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Regulation of Phosphatidate Phosphatase Activity from the Yeast *Saccharomyces cerevisiae* by Nucleotides*

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Regulation of *Saccharomyces cerevisiae* membrane-associated phosphatidate phosphatase (3-*sn*-phosphatidate phosphohydrolase, EC 3.1.3.4) activity by nucleotides was examined using pure enzyme and Triton X-100/phosphatidate-mixed micelles. Adenosine, guanosine, cytidine, and uridine nucleotides inhibited phosphatidate phosphatase activity in a dose-dependent manner. ATP and CTP were the most potent inhibitors of the enzyme. A kinetic analysis was performed to determine the mechanism of enzyme inhibition by nucleotides. The mechanism of inhibition by ATP and CTP with respect to phosphatidate (the substrate) was complex. The dependence of phosphatidate phosphatase activity on phosphatidate was cooperative, and nucleotides affected both V_{max} and K_m. ATP did not inhibit phosphatidate phosphatase activity by binding to the enzyme or to phosphatidate. Phosphatidate phosphatase dependence on Mg^{2+} ions (the cofactor) followed saturation kinetics, and the mechanism of nucleotide inhibition with respect to Mg^{2+} ions was competitive. Thus, the mechanism of enzyme inhibition by nucleotides was the chelation of Mg^{2+} ions. The inhibitor constant for ATP was lower than its cellular concentration in glucose-grown cells. However, the inhibitor constant for ATP was higher than its cellular concentration in glucose-starved cells. Changes in the cellular concentration of ATP affected the proportional synthesis of triacylglycerols and phospholipids. These results were consistent with the regulation of phosphatidate phosphatase activity by ATP through a Mg^{2+} ion chelation mechanism.

PA[1] phosphatase (3-*sn*-phosphatidate phosphohydrolase, EC 3.1.3.4) catalyzes the dephosphorylation of PA to yield diacylglycerol and P_i (1). This enzyme plays a major role in the synthesis of phospholipids and triacylglycerols in eucaryotic organisms (2–5). PA phosphatase also plays a role in mammalian cell signaling mechanisms as part of the phospholipase D-PA phosphatase pathway for phosphatidylcholine-derived diacylglycerol (6). This diacylglycerol is responsible for the sustained activation of protein kinase C (6). Owing to its tractable molecular genetic system, we are using *Saccharomyces cerevisiae* as a model eucaryote to study the regulation of PA phosphatase activity and its role in lipid metabolism.

Membrane-associated PA phosphatase is a highly regulated enzyme in *S. cerevisiae*. Enzyme activity is regulated by growth phase (7–9), inositol supplementation (8, 9), phosphorylation via cAMP-dependent protein kinase (10), and by sphingoid bases (11). Regulation of PA phosphatase activity by phosphorylation (10) and sphingoid bases (11) is attributed to biochemical mechanisms, whereas regulation of activity by growth phase (9) and inositol (9) is attributed to genetic mechanisms. The substrate and product of the PA phosphatase reaction are found at branch points in the pathways leading to the synthesis of phospholipids and triacylglycerols (2) (Fig. 1). Thus, it would be anticipated that the enzyme should play a role in the proportional synthesis of these lipids. Indeed, regulation of PA phosphatase activity by growth phase, inositol, and phosphorylation correlates with changes in the synthesis of phospholipids and triacylglycerols (7, 8, 10, 12).

The nucleotides ATP and CTP are substrates in the pathways leading to the synthesis of the energy-rich intermediates CDP-ethanolamine and CDP-choline (2) (Fig. 1). These intermediates combine with the product of the PA phosphatase reaction, diacylglycerol, to form phosphatidylethanolamine and phosphatidylcholine, respectively. CTP also reacts with the substrate of the PA phosphatase reaction, PA, to form CDP-diacylglycerol (2) (Fig. 1). CDP-diacylglycerol is used in an alternative pathway for phosphatidylethanolamine and phosphatidylcholine synthesis (2). Since nucleotides are used in reactions involving the substrate and product of the PA phosphatase reaction, we questioned whether they played a role in the regulation of PA phosphatase activity. The availability of pure preparations of PA phosphatase from *S. cerevisiae* (9, 13) allowed us to examine the effects of nucleotides on enzyme activity in a well defined system. We found that nucleotides inhibited PA phosphatase activity, with ATP and CTP being the most potent inhibitors. ATP did not inhibit PA phosphatase activity by binding to the enzyme or to its substrate PA. Instead, the mechanism of inhibition was the chelation of the enzyme's cofactor Mg^{2+}. The inhibitor constant for ATP was in the range of its cellular concentration. Moreover, changes in cellular ATP concentrations affected the proportional synthesis of phospholipids and triacylglycerols. These results were consistent with the regulation of PA phosphatase activity by ATP through a Mg^{2+} ion chelation mechanism.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade. Nucleotides, 8-azido-ATP, neutral lipids, phospholipids, Triton X-100, and bovine serum albumin were purchased from Sigma. Dioleoyl-diacylglycerol and dioleoyl-PA were purchased from Avanti Polar Lipids. *Escherichia coli* diacylglycerol kinase was obtained from Lipidex Inc. Radiochemicals and EN^{3}HANCE were purchased from DuPont NEN, and scintillation counting supplies were purchased from National Diagnostics. Sephacryl S-200 was purchased from Pharmacia Biotech Inc. Affi-Gel blue was from Bio-Rad. Silica gel-loaded SG81 chromatography paper was purchased from

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[1] The abbreviation used is: PA, phosphatidate.

