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Unusual effect of *myo*-inositol on phospholipid biosynthesis in *Cryptococcus neoformans*

Vicki L. Vincent and Lisa S. Klig

Author for correspondence: Lisa S. Klig. Tel: +1 310 985 2424. Fax: +1 310 985 2315.
e-mail: lsklig@beach1.csulb.edu

Department of Biological Sciences, California State University Long Beach, Long Beach, CA 90840, USA

***Cryptococcus neoformans* is an opportunistic fungal pathogen which preferentially localizes to the inositol-rich environment of the central nervous system. One of its distinguishing traits is its capacity to catabolize inositol. Inositol is a precursor for the synthesis of phosphatidylinositol (PI). This study demonstrated that *C. neoformans* synthesizes inositol. Three inositol-containing sphingolipids were identified in *C. neoformans*: ceramide-(P-inositol)$_2$mannose, ceramide-P-inositol-mannose, and ceramide-P-inositol. These inositol-containing sphingolipids are typical of fungi but not higher eukaryotes. The effect of inositol on the membrane lipid composition of *C. neoformans* was also examined. In contrast to the nonpathogenic yeast *Saccharomyces cerevisiae*, neither the PI composition nor the synthesis of methylated phospholipids was altered by exogenous inositol. Hence, *C. neoformans* appears to have a metabolic mechanism for maintaining a steady lipid composition regardless of the inositol in its environment.**

Keywords: *Cryptococcus neoformans*, inositol, phospholipids

INTRODUCTION

Cryptococcus neoformans is an opportunistic fungal pathogen that infects humans. It is present but harmless in the respiratory tract of many healthy individuals (Randhawa & Paliwal, 1979). Infections in immunocompromised individuals usually manifest as meningoencephalitis (Kwon-Chung & Bennett, 1992). Localization of this organism to the inositol-rich environment of the central nervous system (CNS) (Spector & Lorenzo, 1975) differs from systemic infections with the fungal pathogens *Candida albicans* (Odds, 1987) and *Aspergillus* spp. (Musial *et al.*, 1988).

Studies of *C. neoformans* have been stimulated by increasing numbers of cryptococcal infections (Kwon-Chung & Edman, 1992) and a lack of effective antifungal therapies (Clark *et al.*, 1990). Four biological characteristics have been implicated in the pathogenicity of *C. neoformans*: (1) polysaccharide capsule (Bulmer *et al.*, 1967; Dykstra *et al.*, 1977; Fromtling *et al.*, 1982; Jacobson *et al.*, 1982; Kozel & Cazin, 1971; Kozel & Mastroianni, 1976; Kozel, 1977; Kwon-Chung & Rhodes, 1986); (2) phenoloxidase activity (Jacobson & Emery, 1991; Kwon-Chung *et al.*, 1982; Kwon-Chung & Rhodes, 1986; Polacheck *et al.*, 1982, 1990; Rhodes *et al.*, 1982; Wang & Casadevall, 1994); (3) ability to grow at 37 °C (Kwon-Chung & Rhodes, 1986; Rhodes *et al.*, 1982); and (4) α mating type (Kwon-Chung & Bennett, 1978; Kwon-Chung *et al.*, 1992; Moore & Edman, 1993). Further identification of unique biochemical and genetic characteristics of this organism may aid in the development of new antifungal treatments.

C. neoformans has the distinctive ability to use inositol as a sole carbon source (Barnett, 1976). This trait has been used clinically for a number of years to identify cryptococcal infections (Konemane & Roberts, 1985). Inositol is a six-carbon sugar and a precursor for the synthesis of phosphatidylinositol (PI), a major membrane phospholipid (Hokin & Hokin, 1953; Paulus & Kennedy, 1960). PI is an essential structural component of membranes (Hill *et al.*, 1990) and is also an anchor for certain membrane proteins (Cross, 1990). Moreover, PI and its phosphorylated metabolites are integral parts of the signal transduction pathway essential for balanced cellular growth (Berridge & Irvine, 1984). PI is also the precursor for the synthesis of ceramide-(P-inositol)$_2$mannose [M(IP)$_2$C], ceramide-P-inositol-mannose (MIPC) and ceramide-P-inositol (IPC), three inositol-containing

Abbreviations: CNS, central nervous system; DAG, diacylglycerol; IPC, ceramide-P-inositol; MIPC, ceramide-P-inositol-mannose; M(IP)$_2$C, ceramide-(P-inositol)$_2$mannose; *N*-MTF, *N*-methyltransferase; PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMME, phosphatidylmonomethylethanolamine; PS, phosphatidylserine; SAM, *S*-adenosylmethionine.

