



MITOGENIC STIMULATION IN ISOPROTERENOL TREATED CELLS OF *DICTYOSTELIUM DISCOIDEUM*

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Amoebae of the cellular slime mould *Dictyostelium discoideum* showed stimulated mitogenic activity when exposed to 200 μ M isoproterenol, an activator of adenylyl cyclase, for 30 min. Approximately 40% increase in cell proliferation was found at 48 h after isoproterenol treatment. A faster and larger plaque formation as well as higher uptake of FITC-labelled *E. coli* indicates greater phagocytotic activity in the treated cells. A concurrent increase in DNA and protein syntheses was also recorded in the treated cells. Administration of 400 μ M caffeine or 200 μ M (+) propranolol brought down the isoproterenol-induced elevation in the cell division rate to control levels. These results are discussed in relation to a precocious activation of adenylyl cyclase in the treated cells leading to a transient but significant increase in cell division in this organism.

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KEYWORDS: cell proliferation; *Dictyostelium*; isoproterenol; propranolol; caffeine.

INTRODUCTION

The binding of growth factor to its corresponding membrane receptor activates intracellular signaling pathways initiating a complex spatio-temporal phosphorylation cascade. This leads to the stimulation of cell growth, cell cycle progression and cell proliferation. The stimulation of cellular receptors by catecholamines regulates a host of physiological responses depending on the cell type. Isoproterenol (Iso), a synthetic catecholamine, binds to β -adrenergic receptors and activates adenylyl cyclase (William and Lefkowitz, 1977). In humans, isoproterenol is known to reduce diastolic blood pressure, increase cAMP level and oxygen consumption (Popa, 1984). In the rat, isoproterenol stimulates the growth of salivary gland cells (Stein and Baserga, 1970a) and also that of cultured proximal tubular cells in the mouse (Wolfe *et al.*, 1996). Stimulation in protein synthesis (Stein and Baserga, 1970b) and DNA synthesis (Byron, 1974) following isoproterenol treatment has also been reported.

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In the cellular slime mould *Dictyostelium discoideum*, adenylyl cyclase plays a crucial role in signal transduction during development. It converts ATP to cAMP that acts as a chemoattractant during cell aggregation of *Dictyostelium*. Adenylyl cyclase and cAMP levels remain low during growth but dramatically increase during development (Klein, 1976). To date there are three types of adenylyl cyclase found in *Dictyostelium*; of these vegetative adenylyl cyclase is expressed during the growth period of this organism (Kim *et al.*, 1998).

In this report we present evidence that isoproterenol treatment stimulates phagocytotic activity, protein and DNA syntheses and cell proliferation in vegetative amoebae of *Dictyostelium discoideum* and that the administration of caffeine or propranolol abolishes mitogenic stimulation in the treated cells.

MATERIALS AND METHODS

Culture methods

Growth in axenic medium: The axenic strain (Ax2) of *Dictyostelium discoideum* was cultured according

to the method of Ashworths and Watts (1970). Cells were cultured in axenic medium in 250 ml flasks and kept in an orbital shaker (120 rpm) at 22°C. Exponentially growing cells were harvested at a density of $5-6 \times 10^6$ cells/ml by centrifuging at 2000 rpm for 2 min.

Growth on nutrient agar: Exponentially growing *Dictyostelium* amoebae were evenly spread on *E. coli* (B/r) seeded on a nutrient agar plate and kept at 22°C.

Cell number

Cell number was determined using a haemocytometer, under a Zeiss (Jena, Germany) microscope. Average of three counts was taken as the cell number.

Isoproterenol treatment

The two different optical forms of isoproterenol (Sigma, St Louis, MO, U.S.A.), dextro (+), laevo (-) and equimolar mixtures of the 2 were used in the present study. Mid log-phase cells (5×10^6 cells/ml) were harvested by centrifugation and washed thoroughly with chilled P-buffer (pH 6.3). They were treated with different enantiomers and concentrations of isoproterenol (100 μ M to 400 μ M) in P-buffer for 30 min. The treated cells were washed three times with ice-cold phosphate buffer and then resuspended in axenic medium and kept shaken as described. Cell counts were taken at regular intervals of 24 h using a haemocytometer. Cell viability was checked by staining the treated and control cells with 0.1% trypan blue.