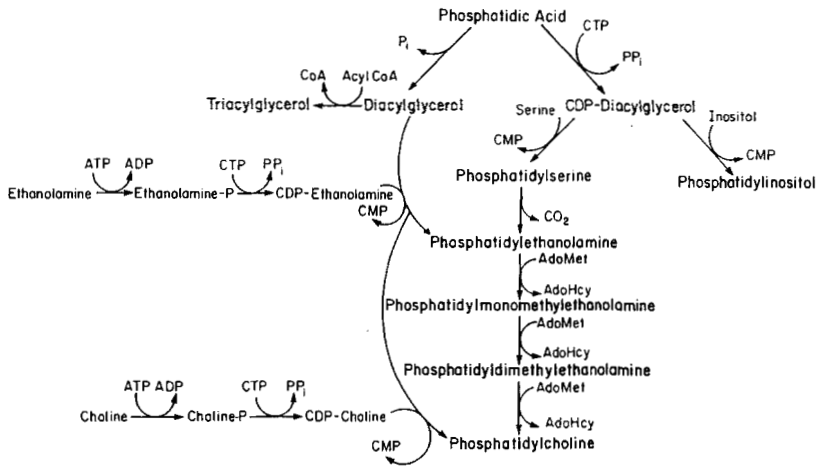

FIG. 1. **Pathways for the biosynthesis of the major phospholipids and triacylglycerols in *S. cerevisiae*.**

Whatman Inc. Growth medium supplies were purchased from Difco Laboratories.

Methods

Purification of PA Phosphatase—Membrane-associated 104- (9, 13) and 45-kDa (9) PA phosphatases were purified to near homogeneity as previously described. The specific activity of the enzymes were 15 and 2.3 μmol/min/mg.

Enzyme Assays and Protein Determination—PA phosphatase activity was measured for 20 min by following the release of water-soluble [^{32}P]P_i from chloroform-soluble [^{32}P]PA (20,000 cpm/nmol) at 30 °C (8). The reaction mixture contained 50 mM Tris-maleate buffer (pH 7.0), 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 1 mM Triton X-100, 0.1 mM PA, and an appropriate dilution of PA phosphatase in a total volume of 0.1 ml. All assays were conducted in triplicate with a typical standard deviation of ±5%. Enzyme assays were linear with time and protein concentration. 1 unit of PA phosphatase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product/min. Specific activity was defined as units/mg of protein. ATPase activity was measured by following the release of [^{32}P]P_i from [γ-^{32}P]ATP (2,000 cpm/nmol) as previously described (14). Protein was determined by the method of Bradford (15), using bovine serum albumin as the standard.

Preparation of ^{32}P-Labeled PA—[^{32}P]PA was enzymatically synthesized from dioleoyl-diacylglycerol and [γ-^{32}P]ATP using *E. coli* diacylglycerol kinase (16) as previously described (13).

Preparation of Triton X-100/PA-mixed Micelles—PA in chloroform was transferred to a test tube, and solvent was removed *in vacuo* for 40 min. Triton X-100/PA-mixed micelles were prepared by adding Triton X-100 to dried PA. The uniformity of the Triton X-100/PA-mixed micelles was determined by gel filtration chromatography (17).

Sephacryl S-200 Chromatography—A Sephacryl S-200 column (1 × 11 cm) was equilibrated with 50 mM Tris-maleate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, and 0.35 mM Triton X-100 at room temperature. Samples (0.2 ml) were applied and eluted from the column in equilibration buffer at a flow rate of 16 ml/h. Elution of Triton X-100 was determined by measuring its absorbance at 275 nm. PA and ATP concentrations were determined by liquid scintillation counting using [^{32}P]PA (300 cpm/nmol) and [γ-^{32}P]ATP (700 cpm/nmol), respectively.

Affi-Gel Blue Chromatography—An Affi-Gel blue column (0.7 × 0.8 cm) was equilibrated with 50 mM Tris-maleate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 20% glycerol, and 1% sodium cholate at 5 °C. Pure PA phosphatase (2.5 nmol/min) was applied to the column at a flow rate of 10 ml/h. The column was then washed with increasing concentrations of ATP. Fractions (0.3 ml) were collected at a flow rate of 10 ml/h.