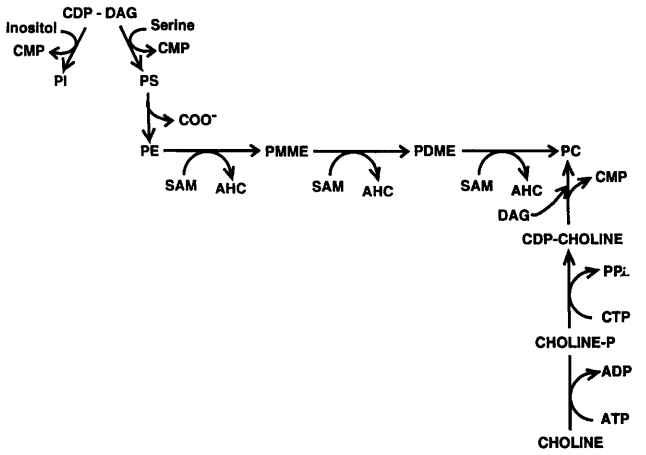

Fig. 1. **Phospholipid biosynthetic pathway. AHC, adenosylhomocysteine.**

sphingolipids identified and characterized in *Saccharomyces cerevisiae* (Smith & Lester, 1974; Steiner & Lester, 1972; Steiner *et al.*, 1969).

Inositol-containing sphingolipids have not been observed in higher eukaryotes, however, other sphingolipids and their degradation products have been shown to affect cellular differentiation and proliferation in mammalian cells (Olivera *et al.*, 1992). In yeast, inability to synthesize sphingolipids results in increased cell density, loss of ability to divide and ultimately loss of viability (Buede *et al.*, 1991; Dickson *et al.*, 1990; Pinto *et al.*, 1992).

The phospholipid biosynthetic pathway (Fig. 1) and its regulation have been studied in the nonpathogenic yeast *S. cerevisiae* and in the pathogenic yeast *C. albicans* (Carman & Henry, 1989). The presence of exogenous inositol in the growth medium of *S. cerevisiae* and *C. albicans* shifts phospholipid biosynthesis toward the formation of PI at the expense of methylated phospholipid synthesis (Bailis *et al.*, 1987; Kelley *et al.*, 1988; Klig *et al.*, 1985; Poole *et al.*, 1986). Both *S. cerevisiae* and *C. albicans* are capable of phosphatidylcholine (PC) synthesis by two pathways: *de novo* PC synthesis via methylation of phosphatidylethanolamine (PE) (Klig *et al.*, 1990; Steiner & Lester, 1972) or reaction of CDP-activated choline with diacylglycerol (DAG) (Kennedy & Weiss, 1956; Klig *et al.*, 1990). In *C. albicans*, the addition of exogenous choline decreases the synthesis of PC by reducing the activity of the *N*-methyltransferases (*N*-MTFs) (Klig *et al.*, 1990). In *S. cerevisiae*, *N*-MTF activity decreases in response to choline only if inositol is present (Klig *et al.*, 1988; Waechter & Lester, 1973; Yamashita *et al.*, 1982).

Prior research has established the overall lipid composition of *C. neoformans* at various growth phases in complex media (Itoh *et al.*, 1975; Itoh & Kaneko, 1977). This study examined the effects of exogenous inositol on growth, lipid synthesis and lipid composition of *C. neoformans*. Inositol biosynthesis by *C. neoformans* is demonstrated. Furthermore, *C. neoformans* membrane phospholipid composition is shown to be unaltered by the availability of inositol. These data suggest that *C. neoformans* utilizes a novel regulatory mechanism for inositol metabolism in a programme of balanced cell growth.

METHODS

Strains and media. All experiments were performed with two serotype D strains of *Cryptococcus neoformans*, C20a and C21α, obtained from Dr J. Edman at the University of California, San Francisco, USA. *Saccharomyces cerevisiae* strain A6 (*ade6* **a**) was used as a control.

All strains were maintained on YEPD (1%, w/v, yeast extract, 2%, w/v, Difco bacto-peptone, 2%, w/v, glucose, 2%, w/v, agar) plates. For experimental procedures, strains were grown in complete synthetic defined (SD) medium (lacking inositol) as described previously (Klig *et al.*, 1990). SD medium was supplemented with 75 μM inositol as indicated. In SD-inositol medium, 110 mM inositol was substituted for glucose as the carbon source. Where indicated in the text, the media were supplemented with 10 mM choline.

Determination of growth parameters. The growth rate of each yeast strain was determined in SD medium with and without inositol and/or choline supplementation, and in SD-inositol medium with and without choline supplementation. Cultures (10 ml) were inoculated and grown overnight at 30 °C with shaking. Cultures were diluted with fresh medium to an OD_{600} of 0·1 (~ 10^6 cells ml^{-1}). The cells were then incubated, shaking, at 30 °C and aliquots were removed at the times indicated. Cell numbers were determined spectrophotometrically (OD_{600}) and by viability plating.

Pulse labelling to examine lipid synthesis. Strains C20a, C21α and A6 were grown at 30 °C to mid-exponential phase. The cultures were diluted to 3–5 × 10^6 cells ml^{-1}. Incubation at 30 °C was continued until growth resumed and the cells underwent at least one doubling. The cells were then pulse labelled for 15 min with 0·1 mCi ^{32}P ml^{-1} (3·7 MBq ^{32}P ml^{-1}) (8500–9120 mCi $mmol^{-1}$; NEN/Dupont) or for 30 min with 0·5 μCi [*methyl*-^{14}C]methionine ml^{-1} (18·5 kBq [*methyl*-^{14}C]-methionine ml^{-1}) (43·4 mCi $mmol^{-1}$; NEN/Dupont) or 1·0 μCi [^{14}C]choline ml^{-1} (37 kBq [^{14}C]choline ml^{-1}) (55 mCi $mmol^{-1}$; ICN).

