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ABSTRACT

Previous results from our group have indicated that arachidonic acid decrease cAMP production through a modification of heterotrimeric G proteins. In the present study, we have characterized the high affinity GTPase activity present in Leydig cell membranes and its regulation by fatty acids. The high-affinity GTPase activity, measured as [γ-32P]GTP hydrolysis rate, was both time and protein concentration dependent. Arachidonic acid elicited a dose-dependent inhibition of enzyme activity with an IC50 = 26.7 ± 1.1 μM. The existence of only two double bonds in linoleic acid is reflected by a decrease in its inhibitory activity (IC50 = 34 ± 2.3 μM). Saturated fatty acids showed no effect at this level. The kinetic analysis as interpreted by Lineweaver-Burk plots, indicated that 50 μM arachidonic acid had no effect on the apparent affinity for GTP, but resulted in a 40% decreases in the maximal velocity of the reaction. Arachidonic acid modulation of GTPase activity was not attenuated by blocking eicosanoid metabolism with inhibitors of 5-lipoxygenase, cyclooxygenase, or epoxygenase P-450. The addition of arachidonic acid to pertussis toxin-treated membranes had no effect on the enzyme activity, indicating that arachidonic acid does not modify the GTPase activity present in Gαi protein. However, ADP-ribosylation with cholera toxin followed by arachidonic acid treatment led to a further 40% inhibition when compared with cholera toxin treatment alone. These results allowed us to postulate that arachidonic acid inhibits the GTPase activity of Gαi protein family. To further analyze the mechanism of arachidonic acid inhibition of GTPase activity, the effect of arachidonic acid on the [35S]GTPγS binding was studied. No effect of this fatty acid on GTP binding was found. Combining our previous results with those found here, we can conclude that arachidonic acid maintains Gαi proteins in their active state, which in turn inhibit adenylate cyclase and results in decrease cAMP levels. (Endocrinology 141: 1093–1099, 2000)

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M ost of the biological functions of unsaturated fatty acids are due to their ability to act as second messengers or modulators of the activities of functionally important proteins. Fatty acids can act on signal transduction pathways by direct and/or indirect means. However, several studies clearly show that fatty acids per se are messenger and modulator molecules that can mediate cell responses to extracellular signals (1). Arachidonic acid (AA), which is esterified to the sn-2 position of membrane phospholipids, is mainly released from phospholipids by activation of phospholipase A2 (PLA2). It can be also produced by activation of phospholipase C (PLC) followed by diacylglycerol hydrolysis by diacylglycerol lipase; phospholipase D (PLD) followed by phosphatidic acid phosphohydrolase and diacylglycerol lipase; phospholipase A1 (PLA1) followed by phosphatidic acid phosphohydrolase and diacylglycerol lipase; and finally, phospholipase A1 (PLA1) followed by lysophospholipase (2).

In Leydig cells, steroidogenesis is regulated by LH, via cAMP and other second messengers such as calcium, chloride ions, and arachidonic acid and/or its metabolites (3, 4). Recent results have shown that LH causes a dose- and time-dependent release of arachidonic acid from Leydig cells (5). Furthermore, arachidonic acid itself has been reported to act as an additional intracellular messenger associated with the hormonal action of LH (6–8). Previous results from our group have shown that in rat Leydig cells that arachidonic acid exerts a dose- and time-dependent biphasic effect on LH- and dibutyryl-cAMP-stimulated testosterone production (9). During short periods of incubation this fatty acid inhibits testosterone synthesis by decreasing cAMP levels. (Endocrinology 141: 1093–1099, 2000)
present work was to determine if the effect of arachidonic acid on G proteins is due to a modification of its GTPase activity.

Materials and Methods

DMEM was obtained from Life Technologies, Inc. (Middlesex, UK). Collagenase (Type I) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Arachidonic acid (free acid, sealed ampoule), linoleic acid (sealed ampoule), stearic acid (free acid), BSA (essentially fatty acid free) (BSA-FAF), ATP, GTP, creatine kinase, creatine phosphate, Percoll, trypsin inhibitor (1% sterile-filtered solution) indomethacin, nordihydroguaiaretic acid (NDGA), clertrimazole, pertussis toxin (PTX), and cholera toxin (CTX), were purchased from Sigma Química (Alcobendas, Spain). [γ-32P] GTP and [35S]GTPγS were obtained from NEN Life Science Products (Madrid, Spain). All other chemicals used were of analytical reagent grade.

