MOLECULAR AND CELLULAR BIOLOGY, Nov. 1989, p. 5260–5264
0270-7306/89/115260-05$02.00/0

Vol. 9, No. 11

Inhibition by Phospholipids of the Interaction between R-ras, Rho, and Their GTPase-Activating Proteins

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Received 3 May 1989/Accepted 1 August 1989

Certain lipids were found to inhibit the interaction between rho and R-ras proteins and their respective GTPase-activating proteins (GAP). Inhibitory lipids were similar for each protein but differed significantly from those previously found to inhibit the interaction between ras protein and GAP activity. These data raise the possibility that ras and related proteins are controlled biologically by interactions between lipids and GAP molecules.

R-*ras* and *rho* are related to the proto-oncogene *ras* by sequence homology as well as by their ability to bind and hydrolyze GTP (11, 12). Ras is part of a signal transduction mechanism leading to cellular proliferation (14), but no function is known for either R-ras or rho. In contrast to control of classical G proteins involved in signal transduction (8), the biological and biochemical control of ras and ras-related proteins is poorly understood. A GTPase-activating protein (GAP) which is a potential negative regulator of ras activity has been characterized (16), but because ras-GAP remains active in proliferating cells its potential role in the control of ras (and therefore cellular proliferation) is unclear (10, 16). Recently, however, certain lipids whose metabolism is known to be altered in mitogenically stimulated cells were shown to disrupt the effect of ras-GAP upon purified ras protein (17). It is now clear that ras-GAP interacts with R-ras, whereas the rho protein interacts with a distinct smaller molecule, rho-GAP (6). In this study, a similar but nonidentical group of lipids is shown to disrupt the interaction between R-ras and GAP and between rho and rho-GAP. The biological significance of these observations is not yet clear, but the data raise the possibility that lipids might normally function to control the activity of ras-related proteins through interactions with GAP molecules.

The ras-related proteins R-ras and rho were purified from a bacterial expression system (6), and their GTPase activities were assayed by measuring the loss of radiolabeled γ-^{32}P from protein-bound GTP. The GTPase activities were relatively slow for rho and R-ras proteins in the absence of added GAP activity as previously observed for ras protein. Addition of GAP activity as a crude cytoplasmic extract increased the GTPase rate severalfold (Fig. 1). The presence of a 29-kilodalton protein with GAP activity specific for rho has been demonstrated in such extracts, along with the 125-kilodalton ras-GAP activity (6, 7). Previous studies indicate that the ability of ras-GAP to stimulate GTPase activity of ras protein was inhibited by certain lipids (17). We were anxious to test the possibility that lipids might also inhibit the GAP activity associated with other ras-related proteins. For this analysis, a variety of lipids were coincubated with rho protein and GAP-containing cell lysates. Certain of the lipids tested were able to inhibit the ability of rho-GAP to stimulate GTPase activity of rho protein (Table 1). In particular, 60 μg of added phosphatidic acid per ml inhibited rho-GAP activity virtually completely and restored the GTPase rate to a level indistinguishable from that of rho protein alone (Fig. 1A). For these studies, purified R-ras or rho proteins (2 μg) were preloaded with γ-^{32}P-labeled GTP by incubation of 22 μl of incubation buffer containing 20 mM Tris hydrochloride (pH 7.5), 25 mM NaCl, 0.1 mM dithiothreitol, 5 mM EDTA, and 14 μm γ-^{32}P-labeled guanosine triphosphate (10 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) at 30°C for 10 min. GTPase activity was then assayed by incubating the γ-^{32}P-labeled GTP-loaded protein (0.2 μg) at 30°C in 30 μl of reaction buffer (20 mM Tris hydrochloride [pH 7.5], 0.2 mM dithiothreitol, 1 mM $MgCl_2$, 1 mg of bovine serum albumin per ml, 3 mM unlabeled GTP, 0.1% octylglucopyranoside). GAP activity was added as a lysate of mouse brain (0.5 mg/ml [17]). The activity of various lysates differed. The activity of each lysate was therefore measured and maintained in the reactions reported at a level able to induce hydrolysis of 70 to 80% of bound, labeled nucleotide. Lipids were added to the reaction mixture as mixed micelles containing 100 μg of dipalmitoyl phosphatidic acid per ml for rho protein or 300 μg/ml for R-ras protein (Fig. 1B) along with 0.1% octylglucopyranoside. The amount of bound radioactivity was determined by filter binding on nitrocellulose (BA 85 [25 mm, 0.4-μm pore size]; Schleicher & Schuell, Inc., Keene, N.H.).

