Long-chain alkyl ester of AMP acts as an antagonist of glucose-induced signal transduction that mediates activation of plasma membrane proton pump in *Saccharomyces cerevisiae*

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One of the long-chain alkyl esters of AMP, adenosine 5'-hexadecylphosphate (AMPC16), exhibited a cytotoxic growth inhibitory effect on cells of various yeast strains. The growth inhibitory effect of AMPC16 on *Saccharomyces cerevisiae* cells was observed only in medium containing Mg^{2+} , which accelerated cellular uptake of the nucleotide analogue. In the presence of Mg^{2+} , AMPC16 completely inhibited glucose-induced extracellular acidification by the intact cells and also interfered with activation of the plasma membrane ATPase, but did not directly inhibit the ATPase activity itself. AMPC16 treatment prevented cells from increasing their intracellular *sn*-1,2-diacylglycerol (DAG) level in response to glucose, whereas the inhibition of proton extrusion by the cells could be largely reversed by the coaddition of a membrane-permeable DAG analogue. The DAG analogue, a physiological activator of protein kinase C (PKC), was not protective against the inhibition of glucose-induced proton extrusion by staurosporine, which is capable of directly interfering with the action of PKC. These results implied that AMPC16 caused a Mg^{2+} -dependent cytotoxic effect on *Sac. cerevisiae* cells by interfering with a phosphatidylinositol type of signal that mediates activation of the plasma membrane proton pump.

Keywords: *Saccharomyces cerevisiae*, plasma membrane H^+ -ATPase, signal transduction, nucleotide analogue

INTRODUCTION

AMP can be structurally modified to contain long-chain alkylphosphate esters, which can inhibit adenylate cyclase (Hynie & Smrt, 1978) and ribonuclease (Yasuda & Inoue, 1983) to varying extents, depending on the length of the alkyl side chain. Among these analogues, adenosine 5'-hexadecylphosphate (AMPC16, see Fig. 1) has a structure quite analogous to that of adenosine 5'-acylphosphates such as adenosine 5'-palmitoyl-

phosphate, the transient intermediate product of the palmitoyl-CoA synthetic reaction combining palmitic acid and ATP. As can be predicted from the structural analogy, AMPC16 inhibits the long-chain fatty acyl-CoA synthetase of rat liver microsomes in a non-competitive fashion (Shiraishi *et al.*, 1994). It remains unknown whether these AMP analogues can influence the cell proliferation of mammals or other organisms via the inhibition of enzyme activities.

In our screening study of chemical antagonists of signal transduction, long-chain alkyl esters containing UMP were synthesized, in addition to those containing AMP, and their activities were evaluated in terms of an inhibitory effect against the pheromone-triggered mating process of *Saccharomyces cerevisiae* (Machida *et al.*, 1997). Uridine 5'-hexadecylphosphate (UMPC16,

Abbreviations: AMPC12, adenosine 5'-dodecylphosphate; AMPC16, adenosine 5'-hexadecylphosphate; AMPC20, adenosine 5'-eicosylphosphate; DAG, diacylglycerol; DES, diethylstilboestrol; PKC, protein kinase C; UMPC12, uridine 5'-dodecylphosphate; UMPC16, uridine 5'-hexadecylphosphate; UMPC20, uridine 5'-eicosylphosphate.

see Fig. 1) specifically inhibited sexual agglutination between cells of the α - and α -mating types initiated via the binding of α and α pheromones with the corresponding receptor molecules which function to activate a GTP-binding protein on the cell surface of the opposite mating type (Manfredi *et al.*, 1996). Similar inhibition was also observed with AMPC16 but it was accompanied by an inhibition against vegetative cell growth, which was in agreement with a previous finding on the role of the AMP analogue as an inhibitor of long-chain acyl-CoA synthetase (Shiraishi *et al.*, 1994). In addition, the growth inhibition by AMPC16 may reflect its interference with a cAMP-dependent cell cycle mechanism (Hynie & Smrt, 1978; Morishita *et al.*, 1995). In the present study, we examined the antimicrobial activities of these nucleotide analogues against a wide variety of bacteria, yeast and fungi, and characterized the mechanism of the Mg^{2+} -dependent cytotoxic action of AMPC16 against *Sac. cerevisiae* cells.