Photoinactivation Experiments with 8-Azido-ATP—Photoinactivation experiments were performed at room temperature in a total volume of 20 μl as described by Nickels and Carman (14). The photoinactivation mixture contained 50 mM Tris-maleate (pH 7.0), 2 mM $MgCl_2$, 3.2 mM Triton X-100, and 0.1 mM PA. PA phosphatase (0.3 nmol/min) was added to photoprobe reaction mixtures and incubated for 2 min in the dark. Samples were then irradiated for 2 min with a UVL-11 lamp (254 nm) at a distance of 6 cm. Following irradiation, samples were diluted 50-fold with 50 mM Tris-maleate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol. The diluted samples were assayed for PA phosphatase activity as described above. The extent of photoinactivation of PA phosphatase was determined relative to that of control samples that were irradiated in the presence of reduced photoprobe (18). Reduced 8-azido-ATP (8-amino-ATP) was prepared by treatment with dithiothreitol (18).

Growth Conditions—Strain *MAT***a** *ade5* (19), which shows normal regulation of phospholipid metabolism (20–23), was used for analysis of lipids in response to glucose starvation and ATP depletion (24). Cells were maintained on 1% yeast extract, 2% peptone, 2% glucose medium plates containing 2% Bacto-agar. Cells were grown in complete synthetic medium (19) containing 2% glucose at 30 °C to the exponential phase of growth (2×10^7 cells/ml). To label lipids, cells were grown in the presence of [2-^{14}C]acetate (0.5 mCi/ml). Half of the culture was washed twice with growth medium containing glucose, and half of the culture was washed twice with growth medium without glucose. Cultures were resuspended in growth medium with and without glucose and incubated for 30 min to deplete ATP in glucose-starved cells (24). Cell numbers were determined by microscopic examination with a hemacytometer or by absorbance at 660 nm.

Analysis of Lipids—Lipids were extracted from cells by the method of Bligh and Dyer (25). The chloroform phase was neutralized and dried *in vacuo*, and the residue was dissolved in chloroform. Lipids were separated by paper chromatography on Na_2EDTA-treated SG81 paper (26). Total phospholipids, diacylglycerol, and triacylglycerol were analyzed by one-dimensional chromatography (27), and PA was analyzed by two-dimensional chromatography (28). The positions of the ^{14}C-labeled lipids on the chromatograms were determined by fluorography and compared with standard lipids after exposure to iodine vapor. The amount of each labeled lipid was determined by liquid scintillation counting of the corresponding spots on chromatograms.

RESULTS

Effect of Adenosine, Guanosine, Cytidine, and Uridine Nucleotides on PA Phosphatase Activity—Purified preparations of the 104- and 45-kDa forms of PA phosphatase from *S. cerevisiae* were used to examine the effects of nucleotides on PA phosphatase activity. In these experiments, PA phosphatase activity was measured with a subsaturating concentration (3 mol %) of PA (17). The concentration of PA was expressed as a surface concentration (in mol %) since PA forms uniform mixed micelles with Triton X-100 (17) and PA phosphatase activity follows surface dilution kinetics with respect to PA (9, 17). A subsaturating concentration of PA (near the K_m value) was used so we could simultaneously screen nucleotides that were inhibitory or stimulatory to enzyme activity. PA phosphatase activity is absolutely dependent on Mg^{2+} ions as a cofactor (9, 13). The Mg^{2+} ion concentration used in these experiments was maintained at a saturating concentration (2 mM) for optimum PA phosphatase activity (9, 13). However, under the assay conditions used in these experiments, Mg^{2+} ions would form complexes with nucleotides (29). Our rationale for not using excessive amounts of Mg^{2+} ions in these experiments was to keep their concentration relatively close to the physiological concentration (0.5 mM) of

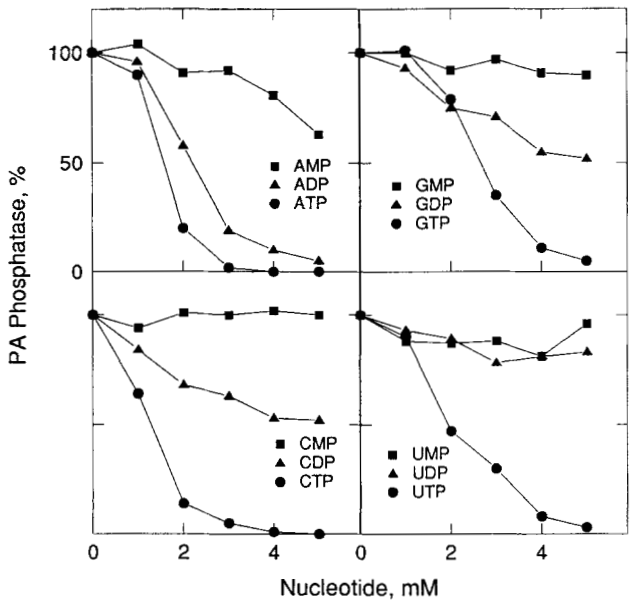

FIG. 2. **Effect of adenosine, guanosine, cytidine, and uridine nucleotides on PA phosphatase activity.** PA phosphatase activity was measured in the presence of the indicated nucleotides. The surface concentration of PA was 3 mol % (bulk concentration of 0.1 mM).

free Mg^{2+} ions found in eucaryotic cells (30). This allowed us to observe effects of nucleotides on PA phosphatase activity under conditions that may occur *in vivo*.