These results indicate that lipids can influence the interaction of both ras and the ras-related rho protein with their respective GAP molecules. There were similarities as well as clear differences in the types of lipids inhibitory for GAP activity associated with the two proteins. Previously, it was shown that phosphatidic acids containing arachidonic acid displayed maximal inhibitory activity for ras-GAP. Phosphatidic acids with saturated fatty acids were virtually inactive (17). With rho protein, on the other hand, phosphatidic acids containing saturated fatty acids were found to be highly active, while those containing unsaturated fatty acids (including arachidonic acid) exhibited less activity. In addition, while neither rho-GAP nor ras-GAP was inhibited by phospholipids present in normal membranes at high concentrations such as phosphatidylcholine or phosphatidylethanolamine, rho-GAP was inhibited to a limited extent by phosphatidylserine, diacylglycerol, and lyso-phosphatidic acid. These last three lipids did not inhibit ras-GAP. Inhibitions by phosphatidylinositol monophosphate and free arachidonic acid had similar efficiencies of inhibition for both ras and rho proteins (Table 1).

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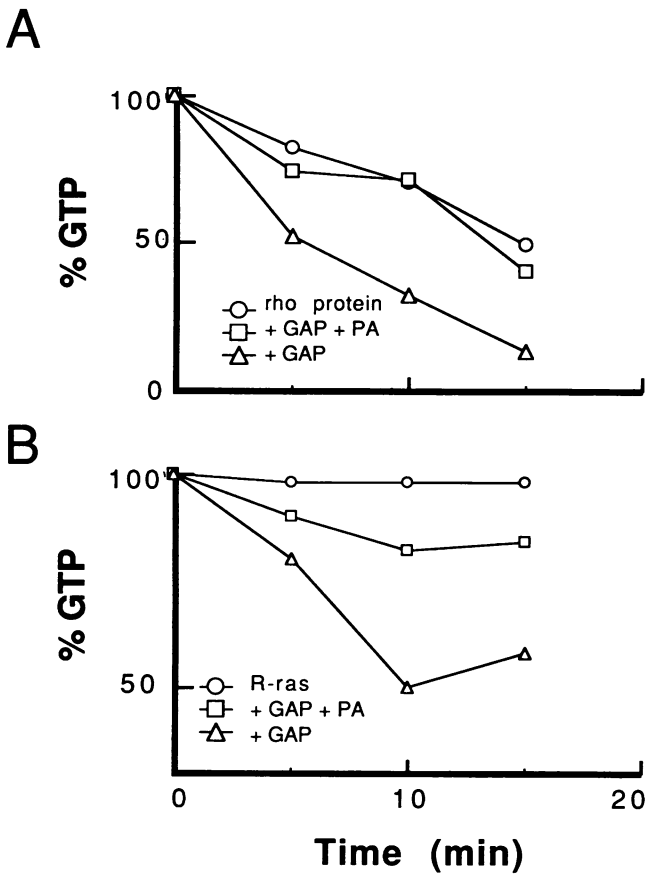

FIG. 1. Time course of inhibition of GTPase activity. Purified, bacterially synthesized rho (A) or R-ras (B) proteins were allowed to bind γ-^{32}P-labeled GTP and were then incubated for the times indicated. Labeled proteins were incubated either alone, in the presence of crude cytoplasmic lysate containing GAP activity, or in the presence of GAP activity along with added dipalmitoyl phosphatidic acid (PA). GAP activity was inhibited by the added lipid for each protein at all times tested. The percentage radioactivity indicates the counts per minute of γ-labeled nucleotide which remained associated with the protein at each time point indicated. GTPase activity would release the labeled phosphate. For the above-described experiments, samples of reaction mixture were removed and filtered at the times indicated. In separate experiments, added lipids were shown to have no direct effect upon the GTPase activity of ras or these ras-related proteins. In the case of ras proteins, extensive studies failed to demonstrate alterations in either GTPase activity or nucleotide exchange rates by added lipid alone.