Caffeine and propranolol treatment

Pre, post, and simultaneous administration of 400 μ M caffeine (Sigma) and 200 μ M (+) propranolol (Sigma) was carried out for 30 min to the isoproterenol treated cells.

Colony blot assay

The colony morphology of *Dictyostelium discoideum* cells was studied by placing the control and treated cells as small droplets on nutrient agar plates, evenly spread with *E. coli*. The cells were allowed to grow at 22°C. When plaques started to appear on the *E. coli* lawn, millipore filters (Whatman, U.K., 0.45 μ m) were gently placed on the top of each plaque for 3 min and then immediately frozen in liquid nitrogen. The filters were stained with 0.2% ponceau s solution (Sigma) for 5 min. These filters were washed thoroughly in

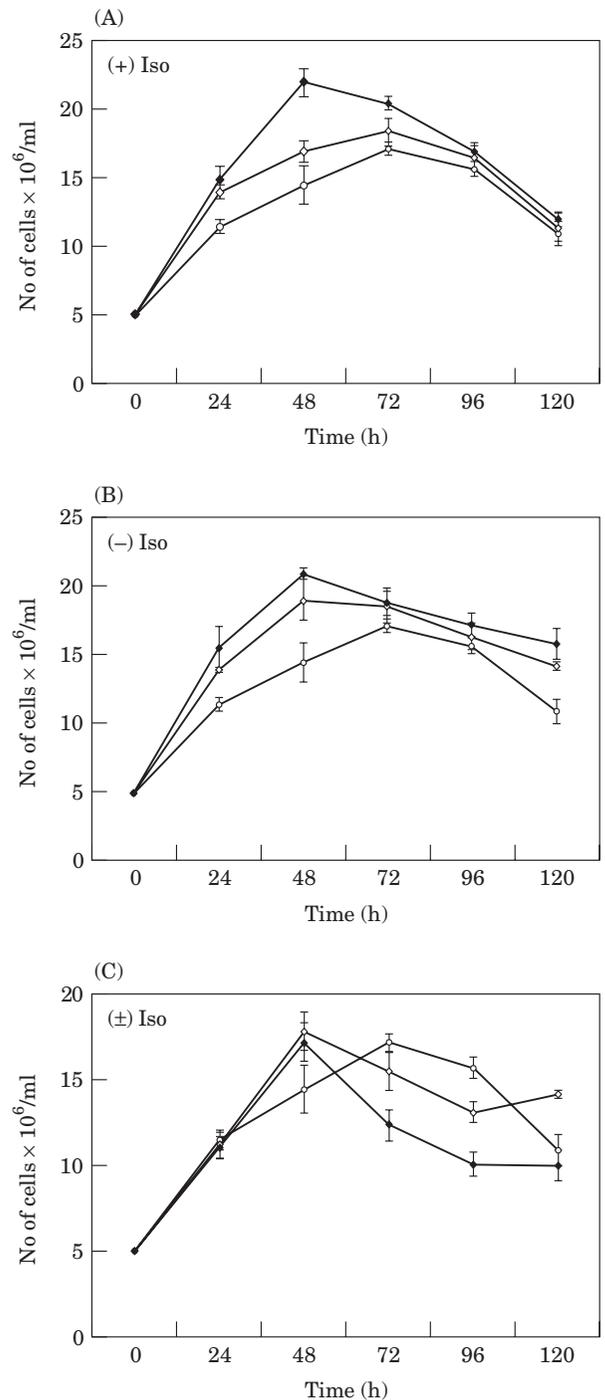


Fig. 1a-c. Growth curves of *Dictyostelium* cells treated with Iso. —○— Control, —◇— 100 μ M Iso, —◆— 200 μ M Iso.

distilled water and then fixed in 3% trichloroacetic acid (TCA) solution for 3 min and then air dried (Bozzaro *et al.*, 1987).