The effects of the mono-, di-, and triphosphorylated derivatives of adenosine, guanosine, cytidine, and uridine on 104-kDa PA phosphatase activity is shown in Fig. 2. Several of these nucleotides inhibited PA phosphatase activity in a dose-dependent manner. This inhibitory effect increased with the number of phosphate groups attached to the ribonucleoside. The most potent nucleotide inhibitors of PA phosphatase activity were ATP and CTP (Table I). ADP, GTP, and UTP were less potent inhibitors of the enzyme (Table I). Similar results were obtained for the 45-kDa form of PA phosphatase activity (data not shown). Since the 104- and 45-kDa forms of PA phosphatase have similar enzymological properties (9, 13, 17), the 45-kDa PA phosphatase activity was not examined further.

Effects of ATP and CTP on the Kinetics of PA Phosphatase Activity with Respect to the Surface Concentration of PA—A detailed kinetic analysis was performed on PA phosphatase to explore the mechanism of ATP and CTP inhibition on enzyme activity. We first examined the effects of ATP and CTP on the kinetics of PA phosphatase activity with respect to the surface concentration of PA. Under the conditions (*i.e.* Triton X-100/PA-mixed micelle concentrations) of these experiments, PA phosphatase activity was dependent on the surface concentration of PA and independent of the bulk PA concentration (17). The kinetic data were analyzed with the EZ-FIT computer program of Perrella (31). As previously described (11), PA phosphatase activity showed cooperative kinetics toward PA (Hill number = 1.9) in the absence of nucleotides (Figs. 3 and 4). PA phosphatase activity was inhibited by ATP (Fig. 3) and CTP (Fig. 4) in a dose-dependent manner at each concentration of PA. Furthermore, PA phosphatase exhibited cooperative kinetics toward PA at each concentration of ATP (Fig. 3*A*) and CTP (Fig. 4*A*). The Hill number for the PA dependence curves at each concentration of ATP and CTP did not vary significantly from the value of 2. We constructed double-reciprocal plots of the data where the PA concentration was raised to the Hill number of 2 (32). As previously reported (11), the K_m value for PA was 2.2 mol %. ATP (Fig. 3*B*) and CTP (Fig. 4*B*) affected both the apparent V_{max} and K_m values for PA phosphatase activity with respect to PA. These results were indicative of a complex mixed type of inhibition mechanism (32). Replots of the 1/*V* intercepts or slopes *versus* the inhibitor concentrations from Figs. 3*B* and 4*B* were nonlinear. This may be a reflection of the cooperative kinetics exhibited by the enzyme and/or the complex mechanism of inhibition. This complexity precluded the determination of true inhibitor constants for ATP and CTP.

TABLE I
Inhibitor constants and cellular concentrations of nucleotides

Inhibitor	IC_{50}[a]	Cellular concentration[b]	
		Glucose	Starved
		mM	
ATP	1.3	2.3	0.57
ADP	1.9	0.4	0.80
CTP	1.1	0.2	
GTP	2.2	0.5	
UTP	1.9	0.6	

[a] IC_{50} values were calculated from plots of the log of the PA phosphatase activity from Fig. 2 *versus* the inhibitor concentration.

[b] Values for the cellular concentrations of nucleotides were from Refs. 36 and 37. These values were determined from cells grown in the presence of glucose. The concentrations of ATP and ADP in glucose-starved cells were based on the data from Refs. 24 and 37.

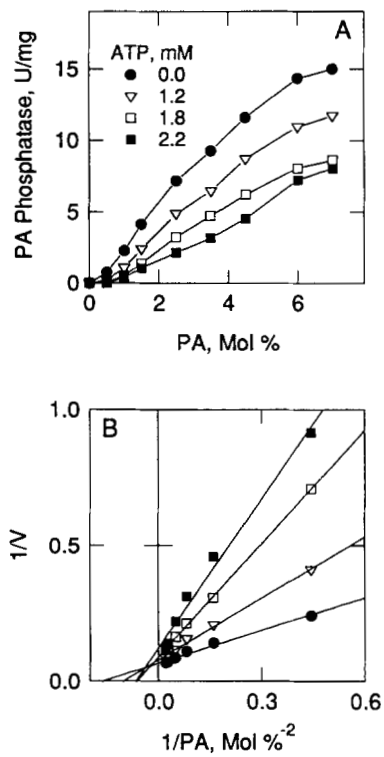

FIG. 3. **Effect of ATP on the kinetics of PA phosphatase activity with respect to the surface concentration of PA.** *A*, PA phosphatase activity was measured as a function of the surface concentration (mol %) of PA (bulk concentration of 0.1 mM) at the indicated set concentrations of ATP. *B*, reciprocal plot of the data in *A* where the PA concentration was raised to the Hill number of 2. The *lines* drawn in *B* were a result of a least-squares analysis of the data.

