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EFFECT OF BIOAMINES ON THE CELLULAR DIFFERENTIATION OF *HARTMANNELLA CULBERTSONI**

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Abstract—Epinephrine, 5-hydroxytryptamine, dopamine and tyramine induce axenic encystation of the free living amoeba *Hartmannella culbertsoni*. Cycloheximide inhibits encystation of amoeba mediated by the amines. The amines uniformly stimulate 3 to 4-fold the ability of the cells to synthesize cyclic 3′,5′-adenosine monophosphate from adenosine triphosphate formed *in situ*. Epinephrine[^{14}C] is bound to membranes of the amoeba (K_m of binding = 6·5 mM epinephrine). When amoebae are exposed to epinephrine, tyramine, dopamine or cAMP in a non-nutrient medium for 6 hr protein kinase activity is stimulated as evident from increased incorporation of ^{32}P into cellular proteins.

Entamoeba histolytica, the causative agent in amoebic dysentery, colitis, appendicitis or hepatitis, coexists with commensal microbial flora, multiplies vigorously and undergoes encystation and excystation in the human intestinal canal.[1,2] Whereas ulceration of the cecum and abscess formation in liver are due to the erosive action of the vegetative form of the pathogen, the cystic form is responsible for the transmission and the chronic clinical condition of the afflicted persons. Multiplication of the vegetative form of *E. histolytica* can be suppressed by 5-chloro-7-idohydroxyquinoline, 1-β-hydroxyethyl-2-methyl-5-nitroimidazole or chloroquine phosphate. Since the cysts of *E. histolytica* are not eliminated by these drugs, there is an urgent need for potent cysticidal agents to conquer the endemic form of the disease.[3]

The design and development of effective cysticidal agents would need a complete understanding of the nature of the cyst wall and the molecular biology of amoebic encystment in general. Biochemical events that trigger encystation of *E. histolytica* have not been adequately explored.[4] Attempts to discover optimal conditions for inducing encystment *in vitro* of axenically grown *E. histolytica* have been hitherto unsuccessful (S. R. Das *et al.*, unpublished observation from this laboratory). Using, however, a free living soil amoeba *Hartmannella culbertsoni* as a model, axenic encystation has been demonstrated in a non-nutrient medium containing taurine and Mg^{2+}.[5] Furthermore, taurine[^{35}S] has been shown to bind to *H. culbertsoni* membranes in the presence of Mg^{2+} [6] and to activate the synthesis of cyclic 3′,5′-adenosine monophosphate (cAMP) in encysting amoebae.[7]

Epinephrine and certain other bioamines can also induce encystment of *H. culbertsoni*. The effects of amines on the synthesis of cAMP, phosphorylation of cellular

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proteins and the binding of epinephrine[^{14}C] to membranes of *H. culbertsoni* are reported in this communication.

EXPERIMENTAL

Cells

Hartmannella culbertsoni[8] obtained from the collection of Dr. B. N. Singh of this Institute was grown in a medium containing 1% (w/v) tryptone (Difco Labs, U.S.A.), 1% (w/v) proteose peptone (Oxoid Labs, England) and 0·5% (w/v) NaCl. The medium was adjusted to pH 6·8 and autoclaved at 15 lbs/in^2 for 20 min. Ten ml of a 6-day-old culture of the organism (10^5 cells) were inoculated into 100 ml sterile medium and allowed to multiply in the stationary phase at 37° for 6 days. The organisms were then collected by centrifugation at 800 *g* for 10 min and washed twice with sterile distilled water by centrifugation as above and the amoebae finally suspended in the appropriate sterile medium as required.[5]

Encystation

This was carried out aseptically by either of the two following methods:

(a) Sterile non-nutrient agar plates containing 15 mM $MgCl_2$ and different concentrations of amines were prepared. Suspensions of amoebae (10^6 cells/plate) were spread on the agar-surface and the plates incubated at 27 ± 2°. Samples taken with a sterile loop at intervals were dispersed in 150 mM NaCl and examined microscopically for the appearance of cysts.