Phagocytosis assay

Phagocytosis was measured according to the method of Vogel (1987). Control and treated cells

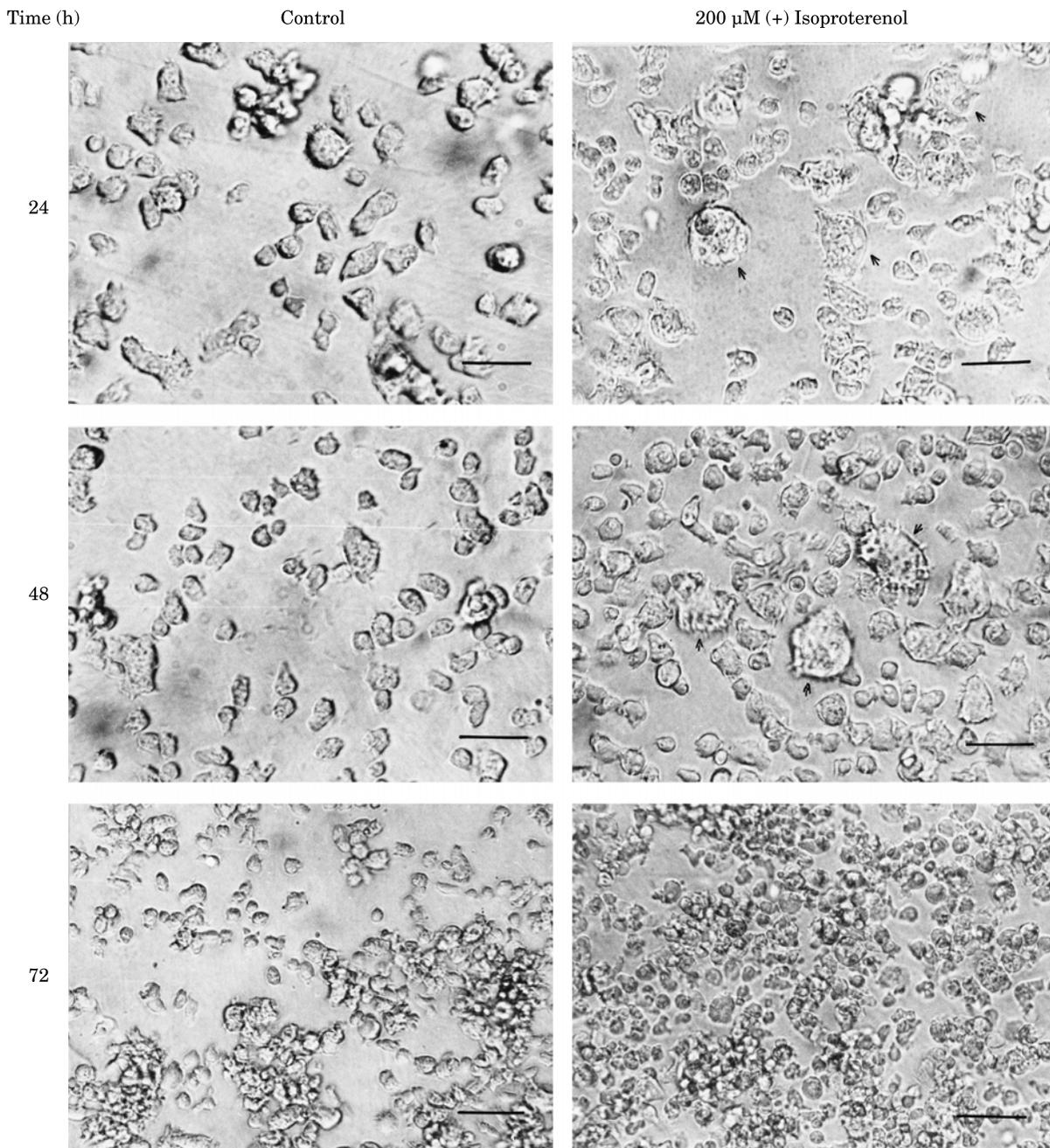


Fig. 2. Photomicrograph of control and Iso treated cells in multiwell test plate. Arrow indicates the larger cell; Bar=50 μ M.

were administered FITC labeled bacteria at a ratio of 1:200 and incubated in an orbital shaker at 120 rpm, at 22°C. One ml of sample was withdrawn at intervals of 30 and 60 min. Phagocytosis was stopped immediately by diluting with 4 ml chilled P-buffer. Uningested bacteria were removed by thoroughly washing with P-buffer by pelleting the cells. The cells were resuspended in P-buffer (pH 9.2) and the cell number was determined. The cells were lysed by treating them with triton X-100 at a final concentration of 0.2%. The fluorescent

intensity was measured in a fluorescent spectrophotometer (RF-450, Shimadzu, Japan) using excitation and emission wavelengths of 470 and 520 nm respectively. The number of bacteria ingested was determined by comparison with a standard curve.

