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Acetylcholine Induces Yeast to Hyphal Form Transition in *Candida Albicans*

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Abstract

Background: In Candida Albicans, yeast to hyphal form transition can be induced by serum, proline, glucose, and N-acetyl glucosamine. Acetylcholine is a neuromodulator which can stimulate both muscarinic and nicotinic acetylcholine receptors in humans. In this study, we are reporting that acetylcholine can induce yeast to hyphal form transition in C. Albicans. The adenylyl cyclase inhibitor, MDL 12, 330A inhibited this transition indicating the role of cAMP. Muscarinic receptors in C. Albicans did not report yet. We have reported that C. Albicans Rrp9 exhibits identity and similarity with the human muscarinic receptor M1. In humans, activation of muscarinic M1 receptor can produce cAMP through inositol phosphate pathway. The inositol phosphate pathway in C. Albicans is already known. We have carried out the local and global alignment sequences between the proteins of humans and C. Albicans which are involved in inositol phosphate pathway. We found considerable identities and similarities between them. Herein, we hypothesize that acetylcholine may activate Rrp9 which may lead to activation of inositol phosphate signalling pathway in C. Albicans. This study suggests that Rrp9 may have a potential role in yeast to hyphal form transition in C. Albicans.

Keywords: Morphogenesis; Acetylcholine; Muscarinic receptors; cAMP; Inositol phosphate pathway; Bioinformatics

Introduction

Communication and sensing processes in eukaryotic cells are governed by their surrounding environment. The first step in sensing of signalling molecules or ligands depends on a receptor [1]. The signal molecule or ligand may bind with its receptor leading to intracellular responses that involve many physiological and biological events [2]. Acetylcholine is a neurotransmitter secreted from nerve cells to send signals to other cells. Acetylcholine stimulates both muscarinic and nicotinic acetylcholine receptors [3]. Muscarinic acetylcholine receptors are typical G-protein coupled receptors that mediate various important physiological and biological functions according to their location and subtype [4]. Five distinct muscarinic receptor subtypes (M1-M5) are known in humans [5]. M1, M3, and M5 receptors can couple with Gαq-protein and stimulate the inositol phosphate pathway. The M2 and M4 receptors act via Gαi-protein to inhibit adenylyl cyclase which results in reducing of intracellular cAMP production [6]. In Candida Albicans, the essential protein, Rrp9 is reported to exhibit identity and similarity with human muscarinic M1 receptor [7]. Activation of human muscarinic M1 receptor can produce cAMP through inositol phosphate pathway. After binding with an agonist, acetylcholine, the activated muscarinic M1 receptor couples with Gq subunit type of heterotrimeric G-alpha protein which leads to stimulation of phospholipase C (PLC) through inositol phosphate pathway. The enzyme, phospholipase C can hydrolyse the phospholipid, phosphatidylinositol 4, 5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG and IP3 are second messengers that regulate diverse cellular processes in human cells. IP3 diffuses through the cytosol to binding with IP3 receptors of calcium channels in the smooth endoplasmic reticulum (ER) to release calcium into cytoplasm. Calcium involved in signal transduction which can catalyse calmodulin (caM) to stimulate adenylyl cyclase production. Adenylyl cyclase produces cAMP from ATP. On the other hand, DAG can activate protein kinase C and in turn stimulate adenylyl cyclase to form cAMP [8-10]. Production of cAMP is involved in many intracellular activities like cell growth, regulation of cell proliferation, skin cell signalling and immune responsiveness [11-15], learning and memory processes [16,17]. Morphogenesis in C. Albicans is considered as a good model system for studying eukaryotic cell differentiation. In C. Albicans, yeast to hyphal form transition can be induced by various external signals such as serum, neutral pH, high temperature, contact, glucose, proline, N-acetyl glucosamine, CO2, and starvation [18-26]. Yeast to hyphal form transition involves many signalling pathways such as cAMP-PKA and Mitogenic-activated protein (MAP) kinase pathways [27,28]. In this study, acetylcholine can induce yeast to hyphal form transition in C. Albicans and the mechanism of induction is hypothesized.

