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INHIBITORY EFFECT OF ARACHIDONIC ACID ON GTPase ACTIVATING PROTEIN IS ANTAGONIZED BY 1-STEAROYL, 2-ARACHIDONOYL GLYCEROL

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The intrinsic GTPase activity of cellular protein $p21^{ras}$ is strongly increased by cytosolic GTPase activating protein (GAP). The activity of this enzyme has been shown to be inhibited by arachidonic acid. We report here that this inhibition is antagonized by the presence of 1-stearoyl, 2-arachidonoyl glycerol. This effect is structure specific and dose dependent, being maximum at 200 µg/ml of diacylglycerol (DG). These results suggest that production of DG in response to hormones or growth factors stimulation could indirectly modulate the interaction between $p21^{ras}$ and GAP.

The Ha-ras proto-oncogene product p21 participates in the transduction of proliferative signals by a mechanism that is largely unknown (1). It is generally accepted that p21 is active when GTP is bound and becomes inactive upon hydrolysis of GTP to GDP, a reaction which is stimulated by GTPase activating protein (GAP) (2). GAP itself may be a target of p21 (3-4) or may regulate the interaction of p21 with its targets (5). GAP is a cytoplasmic protein which is found in almost all cells and tissues (6) with the highest concentration in the brain.

The cellular mechanism by which GAP regulates the hydrolysis of ras-GTP is still not clear. An indication comes from the study of

Abbreviations: GAP, GTPase activating protein; AA, arachidonic acid; DG, diacylglycerol; DAS, stearoyl-arachidonoyl glycerol; DO, dioleoylglycerol; DP, dipalmitoylglycerol; DPS, palmitoyl-stearoyl glycerol; PKC, protein kinase C.

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Downward and colleagues (7) who have demonstrated that stimulation of quiescent cells by phorbol ester (TPA) causes a very rapid increase in ras-GTP level. They, therefore, have suggested that activation of protein kinase C (PKC) is responsible for a decrease in GAP activity. It is however not clear how this interaction takes place. It has also been shown that stimulation of cells by growth factors results in tyrosine phosphorylation of GAP and its association with the growth factor receptor (8-9).

Stimulation of DNA synthesis by ras in many cells requires PKC activation (10). Indeed, a number of ras-transformed cell lines do contain high levels of DG and activated PKC (11-12). Endogenous diacylglycerol, the activator of PKC (13), is generated in a variety of cells in response to hormones or growth factors that stimulate phosphatidylinositol turnover (14). In ras-transformed cells DG has been shown to be produced by the hydrolysis of phosphatidylcholine (15). In many cell types diglycrol lipase catalyses the hydrolysis of DG and the resultant AA may be used for eicosanoid formation.

In vitro experiments (16-17) have shown that some lipids produced after mitogenic stimulation of the cells, e.g. phosphatidylinositol, phosphatidic acid, arachidonic acid (AA) but not DG, inhibit GAP activity, and therefore could be involved in the regulation of ras/GAP interaction. While Tsai et al. (16) have shown that DG does not inhibit GAP activity, high levels of DG under resting conditions and its elevation after cell stimulation raises the possibility that it might also be involved in controlling ras activity. Diacylglycerol may perhaps interfere with the association of these lipids with GAP by either having a synergistic effect on AA (such as the case with PKC) or an antagonistic effect (e.g. sphingosine effect on PKC). In the present study we demonstrate that in fact DG (1-stearoyl, 2-arachidonoyl) does antagonize the inhibitory effect of AA on GAP activity in a dose dependent manner.

Materials and Methods

All chemicals were purchased from Sigma. Rabbit anti-rat immunoglobulin was obtained from UBI (Lake Placid, New York). Ha-ras was over expressed and isolated from E. Coli as described (6) and stored at -80°C at 1 mg/ml. GAP was prepared from rat brain lysate according to Trahey and McCormick (2) and stored at -80°C.

GTPase assay The GTPase activity of GAP was determined by immunoprecipitation of ras bound-nucleotides by monoclonal antibody Y13-259 according to Tsai et al. (16) with modifications.