Steady state *in vivo* labelling to examine lipid composition. Strains C20a, C21α and A6 were grown in the media indicated at 30 °C. The cultures were diluted with fresh media and grown for five to six generations, to mid-exponential phase, in the presence of 0·01 mCi ^{32}P ml^{-1} (0·37 MBq ^{32}P ml^{-1}) (8500–9120 mCi $mmol^{-1}$; NEN/Dupont).

Phospholipid and sphingolipid extraction and separation. The yeast cells were broken and the lipids extracted essentially as described by Atkinson (1984). The samples were dried under a stream of nitrogen gas, resuspended in 40 μl chloroform/methanol (2:1, v/v), and spotted on Whatman SG81 silica-impregnanted paper (dipped in 2%, w/v, EDTA, pH 7·1). The lipids were separated by two-dimensional chromatography. Solvent I for phospholipid separation contained: 198 ml chloroform, 81 ml methanol, 12·5 ml 28·8% (w/v) ammonium hydroxide and 0·6 ml 5% (w/v) butylated hydroxytoluene in chloroform. Solvent II contained: 192 ml chloroform, 24 ml methanol, 6 ml water, 30 ml glacial acetic acid, and 0·6 ml 5% (w/v) butylated hydroxytoluene in chloroform. For sphingolipid separation, solvent I contained: 135 ml chloro-

form, 105 ml methanol, 15 ml water, 10 ml 28·8% (w/v) ammonium hydroxide, and 0·6 ml 5% (w/v) butylated hydroxytoluene in chloroform. Solvent II contained: 150 ml chloroform, 61 ml methanol, 40 ml glacial acetic acid, 16 ml water, and 0·54 ml 5% (w/v) butylated hydroxytoluene in chloroform. ^{14}C-labelled methylated lipids were separated by one-dimensional chromatography. The solvent consisted of chloroform/methanol/28·8% (w/v) ammonium hydroxide (66:17:3, by vol.).

Autoradiographs, obtained by exposing the chromatograms to Kodak AR-5 X-ray film, were used to locate the labelled lipids. Lipids were identified by their migration patterns relative to standards. The samples were cut from the chromatograms and counted by liquid scintillation.

Inositol excretion assay. C20a or C21α was grown on SD-inositol medium for up to 1 week. Cells were then transferred to complete SD plates (lacking inositol), sprayed with *S. cerevisiae* indicator strain AID (*ade-1/ade-1, inol-13/inol-13, lys-2/+, +/ade5,* a/α) (Greenberg *et al.*, 1982) and incubated at 30 °C. Growth of the indicator strain, which produces red colonies in the presence of inositol, was evaluated.

Analysis of data. c.p.m. incorporated into each lipid was determined. The percentage of each lipid relative to the total amount of label incorporated in all lipids was then calculated. The mean ± one standard error of the mean for all experimental trials was determined. One-way or two-way analysis of variance was used to determine the significance of the effects of the precursors on phospholipid synthesis or composition.

RESULTS

Growth parameters of *C. neoformans*

Two *C. neoformans* strains, C20a and C21α, and *S. cerevisiae* strain A6, were grown at 30 °C in SD medium with or without inositol supplementation and in SD-inositol medium without glucose. The doubling times for *C. neoformans* strains C20a and C21α in SD medium with or without inositol supplementation (75 μM) were approximately 3 h (Table 1). Both strains entered stationary phase at approximately 3×10^7 cells ml^{-1} in SD medium regardless of inositol supplementation. The growth rates were not altered by the addition of choline to the growth media. In SD-inositol medium (no glucose) the doubling times of both strains of *C. neoformans* were approximately 8 h, and there were approximately 2×10^7 cells ml^{-1} at stationary phase. When choline was added to SD-inositol medium, the growth rate of both *C. neoformans* strains was drastically decreased, to a doubling time of approximately 12 h. Similar to *C. neoformans*, *S. cerevisiae* strain A6 doubled approximately once every 3 h in SD medium with or without inositol supplementation (75 μM). However, *S. cerevisiae* did not grow in SD-inositol medium without glucose.

Table 1. Growth parameters of *C. neoformans* and *S. cerevisiae*

The doubling times in mid-exponential phase are shown.

Yeast strain	Glucose concn	Inositol concn	Growth rate (h)	
			Without choline	With choline (10 mM)
A6	110 mM	0	3	3
	110 mM	75 μM	3	3
C20a	110 mM	0	3	3
	110 mM	75 μM	3	3
	0	110 mM	8	12
C21α	110 mM	0	3	3
	110 mM	75 μM	3	3
	0	110 mM	8	12