It appears, therefore, that within the set of lipids tested, those which inhibit ras-GAP are also inhibitory for rho-GAP while other lipids are specifically inhibitory for rho-GAP. To further characterize the inhibition of rho-GAP, a dose-response analysis was performed with dipalmitoyl phosphatidic acid and rho protein. One-half maximal inhibition of rho-GAP activity was observed with approximately 5 μg of phosphatidic acid per ml, while maximal inhibition was observed with 45 μg/ml, resulting in complete inhibition of the ability of GAP to activate rho GTPase activity (Fig. 2A). No concentration of phosphatidic acid was observed to further reduce GTPase activity (below that observed with rho protein in the absence of added GAP activity). This indicates that the lipid is able to efficiently inhibit rho-GAP but cannot influence the basal GTPase rate of rho protein itself. A similar observation was previously observed with

TABLE 1. Inhibitions of GAP activity for different proteins by various lipids

Lipid[a]	% inhibition[b] of protein		
	ras[c]	rho	R-ras
Phospholipids			
Phosphatidylserine	0	50	0
Phosphatidylcholine	0	0	0
Phosphatidylethanolamine	0	0	0
Phosphatidic acid (arachidonyl, steroyl)	100	64	13
Phosphatidic acid (dipalmitoyl)	0	94	41
Phosphatidylinositol monophosphate	78	64	33
Diacylglycerol (dilinolein)	0	12	0
Lyso-phosphatidic acid	0	18	0
Fatty acids[d]			
Arachidonic acid (20:4)	100	56	49
Linoleic acid (18:3)	80	60	56
Arachidic acid (20:0)	0	0	0
Palmitic acid (16:0)	0	16	30

[a] Lipid concentrations were 100 μg/ml, except for R-ras, with 300 μg/ml. Lipids were prepared in 1% octylglucopyranoside and diluted 10-fold into the reaction mixture.
[b] Calculated as described in Fig. 2.
[c] Incubations involving ras protein included 0.15 M NaCl.
[d] Composition of fatty acids is indicated in parentheses by the number of carbon atoms followed by the number of double bonds.

ras protein and phosphatidic acid containing arachidonic acid (17). Each of the experiments described above involved GAP activity in the form of crude cytoplasmic lysate. To confirm that the activity observed was the result of the interaction of lipids directly with rho-GAP protein, an experiment was performed with 500-fold-purified (A. Hall, manuscript in preparation) human rho-GAP. We observed that dipalmitoyl phosphatidic acid was able to inhibit the interaction of this protein with purified rho protein as had been observed with the crude cytoplasmic lysates.

Mutational analysis of ras has identified a region of the protein which might function as an effector binding domain (15, 18). This region (amino acids 30 to 40) has been shown to be required for the interaction between ras protein and ras-GAP (1, 4). The fact that this sequence is different in the rho protein might explain why rho interacts with a separate GAP, which we have shown here is influenced by a distinct group of lipids. In contrast, although *ras* and R-*ras* exhibit only 55% overall homology, R-ras contains the same effector binding domain as ras protein (11), and both proteins interact with the same GAP molecule (6). We therefore thought it of particular interest to see whether the same lipids affected the interaction of ras-GAP with ras and R-ras proteins. R-ras protein was allowed to bind radiolabeled GTP and was then incubated with cytoplasmic extracts containing GAP activity in the presence or absence of various lipids. As with the two related proteins ras and rho, some lipids did inhibit the ability of GAP to stimulate the GTPase activity of R-ras protein. The extent of inhibition initially observed, however, was not as great as observed with ras protein or rho protein (Fig. 1B, Table 1). None of the lipids initially tested inhibited GAP activity by more than 50%.