Macromolecular synthesis

³H-thymidine and ³H-leucine incorporation was used as a measure of DNA and protein synthesis respectively. The treated and control cells were

labeled with 25 $\mu\text{Ci/ml}$ of ^3H -thymidine (Sp act. 629 GBq/mM; BARC, Mumbai, India) and 20 $\mu\text{Ci/ml}$ of ^3H -leucine (Sp act. 1850 GBq/mM; BARC, India) respectively for 1 h. The labeled cells were lysed and precipitated with 10% TCA on glass fiber filters (Millipore, 0.45 μm). After thoroughly washing with an excess of cold 5% TCA, the filters were air dried and counted in 10 ml scintillation fluid in a Beckman scintillation counter following the usual procedure. The incorporation of radioactive label into the TCA insoluble fraction was calculated as cpm/ 10^6 cells.

Microscopic studies

The morphology of control and treated cells was studied and photomicrographs were taken in an Axiovert-10 (Zeiss) microscope. The cell size and plaque size were analysed by using the UTHSCSA image tool software (San Antonio, U.S.A.). All the experiments were repeated at least three times and adequate controls were included in each case.

RESULTS

Isoproterenol acts as a mitogen in a dose dependent manner

Exponentially growing *Dictyostelium* cells showed negligible lethality when treated with 200 μM isoproterenol. Among the various doses of (+) and (-) isoproterenol and its racemic mixtures (equimolar mixtures of (+) and (-) forms), (+) 200 μM isoproterenol treatment for 30 min caused maximum mitogenic stimulation in *Dictyostelium* cells. Therefore this dose was selected as an operational dose for all further experiments. There was an approximately 40% increase in cell number at 48 h with (+) 200 μM isoproterenol treatment as compared to the control cells. However, this increased mitogenic activity declined after 96 h of growth back to the control levels (Fig. 1a-c). The treatment with isoproterenol (-) or equimolar mixtures of enantiomers (\pm) of various doses and durations showed that they were less effective in providing mitogenic stimulation to *Dictyostelium* cells when compared to the (+) isomer (data not shown). Further, isoproterenol treatment caused neither any mitogenic stimulation to stationary phase cells, nor could it induce cell division in developing *Dictyostelium* cells.

Cytomorphological studies

Light microscopic observations showed that the treated cells assumed spherical shapes and

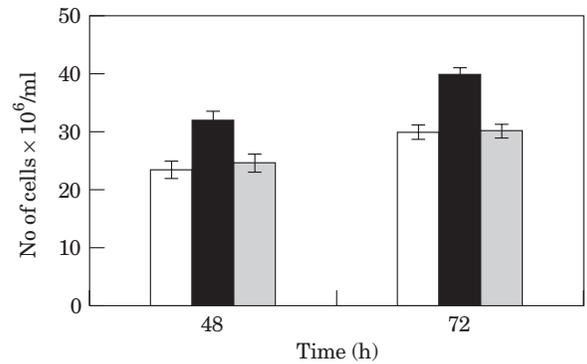


Fig. 3. Effects of 400 μM caffeine and 200 μM (+) Iso on *Dictyostelium*. □ Control, ▨ simultaneous Caffeine and Iso, ■ Iso.

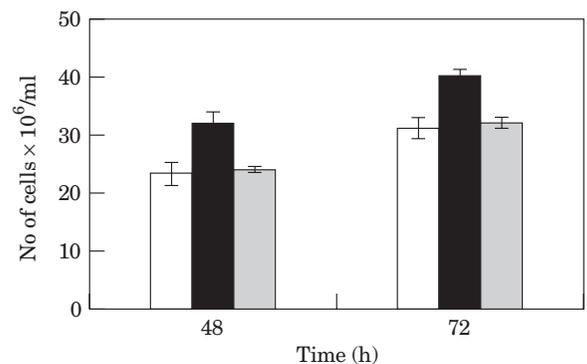


Fig. 4. Effects of 400 μM (+) propranolol and 200 μM (+) Iso on *Dictyostelium* cells. □ Control, ▨ post Iso, ■ Iso.

remained loosely bound to the substratum immediately after treatment. At 48 h onward many large and rounded cells were observed among the treated cell population. The surface area of cells was calculated. The size of large cells was $1896 \pm 238 \mu\text{m}^2$ at 48 h as compared to $859 \pm 213 \mu\text{m}^2$ control cells, i.e. double the size of the control cells. The cell number increased dramatically in the treated cells up to 48 h (Fig. 2).