Phospholipid synthesis by *C. neoformans*

C. neoformans strains C20a and C21α synthesized typically eukaryotic phospholipids. Both strains synthesized the four major phospholipids PI, PS, PC and PE (Table 2). Trace amounts of the methylated phospholipids PMME and PDME were also synthesized. Pulse labelling with ^{32}P for 15 min demonstrated the synthesis of PI by strains C20a and C21α regardless of the inositol concentration in the growth medium (Table 2). In *C. neoformans* strain C20a, PI accounted for approximately 30% of the total phospholipids synthesized during a period of 15 min. There was not a statistically significant difference in the phospholipids synthesized in strain C20a regardless of whether inositol was absent from the growth medium, present in micromolar amounts, or provided in millimolar amounts as the sole energy source (PI, $P = 0{\cdot}077$; PS, $P = 0{\cdot}411$; PC, $P = 0{\cdot}257$; PE, $P = 0{\cdot}472$). In strain C21α, inositol provided as the sole carbon source to media lacking glucose had a slight but statistically significant effect on the synthesis of PI ($P = 0{\cdot}013$) and PS ($P = 0{\cdot}012$). In contrast, in *S. cerevisiae* micromolar amounts of inositol added to media with glucose as a carbon source had a drastic effect on the synthesis of PI ($P < 0{\cdot}0001$), PC ($P = 0{\cdot}001$) and PE ($P = 0{\cdot}003$). PI represented 27·6% of the phospholipids synthesized in 15 min by *S. cerevisiae* grown in medium without inositol supplementation, whereas in growth medium containing 75 μM inositol PI accounted for 51·6% of the phospholipids synthesized.

Phospholipid composition of *C. neoformans*

To examine the effect of inositol on the phospholipid composition of *C. neoformans*, cultures were steady-state labelled with ^{32}P for five to six generations (Fig. 2). The PI percentage of total membrane phospholipids was not altered by exogenous inositol in either strain C20a or C21α (Table 3). Even when inositol was provided as the sole cabon source, PI remained approximately 8% of the total phospholipids labelled. The percentage of PI in the membranes of *C. neoformans* was similar to that of *S. cerevisiae* grown without inositol. In *C. neoformans*, the methylated phospholipid pathway which results in the synthesis of PC was also relatively unaltered by exogenous inositol, although some fluctuation is noted in strain C21α ($P = 0{\cdot}017$). In contrast, inositol caused a significant increase in the proportion of PI ($P < 0{\cdot}0001$), and a

Table 2. **Analysis of phospholipid synthesis in *C. neoformans* by pulse labelling**

Cells were labelled for 15 min with $^{32}P_i$ in defined media supplemented as indicated. Results show the mean ± one standard error of the mean for at least three experiments.

Yeast strain	Glucose concn	Inositol concn	Phospholipid (%)				
			PI	PS	PC	PE	Other*
A6	110 mM	0	27·6 ± 2·1	30·4 ± 5·6	4·4 ± 0·8	8·1 ± 0·9	20·9 ± 5·4
	110 mM	75 μM	51·6 ± 1·2	28·3 ± 1·1	1·2 ± 0·3	12·0 ± 0·5	6·8 ± 0·6
C20a	110 mM	0	28·7 ± 0·9	38·3 ± 2·6	13·0 ± 2·0	7·3 ± 0·7	11·9 ± 2·2
	110 mM	75 μM	27·8 ± 1·2	34·9 ± 3·1	15·3 ± 2·8	7·0 ± 0·7	11·8 ± 2·5
	0	110 mM	32·2 ± 1·7	32·0 ± 4·1	9·5 ± 1·6	9·0 ± 2·0	17·4 ± 3·6
C21α	110 mM	0	26·4 ± 1·4	41·4 ± 1·5	12·1 ± 2·2	8·6 ± 0·8	11·2 ± 1·2
	110 mM	75 μM	28·5 ± 1·5	40·1 ± 1·5	12·6 ± 1·5	8·7 ± 0·3	9·2 ± 1·5
	0	110 mM	36·9 ± 2·9	31·0 ± 3·3	11·4 ± 2·6	7·9 ± 0·2	15·6 ± 1·9

* Includes the pooled percentages of the minor phospholipids, cardiolipin, phosphatidylglycerol, CDP-DAG, phosphatic acid, PMME, PDME and other unidentified lipids present in trace amounts.

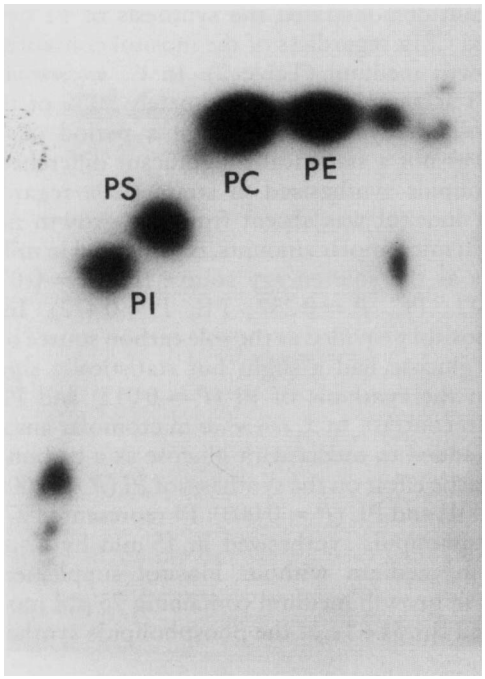

Fig. 2. **Phospholipid composition. Autoradiogram of a two-dimensional chromatographic separation of ^{32}P steady-state labelled phospholipids from strain C20a grown in SD-glucose medium.**

decrease in the proportion of PC ($P = 0{\cdot}005$) in *S. cerevisiae* strain A6.