Effects of ATP and CTP on the Kinetics of PA Phosphatase Activity with Respect to the Concentration of Mg^{2+} Ions—We next examined the effects of ATP and CTP on the kinetics of PA phosphatase activity with respect to the concentration of Mg^{2+} ions. In these experiments, PA phosphatase activity was measured using a saturating surface concentration (9 mol %) of PA. PA phosphatase activity exhibited saturation kinetics with re-

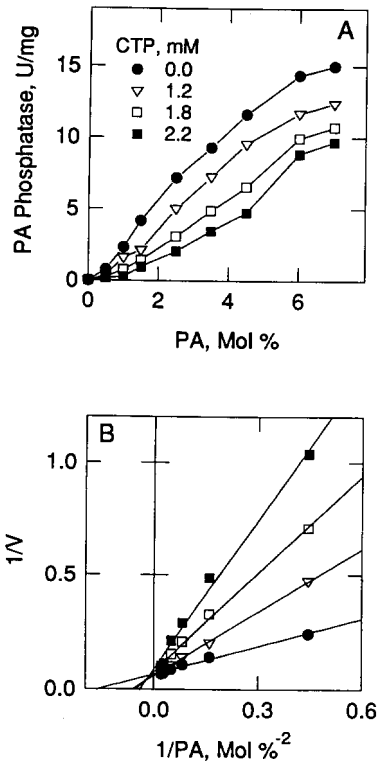

FIG. 4. **Effect of CTP on the kinetics of PA phosphatase activity with respect to the surface concentration of PA.** *A*, PA phosphatase activity was measured as a function of the surface concentration (mol %) of PA (bulk concentration of 0.1 mM) at the indicated set concentrations of CTP. *B*, reciprocal plot of the data in *A* where the PA concentration was raised to the Hill number of 2. The *lines* drawn in *B* were a result of a least-squares analysis of the data.

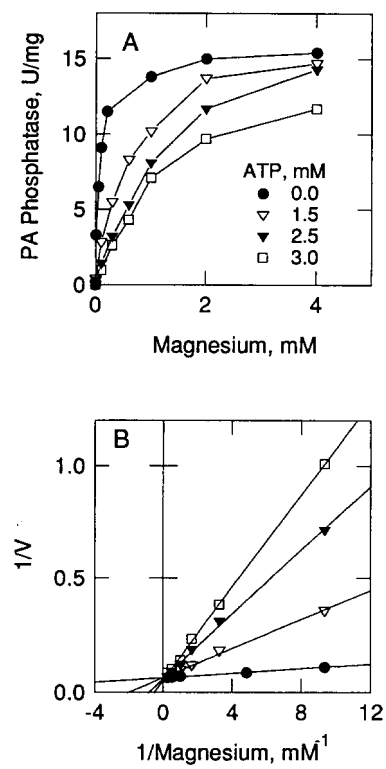

FIG. 5. **Effect of ATP on the kinetics of PA phosphatase activity with respect to the concentration of magnesium ions.** *A*, PA phosphatase activity was measured as a function of the concentration of $MgCl_2$ at the indicated set concentrations of ATP. The surface concentration of PA was 9 mol % (bulk concentration of 0.1 mM). *B*, reciprocal plot of the data in *A*. The *lines* drawn in *B* were a result of a least-squares analysis of the data.

spect to Mg^{2+} ions in the absence and presence of various set concentrations of ATP (Fig. 5*A*) and CTP (Fig. 6*A*). Double-reciprocal plots of the data showed that ATP (Fig. 5*B*) and CTP (Fig. 6*B*) did not affect the apparent V_{max} values but did cause an increase in the apparent K_m value (80 μM) for Mg^{2+} ions. These results were consistent with ATP and CTP being competitive inhibitors (32) of PA phosphatase activity with respect to Mg^{2+} ions.

Effects of ADP, GTP, and UTP on the Kinetics of PA Phosphatase Activity—A less extensive kinetic analysis was performed on PA phosphatase activity to examine the mechanism of ADP, GTP, and UTP inhibition on enzyme activity. The enzyme followed cooperative kinetics with respect to the surface concentration of PA in the presence of ADP, GTP, and UTP with Hill numbers near 2 (Fig. 7*A*). Double-reciprocal plots of the kinetic data indicated that these nucleotides inhibited PA phosphatase activity by either mixed-type or noncompetitive mechanisms. The enzyme followed saturation kinetics with respect to Mg^{2+} ions in the presence of ADP, GTP, and UTP (Fig. 7*B*). The mechanism of PA phosphatase inhibition with respect to Mg^{2+} ions was competitive.