Materials and Methods

Chemicals and media

Acetylcholine chloride was purchased from TCI chemicals Pvt. Ltd., India. Adenylyl cyclase inhibitor, MDL 12,330A was purchased from Sigma-Aldrich, India. Micro titre plates and other media were purchased from HiMEDIA Chemicals Ltd., Mumbai, India.

Culture of *Candida Albicans*

Candida Albicans (ATCC 90028) was obtained from the Institute of Microbial Technology (IMTECH) Chandigarh, India. The culture was maintained on Yeast extract -Peptone –Dextrose (YPD) agar slant at 4°C and propagated by inoculating a single colony from the YPD agar

plates (Yeast extract 1%, Peptone 2%, Dextrose 2% and Agar 2.5%) into 50 ml YPD broth in a 250-ml conical flask. Flasks were incubated overnight at 30°C at 100 rpm on an orbital shaking incubator. The cells were harvested by centrifugation at 2000 rpm and washed thrice with sterile 0.1 M Phosphate-Buffered Saline (PBS), pH 7.4 and the cell density was determined by a haemocytometer count. Finally cells were suspended in sterile PBS.

Yeast to hyphal form transition

Yeast to hyphal form transition assay was carried out in 96-well micro titre plates [29]. C. Albicans cells stock was diluted to 1 × 106 cells/ml in PBS buffer. Various concentrations of acetylcholine chloride were prepared and were added in each well. Wells without acetylcholine were kept as control. The final volume was kept at 200 µl in each well. Triplicate wells were run. The micro titre plates were incubated at 37°C at 120 rpm on an orbital shaker incubator for 2 h. After incubation period, the cells were observed microscopically by using an inverted light microscope (Metzer, India). Hundred cells were counted and numbers of yeast and hyphal forms were noted. Three counting were taken. The main value was used to determine hyphal form formation.

Inhibition of Morphogenesis

C. Albicans cells stock was diluted to 1 × 106 cells/ml in 1% of acetylcholine chloride. Various concentrations of adenylyl cyclase inhibitor, MDL 12,330A were prepared and ranged 200-3.1 µg/ml and were added in each well. Wells without MDL 12,330A were kept as a control. The final volume was kept at 200 µl in each well. The micro titre plates were incubated at 37°C at 120 rpm on shaking incubator for 3 h. After incubation period cells were observed microscopically by using inverted light microscope (Metzer, India). The concentration which inhibited hyphae formation by 50% was compared to the control and was considered as the Minimum inhibitory concentration (MIC) for morphogenesis. All experiments were done in triplicate.

Bioinformatics study

The local and global alignment sequences between human and Candida Albicans proteins involved in inositol phosphate pathway.

The FASTA sequences of human proteins Guanine nucleotide-binding protein G (q) subunit alpha (Gαq protein), Calmodulin (caM), Protein kinase C theta type (PKC-θ), Protein kinase C epsilon type (PKCε), Phospholipase C gamma types 1 & 2 (PLCG1 and PLCG2), Phospholipase C delta types 1, 3 & 4 (PLCD1, PLCD3, and PLCD4) and Phospholipase C β1 were retrieved from Uniprot database with accession numbers of P50148, P62158, Q04759, Q02156, P19174, P16885, P51178, Q8N3E9, Q9BRC7 and Q9NQ66 respectively. The FASTA sequences of Candida Albicans proteins G protein alpha subunit (Gpa2), Calmodulin (caM), Phospholipase C1 (PLC1), Protein kinase C-like 1(PKC1) were obtained from Uniprot database with accession numbers of A0A1D8PJG1, P23286, O13433 and P43057 respectively. The alignment was carried out between human and C. Albicans proteins by using Smith-waterman method for local alignment and by using Needleman-Wunsch method for global alignment as follows:

- Between G protein alpha subunit (Gpa2) (A0A1D8PJG1) from C. Albicans and human Gαq protein (P50148).
- Between Calmodulin (caM) (P23286) from C. Albicans and human Calmodulin (caM) (P62158).
- Between C. Albicans PLC1 (O13433) and human PLCG1 (P19174), PLCG2 (P16885), PLCD1 (P51178), PLCD3 (Q8N3E9), PLCD4 (Q9BRC7) and Phosphlipase C β1 (Q9NQ66) separately.
- Between C. Albicans PKC1 (P43057) and human PKC-θ (Q04759) and PKCε (Q02156) separately.