METHODS

Microbial strains and media. *Sac. cerevisiae* IFO 0203 was mainly used in addition to some other yeast strains (see Table 1) and the bacterial strains such as *Escherichia coli* IFO 3545, *Staphylococcus aureus* NCTC 8530 and *Bacillus subtilis* IFO 3007. The following strains of filamentous fungi were also used: *Aspergillus niger* ATCC 6275, *Penicillium chrysogenum* IFO 4626 and *Mucor mucedo* IFO 7684. The growth properties of *Sac. cerevisiae* IFO 0203 cells were examined in YPD medium which contained 1% yeast extract, 2% peptone and 2% glucose at 30 °C. YPG medium consisted of 1% yeast extract, 2% peptone and 3% glycerol.

Measurement of yeast cell growth. Antimicrobial activities of nucleotide analogues were assayed by the serial broth dilution method which has been conventionally used in our studies on naturally occurring antibiotics (Akeda *et al.*, 1995; Ueki *et al.*, 1996). In this method, the MIC represents the minimum concentration of nucleotide analogue which can absolutely prevent the turbidity increase due to cell growth (Baltch *et al.*, 1998; Kohner *et al.*, 1997). Cells of *Sac. cerevisiae* IFO 0203 were grown overnight in YPD medium with vigorous shaking and were inoculated into freshly prepared medium to give an initial cell density of approximately 10^6 cells ml^{-1} . Cells were

then grown with or without AMPC16 with vigorous shaking and portions were withdrawn at various times to measure the cell density at OD_{610} and to measure the c.f.u. as a measure of the viable cell number (Machida *et al.*, 1999). Cell viability was also determined by the methylene blue staining method (Rodriguez & Parks, 1981) with a microscopic examination, in which the initial cell density was adjusted to 10^7 ml^{-1} .

Synthesis of uridine and adenosine 5'-alkylphosphates. The following nucleotide analogues were synthesized as described previously (Machida *et al.*, 1997): uridine 5'-dodecylphosphate (UMPC12), UMPC16, adenosine 5'-dodecylphosphate (AMPC12) and AMPC16. In the present study, uridine 5'-eicosylphosphate (UMPC20) was synthesized by exactly following the procedure for UMPC16 synthesis except that eicosyl alcohol was used as the alkyl donor in the reaction. Shiraishi *et al.* (1994) have reported the synthesis of adenosine 5'-eicosylphosphate (AMPC20) by a condensation reaction between AMP and eicosyl alcohol, but we synthesized it by following our previously described method using AMP and the corresponding alkylbromide. 1H - and ^{13}C -NMR spectral data of UMPC20 and AMPC20 were obtained using a JEOL JNM-400GX spectrometer in $DMSO-d_6$ and showed good correlation to those obtained with UMPC16 (Machida *et al.*, 1997) and AMPC20 (Shiraishi *et al.*, 1994), respectively. The absolute chemical structures of UMPC20 and AMPC20 were ultimately confirmed by their mass spectra, with m/z 603 for $C_{29}H_{52}N_2O_9P$ (M^+-1) and m/z 626 for $C_{30}H_{53}N_5O_7P$ (M^+-1), respectively. Fig. 1 shows the chemical structures of these AMP analogues together with the structure of UMPC16.

HPLC analysis of AMPC16. Cells of *Sac. cerevisiae* IFO 0203 were grown in YPD medium overnight at 30 °C, harvested and washed with 50 mM succinate buffer (pH 6.5). Washed cells were then suspended at the cell density of 10^7 ml^{-1} in the same buffer containing AMPC16 at 12.5 μg ml^{-1} and incubated with shaking at 30 °C. At various times, portions of the cell suspension were withdrawn and subjected to centrifugal filtration in a membrane-equipped tube (ULTRAFREE-MC, Millipore). Each filtrate was then assayed for its content of AMPC16 by HPLC on an ODS reverse phase column (6×150 mm) at the wavelength of 260 nm. Isocratic elution was done at room temperature with 5 mM $(NH_4)_2HPO_4$ /methanol (20:80, v/v) and a flow rate of 1.0 ml min^{-1} .