Cell isolation and purification

Leydig cells were isolated from 200–300 g Sprague Dawley rats from the Animal House of the University of Alcala. All animals were cared for according to the guidelines of the University Committee of Animal Resources at the University of Alcalá. The testes were decapsulated and subjected to longitudinal shaking (65 strokes/min) with collagenase (0.5 mg/ml) and trypsin inhibitor (20 μl/ml) for 40 min at 37°C. The cells were filtered through 60 μm nylon gauze to remove fragments of seminiferous tubules and subjected to centrifugal elutriation followed by Percoll density gradient (0–90% vol/vol) centrifugation (12). Leydig cell purity was routinely >95%, as determined by 3β-hydroxysteroid dehydrogenase cytochemistry (12).

Membrane preparation

Purified Leydig cells were disrupted by freeze-thawing (4 times) in a buffer containing Tris-HCl 50 mM, pH 7.5, 3 mM EDTA, 5 μg/ml soybean trypsin inhibitor, and 0.1 mM phenylmethylsulfonyl fluoride. Broken cells were centrifuged at 400 × g for 2 min. at 4°C and the supernatant was centrifuged at 100,000 × g for 15 min at 4°C. The final pellet containing the crude plasma membranes were suspended in the same buffer and 50 μl aliquots were stored at −70°C.

Toxin treatments (PTX and CTX)

For in vitro ADP ribosylation, the toxins were activated by incubation with 100 mM DTT for 30 min at 37°C.Activated toxins were diluted in ribosylation buffer as described in Ref. 13 with 5 mM NAD. Samples were exposed to the toxins, 0.125 μg PTX per 1.5 μg proteins and 0.3 μg CTX per 1.5 μg proteins for 30 min at 30°C, before performing the GTPase assays. The time course of ribosylation was followed with [32P]NAD in the presence of PTX or CTX as described previously (13). Labeled proteins were separated on SDS/PAGE gels, dried, and autoradiography was performed by exposing the gels to x-ray film. The results of these experiments indicated that a 30 kDa band was maximally labeled at 30 min with PTX treatment. At this same time, two bands with molecular weights of 50 and 52 kDa were maximally labeled with CTX and were chosen for subsequent ribosylation experiments (data not shown).

GTPase assay

Hydrolysis of [γ-32P]GTP was measured essentially as described by Schepers et al. (14). Briefly, the reaction mixture (70 μl) contains: 50 mM triethanolamine-HCl, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 5 mM MgCl2, 100 μM ATP, 100 mM NaCl, 0.4 μg/ml creatine kinase, 5 μM creatine phosphate, 2 μg/ml BSA, 1 μM ouabain, 200 mM GTP, and 30 mM [γ-32P]GTP plus 10 μl of the appropriate concentration of fatty acid or vehicle. The reaction was initiated by the addition of 20 μl of membrane protein (1.5 μg) to each tube (except the protein concentration experiments). The reaction was carried out at 30°C for 10 min (except for the time-course experiments) and terminated by the addition of 700 μl of ice cold activated charcoal (5% in 20 mM phosphoric acid, pH 2.5) after 10 min at 4°C. Assay tubes were centrifuged for 15 min at 4°C at 3,000 × g. Radioactivity in the supernatant was assayed by liquid-scintillation counting to determine the release of 32Pi. Low-affinity hydrolysis of [γ-32P]GTP was assessed by parallel incubation of membranes with excess (250 μM) GTP. The low-affinity activity was subtracted from the total to calculate high-affinity GTPase activity. Results were expressed as pmol of Pi released per min per mg protein.

GTPγS binding assay

Guanine nucleotide binding by G protein α subunit was determined by incubating membranes (2 μg of proteins) in 100 μl total volume of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM DTT, 5 mM MgCl2, 1 mM EDTA, 0.1 μM [35S]GTPγS, and increasing concentrations of unlabeled GTPγS. The reaction was carried out at 30°C for 45 min (equilibrium conditions), and samples were filtered through a Whatman GF/C filter, and washed with ice-cold (2 × 2 ml) buffer consisting of 50 mM Tris-HCl pH 7.4, 5 mM MgCl2. Nonspecific binding to proteins, assessed by quantitating the binding in the presence of 100 μM unlabeled GTPγS, was subtracted from each value to calculate the specific binding. The radioactivity bound to the filter was measured by liquid scintillation counting. The results were expressed as GTP bound (% of total radioactivity added).