Antagonist treatment nullifies mitogenic stimulation

Pre, post, and simultaneous treatments with 400 μM caffeine inhibited the increased cell proliferation in the isoproterenol treated cells (Fig. 3). Similarly, pre, post, and simultaneous treatments with 400 μM propranolol also suppressed mitogenic stimulation in these cells (Fig. 4).

Colony blot size increases in treated cells

In isoproterenol treated cells, the colony size increased, beginning at 48 h, and became noticeably

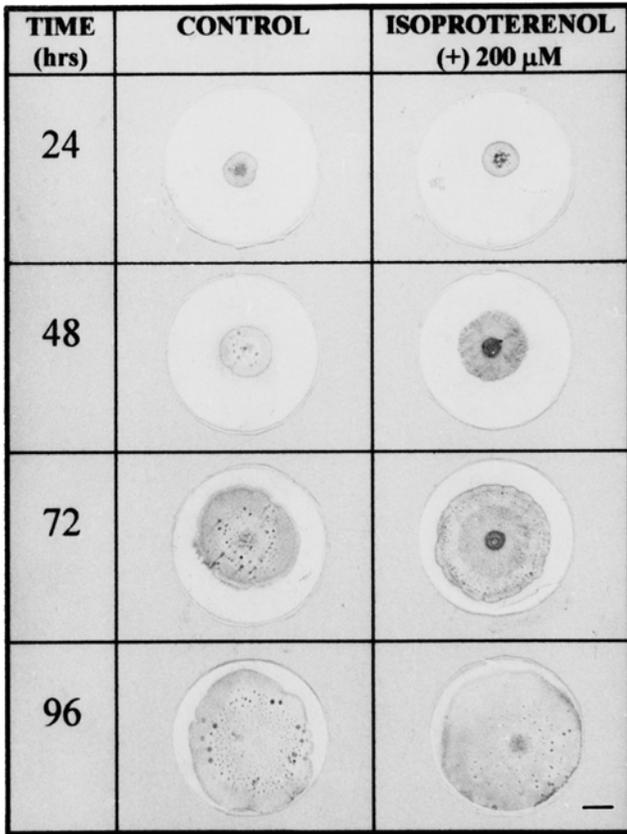


Fig. 5. Colony blots of 200 μ M (+) Iso treated and control cells. Bar=5 mm.

larger at 72 h of growth compared to the control cells (Fig. 5). This indicated faster phagocytic activity, and hence explains the increased cell numbers among the treated cells.

Treated cells show enhanced phagocytosis and macromolecular syntheses

The uptake of FITC labelled *E. coli* was found to be greater in the treated cells (Fig. 6) indicating enhanced phagocytic activity. Isoproterenol treated cells showed approximately 10%, 90%, and 50% enhanced DNA synthesis at 0, 24, and 48 h respectively as was evident from their ³H-thymidine incorporation profiles (Fig. 7). There was approximately 40%, 30%, and 15% increased protein synthesis at 0, 24, and 48 h respectively, as was evident from their ³H-leucine incorporation profiles (Fig. 8).

DISCUSSION

The mitogenic pathways for cells are complex and varied. There are multiple extracellular cues acting

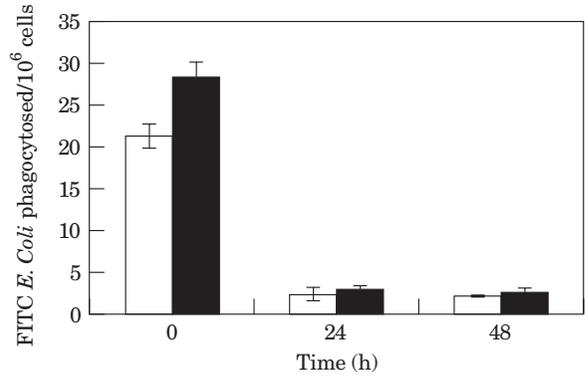


Fig. 6. Phagocytic uptake of FITC labelled *E. coli* in Iso treated and control cells. □ Control, ■ 200 μ M (+) Iso (0 h indicates approx. 90 min after treatment).