Assay of glucose-induced proton extrusion. Plasma membrane H^+ -ATPase activity was assayed *in vivo* by measuring the extracellular acidification due to glucose-induced proton extrusion by the intact cells, as described by Brandão *et al.* (1994). Cells were grown overnight in YPG medium at 30 °C, collected by centrifugation and washed several times with distilled water. Washed cells were then suspended in distilled water at the cell density of 10^8 ml^{-1} and the pH of the cell suspension was adjusted to 7.0 with dilute sodium hydroxide. After preincubating the cell suspension at 30 °C for 10 min, glucose was added at the final concentration of 100 mM together with each of the other chemicals at the indicated concentrations. The pH of the cell suspension was measured using a Horiba pH meter model D-23 at 30 °C.

Preparation of plasma membrane fraction. Cells were grown in YPG medium at 30 °C to mid-exponential phase, washed and suspended in 100 mM N-[tris(hydroxymethyl)methyl]-glycine buffer containing 5 mM EDTA and 2 mM DTT at the cell density of 10^8 ml^{-1} . The cell suspension was incubated at 30 °C for 10 min with supplementation with 100 mM glucose to fully activate the plasma membrane H^+ -ATPase (Brandão *et al.*, 1994). Cells were then collected, washed and resuspended in the above buffer at the cell density of

Table 1. MICs of AMPC16 on yeast cell growth

Test organism	MIC (μg ml^{-1}) with	
	None	Mg^{2+} *
<i>Saccharomyces cerevisiae</i> IFO 0203	> 100	6.25
<i>Schizosaccharomyces pombe</i> IFO 0342	6.25	3.13
<i>Candida albicans</i> IFO 1061	> 100	25.0
<i>Candida saitoana</i> IFO 0768	> 100	12.5
<i>Rhodotorula rubra</i> IFO 0001	> 100	> 100
<i>Hansenula anomala</i> IFO 0136	> 100	12.5

* Assayed in the presence of 10 mM $MgSO_4$.

Fig. 1. Structures of UMPC16, AMPC12, AMPC16 and AMPC20.

$1.5\text{--}2.0 \times 10^8 \text{ ml}^{-1}$, and then immediately frozen in liquid nitrogen and stored until use. The plasma membrane fraction was purified by ultracentrifugation according to the procedure described by Becher dos Passos *et al.* (1992). To measure the plasma membrane H^+ -ATPase activity in the cells with or without AMPC16 treatment, the plasma membrane fractions were prepared from the cells that had been incubated in distilled water containing both 100 mM glucose and 10 mM MgSO_4 (adjusted to pH 7.0) at 30 °C for 10 or 30 min as well as from cells that had been further supplemented with 25 $\mu\text{g AMPC16 ml}^{-1}$.

Assay of plasma membrane H^+ -ATPase. The enzyme activity was assayed on the basis of ATP hydrolysis by the plasma membrane preparation according to the method of Rosa & Sá-Correia (1991) with a slight modification as follows. The standard reaction mixture contained 10 $\mu\text{mol ATP}$, 10 $\mu\text{mol MgSO}_4$, 50 $\mu\text{mol KCl}$ and plasma membrane preparation equivalent to 0.3–0.4 mg protein in 1.0 ml 50 mM Tris/Mes buffer (pH 6.0). KNO_3 , NaN_3 or ammonium molybdate was added at 200 μM to inhibit vacuole-type ATPase, mitochondrial ATPase and acid phosphatase, respectively. After incubating the mixture at 30 °C for 10 min, inorganic phosphate liberated in the reaction was measured with a colorimetric phosphate assay kit (Wako Pure Chemicals). One unit of ATPase activity was defined as the amount of enzyme that liberated 1.0 nmol inorganic phosphate $\text{min}^{-1} (\text{mg protein})^{-1}$. Protein was measured according to the method of Bradford (1976) using BSA as a standard.