Lack of Evidence for an ATP Binding Site on PA Phosphatase or a PA·ATP Complex—We questioned whether nucleotides inhibited PA phosphatase activity by a mechanism involving the direct interaction of the nucleotide with the enzyme. ATP was used as a representative nucleotide inhibitor of PA phosphatase because all of the nucleotides inhibited the enzyme by similar kinetic mechanisms. We examined the possibility that ATP inhibited PA phosphatase activity because the enzyme used ATP as a substrate. However, PA phosphatase did not catalyze the hydrolysis of ATP.

Affi-Gel blue chromatography resin is commonly used to bind nucleotide-requiring enzymes and is also used to purify PA phosphatase (13). We reasoned that if PA phosphatase binds to Affi-Gel blue through a nucleotide-binding site, the enzyme should be specifically eluted with a nucleotide. Pure PA phosphatase was applied to an Affi-Gel blue column followed by successive washing steps with equilibration buffer containing increasing concentrations of ATP. PA phosphatase could not be eluted from the column with concentrations of ATP up to 5 mM. However, as previously described (13), PA phosphatase was eluted from the Affi-Gel blue column with equilibration buffer containing NaCl. Thus, PA phosphatase may bind to the Affi-Gel blue resin through other interactions.

We also addressed the question of whether PA phosphatase has an ATP binding site by using the photoaffinity probe 8-azido-ATP. 8-Azido-ATP has been used to label a variety of nucleotide-binding enzymes (33). Azidonucleotides form reactive nitrenes when irradiated with UV light and insert covalently into nearby amino acid residues (33). This interaction is primarily due to the nature of the enzyme-nucleotide complex (33). The interaction of 8-azido-ATP with PA phosphatase was investigated according to the criteria that must be fulfilled (33) to validate the use of 8-azido-ATP as a specific photoaffinity probe for PA phosphatase. 8-Azido-ATP inhibited PA phosphatase activity in a dose-dependent manner with an IC_{50} value (1.3 mM) similar to that for ATP. 8-Azido-ATP was then examined for its ability to photoinactivate PA phosphatase activity in dose-dependent and time-dependent manners. We could not demonstrate the photoinactivation of PA phosphatase activity by 8-azido-ATP using concentrations of the photoprobe of up to 3 mM.

We questioned if ATP was inhibiting PA phosphatase activity because it formed a complex with PA. The formation of a

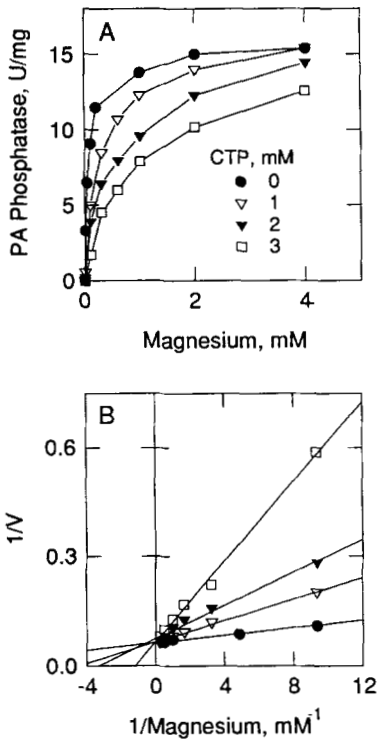

FIG. 6. **Effect of CTP on the kinetics of PA phosphatase activity with respect to the concentration of magnesium ions.** *A*, PA phosphatase activity was measured as a function of the concentration of $MgCl_2$ at the indicated set concentrations of CTP. The surface concentration of PA was 9 mol % (bulk concentration of 0.1 mM). *B*, reciprocal plot of the data in *A*. The *lines* drawn in *B* were a result of a least-squares analysis of the data.