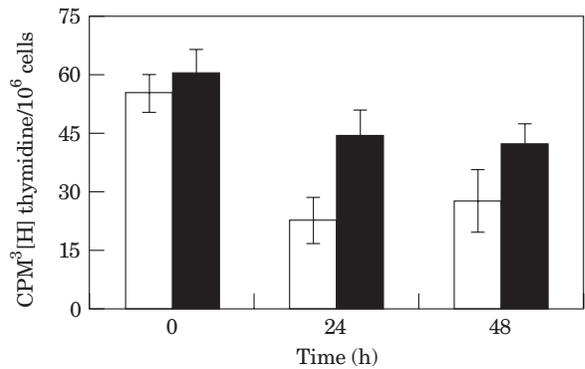


Fig. 7. Incorporation of ³H-thymidine in treated and control cells. □ Control, ■ 200 μ M (+) Iso.

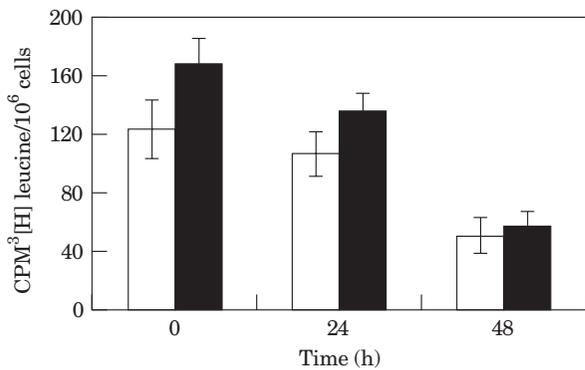


Fig. 8. Incorporation of ³H-leucine in treated and control cells. □ Control, ■ 200 μ M (+) Iso.

on various cell surface receptors for mitogens, which regulate mitogenesis (Schwartz and Baron, 1999). Extracellular mitogens activate an intracellular signaling pathway, which stimulates protein synthesis and cell division. Although many biochemical interactions involved in these pathways have been elucidated, many mechanisms are not yet understood.

The present experimental assays demonstrate that treatment of the growing *Dictyostelium discoideum* cells with 200 μ M (+) isoproterenol elicits dose dependent stimulation of cell proliferation when compared to the control cells. The proliferative effects of isoproterenol have been demonstrated in peripheral adenocarcinoma of the lung (Park *et al.*, 1995), and osteoblast-like cells (Suzuki *et al.*, 1998). This stimulated cell division was accompanied by an increase in cell growth as was reflected in larger cell size, increased phagocytosis and enhanced protein and DNA syntheses in the treated cells. Increased size cells may indicate active mitosing phase cells. A similar stimulation in protein and DNA syntheses has been reported previously (Byron, 1974; Stein and Baserga, 1970a) in isoproterenol treated cells of rat salivary glands.

Dictyostelium amoebae communicate with each other and their extracellular environment through a well-knit signaling network involving adenylyl cyclase, cyclic AMP, cAMP-phosphodiesterase, cAMP-dependent protein kinase, etc (Firtel, 1996). It is well known that isoproterenol activates adenylyl cyclase and raises the intracellular cAMP level (Prasad *et al.*, 1993; Puyushotham *et al.*, 1994). An increased cAMP level has been shown to stimulate growth in thyroid epithelial cells (Miller *et al.*, 1998). Although adenylyl cyclase remains highly active only in the developmental phase of *Dictyostelium discoideum*, the presence of a growth specific adenylyl cyclase (ACB) has been recently identified (Kim *et al.*, 1998). Many of the cellular mechanisms in *Dictyostelium* are sensitive to caffeine and its different effects are dependent on experimental conditions (Janssens and Van Haastert, 1987). In our experiments, simultaneous treatment with 400 μ M caffeine, a known inhibitor of adenylyl cyclase in *Dictyostelium* (Brenner and Thoms, 1984; Dottin *et al.*, 1991), abolishes the stimulated cell proliferation activity in isoproterenol-treated *Dictyostelium* cells.

Thus it seems that there is a precocious activation of adenylyl cyclase in isoproterenol-treated growing *Dictyostelium* cells leading to a transient mitogenic stimulation in this organism. The specificity of the isoproterenol action can be shown by simultaneous administration of 200 μ M (+) propranolol, a known antagonist of isoproterenol (Levitzki *et al.*, 1974), which inhibits the enhanced cell division. To our knowledge this is the first report of mitogenic stimulation by isoproterenol in any lower eukaryotic microbe.

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