Measurement of intracellular DAG level. Cells were grown in YPG medium at 30 °C to mid-exponential phase, washed and suspended in distilled water containing both 100 mM glucose and 10 mM MgSO_4 (adjusted to pH 7.0) at the cell density of $10^7 \text{ cells ml}^{-1}$ and incubated at 30 °C with or without further supplementation with AMPC16 at 25 $\mu\text{g ml}^{-1}$. At various

times, 30 ml portions were withdrawn and the cells were collected by centrifugation. Lipids were extracted from the cell pellets by following our previously described method (Machida *et al.*, 1999) for measurement of the DAG content using a radioenzymic assay (Preiss *et al.*, 1986).

Chemicals. The following chemicals were purchased from Sigma: AMP, UMP, cerulenin, diethylstilboestrol (DES), amphotericin B, staurosporine and 1-oleoyl-2-acetyl-*sn*-glycerol (a membrane-permeable DAG analogue). The other chemicals were of analytical reagent grade.

RESULTS AND DISCUSSION

Antimicrobial activities of nucleotide analogues

In contrast to our previous finding on the growth inhibitory effect of AMPC16 on *Sac. cerevisiae* cells in the sexual agglutination assay (Machida *et al.*, 1997), the nucleotide analogue did not inhibit the growth of yeast cells in YPD medium. The antimicrobial activity assays of AMPC16 were thus repeated in each medium with or without supplementation with 10 mM MgSO_4 since the YHG medium used in the yeast mating process contained the magnesium salt at around 8 mM. No antimicrobial activity was observed with any UMP or AMP analogue, including AMPC16, against the bacterial and fungal strains tested at 100 $\mu\text{g ml}^{-1}$, even in the presence of Mg^{2+} . In contrast, all yeast strains tested except *Rhodotorula rubra* IFO 0001 were sensitive to AMPC16 with MIC values of 3.13–25.0 $\mu\text{g ml}^{-1}$ when the cells were grown in YPD medium supplemented with MgSO_4 , as summarized in Table 1. *Schizosaccharomyces pombe* showed a different pattern of sensitivity to AMPC16, with growth inhibition in the absence as well as in the presence of Mg^{2+} , although the presence of this divalent cation slightly increased the cytotoxic effect of AMPC16, as reflected by the lower MIC value. The growth inhibitory effects of AMP analogues on yeast cells critically depended on the length of the alkyl side chain since AMPC12 and AMPC20 were inactive even at 100 $\mu\text{g ml}^{-1}$ in the medium with Mg^{2+} . The turbidometric measurement of cell growth also revealed a potent growth inhibitory effect of AMPC16 on *Sac. cerevisiae* cells with a MIC value of 6.25 $\mu\text{g ml}^{-1}$ (Fig. 2). Under these conditions, the number of c.f.u. was gradually decreased with increasing time of incubation, reflecting the strong fungicidal activity of AMPC16. The Mg^{2+} -dependent cytotoxic effect of AMPC16 on *Sac. cerevisiae* cells could be confirmed by a concomitant increase in the number of methylene blue stained cells (data not shown).

Role of Mg^{2+} in the growth inhibitory effect of AMPC16

In shaken cultures in YPD medium containing AMPC16 at 6.25 $\mu\text{g ml}^{-1}$, the yeast cell growth was completely inhibited when either MgSO_4 or MgCl_2 was added at a concentration of at least 8 mM but each magnesium salt was only partially effective at 5 mM. AMPC16 could not inhibit the growth of yeast cells in the absence of magnesium salts and the presence of CaCl_2 or BaCl_2 at

Fig. 2. Growth inhibitory effects of AMPC16 on *Sac. cerevisiae*. Cells were grown in YPD medium containing AMPC16 at 0 (○), 3.125 (●), 6.25 (□) or 12.5 μg ml⁻¹ (■). Each medium was further supplemented with 10 mM MgSO₄. Cell growth was measured by the turbidometric assay (a) and the viability assay, in which viable cell number was expressed as the number of c.f.u. (b).