Reduced inhibition of the GAP activity associated with R-ras might indicate that the most active lipids had not been tested, that optimal conditions were not employed, or that there were fundamental differences between ras and R-ras proteins in their interactions with GAP. An attempt was

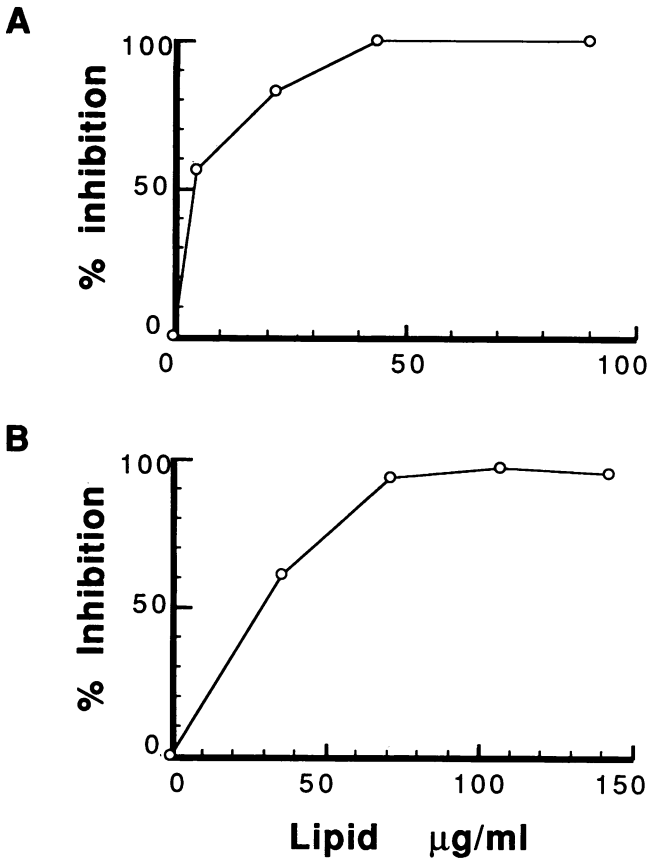

FIG. 2. Dose-response analysis of GAP inhibition by lipid. Purified rho (A) or R-ras (B) proteins were allowed to bind γ-^{32}P-labeled GTP and then incubated with GAP activity in the presence of the indicated concentrations of dipalmitoyl phosphatidic acid (A) or linolenic acid (B). In each case, there was a direct relationship between lipid concentration and inhibition. $MgCl_2$ (5 mM) was used in reactions with R-ras protein. Inhibition of 100% indicated that added lipid had restored the GTPase activity in the presence of added GAP protein to that observed in the absence of GAP. (The percent inhibition is calculated by determining the difference in the amount of label remaining associated with protein alone compared to that after incubation with both lipid and GAP. This value is divided by the difference in label associated with protein alone and after incubation with added GAP only.)

therefore made to determine whether more efficient inhibition of R-ras-GAP activity could be observed under other assay conditions. It was found that either increasing the magnesium ion concentration or presentation of the lipids as liposomes (see below) resulted in more efficient inhibitions of R-ras-GAP activity by added lipid. For example, 190 μg of dipalmitoyl phosphatidic acid per ml inhibited GAP activity associated with R-ras protein by 27% in 1 mM and by 77% in 5 mM $MgCl_2$. Under similar conditions, lipid (phosphatidic acid, arachidonoyl, steroyl [35 μg/ml]) inhibited GAP activity associated with ras protein by 81% at the lower magnesium concentration but by only 58% in 5 mM $MgCl_2$. Increased salt concentrations are known to inhibit GAP activity associated with ras protein. This was not found to be the case, however, with R-ras protein, for which increasing NaCl concentration by 0.1 M above standard conditions resulted in only a 5% decrease in GAP activity, compared to a 38% decrease with ras protein. It is clear that the inhibitions reported above required comparatively high phospholipid concentrations. However, fatty acids, particularly linolenic acid, appeared to have a much greater inhibitory activity for R-ras-associated GAP activity than the phospholipids tested. For the dose-response analysis presented for R-ras, 5 mM $MgCl_2$ was used (Fig. 2B). Under these conditions, 50% inhibition of R-ras-GAP activity was observed at approximately 30 μg of linolenic acid per ml, and complete inhibition of GAP activity was observed with 100 μg of lipid per ml. This was not as sensitive as inhibitions observed with rho protein but was similar to those previously reported for ras protein (17). For comparison, the in vitro concentration of diolein required to stimulate protein kinase C is near 1 μg/ml in a total lipid concentration of 20 μg/ml (5). Although the lipids found to inhibit GAP activity are known to increase within cells following a variety of stimuli, it is not known how their concentrations within membrane microenvironments might compare to the in vitro conditions described here. For purposes of comparison, all reactions (including those involving R-ras), other than the dose-response analysis presented above (Fig. 2B), were carried out under the uniform conditions found to be optimal for rho and ras proteins.