Briefly, purified bacterially synthesized Ha-ras was incubated with [α-^{32}P]GTP (3000 Ci/mmol, NEN) in a buffer containing 20 mM Tris-HCl, 1 mM DTT, 0.5 mM EDTA and 5 μM cold GTP for 10 min at 30°C. Lipid stocks were dissolved in Ch/M (2:1 by vol.) and stored at -20°C. Aliquots of lipids were dried under stream of N2 and redissolved in assay buffer (20 mM Tris-HCl, pH= 7.5) and dispersed by brief sonication, (Fisher, Model 300 Sonic Dismembrator). To this solution was added $MgCl_2$, BSA, NaCl, and cold GTP at final concentrations of 5 mM, 80 μg/ml, 115 mM, 5 μM respectively, 40 μl of brain lysate (produced about 80% hydrolysis) and [α-^{32}P]GTP-loaded ras (30 nM, final volume of 120 μl) and incubated for 20 min at 30°C.

Reactions were stopped by cooling to 4°C and ras was immunoprecipitated by the addition of ras antibody (Y13-259) and incubated at 4°C for 30 min. Protein A sepharose coated with rabbit anti-rat immunoglobulin was then added to the reaction mixtures and incubated for 30 min in cold (4°C) for the separation of ras-bound nucleotides. After washing the beads with washing buffer (20 mM Tris-HCl, 5 mM NaCl) the bound nucleotides were released from the beads by adding SDS (0.35%)- EDTA (1 mM) and boiling for 2 min. Eluted nucleotides were separated on PEI-Cellulose plates (EM Science) and developed in 1M KH_2PO_4 (pH=3.4). Corresponding GTP and GDP spots were then visualized and analyzed with a phosphorimager (Molecular Dynamics, CA).

Results and Discussion

Figure 1 shows the effect of AA and DG (1-stearoyl, 2-arachidonoyl) on GAP activity. Arachidonic acid at 200 μg/ml produced >90% inhibition of GAP activity. However, same concentration of DG was without effect. These results are in agreement with published data (16-18). Fig. 2 demonstrates that the inhibitory effect of AA is dose dependent; slight inhibition was obtained at AA concentration less than 50 μg/ml. The inhibition (about 90% of GAP activity) reached a plateau at ~200 μg/ml of AA. Previously, published data have reported different level of inhibition with AA (16-18), this might be due to the source of GAP and whether it is full length or a truncated protein.

The inhibitory effect of DG on GAP activity was tested using DG containing fatty acids with different chain length. Fig. 3 shows that none of the DG had any effect on GAP activity. However, when used in combination with arachidonic acid at 200 μg/ml, 1-stearoyl,2-arachidonoyl glycerol (DAS) decreased the inhibitory effect of AA on GAP activity (66%). The very slight decrease observed with 1-stearoyl, 2-palmitoyl glycerol (DPS) was not significant. To determine whether DG has any effect on ras protein itself, different