Fig. 3. Time course of the change of AMPC16 level in the suspension of *Sac. cerevisiae* cells. Cells were incubated in 50 mM succinate buffer (pH 6.5) containing AMPC16 (12.5 μg ml⁻¹) with no addition (□), 10 mM CaCl₂ (○) or 10 mM MgCl₂ (●). Values are means ± SD of triplicate assays.

various concentrations up to 100 mM and 25 mM, respectively. The effect of MnCl₂ was examined at lower concentrations because of its own cytotoxic effect, and this salt was found to be ineffective when added at up to 5.0 mM. Thus, the fungicidal activity of AMPC16 was highly dependent on the presence of Mg²⁺. Mg²⁺ is normally present in both the plasma membrane and cytoplasm of yeast cells and participates in a number of enzymic reactions using ATP as a substrate or product. Addition of exogenous Mg²⁺ may increase its concentrations in the above fractions so that it can accelerate the interaction between AMPC16 and the corresponding target molecule. The cytotoxic effect of AMPC16 was re-examined by the methylene blue method using the yeast cells which had been preincubated with YPD medium containing 10 mM MgSO₄ at 30 °C for 30 min and washed with freshly prepared YPD medium lacking Mg²⁺. Non-viable cells did not appear during a subsequent 2 h incubation of the Mg²⁺-pretreated cells in medium containing only AMPC16 at 25 μg ml⁻¹, but did

appear when Mg²⁺ was re-added. This indicates that the Mg²⁺-pretreated cells lost their susceptibility to AMPC16 easily upon simple washing, suggesting that Mg²⁺ promotes the cytotoxic action of AMPC16 via a reversible interaction with a cell surface component. HPLC analysis detected AMPC16 at almost the original level throughout the incubation with or without 10 mM Ca²⁺, as shown in Fig. 3. In contrast, the addition of Mg²⁺ accelerated the disappearance of AMPC16 from the incubation mixture, possibly by enabling its incorporation into or uptake across the plasma membrane. It remains to be determined how Mg²⁺ could stimulate the cellular uptake of AMPC16 in *Sac. cerevisiae* cells, whilst the uptake occurred in *Sch. pombe* cells without the aid of exogenous Mg²⁺ (see Table 1).

Inhibitory effects of AMPC16 on glucose-induced extracellular acidification

Inhibition of long-chain acyl-CoA synthetase seems likely to influence the cell growth more seriously than inhibition of adenylate cyclase and ribonuclease, since long-chain acyl-CoA is essential for the biosynthetic reactions of phospholipids, major constituents of the plasma membrane (Tomoda *et al.*, 1991). In *Sac. cerevisiae* cells, however, fatty acid synthetase can substitute for long-chain acyl-CoA synthetase since the enzyme can produce long-chain fatty acids in the form of the corresponding CoA derivative using acetyl-CoA and malonyl-CoA as starting materials (Johnson *et al.*, 1994). Cerulenin is a typical inhibitor of both fatty acid synthetase and 3-hydroxy-3-methylglutaryl-CoA synthetase, a key enzyme for ergosterol biosynthesis (Omura, 1976). This antibiotic restricted the growth of *Sac. cerevisiae* cells after several hours of incubation during which exhaustion of fatty acids or lipid components pre-existing in the YPD medium may have occurred, as deduced from the protective effects of exogenous long-chain fatty acids against cerulenin-induced growth inhibition (Knoll *et al.*, 1995). The growth-inhibition pattern of cerulenin was characterized by a lack of appearance of non-viable cells, in contrast to the pattern of AMPC16 (see Fig. 2). Instead of focusing on these cytoplasmic enzymes, we focused