The protein concentrations were determined by the method of Bradford (15), using BSA as a standard.

All data are expressed as mean ± SEM from, at least, three experiments, each of which was performed in duplicate. Each experiment is carried out with membranes obtained from a new isolation. Student’s t test was used for statistical analysis and differences were considered significant when P < 0.05

Results

Characterization of G protein-linked GTPase activity in Leydig cell membranes

The high-affinity GTPase activity was determined by measuring the amount of 32P released at 30°C following incubation for different time periods. The GTPase reaction was found to be linear for 15 min. (Fig. 1A). There was also a linear relationship between membrane protein and GTP hydrolysis up to concentrations of 2 μg of membrane protein, reaching a plateau with higher protein concentrations and 10 min incubation times (Fig. 1B). Based on these findings, subsequent experiments were carried out using 1.5 μg of protein and an incubation time of 10 min. Under these conditions, the basal activity was about 50 pmol per min per mg protein.

Arachidonic acid inhibition of high-affinity GTPase activity

Leydig cell membranes were preincubated with arachidonic acid (1, 50, and 100 μM) for different time periods (1–10 min) and GTPase activity was determined. As illustrated in Fig. 2A, there was a time-dependent inhibition of GTPase activity both at 50 μM and 100 μM arachidonic acid. No inhibitory effect was found with 1 μM arachidonic acid at any of the preincubation times assayed. One minute of preincubation with 100 μM arachidonic acid produced approximately a 50% inhibition of the GTPase activity, whereas 2.5 min was necessary to obtain the same level of inhibition when 50 μM of the fatty acid was used. A 60.5 ± 3.7% (n = 18) inhibition of GTPase activity was obtained when the membranes were preincubated for 5 min with 50 μM arachidonic acid and these conditions were used for the subsequent experiments. The dose response effect of arachidonic acid on GTPase activity was studied using doses between 1 and 100 μM and 5 min of preincubation (Fig. 2B). This fatty acid inhibited the high-affinity GTPase activity in a concen-
concentration-dependent manner with an IC₅₀ value of 26.7 ± 1.1 μM. The kinetic analysis of the high-affinity GTPase activity, and its modification by arachidonic acid, was performed by employing Lineweaver-Burk plots of the data obtained as a function of GTP concentrations. A linear relationship obtaining a maximum velocity (Vₘₐₓ) of 3.06 ± 0.3 nmol per min per mg protein was observed. A Michaelis Menten constant value (Kₘ) of 3.36 ± 0.48 μM for control membranes was also observed. Arachidonic acid at a concentration of 50 μM, had no effect on the apparent affinity (Kₘ = 3.45 ± 0.24 μM), but produced a 40% of reduction of the maximal velocity (Vₘₐₓ = 1.88 ± 0.15 nmol per min per mg protein (Fig. 3).

FIG. 1. High-affinity GTP hydrolysis as a function of reaction time and protein concentration. A, The amount of ³²P released from 30 nM [γ³²P]GTP was determined after different incubations times at 30 C with 1.5 μg of protein. B, The amount of ³²P released from 30 nM [γ³²P]GTP was determined after 10 min of incubation with different proteins concentrations. The high-affinity GTPase activity was calculated by subtracting the amount of [γ³²P]GTP hydrolyzed in the presence of 250 μM unlabeled GTP from the total amount of ³²P released and is expressed as pmol of Pi released/mg protein (A) and pmol of Pi released/min (B). All data are the mean ± SEM of three separate experiments, each of which was performed in duplicate. Each experiment was carried out with membranes obtained from a new isolation.

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FIG. 2. Arachidonic acid inhibition of high-affinity GTPase activity in Leydig cell membranes. A, Leydig cell membranes (1.5 μg) were pre-incubated with arachidonic acid (1, 50, and 100 μM) at indicated time periods, and the high-affinity GTPase activity was determined after 10 min of incubation. B, Leydig cell membranes (1.5 μg) were pre-incubated during 5 min with increasing concentrations of arachidonic acid (1–100 μM), and the high-affinity GTPase activity was determined after 10 min of incubation. The high-affinity GTPase activity was calculated by subtracting the amount of [γ³²P]GTP hydrolyzed in the presence of 250 μM unlabeled GTP from the total amount of ³²P released and is expressed as pmol of Pi released/min per mg protein. All data are the mean ± SEM of four separate experiments, each of which was performed in duplicate. Each experiment was carried out with membranes obtained from a new isolation.

