperbolic. We do not know the explanation for this phenomenon, but similar behavior has been seen previously with arachidonic acid (23, 24). It is possible that self-association of arachidonic acid to structures smaller than full micelles is required for maximal inhibitory effects.

A further deviation from a simple competitive pattern was seen from the kinetic analysis (Fig. 3), which showed that arachidonic acid not only raised $K_{m}$ but also reduced $k_{cat}$. The effect on $k_{cat}$ is consistent with an irreversible inhibitory component for which we have some additional evidence (data not shown). In the scintillation proximity assay, we noted that when certain lipids (arachidic acid and phosphatidic acid, but not noticeably arachidonic acid) were added to NF1 before addition of Ras they were more potent inhibitors than when added after Ras, suggesting that the inhibitory mechanism was not always rapidly reversible on the time-scale of these experiments. Also, arachidonic acid caused slow time-dependent effects on the fluorescence of DPH-COOH bound to GAP, consistent with denaturation, which were significantly prevented by the inclusion of Ras.

Although these non-competitive effects occur, the predominately competitive nature of the interaction makes us conclude that arachidonic acid does not act in the way that phosphatidic acid was reported to do by Bollag and McCormick (16). They reported two mainst lines of evidence that phosphatidic acid does not block binding of Ras to NF1 (16). First, the inhibitor displayed non-competitive kinetics, in which the inhibitor reduced $V_{max}$ without affecting $K_{m}$, typical of the depletion of both substrate-bound and free enzyme forms by inhibitor. However, such an inhibitory pattern is also seen with an irreversible or slowly reversible type of action. Second, phosphatidic acid did not block binding of Ras-GTP to NF1 immobilized on beads (16).
No detailed quantitation (e.g. yield of bound proteins) or controls were given for this assay other than that SDS blocked binding. Experiments from our own laboratory (27, 28) showed that the rate of dissociation of NF1 from Ras is fast, such that no significant binding of Ras to NF1 should have been observed under the experimental conditions of Bollag and McCormick (16). Thus, the binding observed might not have been true reversible binding of NF1 to Ras. We found that dodecylmaltoside blocks binding of Ras to NF1 (28), again supporting the hypothesis that a primary mode of inhibition of amphiphilic molecules such as lipids and detergents might be by preventing Ras binding to GAPs.

On the basis that certain lipids differentially inhibited p120-GAP and NF1 and on the assumption that one could extrapolate from data with phosphatidic acid that lipids in general do not block binding of Ras to GAPs, Bollag and McCormick (16) suggested a hypothesis with these key features. (a) p120-GAP and NF1 are differentially regulated by lipids. (b) Lipids activate Ras signaling by inhibiting NF1 GTPase stimulating activity. (c) Lipid-inhibited NF1 binds to Ras-GTP propagating a signal through NF1. (d) NF1 and p120-GAP are alternative effectors for Ras. (e) The mitogenic activity of certain lipids is through their modification of GAP activity in the cell. However, the data presented in this paper are not in accordance with this hypothesis, as (a) no significant differential inhibition of activity was observed, and (b) lipid-inhibited GAPs do not bind to Ras. We would suggest two alternative hypotheses consistent with our data. If GAPs are indeed Ras effectors for a mitogenic pathway, the mitogenic activity of arachidonic acid cannot be accounted for through inhibition of GAP catalytic activities, since arachidonic acid also blocks binding between Ras and these putative effectors. Alternatively, the mitogenic activity of arachidonic acid may be accounted for by inhibition of GAP catalytic activity, but in this case GAPs are unlikely to be effectors for a mitogenic pathway.

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