Fig. 4. Effects of AMPC16 (a), DES (b), cerulenin (c) and amphotericin B (d) on glucose-induced extracellular acidification by cells of *Sac. cerevisiae*. Cells were incubated in distilled water (adjusted to pH 7.0) as a control (○) and incubated in water containing 100 mM glucose alone (●) or both 100 mM glucose and each of the above chemicals at the indicated concentrations (□, ■). In (a), the cell suspensions (○, ●, □, ■), except (△), were further supplemented with 10 mM MgSO_4 . Arrows indicate the time of addition of glucose and each chemical. The pattern of extracellular acidification in each column represents the most representative one from triplicate assays.

on the proton extrusion catalysed by the ATP-dependent reaction associated with the plasma membrane phospholipid bilayer, which has been evaluated as a target of cytotoxic agent (Monk *et al.*, 1995). This enzymic reaction is essential for cell viability because of its function as a proton pump that regulates cytoplasmic pH and provides the driving force for nutrient uptake (Serrano *et al.*, 1986; Serrano, 1989).

Lapathitis & Kotyk (1998) reported that the glucose-induced extracellular acidification depends on both proton extrusion resulting from the action of the plasma membrane H^+ -ATPase and the cellular excretion of glucose-derived carbon dioxide and organic acids. In that study, the extent of contribution of the latter process was examined by using strain Y55 and its isogenic mutant, *pmal-105*, with a genetic lack of the enzyme. This process was estimated to contribute only 6.9% of the overall glucose-induced acidification on the basis of the rate of increase in the extracellular proton concentration predicted from the pH value. As shown in Fig. 4(a), the glucose-induced extracellular acidification initially proceeded without apparent inhibition in AMPC16-treated cells, but was later drastically inhibited in a dose-dependent manner. As can be deduced from the pH values (predicted proton concentrations) at the 30 min incubation, the extracellular acidification was inhibited by $25 \mu\text{g ml}^{-1}$ AMPC16 much more than expected from an inhibition of the glucose-induced process with no relation to plasma membrane H^+ -ATPase. The extracellular pH change was not provoked by incubating the cells only with AMPC16 at $25 \mu\text{g ml}^{-1}$ and 10 mM MgSO_4 , but was initiated with a similar inhibition pattern after addition of glucose. AMPC16 could cause complete cell death at $25 \mu\text{g ml}^{-1}$ in such an experiment using an increased cell density

($10^8 \text{ cells ml}^{-1}$), suggesting a correlation between its inhibitory effect on the action of plasma membrane H^+ -ATPase and the Mg^{2+} -dependent cytotoxicity. The glucose-induced extracellular acidification was also markedly inhibited by 200 μM DES (Fig. 4b), which can directly interfere with the action of plasma membrane H^+ -ATPase (Wach *et al.*, 1990). The extent of extracellular acidification, possibly due to excretion of carbon dioxide and organic acids, was likewise estimated to be less than 10% after a 30 min incubation. AMPC16 was not likely to exhibit a protonophoric activity such as promoting passive influx of extracellular protons since the extracellular pH remained unchanged in the cell suspension with no glucose when the initial pH was adjusted to 5.0. These findings indicate that the inhibition of glucose-induced extracellular acidification is due to the inhibitory effect of AMPC16 on the proton pumping function of the plasma membrane H^+ -ATPase. As already suggested, based on its mode of action, cerulenin did not cause any inhibition of the glucose-induced process during 30 min incubation (Fig. 4c). Amphotericin B exerts an antifungal effect by causing damage of the plasma membrane phospholipid bilayers as a result of its complex formation with ergosterol (Herve *et al.*, 1989). Its inhibitory effect on the cellular proton extrusion thus could be a secondary effect resulting from the plasma membrane disruption (Fig. 4d).

Interference with activation of plasma membrane H^+ -ATPase by AMPC16

In the present study, the ATPase activity in the plasma membrane fraction was assayed in order to examine whether AMPC16 can directly inhibit ATP hydrolysis.