Arachidonic acid modulation of high-affinity GTPase activity is direct and not mediated via its metabolites

To examine if the inhibition of GTPase activity by arachidonic acid is due to the fatty acid itself or to its transformation to active metabolites, we added inhibitors of the arachidonic acid metabolizing enzymes, namely: 5 μM indomethacin for cyclooxygenase, 5 μM NDGA for lipoxygenase, and 5 μM Clotrimazole for epoxygenase P450 (16) together with arachidonic acid. The high-affinity GTPase activity was inhibited by arachidonic acid (50 μM) by about 60%, and the same percentage of inhibition was obtained in the presence of inhibitors of arachidonic acid metabolism. The high-affinity GTPase activity was similar to control when the three in-
Hibitors were assayed together without arachidonic acid.

(Fig. 4)

Relationship between the insaturation degree and GTPase activity

The dose response inhibitory action of arachidonic acid on GTPase activity was then compared with that of other fatty acids having different degrees of unsaturation, mainly linoleic acid (18:2) and stearic acid (18:0). Arachidonic acid was the most potent inhibitor of GTPase activity with an IC\textsubscript{50} \(\approx 26.7 \pm 1.1 \) \(\mu\)M. For linoleic acid, the possession of only two double bonds, was apparently reflected in its diminished activity (IC\textsubscript{50} \(\approx 34.0 \pm 2.3 \) \(\mu\)M). Stearic acid with no double bonds, showed little effect on high-affinity GTPase activity, suggesting a role for the degree of unsaturation in the inhibition of GTPase activity (Fig. 5).

Arachidonic acid modulates GTPase activity of \(G_i\) proteins

We then studied the modification of GTPase activity after treatment of membranes with pertussis toxin (PTX). This toxin catalyzes the ADP-ribosylation of a specific cysteine residue in the \(G_\alpha\) family. This covalent modification inactivates these \(\alpha_i\)-subunits, such that they cannot exchange GDP for GTP (17). Under these conditions, the GTPase activity detected in the assay is only due to PTX-insensitive G proteins. In fact, the GTPase activity present in PTX-treated membranes was about 30% lower than that found in the control. The addition of arachidonic acid to PTX-treated membranes had no effect on the enzyme activity, a clear indication that arachidonic acid does not modify the GTPases activity present in PTX-insensitive G proteins (Fig 6). To analyze the involvement of \(G_i\) proteins in arachidonic acid action, we studied the GTPase activity after treatment of membranes with cholera toxin (CTX), which catalyzes the ADP-ribosylation of a specific arginine residue in \(G_\alpha\) family (17). This covalent modification inhibits the intrinsic GTPase activity of these \(\alpha_i\)-subunits so that, under these conditions, the GTPase activity detected in the assay is only due to CTX-insensitive G proteins (G\(\alpha\) proteins). As shown in Fig. 6, ADP-ribosylation with CTX followed by arachidonic acid treatment led to a 40% of inhibition when compared with CTX-treatment alone. These results clearly indicate that arachidonic acid inhibits the GTPase activity of the \(G_i\) family of proteins.
Lack of effect of arachidonic acid on GTP\(\gamma\)S binding

The inhibitory effect of arachidonic acid on GTPase activity might be due to either direct effects on the intrinsic GTPase itself, or a decrease in GTP binding. To test this second possibility, we studied the \([35S]GTP\gamma S\) binding in Leydig cell membranes. Under optimized conditions, GTP\(\gamma\)S binding was linear up to a protein concentration of 5 \(\mu g/tube\) and was also time dependent (at 30 min; data not shown). Increasing concentrations of GTP competitively inhibited \([35S]GTP\gamma S\) binding to membranes and arachidonic acid had no effect on this binding. Thus, inhibition of GTPase activity by arachidonic acid cannot be attributable to an inhibition in GTP binding (Fig. 7).