In addition to the reaction conditions, the structure of inhibitory lipids differed for the GAP activity of R-ras and ras proteins. As in the case of ras protein, neither phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, diacylglycerol, nor lysophosphatidic acid inhibited the effect of GAP upon R-ras. Each of the last three exhibited limited but reproducibly observable inhibition for rho protein. On the other hand, R-ras protein was similar to rho protein in that phosphatidic acid with saturated fatty acids was highly inhibitory, much more so than phosphatidic acid containing arachidonic acid. These results with R-ras have been duplicated with purified ras-GAP (together with M. S. Marshall and J. B. Gibbs, unpublished data). In general, it appears that phosphatidic acids with saturated fatty acids are more inhibitory for GAP activity associated with both rho and R-ras proteins, while the association of ras protein with GAP is more sensitive to phosphatidic acids with unsaturated fatty acids (Fig. 3). It is clear from these results, however, that GAP activity is inhibited by a limited set of lipids for each of the three members of this family of proteins tested to date. Arachidonic acid and phosphatidylinositol monophosphate are inhibitory to a similar degree for each of the three proteins, while saturated fatty acids had little inhibitory activity (Table 1). Since R-ras and ras proteins share the peptide sequence required for GAP activity and both interact with the same GAP molecule, the differences in the structure of inhibitory lipids must imply that protein sequences other than the conserved effector-binding domain influence the interaction with GAP.

In each of the experiments described above, the lipids were prepared in the form of mixed micelles with octylglucopyranoside (1%) to ensure that the physical form of the lipid did not vary between individual preparations or for separate types of lipid molecules. It was therefore important to demonstrate that lipids presented to rho and R-ras proteins in the form of liposomes could inhibit GAP activity. Independent studies confirmed that when phosphatidic acids were dried and then sonicated in buffer, liposomes with the ability to entrap buffer were formed. When liposomal phosphatidic acid was presented to rho protein, inhibitions similar to those reported above were observed. Liposomes appeared to be even more inhibitory for R-ras protein-associated GAP activity than the mixed micelles were. Presentation of dipalmitoyl phosphatidic acid (210 μg/ml) to