PC synthesis by *C. neoformans*

C. neoformans cells were pulse labelled with [^{14}C]choline to establish the existence of an auxiliary pathway for the synthesis of PC. This pathway was originally described in *S. cerevisiae* (Kennedy & Weiss, 1956). After a 30 min pulse with [^{14}C]choline, 49000 and 65000 c.p.m. were incorporated into PC by 1·45 and 2·45 × 10^7 cells of strains C20a and C21α, respectively. The total ^{14}C incorporation into PC was not altered by the presence of inositol.

Methylated phospholipid synthesis by *C. neoformans*

To examine the effects of inositol and choline on *N*-MTF activity in *C. neoformans*, pulse labelling experiments with [*methyl*-^{14}C]methionine were performed (Fig. 3). [*methyl*-^{14}C]Methionine can be taken up by cells and converted to [^{14}C]*S*-adenosylmethionine (SAM). The [^{14}C]SAM can provide the methyl group for the synthesis of PMME, PDME and PC. The relative proportions of ^{14}C-labelled PMME, PDME and PC synthesized by C20a and C21α were not significantly altered by the presence or absence of inositol in the growth medium (Table 4). Addition of choline to the growth media, however, resulted in a statistically significant decrease in the proportion of PC (C20a, $P < 0{\cdot}0001$; C21α, $P = 0{\cdot}018$) and an increase in the proportion of PMME (C20a, $P < 0{\cdot}0001$; C21α, $P < 0{\cdot}024$). Inositol did not have a significant effect on methylated lipid synthesis in *C. neoformans*. In *S. cerevisiae*, choline significantly decreased PC ($P < 0{\cdot}0001$) and increased PMME ($P < 0{\cdot}0001$) synthesis. The addition of both inositol and choline to the growth medium of *S. cerevisiae* further decreased the proportion of PC ($P = 0{\cdot}003$) and increased the proportion of PMME ($P < 0{\cdot}0001$) and PDME ($P < 0{\cdot}0001$).

Direct comparison of the total c.p.m. incorporated by the *N*-MTFs into the methylated phospholipids clearly revealed the effects of choline and inositol (Table 5). *N*-MTF activity of strain C20a was repressed more than threefold in the presence of choline. Similarly the *N*-MTF activity in C21α was repressed more than sixfold by choline. This repression was not dependent on or affected by inositol. In contrast, the repression of total *N*-MTF activity in *S. cerevisiae* by either exogenous inositol or choline was less than twofold. However, in the presence of both inositol and choline *N*-MTF activity in *S. cerevisiae* was repressed 25-fold.

Table 3. Analysis of phospholipid composition of *C. neoformans* by steady-state labelling

Cells were labelled for five to six generations with $^{32}P_i$ in defined media supplemented as indicated. Results show the mean ± one standard error of the mean for at least four experiments.

Yeast strain	Glucose concn	Inositol concn	Phospholipid (%)				
			PI	PS	PC	PE	Other*
A6	110 mM	0	9·2±0·8	10·7±1·3	49·2±2·7	17·8±2·0	20·8±2·6
	110 mM	75 μM	28·1±0·1	9·0±0·9	30·4±1·8	23·8±1·7	13·0±2·2
C20a	110 mM	0	7·6±0·2	11·8±0·6	42·3±1·6	27·7±2·3	13·4±2·2
	110 mM	75 μM	10·0±1·8	11·6±0·7	41·7±2·3	24·2±0·8	11·2±2·5
	0	110 mM	7·9±0·5	11·2±0·5	41·5±3·0	27·0±2·9	12·0±2·4
C21α	110 mM	0	6·9±0·5	8·0±0·5	45·1±1·7	23·8±2·7	15·6±4·5
	110 mM	75 μM	8·7±0·6	7·8±1·2	40·1±2·5	28·1±1·9	20·4±2·9
	0	110 mM	8·0±0·1	8·1±1·6	53·1±3·5	21·2±1·6	9·2±1·7

*Includes the pooled percentages of the minor phospholipids, cardiolipin, phosphatidylglycerol, CDP-DAG, phosphatic acid, PMME, PDME and other unidentified lipids present in trace amounts.