Statistical analysis

Values of samples were compared by using Student's t-test. A value of $P < 0.05$ was considered statistically significant.

Results

Acetylcholine induces yeast to hyphal form transition in C. Albicans

Acetylcholine induced yeast to hyphal form transition in C. Albicans (ATCC 90028) after 2 h. At concentration of 1% of acetylcholine, hundred percentages of hyphal formation was showed (Figure 1a; Figure 2a). A 95%, 85%, 80% and 35% of yeast to hyphal form transition was showed at concentrations of 0.5%, 0.25%, 0.125% and 0.062% respectively. At concentration of 0.031% of acetylcholine, 15% of hyphal formation was showed.

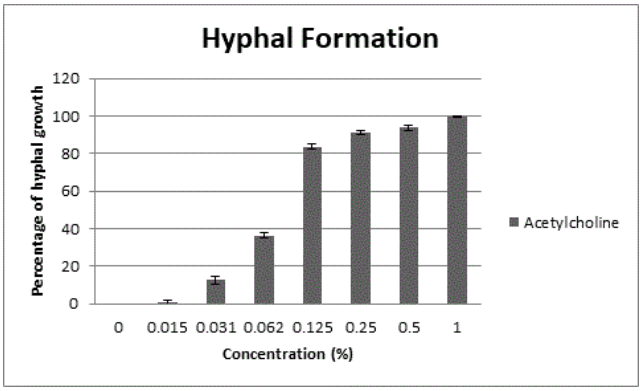

Figure 1a: Acetylcholine inducing of yeast to hyphal form transition in *Candida Albicans* at 37 °C after 2h.

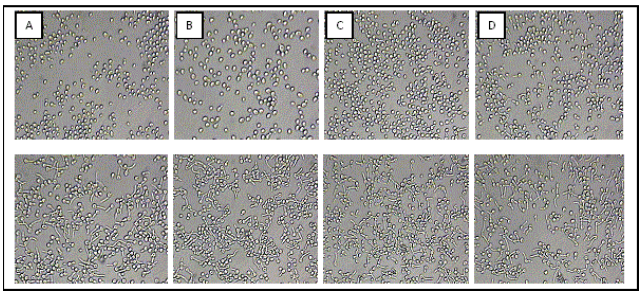

Figure 2a: Acetylcholine induces yeast to hyphal form transition in *Candida Albicans*; A) Control; B) 0.015%; C) 0.062%; D) 0.031%; E) 0.125%; F) 0.25%; G) 0.5%; H) 1%.

Adenylyl cyclase inhibitor, MDL 12,330A inhibits yeast to hyphal formation induced by acetylcholine in *C. Albicans*

The human adenylyl cyclase inhibitor, MDL 12,330A inhibited yeast to hyphal form transition at 12.5 µg / ml and above this concentration. Fifty percentage of hyphal formation was inhibited at 6.2 µg / ml and considered as the Minimum inhibitory concentration (MIC) (Figure 2b).

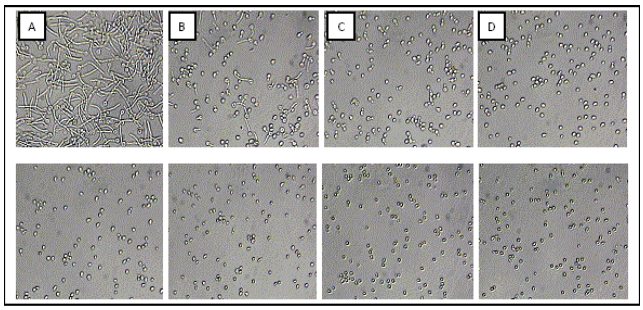

Figure 2b: Effect of adenylyl cyclase inhibitor, MDL 12,330A on acetylcholine induced yeast to hyphal formation in *Candida Albicans* (ATCC 90028). A) Control; B) 3.1 µg/ml C) 6.2 µg/ml; D) 12.5 µg/ml; E) 25 µg/ml; F) 50 µg/ml; G) 100 µg/ml; H) 200 µg/ml.