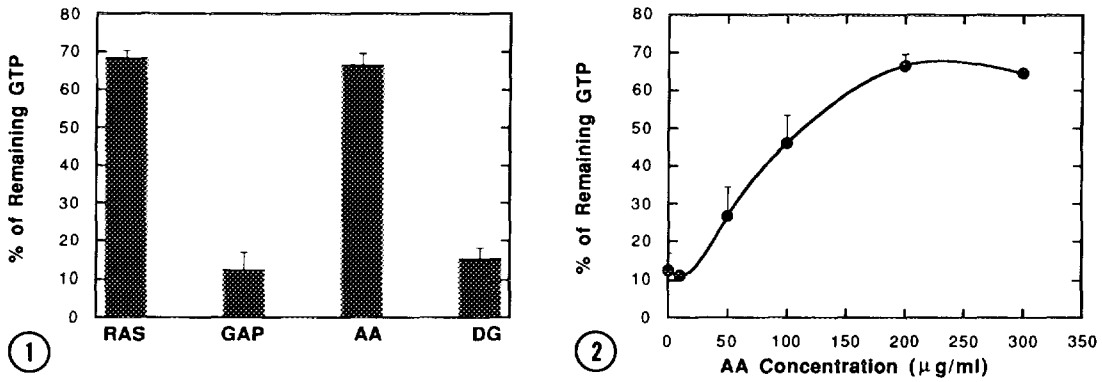


Fig. 1. Inhibition of GAP activity by AA and DG. Incubations were performed in the presence [α-^{32}P]GTP-bound ras alone (Ras), plus 40 μl of brain lysate (GAP) and in the presence of GAP and 200 μg/ml of arachidonic acid (AA) or 1-stearoyl, 2-arachidonoyl glycerol (DG). Data are the average of three separate determinations +/- SEM.

Fig. 2. Dose response inhibition of GAP by AA. The ability of GAP to stimulate hydrolysis of [α-^{32}P]GTP-bound ras decreased gradually as the concentration of AA increased in the incubation medium (50-300 μg/ml AA). Data are the average of three separate determinations +/- SEM.

concentrations of DG were incubated in the presence of [α-^{32}P]GTP-loaded ras without the addition of GAP. Figure 4 shows that DG did not stimulate nucleotide hydrolysis from [α-^{32}P]GTP-bound ras. The

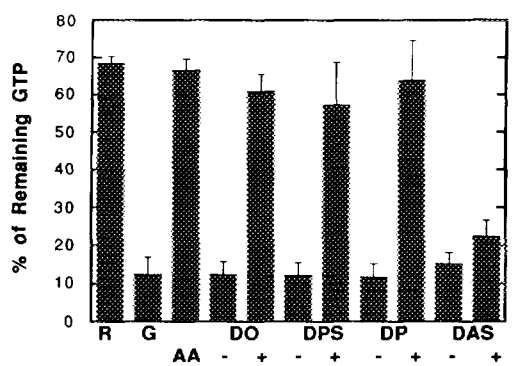


Fig. 3. Effect of diacylglycerol containing fatty acids with different chain length on GAP activity. Incubations were carried out in the presence of [α-^{32}P]GTP-bound ras alone (R), plus GAP (G) in the absence or presence of arachidonic acid (AA), and diacylglycerols; 1-stearoyl, 2-arachidonoyl (DAS), dioleoyl (DO), 1-stearoyl, 2-palmitoyl (DPS) and dipalmitoyl (DP) glycerol. Lanes with (+) sign indicate that the effect of diacylglycerols on GAP activity was measured in the presence of arachidonic acid. All substrates were used at 200 μg/ml. The experiment was repeated two times.

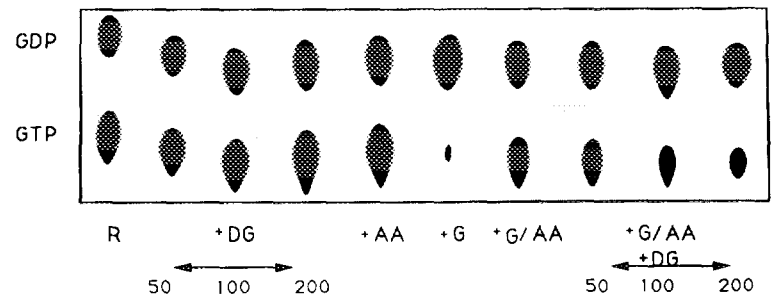
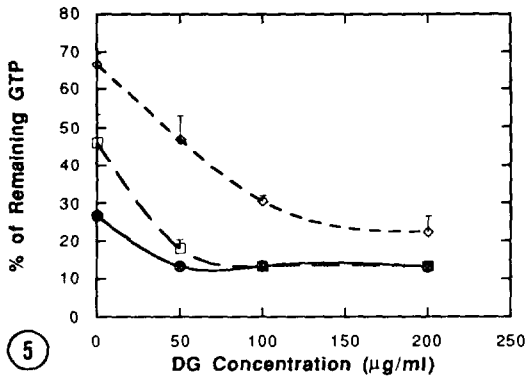