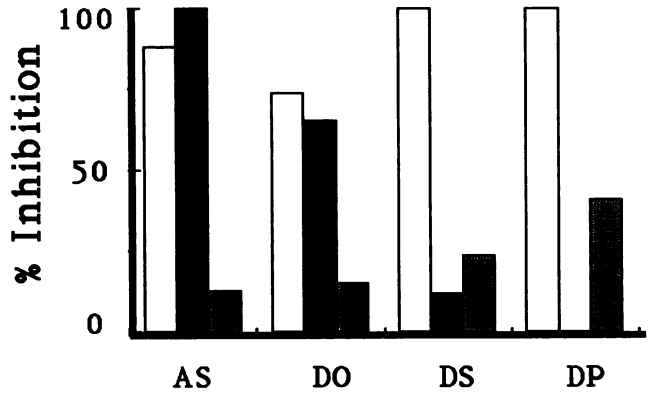

FIG. 3. Comparisons of GAP inhibition by different phosphatidic acids with different proteins. The percentage of inhibition of GAP activity in the presence of 100 μg of phosphatidic acid per ml was determined for ras (■), rho (□), and R-ras (▨) proteins. Phosphatidic acids differed in fatty acid composition as indicated and included two with exclusively saturated fatty acids (DP, dipalmitoyl; DS, disteroyl), one with unsaturated fatty acids (DO; dioleoyl), and a fourth (AS) with arachidonic acid and steric acid. R-ras and rho proteins share the ability to be inhibited most efficiently by phosphatidic acids with saturated fatty acids, while ras-associated GAP activity is inhibited minimally by these lipids and is more sensitive to phosphatidic acids containing unsaturated fatty acids. This observation is particularly interesting since R-ras and ras proteins can interact with the same GAP molecule. Calculations of percent inhibition are described in the legend to Fig. 2.

R-ras protein in the form of mixed micelles with octylglucopyranoside resulted in 60% inhibition of GAP activity, while the same amount of lipid in the form of liposomes resulted in 86% inhibition of GAP activity. For the preparation of lipid mixed micelles, 0.5 to 1.0 mg lipid dissolved in chloroform was dried under vacuum in a glass tube (12 by 75 cm). The thin layer of lipid coating the glass was then suspended in 1 ml of 1% octylglucopyranoside in 0.1 M Tris hydrochloride (pH 7.5) and briefly sonicated on ice with a probe sonicator. Liposomes of phosphatidic acid were prepared as described above except that octylglucopyranoside was omitted and sonication was more extensive (17). Inclusion of labeled nucleotide in the sonication buffer followed by passage of the product over a molecular exclusion column confirmed that liposomes had formed. In separate experiments, liposomes of phosphatidic acid or phosphatidylcholine were passed over Sepharose 4B molecular exclusion columns in the presence of ras-GAP-containing cytoplasmic lysates. The elution of GAP protein was determined by blotting and staining with anti-GAP antibodies (provided by J. Gibbs). GAP protein was not observed to associate with lipids under these conditions (unpublished data). It should be noted, however, that the bacterially expressed proteins analyzed here would not contain the types of lipid modifications recently described for ras proteins (9). Lipids were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Avanti Polar Lipids (Pelham, Ala.).

It is clear that for each of the proteins tested here, GAP activity can be inhibited by a limited set of lipids. The subtle differences in structural requirements of inhibitory lipids for each of the three proteins tested serves to emphasize the individuality of the interactions observed. Similar observations must be made for other ras-related proteins, but these observations raise the possibility that ras and related proteins might function in separate signal transduction pathways each controlled by interactions with lipids and GAP molecules. Because of the variety of ras-related proteins, these potential signal transduction pathways could be numerous. The fact that phosphatidic acids appear to be inhibitory to GAP activity in each case might explain the profound and varied effects of phosphatidic acids added to a variety of cells (13) and the fact that phosphatidic acids are produced by varied stimuli in different cell types (3).

The hypothetical signal transduction pathways described above would share the common feature that any means of controlling the production of active lipids in the correct membrane microenvironment would potentially influence the activity of the critical ras-related protein and therefore propagation of the corresponding signal. These signaling systems would therefore potentially be subject to both positive and negative control by multiple lipid metabolic enzymes whose net influence upon membrane composition would be reflected in the activity of the relevant ras-related protein. If ras participates in such a signal transduction pathway, it might be the focus of positive as well as negative proliferative signals which are known to influence cells. The initial observations that the interaction between ras and GAP can be controlled by lipids was suggested by biological experiments suggesting that ras activity is normally controlled by lipid metabolism (19). Other than this observation, however, the biological significance of the observations reported here await further investigation. It is generally believed, however, that ras and related proteins are biologically active only when bound to GTP (2). GAP activity would therefore be biologically inhibitory, while according to the model presented above, lipids would block the activity of GAP and maintain the ras-related proteins in an active state.

We thank Margaret Roudebush for technical assistance.

This work was supported in part by a Public Health Service grant from the National Institutes of Health.

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