Proteins of humans and *Candida Albicans* which are involved in the inositol phosphate pathway share significant identities and similarities.

Local alignments

The local alignment between human Gαq protein and C. Albicans G protein alpha subunit (Gpa2) showed that human Gαq protein has 38% identity and 56% similarity with C. Albicans G protein alpha subunit (Gpa2) at the amino acid level at an overlap of 370 amino acids (Table 1; Figure 3a). The local alignment between human calmodulin (caM) and C. Albicans calmodulin (caM) showed that the human calmodulin (caM) has 71% identity and 89% similarity to C. Albicans calmodulin (caM) at an overlap of 149 amino acid (Table 1). The local alignment between, human Protein kinase C epsilon type (PKCε) and *Candida Albicans* protein kinase C-like 1(PKC1) revealed that human PKCε has 35% identity and 51% similarity to C. Albicans PKC1 at an overlap of 757 amino acids (Table 1). Also, the local alignment between human protein kinase C theta type (PKC-θ) and C. Albicans PKC1 showed that human PKC-θ has 34% identity and 54% similarity to C. Albicans PKC1 at an overlap of 756 amino acids (Table 1). The local alignment between human Phospholipase C delta types (PLCD1, PLCD3, and PLCD4) and C. Albicans Phospholipase C1 (PLC1) represented that human PLCD1 and PLCD3 have 30% identity with similarities 49% and 47% respectively to C. Albicans PLC1 at an overlap of 699 and 634 amino acids respectively, the human PLCD4 also has 33% identity and 48% similarity with C. Albicans PLC1 at an overlap of 635 amino acids (Table 1). The local alignment between human Phospholipase C gamma types (PLCG1and PLCG2) to C. Albicans PLC1 revealed that human PLCG1 has 42% identity and 61% similarity with C. Albicans PLC1 at amino acids level of 159 amino acids, while PLCG2 has 44% identity and 61% similarity to CaPLC1 at an overlap of 159 amino acids (Table 1). The local alignment between human Phospholipase C beta 1(PLCβ1) and C. Albicans PLC1 showed that C. Albicans PLC1 has 36% identity and 55% similarity with human PLCβ1 at an overlap of 363 amino acids (Table 1; Figure 3b).

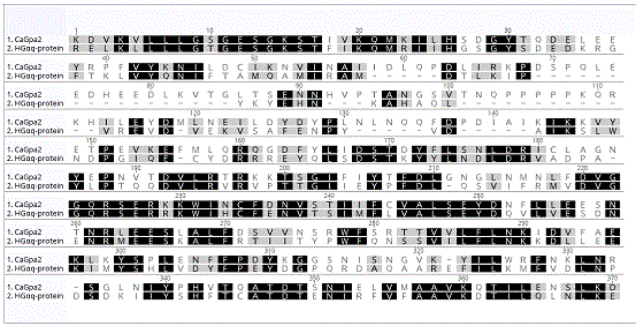

Figure 3a: Pairwise alignment between amino acid sequences of *Candida Albicans* Gαq (CaGpa2) and human Gαq-protein (HGαq) showing 38% identity and 56% similarity; Identical residues; Similar residues.

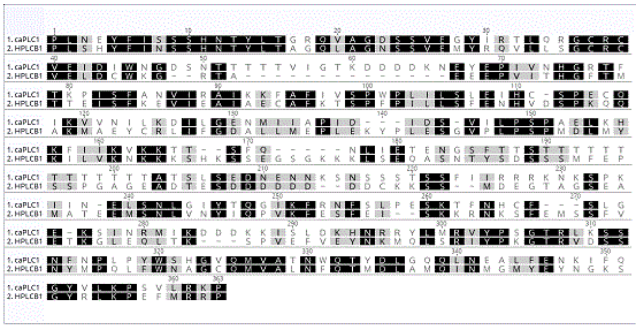

Figure 3b: Pairwise alignment between amino acid sequences of Candida Albicans PLC1 (CaPLC1) and human PLCB1 (HPLCB1) showing 36% identity and 55% similarity; Identical residues; Similar residues.