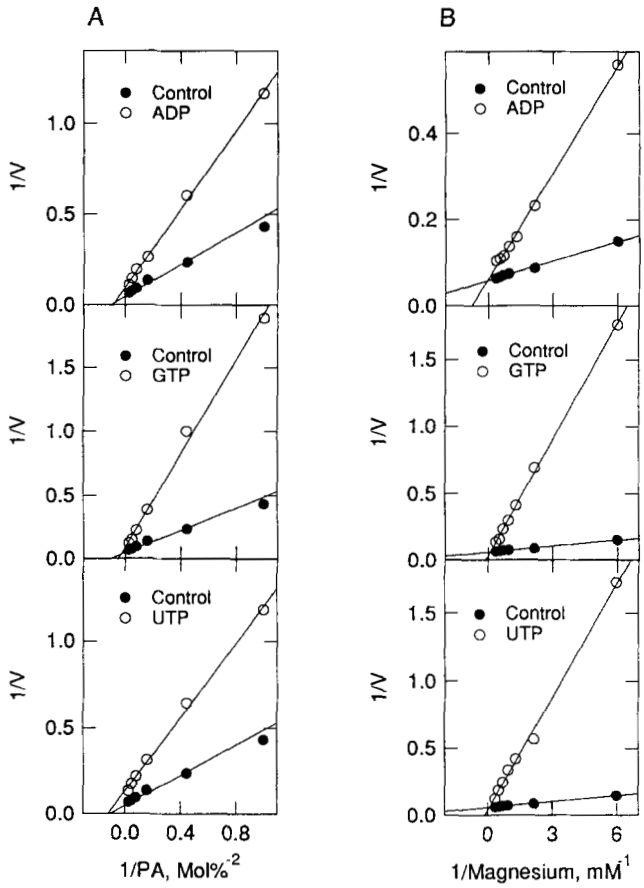

FIG. 7. **Effects of ADP, GTP, and UTP on the kinetics of PA phosphatase activity with respect to the surface concentration of PA and the concentration of magnesium ions.** *A*, PA phosphatase activity was measured as a function of the surface concentration (mol %) of PA (bulk concentration of 0.1 mM) in the absence (*control*) and presence of 2.5 mM ADP, 2.5 mM GTP, and 2.5 mM UTP as indicated. The data are plotted as 1/*V* (units/mg) *versus* the reciprocal of the PA surface concentration raised to the Hill number of 2. *B*, PA phosphatase activity was measured as a function of the concentration of $MgCl_2$ in the absence (*control*) and presence of 2.5 mM ADP, 2.5 mM GTP, and 2.5 mM UTP as indicated. The surface concentration of PA was 9 mol % (bulk concentration of 0.1 mM). The data are plotted as 1/*V* (units/mg) *versus* the reciprocal of the $MgCl_2$ concentration. The *lines* drawn in *B* were a result of a least-squares analysis of the data.

PA·ATP complex was examined using gel filtration chromatography with Sephacryl S-200. When a mixture of 1 mM Triton X-100, 0.1 mM PA, and 2 mM ATP was applied to a Sephacryl S-200 column, Triton X-100/PA-mixed micelles and ATP were well separated from each other. The V/V_0 values for Triton X-100/PA-mixed micelles and ATP were 1.23 and 2.27, respectively. When pure PA phosphatase (0.3 nmol/min) was subjected to Sephacryl S-200 chromatography in the presence of 1 mM Triton X-100, 0.1 mM PA, and 2 mM ATP, the enzyme coeluted with Triton X-100/PA-mixed micelles as previously described (17). ATP did not associate with Triton X-100/PA/PA phosphatase-mixed micelles. The absence of Mg^{2+} ions in the chromatography buffer in these experiments had no effect on the elution profiles of Triton X-100/PA-mixed micelles or ATP.

Effect of Glucose Starvation on Lipid Composition—Talwalkar and Lester (24) have shown that upon glucose starvation, the cellular levels of ATP decrease 4-fold (Table I). By starving cells for glucose (24), we manipulated cellular ATP levels and examined the effect of ATP on PA phosphatase activity *in vivo*. Regulation of the enzyme by ATP should be reflected in changes in the concentrations of the substrate and product of the PA phosphatase reaction. Glucose-supplemented cells were labeled to steady-state with [2-^{14}C]acetate to analyze total lipids. The labeled cells were washed and then incubated for 30 min in fresh growth medium lacking glucose and [2-^{14}C]acetate. Following the incubation, the lipid composition of the cells was analyzed. Indeed, upon glucose starvation and ATP depletion, the level of diacylglycerol increased 1.5-fold, and the level of PA decreased 1.5-fold relative to glucose-supplemented cells (Fig. 8). Previous studies have shown that the proportional synthesis of triacylglycerols and phospholipids is governed by the regulation of PA phosphatase activity (7–9). Glucose starvation and ATP depletion resulted in a 1.5-fold increase in triacylglycerols and a 1.46-fold decrease in total phospholipids (Fig. 8). This was consistent with the regulation of PA phosphatase activity by ATP.

DISCUSSION

In this work, we examined the regulation of PA phosphatase activity by nucleotides. This work was prompted because of the prominent role nucleotides play in phospholipid metabolism (Fig. 1). ATP has been shown to inhibit PA phosphatase activity from rat liver (34). However, definitive conclusions about the regulation of rat liver PA phosphatase activity by ATP cannot be made because the previous study was performed with impure enzyme and a poorly defined substrate environment. We carried out systematic kinetic experiments using pure enzyme and well defined Triton X-100/PA-mixed micelles. PA phosphatase was inhibited by adenosine, guanosine, cytidine, and uridine nucleotides. ATP and CTP were the most potent inhibitors of the enzyme. The analysis of the mechanism of inhibition of activity by ATP and CTP was not straightforward because the enzyme followed cooperative kinetics toward PA. However, by raising the PA concentration to the Hill number of 2, linear double-reciprocal plots were obtained. These plots indicated