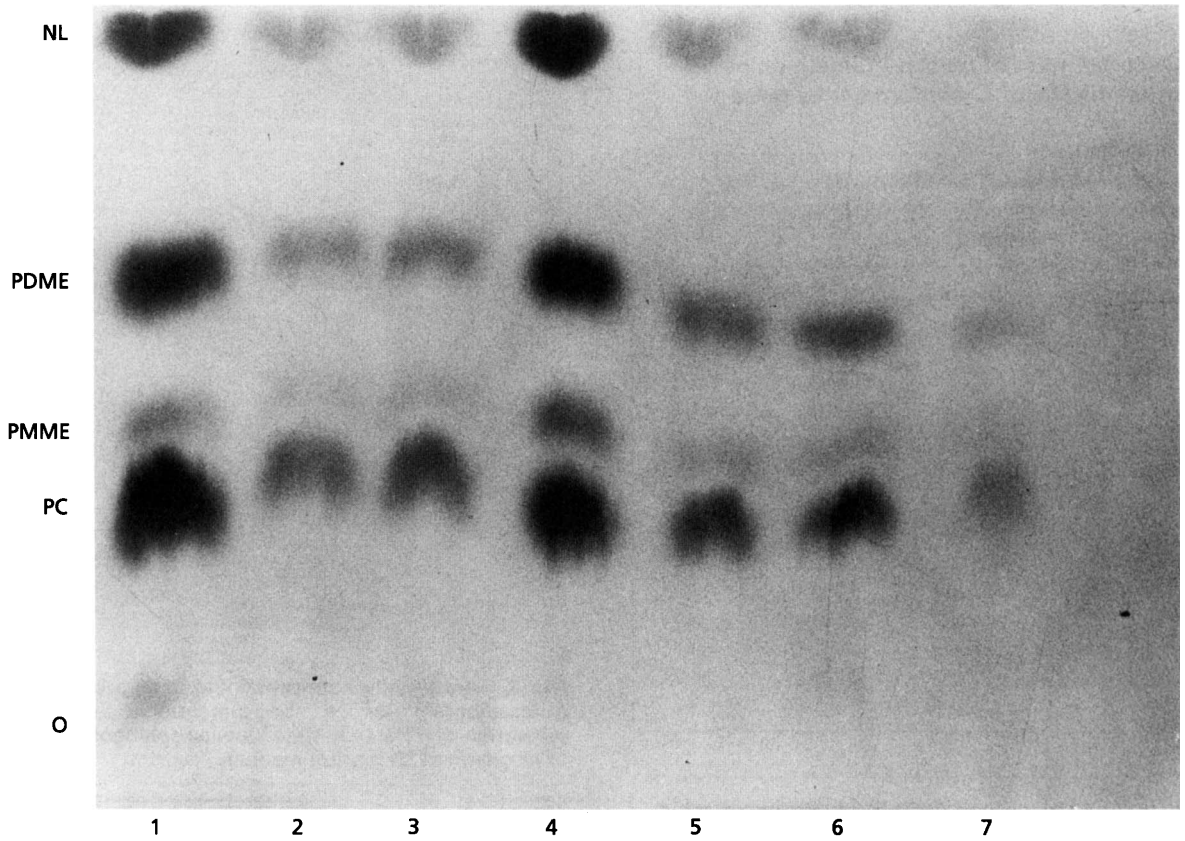

Fig. 3. Methylated phospholipid synthesis. Autoradiogram of a one-dimensional chromatographic separation of [*methyl*-^{14}C]methionine-labelled methylated phospholipids. Lipids were extracted from yeast grown in SD medium without inositol (lane 1, A6; lane 2, C20a; lane 3, C21α), in SD medium with 75 μM inositol (lane 4, A6; lane 5, C20a; lane 6, C21α) or in SD-inositol medium (lane 7, C20a). Abbreviations: O, origin; NL, neutral lipids.

Inositol-phosphate-containing sphingolipids in *C. neoformans*

The presence of inositol-phosphate-containing sphingolipids in *C. neoformans* was established by steady-state labelling of the lipids with ^{32}P. ^{32}P-labelled lipids extracted from *C. neoformans* were identified by their migration relative to the standards IPC, MIPC and $M(IP)_2C$ (Fig. 4). These three inositol-containing sphingolipids were found in the membranes of *C. neoformans* regardless of inositol

Table 4. Analysis of methylated phospholipid synthesis in *C. neoformans* by pulse labelling

Cells were labelled for 30 min with [*methyl*-^{14}C]methionine in defined media supplemented as indicated. Results show the mean ± one standard error of the mean of at least four experiments. NA, Not available.

Yeast strain	Glucose concn	Inositol concn	Phospholipid (%)					
			Without choline			With choline (10 mM)		
			PC	PMME	PDME	PC	PMME	PDME
A6	110 mM	0	73·7 ± 4·0	4·7 ± 1·2	20·9 ± 2·9	56·4 ± 2·2	10·1 ± 1·2	32·6 ± 1·1
	110 mM	75 μM	73·2 ± 2·6	6·4 ± 1·5	24·3 ± 3·4	33·6 ± 2·2	26·7 ± 1·0	38·2 ± 0·8
C20a	110 mM	0	58·4 ± 1·8	14·6 ± 1·1	27·0 ± 0·8	49·0 ± 2·6	24·0 ± 3·8	34·5 ± 3·3
	110 mM	75 μM	56·0 ± 1·7	14·2 ± 1·3	31·0 ± 2·7	44·1 ± 1·8	22·0 ± 1·2	33·9 ± 1·7
	0	110 mM	58·4 ± 3·5	15·3 ± 2·0	25·8 ± 1·8	NA	NA	NA
C21α	110 mM	0	55·0 ± 2·0	15·9 ± 1·8	29·4 ± 1·5	45·2 ± 2·8	21·0 ± 1·7	33·7 ± 1·7
	110 mM	75 μM	56·0 ± 3·0	14·5 ± 1·5	29·4 ± 2·4	52·6 ± 1·8	14·1 ± 0·8	33·3 ± 2·4
	0	110 mM	53·2 ± 2·2	19·2 ± 1·4	27·4 ± 1·3	NA	NA	NA

Table 5. Incorporation of *methyl*-^{14}C]methionine into the methylated lipids of *C. neoformans* by pulse labelling

c.p.m. were counted by liquid scintillation. Results show c.p.m. incorporation into methylated phospholipids for a typical experiment. NA, Not available.