Global alignments

The global alignment between human Gαq protein and C. Albicans G protein alpha subunit (Gpa2) showed that human Gαq protein has 30% identity and 45% similarity with C. Albicans G protein alpha subunit (Gpa2) at the amino acid level at an overlap of 505 amino acids (Table 2). The global alignment between human calmodulin (caM) and C. Albicans calmodulin (caM) showed that the human calmodulin (caM) has 71% identity and 89% similarity with C. Albicans calmodulin (caM) at an overlap of 149 amino acids (Table 2; Figure 3c). The global alignment between human Protein kinase C epsilon type (PKCε) and C. Albicans Protein kinase C-like 1(PKC1) revealed that human PKCε has 27% identity and 42% similarity with C. Albicans PKC1 at an overlap of 1118 amino acids (Table 2; Figure 3d). Also, the global alignment between human Protein kinase C theta type (PKC-θ) and C. Albicans PKC1 showed that human PKC-θ has 27% identity and 40% similarity with C. Albicans PKC1 at an overlap of 1115 amino acids (Table 2). The global alignment between human Phospholipase C delta types (PLCD1, PLCD3, and PLCD4) and C. Albicans Phospholipase C1 (PLC1) showed that human PLCD1 and

PLCD4 have 25% identity with similarities of 39% and 38% respectively to C. Albicans PLC1 at an overlap of 1119 and 1118 amino acids respectively (Table 2), while human PLCD3 has 24% identity and 40% similarity with CaPLC1 at an overlap of 1111 amino acids (Table 2). The global alignment between human Phospholipase C gamma types (PLCG1and PLCG2) with C. Albicans PLC1 revealed that human PLCG1and PLCG2 have 21% identity with similarities of 36% and 37% respectively to CaPLC1 at an overlap of 1403 and 1360 amino acids respectively (Table 2).

	Protein names	Candida Albicans											
		G-protein alpha subunit (Gpa2)			PKC1			Calmodulin (caM)			(PLC1)		
		Identity %	Similarity%	A.As overlap	Identity %	Similarity%	A.As overlap	Identity %	Similarity%	A.As overlap	Identity %	Similarity%	A.As overlap
Human	Gαq-protein	38	56	370									
	PKC-θ				34	54	756						
	PKCε				35	51	757						
	Calmodulin (caM)							71	89	149			
	PLCD1										30	49	699
	PLCD3										30	47	634
	PLCD4										33	48	635
	PLCG1										42	61	159
	PLCG2										44	61	159
	PLCB1										36	55	363

Table 1: Local alignment between humans and *Candida Albicans* proteins which are involved in the inositol phosphate pathway.

	Candida Albicans											
	Protein names	G-protein alpha subunit (Gpa2)			PKC1			Calmodulin (caM)			(PLC1)		
		Identity %	Similarity%	A.As overlap	Identity%	Similarity %	A.As overlap	Identity%	Similarity %	A.As overlap	Identity%	Similarity %	A.As overlap
Human	Gαq-protein	30	45	505									
	PKCQ				27	40	1115						
	PKCE				27	42	1118						
	Calmodulin (caM)							71	89	149			
	PLCD1										25	39	1119
	PLCD3										24	40	1111
	PLCD4										25	38	1118
	PLCG1										21	36	1403
	PLCG2										21	37	1360
	PLCB1										21	32	1498

Table 2: Global alignment between humans and Candida Albicans proteins which are implicated in the inositol phosphate pathway.

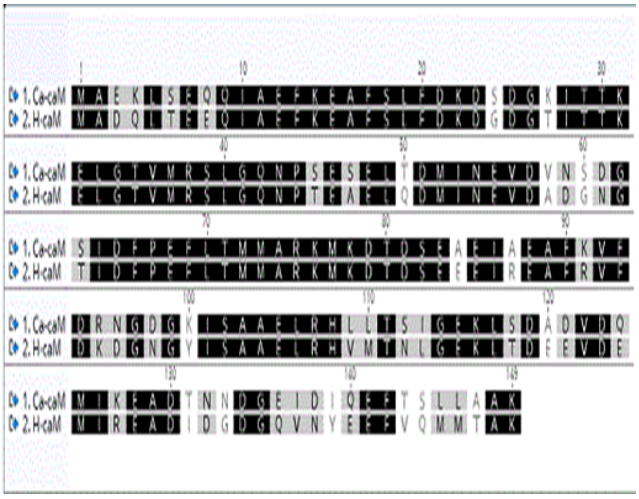

Figure 3c: Pairwise alignment between amino acid sequences of *Candida Albicans* calmodulin (Ca-caM) and human calmodulin (H-caM) showing 71% identity and 89% similarity; Identical residues; Similar residues.