Table 2. Effects of AMPC16 on ATPase activity of the plasma membrane fraction

Chemical	Concn	ATPase activity*	Relative activity (%)
None		417 ± 51	100
AMPC16	25 µg ml ⁻¹	448 ± 60	107
	250 µg ml ⁻¹	423 ± 50	101
DES	20 µM	273 ± 37	65
	200 µM	62 ± 20	15
Amphotericin B	25 µg ml ⁻¹	117 ± 31	28

* Values are shown as nmol min⁻¹ (mg protein⁻¹) and are means ± SD of triplicate assays.

As summarized in Table 2, the enzyme activity was significantly inhibited by either DES or amphotericin B, but not by AMPC16. Plasma membrane H⁺-ATPase purified from bakers' yeast exhibited a loss of catalytic ability to hydrolyse ATP, but it was found to recover the original activity after reconstitution with a phospholipid component such as phosphatidylserine (Koland & Hammes, 1986). By analogy, the amphotericin B-mediated inhibition of the enzyme activity may be caused by an inhibition of the functional interaction between the enzyme protein and phospholipids in the isolated plasma membrane fraction.

The plasma membrane fractions were also prepared from cells which had been incubated under the same conditions as in Fig. 4(a) to examine the effect of AMPC16 on the level of active H⁺-ATPase. In triplicate assays, the enzyme activity was detected at a level of 424 ± 75 units (mg protein)⁻¹ after 10 min incubation with 100 mM glucose alone and it was then significantly reduced to the level of 250 ± 53 units (mg protein)⁻¹ during another 20 min incubation. The enzyme activity was detected at the significantly reduced level of 42 ± 21 units (mg protein)⁻¹ in AMPC16-treated cells at 10 min incubation and remained within a similar range during another 20 min incubation. The mechanism of the effect of AMPC16 on the plasma membrane H⁺-ATPase should have involved inhibition of the enzyme activity *in vivo* or interference with its activation.

Relationship between AMPC16-induced events and DAG

The plasma membrane H⁺-ATPase from *Sac. cerevisiae* is phosphorylated on multiple serine and threonine residues during its movement into the cell surface from the endoplasmic reticulum (Chang & Slayman, 1991). Phosphorylation of the enzyme is associated with an increased ATPase activity during growth on glucose. Upon glucose starvation, dephosphorylation occurs, together with a decrease in enzymic activity, and both are rapidly reversed upon re-addition of glucose. Site-specific phosphorylation is likely to regulate the ATPase activity in response to nutritional signals. It is known that the cellular cAMP level shows a rapid increase

under the above conditions in which H⁺-ATPase-dependent proton extrusion occurs at the fully stimulated rate (Morishita *et al.*, 1995), suggesting a cAMP-dependent mechanism for the site-specific phosphorylation of the enzyme. However, yeast strains that are specifically deficient in the glucose-induced cAMP increase still show normal activation of plasma membrane H⁺-ATPase (Becher dos Passos *et al.*, 1992). Moreover, yeast mutants with widely divergent levels of cAMP-dependent protein kinase activity display very similar levels of activation of the enzyme.

On the other hand, the addition of a membrane-permeable DAG analogue or other activators of protein kinase C (PKC) (Ogita *et al.*, 1990) to intact cells activates the H⁺-ATPase and at the same time causes a stimulation of proton extrusion from the cells (Brandão *et al.*, 1994). Both effects are reversed by the addition of staurosporine, a PKC inhibitor, which can cause a more potent inhibition of the enzyme activation when added together with calmidazolium, an inhibitor of Ca²⁺/calmodulin-dependent protein kinase (Brandão *et al.*, 1994). These results have suggested a model that explains glucose-induced activation of the enzyme via a phosphatidylinositol-type signalling pathway triggering phosphorylation of the enzyme by both PKC and Ca²⁺/calmodulin-dependent protein kinase. As shown in Fig. 5(a), the exogenous addition of DAG analogue could largely cancel the inhibitory effect of AMPC16 on cellular proton extrusion although its protective effect was not enhanced at a higher concentration (data not shown). The DAG analogue did not protect cells against the inhibition of glucose-induced proton extrusion by staurosporine (Fig. 5b). These results agree with the above model in which AMPC16 acts as an antagonist of phosphatidylinositol-type signalling that mediates glucose-induced activation of plasma membrane H⁺-ATPase.