Yeast strain	Glucose concn	Inositol concn	Total c.p.m. incorporation	
			Without choline	With choline (10 mM)
A6	110 mM	0	20632·4	14215·0
	110 mM	75 μM	14164·8	816·0
C20a	110 mM	0	1071·1	309·4
	110 mM	75 μM	2086·8	641·8
	0	110 mM	594·8	NA
C21α	110 mM	0	4681·1	696·2
	110 mM	75 μM	3728·2	566·8
	0	110 mM	491·6	NA

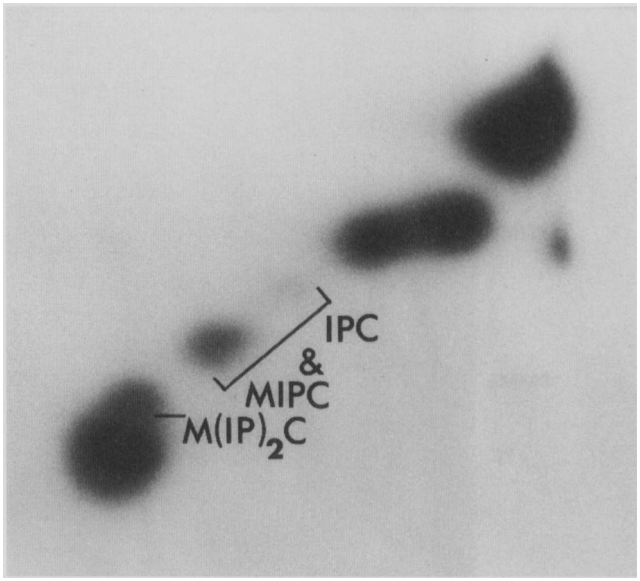

Fig. 4. Inositol-phosphate-containing sphingolipid composition. Autoradiogram of a two-dimensional chromatographic separation of ^{32}P steady-state labelled sphingolipids from strain C21α grown in SD-inositol medium.

supplementation of the growth medium (Table 6). Growth of *C. neoformans* in SD-inositol medium (no glucose), however, led to a decrease in the amount of label incorporated into the inositol-containing sphingolipids.

Inositol excretion assay

To determine if the phospholipid composition was regulated independently of exogenous inositol due to excretion of internal inositol, strains C20a and C21α were tested for inositol excretion. An *S. cerevisiae* strain AID (*ade-1/ade-1, ino-1/ino-1, lys-2/+, his-3/+, +/ade-5*, a/α) was used as an indicator for the excretion of inositol by *C. neoformans*. In the presence of inositol this diploid *S. cerevisiae* strain produces red colonies. *C. neoformans* cells grown in SD-inositol medium were transferred to SD-glucose medium lacking inositol with the indicator strain AID. Growth of red *S. cerevisiae* colonies was not observed after 2 weeks of incubation with *C. neoformans*. This demonstrates that *C. neoformans* does not excrete inositol which may have accumulated intracellularly.

Table 6. Analysis of inositol-containing sphingolipids in *C. neoformans* by steady-state labelling

c.p.m. were counted by liquid scintillation. Results show c.p.m. incorporation into sphingolipids for a typical experiment.

Yeast strain	Glucose concn	Inositol concn	Total c.p.m. incorporation	
			$M(IP)_2C$	MIPC + IPC
A6	110 mM	0	731·9	1852·9
	110 mM	75 μM	288·6	773·7
C20a	110 mM	0	2574·5	7251·5
	110 mM	75 μM	1245·3	2403·3
	0	110 mM	1108·2	2175·3
C21α	110 mM	0	1629·3	6354·5
	110 mM	75 μM	1414·0	5489·8
	0	110 mM	993·1	2151·8

DISCUSSION

Inositol is a key cellular metabolite which can be used by *C. neoformans* for the synthesis of membrane lipids and as an energy source for growth. This study examined the effects of exogenous inositol and choline on cell growth, lipid synthesis and lipid composition in two wild-type strains of *C. neoformans*, C20a and C21α. Both **a** and α mating type strains grew at similar rates in SD medium with or without inositol and/or choline supplementation (Table 1). However, the growth rate decreases approximately twofold in SD-inositol medium (without glucose). The growth rate is further decreased by the presence of choline in this medium. *C. neoformans* was demonstrated to synthesize typically eukaryotic phospholipids by pulse labelling experiments with ^{32}P, [*methyl*-^{14}C]methionine and [^{14}C]choline (Tables 2 and 4). These experiments also showed that PC synthesis occurs via both the *de novo* and auxiliary pathways (Fig. 1). Most importantly, the PI percentage of total phospholipid synthesized is relatively unaffected by the concentration of exogenous inositol in the growth medium. Steady-state labelling experiments revealed that the major phospholipids in the membranes of *C. neoformans* are PI, PS, PE and PC (Table 3). *C. neoformans* was shown to have the inositol-containing sphingolipids IPC, MIPC and $M(IP)_2C$ which are unique to yeast. The phospholipid composition data are in agreement with previous studies of *C. neoformans* lipids which were performed using complex growth medium (Itoh *et al.*, 1975; Itoh & Kaneko, 1977). Another report on the lipid composition of *C. neoformans* was unable to identify PS or sphingolipids (Rawat *et al.*, 1984). This discrepancy may be due to differences in the extraction and/or separation techniques used for each study. The phospholipid composition analyses presented here indicate that exogenous inositol does not affect the proportion of inositol-containing phospholipids in the membranes of *C. neoformans*.