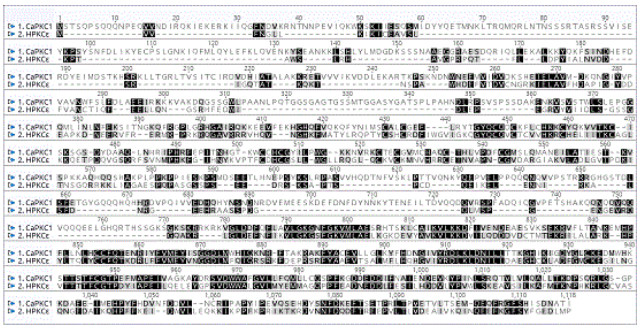

Figure 3d: Pairwise alignment between amino acid sequences of *Candida Albicans* PKC1 (CaPKC1) and human PKCε (HPKCε) representing 27% identity and 42% similarity; Identical residues; Similar residues.

Discussion

In this study, effect of acetylcholine on C. Albicans morphogenesis is tested. Acetylcholine induced yeast to hyphal form transition in a concentration dependent manner (Figure 1a; Figure 2a). The adenylyl cyclase inhibitor, MDL 12,330A inhibited this transformation (Figure 1b; Figure 2b) indicating the role of cAMP. In C. Albicans, cAMP-mediated signalling pathway is involved in the yeast-to-hyphal form conversion [30]. Muscarinic receptors in C. Albicans are not reported to exist. Rrp9 protein in C. Albicans is reported to exhibits identity and similarity with human muscarinic M1 receptor [7]. Acetylcholine also is reported to binds with Rrp9 protein [31]. In humans, activation of muscarinic M1 receptor leads to production of cAMP through inositol phosphate pathway. The inositol phosphate pathway in C. Albicans is reported to exist. Roy and Datta (1987) showed that calmodulin inhibitor, trifluoperazine (TFP) inhibited yeast to germ tube formation of C. Albicans induced by N-acetyl glucosamine [32]. Trifluoperazine (TFP) is known to be a protein kinase-C inhibitor [33]. Gadd and Foster (1997) found that inositol 1, 4, 5-trisphosphate (IP3) was produced during yeast form and germ tube formation in C. Albicans [34]. Sato et al. (2004) found that hyphae formation in C. Albicans grown on Sabouraud's medium containing 10% FBS was inhibited by calmodulin inhibitor, (TFP or W-7) and adenylyl cyclase inhibitor MDL 12,330A [35]. They also found that the relative expressions of hyphae-specific mRNAs of ALS3, ALS8 in C. Albicans were inhibited by the addition of TFP and MDL-12-330A [35]. The expression of adhesion proteins, AL3 and ALS8 was also controlled by the RAS-cAMP pathway [36, 37]. These findings suggest that the Ca2+/calmodulin signal pathway is associated with the RAS-cAMP pathway which regulates the transformation of C. Albicans cells. The second messengers, cAMP and $Ca2^{+}$ CaM can transmit their effect through various cellular signalling pathways [38]. When a muscarinic M1 receptors is activated by acetylcholine. This is lead to production of cAMP via inositol triphospate pathway. The bioinformatics study showed considerable identities and similarities between the proteins of humans and C. Albicans which are involved in the inositol phosphate pathway (Table 1; Table 2; Figure 3(a-d). Herein, it is hypothesized that acetylcholine may induce yeast to hyphal form conversion in C. Albicans through inositol phosphate pathway by activation of muscarinic M1 receptor like protein, Rrp9. Activation of Rrp9 by acetylcholine in C. Albicans may couple to Gα-protein (CaGpa2) which can lead to stimulation of C. Albicans phospholipase C1 (CaPLC1) via inositol phosphate pathway. Phospholipase C (CaPLC1) may hydrolyze phosphatidylinositol 4, 5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG may stimulate C. Albicans protein kinase-C1 (CaPKC1) that leads to activation of C. Albicans adenylyl cyclase (CDC35) for producing cAMP. Also, IP3 in turn stimulates the releasing of calcium from cytosol into cytoplasm to couple with C. Albicans calmodulin (caM) for activation of adenylyl cyclase (CDC35) which leads to production of cAMP (Figure 4). This pathway may produce cAMP that may induce yeast to hyphal form transition and this transition can be inhibited by adenylyl cyclase inhibitor, MDL 12,330A (Figure 4). This study suggests that C. Albicans Rrp9 may have a potential role in C. Albicans morphogenesis.