Discussion

Previous results from our group have indicated that inhibition of steroidogenesis by arachidonic acid in Leydig cells result from a suppression of hormone-stimulated cAMP levels by activation of \(G\) \(_i\) proteins (10). In the present work, we have characterized the high-affinity GTPase activity of \(G\) proteins in Leydig cell membranes and their regulation by arachidonic acid. Several reports have postulated that arachidonic acid regulates GAP (GTPase activating protein)-stimulated GTPase activity, with important regulatory effect on cellular function (18–23). However, little is known about the regulation of high-affinity GTPase activity by fatty acids.

Our present results clearly indicate that arachidonic acid produces a time- and dose-dependent inhibition of high-affinity GTPase activity with an IC\(_{50}\) of 26.7 \(\mu M\). In fetal guinea-pig lung membranes, arachidonic acid seems to play an important role in the developing organ by decreasing GTPase activity (16), but with a IC\(_{50}\) = 48 \(\mu M\). In rat and human islet membranes (24), high arachidonic acid concentrations (330 \(\mu M\)) produce insulinotropic effects due, in part, to its ability to maintain G proteins in their active configuration by decreasing GTP hydrolysis.

It is well known that arachidonic acid acts indirectly via its metabolites (21), and directly by modulating G protein-mediated signals (2, 18, 20). In this context, our results clearly indicate that the action of arachidonic acid on G proteins in Leydig cell membranes is direct and not due to its transformation to its active metabolites, because the inhibitory effect is not reversed in the presence of arachidonic acid metabolism inhibitors. This is consistent with our previous report showing that arachidonic acid itself decreases the LH-stimulated cAMP production in Leydig cells (9) and by others, indicating that unsaturated fatty acids can interact with signaling proteins \textit{in vitro} and modulate their activities. For example, arachidonate and related unsaturated fatty acids physically associate with and inhibits the activity of the Ras GTPase activating protein known as GAP (20). Such lipids can also regulate the association of the Ras-related protein, Rac, with a specific GDP dissociation inhibitor (18). In addition, an apparent relationship between the potency of fatty
acid inhibition of GTPase activity and the number of double bonds in the fatty acid molecule has been noted by other workers and is consistent with the data presented herein. For example, when bacterially synthesized c-Ha-ras protein (Ras) was incubated with GTPase-activating protein in the presence of saturated or unsaturated fatty acids, 150 μM arachidonic acid blocked GTPase-activating protein activity by 88%, whereas linoleic acid (18:2) was 33% inhibitory and saturated fatty acids (palmitic, stearic) showed no effect at similar concentrations (20).

The GTPase assay, performed in ADP-ribosylated membranes, clearly indicated that arachidonic acid acts on a CTX-insensitive (PTX-sensitive) G_i family of proteins. The mechanism by which arachidonic acid inhibits GTPase activity is not yet known, but present results indicate that affinity for GTP binding is maintained by arachidonic acid.

Previous results from our group have indicated that arachidonic acid inhibits LH-stimulated cAMP production in Leydig cells (9) by activation of a G_i protein (10). The results presented in this paper indicate that arachidonic acid inhibits the GTPase activity of G_i proteins without a modification of GTP binding. This implies that arachidonic acid maintains G_i proteins in their activated state, and thereby, are able to inhibit adenylate cyclase. It is reasonable to postulate that this mechanism by which arachidonic acid decreases the LH-stimulated cAMP and testosterone accumulation previously reported by us (9, 10). It is not completely clear whether the LH receptor is coupled to G_i, but several reports have indicated that G_i, in addition to G_s, may also have a role in the control of Leydig cell cAMP production (27–29). More recently Rajagopalan-Gupta et al. (30) have demonstrated that activation of LH receptor promotes activation of G_s in addition to G_i. Therefore, the present results together with those indicating that arachidonic acid is released after LH-receptor interaction (5) clearly point out the mechanism by which arachidonic acid acts as a second messenger in steroidogenesis in Leydig cells.

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References

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Fig. 7. Lack of effect of arachidonic acid on GTP binding. Leydig cell membranes (2 μg) were incubated with [35S]GTPγS (0.1 μM) and increasing concentrations of unlabeled GTPγS (10−2–10−4 μM), in the presence and absence of 50 μM of arachidonic acid during 45 min. The GTP binding was expressed as percentage of total radioactivity added. All data are the mean ± SEM of six separate experiments, each performed in duplicate. Each experiment was carried out with membranes obtained from a new isolation.
accompany the activation of all heterotrimeric G proteins? Cell Signal 9:141–151