Our findings suggested that AMPC16 inhibited the cellular generation of *sn*-1,2-DAG in response to a nutritional signal from glucose rather than directly interfering with the action of PKC. A radioenzymic assay has been used for the measurement of the intracellular level of DAG and has revealed a decrease of DAG along with apoptosis of various mammalian cells (Melnykovich *et al.*, 1992; Haug *et al.*, 1994). As shown in Fig. 6, the intracellular DAG level showed an immediate response to the addition of glucose, being increased to a maximum value at 10 min incubation, with a subsequent decrease to the original level. In contrast, in AMPC16-treated cells, the DAG level did not increase in response to glucose; instead DAG was decreased to a minimum level during the initial 10 min incubation. A mutant lacking *PLC1*-encoded phospholipase C gave clearer evidence showing the participation of a phosphatidylinositol-type signal in glucose-induced activation of plasma membrane H⁺-ATPase (Coccetti *et al.*, 1998). In cells of the mutant strain, the glucose signal did not provoke the increase in the DAG level and failed to activate the plasma membrane H⁺-ATPase.

In previous studies, we found a potent growth-inhibitory

Fig. 5. Protective effects of a membrane-permeable DAG analogue on the inhibition of glucose-induced proton extrusion by AMPC16 (a) and staurosporine (b). Cells were incubated in distilled water (adjusted to pH 7.0) as a control (○). In (a), cells were incubated in distilled water (adjusted to pH 7.0) containing 100 mM glucose and 10 mM MgSO₄ with (□, ■) or without (●) further addition of AMPC16 at 25 µg ml⁻¹. In (b), cells were incubated in distilled water (adjusted to pH 7.0) containing 100 mM glucose with (□, ■) or without (●) further addition of staurosporine at 50 µM. In both experiments, the DAG analogue was added at 30 µM (■). Arrows indicate the time of addition of glucose and each chemical. Each extracellular acidification curve is the most representative one from triplicate assays.

effect of the isoprenoid farnesol on *Sac. cerevisiae* cells, which was characterized by a transition of the budded to unbudded phase together with a decrease of intracellular DAG (Machida *et al.*, 1999). Unlike the farnesol-induced apoptosis of mammalian cells (Haug *et al.*, 1994; Voziyan *et al.*, 1995), farnesol-treated yeast cells were still viable but were prevented from progressing through the cell cycle for a very long period (Machida *et al.*, 1998, 1999). This means that the simple loss of DAG could not be the cause of the death in *Sac. cerevisiae* cells. As mentioned, complete inhibition of glucose-induced activation of plasma membrane H⁺-ATPase was achieved by the combined addition of staurosporine and calmidazolium (Brandão *et al.*, 1994). On the other hand, AMPC16 alone maximally inhibited glucose-induced proton extrusion so that its inhibition could not be completely reversed by exogenous addition of DAG (see Fig. 5). AMPC16 should exert an additional inhibitory effect on a DAG-independent mechanism for activation of plasma membrane H⁺-ATPase, on the assumption that this inhibitory effect can account for the cell death. Alternatively, the fungicidal effect of AMPC16 may arise from the combined inhibition of H⁺-ATPase and some other enzyme such as long-chain acyl-CoA synthetase or adenylate cyclase, which can be a target of this nucleotide analogue in other organisms (Shiraishi *et al.*, 1994; Hynie & Smrt, 1978). These possibilities are currently under investigation.

Fig. 6. Time course of the change of the intracellular DAG level during incubation with or without AMPC16. Cells were incubated in distilled water (adjusted to pH 7.0) containing 100 mM glucose and 10 mM MgSO₄ (○) or in this solution further supplemented with AMPC16 at 25 µg ml⁻¹ (●). Lipid fractions were prepared and the DAG in each fraction was measured as described in Methods. Values are means ± SD of triplicate assays.

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