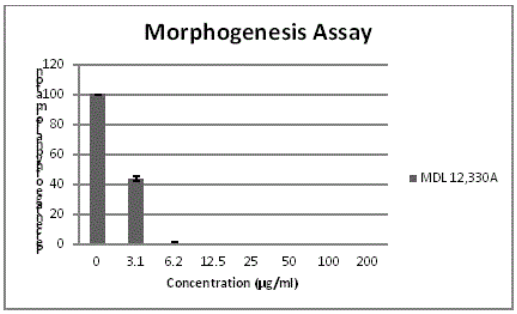

Figure 1b: Effect of adenylyl cyclase inhibitor, MDL 12,330A on acetylcholine induced yeast to hyphal formation in *Candida Albicans* (ATCC 90028).

Conclusion

The neurotransmitter, acetylcholine can activate muscarinic M1 receptor through inositol phosphate pathway which leads to cAMP production. In humans, the second messenger cAMP is implicated in various intracellular activities such as cell growth, regulation of cell proliferation, skin cell signalling and immune responsiveness. In C. Albicans, cAMP production is known to involve in yeast to hyphal formation. In this study, acetylcholine can induce yeast to hyphal form transition and the adenylyl cyclase inhibitor inhibited this transition representing role of cAMP. Inositol phosphate pathway is reported to know in C. Albicans. The bioinformatics study exhibits identities and similarities between humans and C. Albicans proteins which are involved in inositol phosphate pathway. This study indicates that C. Albicans may have a protein like muscarinic receptors. It is suggested that C. Albicans Rrp9 protein may have a potential role in yeast to hyphal form conversion.

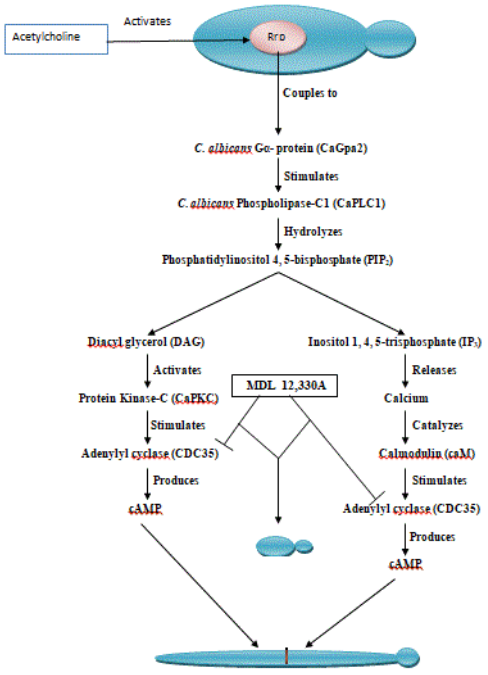

Figure 4: A hypothetical model for the mechanism action of muscarinic receptor agonist, acetylcholine and adenylyl cyclase inhibitor, MDL 12,330A on *Candida Albicans* (ATCC 90028) morphogenesis. We hypothesize that acetylcholine induces yeast to hyphal form transition by activating the muscarinic M1 receptor like protein, CaRrp9. Activated CaRrp9 stimulates C. Albicans adenylyl cyclase (CDC35) through inositol phosphate signalling pathway, while adenylyl cyclase inhibitor, MDL 12,330A inhibits this transformation by blocking adenylyl cyclase.

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