This study establishes that *C. neoformans* synthesizes inositol *de novo*. Specifically, pulse labelling experiments confirm that PI was synthesized in the membranes of cells grown for many generations in medium without inositol. Even though inositol prototrophy is typical of most eukaryotic cells, some fungi are natural inositol auxotrophs (Fernandez *et al.*, 1986). Interestingly, *C. neoformans* is one of a few yeasts that use inositol as a carbon source (Barnett, 1976). Thus, this organism can serve as a model to study the coordination of inositol synthesis, uptake and catabolism.

C. neoformans, unlike most other yeasts, has the ability to grow on SD-inositol medium without glucose (Barnett, 1976). When choline is added to this medium, however, the cells' growth rate decreases. In contrast, choline does not affect the growth rate of *C. neoformans* in SD media with glucose as an energy source. Choline may be interfering with inositol uptake, inositol catabolism or lipid synthesis.

The addition of inositol and choline to the growth medium of *S. cerevisiae* alters the proportion of methylated lipids synthesized and decreases total *N*-MTF activity (Tables 3 and 4). The addition of choline with or without inositol to the growth medium of *C. albicans* alters the proportions of the methylated lipids and decreases *N*-MTF activity (Klig *et al.*, 1990). Micromolar amounts of exogenous inositol in the growth medium of *C. neoformans* do not alter the relative proportion of the methylated phospholipids synthesized (Table 4) or the total amount of *N*-MTF activity (Table 5). The apparent decrease in total c.p.m. incorporation into methylated lipids when the cells are grown in SD-inositol medium is probably due to the decreased growth rate. Addition of choline only slightly alters the proportions of methylated lipids synthesized (Table 4); however, total *N*-MTF activity is decreased (Table 5). Thus, *N*-MTF activity in *C. neoformans* appears to respond to choline and is unaffected by inositol.

PI synthesis by *C. neoformans* strain C20a is not altered by exogenous inositol in the medium (Table 2). Strain C21α demonstrated a slight increase in PI synthesis when inositol was provided as the sole carbon source (in millimolar amounts). Supplementation of the growth medium of strain C21α with micromolar amounts of inositol, however, does not result in a significant change in PI synthesis. In contrast, there is a dramatic increase in PI synthesis in *S. cerevisiae* when the growth medium is supplemented with even micromolar amounts of inositol. Steady-state lipid labelling experiments demonstrated that the overall phospholipid composition of both strains of *C. neoformans* is not affected by inositol, even when it is provided as the carbon source (Table 3). In contrast, the PI percentage of total membrane lipids in *S. cerevisiae* and *C. albicans* varies with inositol supplementation of the media (Culbertson *et al.*, 1976; Klig *et al.*, 1990). These data suggest that *C. neoformans* may possess a novel regulatory mechanism for stabilizing its membrane composition.

Three models offer possible mechanisms of a steady *C. neoformans* phospholipid composition despite variable

environments. The first model proposes that *de novo* inositol synthesis results in an internal inositol concentration sufficient to fully saturate PI synthase. Addition of inositol to the growth media would have no effect on the already saturated PI synthase. The second model also involves *de novo* inositol synthesis, proposing that the internal inositol concentration is consistently sufficient to repress the *N*-MTFs. CDP-DAG is the common precursor for the synthesis of PI and the methylated phospholipids. Maintenance of repressed *N*-MTFs would result in a steady amount of CDP-DAG available for PI synthesis. Addition of inositol to the growth media would, again, have no effect on the already repressed *N*-MTFs resulting in a steady phospholipid composition. The third model proposes that *C. neoformans* maintains a constant internal inositol concentration. This pool size is not necessarily at saturating or repressing levels. This study suggests that the intracellular inositol concentration does not appear to be regulated by excretion. However, the internal inositol pool size could be controlled by regulating either inositol uptake or catabolism. A constant internal pool of inositol, regardless of inositol availability, would result in a steady phospholipid composition.

C. neoformans is surrounded by a polysaccharide capsule which has been shown to decrease the phagocytosis of *C. neoformans* by leukocytes (Kozel & Cazin, 1971; Kozel & Mastroianni, 1976; Kozel, 1977). The capsule is composed of a mannose backbone with xylose and glucuronic acid side chains (Bhattacharjee *et al.*, 1979). In plants, inositol is a precursor of glucuronic acid and xylose (Moore & Edman, 1993). It is interesting to speculate that in *C. neoformans* inositol catabolic products may be used for capsule synthesis.

Cryptococcal infections in immunocompromised individuals preferentially localized to the CNS where the concentration of inositol is high (Spector, 1976; Spector & Lorenzo, 1975). Two unusual traits of *C. neoformans* are the utilization of inositol as a carbon source and maintenance of a constant PI percentage in its membranes. These traits may influence its localization to the inositol-rich environment of the CNS.

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