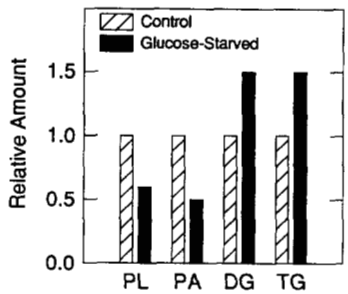

FIG. 8. **Lipid composition of cells starved for glucose.** Glucose-supplemented cells were grown in the presence of [2-^{14}C]acetate (0.5 μCi/ml) for steady-state labeling of lipids as described in the text. Cells were washed and incubated for 30 min in fresh growth medium without glucose. Control cells were incubated in growth medium with glucose. Following the 30-min incubation, lipids were extracted from the glucose-supplemented (*control*) and glucose-starved cells and analyzed as described in the text. The incorporation of [2-^{14}C]acetate into total lipids was 55,000–60,000 cpm. The amounts of phospholipids (*PL*), PA, diacylglycerol (*DG*), and triacylglycerol (*TG*) were 33, 0.8, 1.1, and 12.6 mol %, respectively, and were set at 1 in the figure. The data shown are representative of two independent experiments.

that ATP and CTP inhibited PA phosphatase activity by a complex mixed type of mechanism.

The mechanism of PA phosphatase inhibition by ATP was examined further. PA phosphatase did not catalyze the hydrolysis of ATP. Thus, ATP did not inhibit PA phosphatase through a competitive mechanism in which the enzyme used ATP as a substrate. ATP did not appear to inhibit PA phosphatase activity by binding to the enzyme or to its substrate PA. This was supported by the following results. 1) We could not purify PA phosphatase by Affi-Gel blue chromatography using substrate-specific elution with ATP; 2) ATP did not associate with PA phosphatase in Triton X-100/PA-mixed micelles; 3) PA phosphatase could not be photoinactivated with the photoaffinity probe 8-azido-ATP; and 4) ATP did not associate with PA in Triton X-100/PA-mixed micelles.

Kinetic experiments showed that nucleotides inhibited PA phosphatase activity by a competitive mechanism with respect to Mg^{2+} ions. As the Mg^{2+} ion concentration was increased, the inhibition by nucleotides was overcome. Thus, PA phosphatase activity was inhibited by nucleotides by a simple chelation mechanism. This mechanism was also reflected in the inhibition being dependent on the number of phosphate groups attached to the ribonucleoside of nucleotides (29). It is unclear whether PA phosphatase has a Mg^{2+} ion requirement (9, 13) because it has a Mg^{2+} binding site or whether Mg^{2+} ions are required to form a Mg·PA substrate complex. The K_m value for PA increased in the presence of ATP (Fig. 2*B*). This kinetic pattern could result if a Mg·PA complex was required for activity and the concentration of the complex was reduced due to the chelation of Mg^{2+} ions by ATP.

Could such a simple and obvious mechanism of PA phosphatase regulation by ATP be physiologically relevant? If the regulation of PA phosphatase activity by a nucleotide was to be relevant *in vivo*, the inhibitor constant for the nucleotide should be within the range of its cellular concentration. The inhibitor constants for ADP, CTP, GTP, and UTP were 2–5-fold higher than their cellular concentrations (Table I). Therefore, it is unlikely that these nucleotides play a role in the regulation of PA phosphatase activity *in vivo*. However, the inhibitor constant for ATP was 1.8-fold lower than its cellular concentration in glucose-grown cells (Table I). Thus, PA phosphatase activity would be expected to be inhibited. When cells are starved for glucose, the cellular concentration of ATP decreases 4-fold (24), bringing its concentration 2.2-fold below the inhibitor constant for ATP (Table I). This concentration of ATP would not have a significant effect on PA phosphatase activity (Fig. 2). Upon ATP depletion, the free Mg^{2+} ion concentration in the cell would be expected to rise, and the activity of PA phosphatase could increase. Changes in the cellular concentration of ATP should affect PA phosphatase activity *in vivo*. Indeed, the analysis of lipids upon glucose starvation showed that the diacylglycerol concentration increased whereas the PA concentration decreased. This result was consistent with an increase in PA phosphatase activity when the ATP level decreased. Glucose starvation also caused an increase in triacylglycerols and a decrease in phospholipids. These results raised the suggestion that ATP affected the proportional synthesis of triacylglycerols and phospholipids through the regulation of PA phosphatase activity. ATP has also been shown to play a role in the regulation of phosphoinositide metabolism in *S. cerevisiae*. Decreases in the levels of ATP cause decreases in the synthesis of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate (24, 35). These effects have been attributed to the regulation of membrane-associated phosphatidylinositol 4-kinase activity by ATP and ADP (36). This further supports the notion that ATP plays a role in the coordinate synthesis of triacylglycerols and phospholipids.

The explanation given for the mechanism of PA phosphatase inhibition by ATP was a first approximation for the regulation that may occur *in vivo*. Our studies did not rule out the possible regulation of PA phosphatase activity by other factors that could have changed in response to glucose starvation. Several mechanisms are known to exist in *S. cerevisiae* that control the activity of PA phosphatase (7–11), and these mechanisms must be interrelated.

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