Fig. 4. The effect of 1-stearoyl, 2-arachidonoyl glycerol (DG) on hydrolysis of [α-^{32}P]GTP-bound ras. Incubation mixture contained [α-^{32}P]GTP-bound ras alone (R), ras with increasing concentrations of DG (+DG, 50-200 μg/ml) , ras plus 200 μg/ml arachidonic acid (+AA), ras plus GAP (+G) and ras plus GAP and 200 μg/ml of AA without (+G/AA) or with increasing concentrations of DG (+G/AA/DG). The experiment is repeated three times +/- SEM.

same observations was made in the presence of AA. When GAP and AA were added to the medium, DG reduced the inhibitory effect of AA in a dose dependent manner with the highest reduction at 200 μg/ml of DG. When we tested the effect of different concentration of AA in the presence of increasing concentration of DG (Fig. 5), it appeared that at equal concentration, DG blocks the inhibitory effect of AA on GAP activity. On the other hand, at fixed concentration of DG (100 μg/ml, Fig 6), increasing the level of AA (above 100 μg/ml) would restore the inhibitory effect of AA on GAP activity. It, therefore, seems that DG competes with AA for the lipid binding domain of GAP.

Although in vitro studies clearly demonstrate that AA and some other long chain polyunsaturated fatty acids (unpublished data) inhibit GAP activity, the physiological significance of these inhibitions remains to be determined. According to Price et al. (15), loading of cells with oncogenic ras results in elevation of DG prior to the release of AA. With regard to this, high concentrations of AA should be produced to overcome the effect of DG and inhibit GAP activity.

In many types of cells DG is generated in response to stimulation by hormones or growth factors. The endogenous DG is thought to signal cellular events by serving as PKC activator (13). However, it has been shown that the accumulation of DG in the cellular membrane could perturb bilayer structure and also affect several membrane enzymes (phospholipase A_2, phospholipase D) and non-PKC dependent cell membrane trafficking (19-20). The present



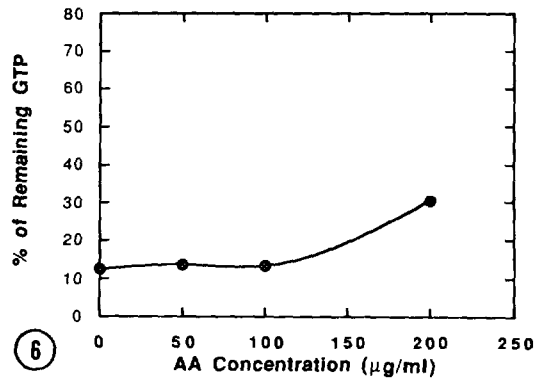


Fig. 5. Effect of 1-stearoyl, 2-arachidonoyl glycerol (DG) on hydrolysis of [α-^{32}P]GTP-bound ras in the presence of different levels of arachidonic acid. Hydrolysis of [α-^{32}P]GTP-bound ras was measured in the presence of GAP, increasing concentrations of DG and 50 (•-•), 100 (□-□) and 200 (◊-◊) µg/ml of AA. Data are the average of three separate determinations +/- SEM.

Fig. 6. Effect of increasing concentration of AA on GAP activity in the presence of 100 µg/ml DG (1-stearoyl, 2-arachidonoyl). Hydrolysis of [α-^{32}P]GTP-bound ras was determined in the presence of GAP, 100 µg/ml of DG and increasing concentration of AA. Data are the average of two separate assays.

data indicate that accumulation of DG in the membrane might also be involved in (perhaps indirect) regulation of GAP activity and thereby regulate cellular proliferation through p21^{ras}.

It has been suggested that stimulation of cells by growth factors (EGF, PDGF) causes phosphorylation of GAP and its association with growth factor receptors (8-9). Sequestration of GAP to the receptor may serve to remove GAP from cytoplasm and modify its action, in some way allowing ras to remain in its GTP-bound form. Our data suggest that once bound to the membrane receptor the activity of GAP could also be modulated by membrane lipids.

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