(b) suspension of amoebae (10^6 cells/flask) was inoculated into 10 ml medium containing 150 mM NaCl, 15 mM $MgCl_2$ and the required concentration of amine. The flask holding the suspension was shaken at 37 ± 1° for 6 hr (80 horizontal strokes/min, amplitude 1·5 cm). The amoebae were then recovered by centrifugation, washed twice with sterile 80 mM NaCl, plated on plain non-nutrient agar plates, incubated and examined microscopically for appearance of cysts as under (a).

Counts of trophozoites and mature double walled cysts were obtained from triplicate samples by haemocytometry and the viability of cysts was tested by negative eosine staining.[9]

Uptake of epinephrine

Cells were incubated in the presence of epinephrine[^{14}C] for 20 min at 37 ± 1° and then washed twice with cold sterile 80 mM NaCl. The cells were then homogenized manually in 80 mM NaCl in a Potter–Elvehjem homogenizer fitted with a Teflon pestle. Microscopic examination showed that the morphology of the amoeba was destroyed by this procedure. A particulate fraction was prepared by centrifugation at 100,000 *g* for 60 min at 0°.

Synthesis of cAMP

Freshly harvested 6-day-old amoebae were dispersed in medium containing glucose, NaCl, $MgCl_2$, adenine-8[^{14}C] and antibiotics and incubated with shaking at 37° for 2·5 hr. By this time *in situ* synthesis of ATP[^{14}C] was optimal. Theophylline was then added and the suspension incubated for a further 2·5 hr. The cells were harvested and processed for the recovery of nucleotides. Labelled cAMP was separated and identified

by chromatography in three different solvent systems and by recovery of predicted decomposition products by chemical and enzymatic treatments.[9,10]

Phosphorylation of cellular proteins

Phosphorylation of proteins, measured by the incorporation of ^{32}P, was carried out as described for mammalian cells by Sahib *et al.*[11]

RESULTS

Encystation induced by amines

Bioamines induced encystation of *H. culbertsoni* whether present in the non-nutrient agar throughout or when in contact with amoebae for a period of 6 hr only prior to plating on non-nutrient agar. Typical results are summarized in Table 1 as means of per cent encystation induced under either of the conditions. The inhibitory effect of cycloheximide on encystment induced by the amines is also shown in Table 1.

TABLE 1. ENCYSTATION OF *H. culbertsoni* INDUCED BY BIOAMINES

Experimental conditions	Addition	Per cent conversion of vegetative form of viable mature cysts		
		24 hr	48 hr	72 hr
Expt. I	None	0	8	11
Amines present in non-nutrient agar throughout	5 mM Epinephrine	12	45	100
	2 mM 5-hydroxy-tryptamine	2	23	62
	4 mM Tyramine	0	35	69
	4 mM Dopamine	0	20	58
Expt. II	None	0	8	11
Amoebae exposed to different amines for 6 hr washed and plated on non-nutrient agar	5 mM Epinephrine	15	55	100
	2 mM 5-hydroxy-tryptamine	32	72	80
	4 mM Tyramine	18	51	100
	4 mM Dopamine	27	48	82
Expt. III	5 mM Epinephrine	12	45	100
Amines present along with cycloheximide in non-nutrient agar throughout	5 mM Epinephrine + 0·1 mM cycloheximide	0	0	14
	2 mM 5-Hydroxy-tryptamine	2	23	62
	2 mM 5-Hydroxytryptamine + 0·1 mM cycloheximide	0	0	0
	4 mM Tyramine	0	35	69
	4 mM Tyramine + 0·1 mM cycloheximide	0	0	16

Conditions as described in Experimental.

Expt. I and III—Values are means of three identical replicates.

Expt. II—Values are mean of two identical replicates. In each set, duplicate plates were run and haemocytometric counts were made on three samples from each plate individually.

The difference in results obtained in Experiment I and II was due to some inhibitory action of 5-hydroxy-tryptamine, tyramine or dopamine on the amoebae when present throughout the encystation period studies as in Experiment I or III and the presumable absence of such effects when the amoebae were first exposed to these amines in liquid medium and washed free from them before plating them on non-nutrient agar plates.

Uptake of epinephrine

Results presented in Table 2 indicate that the amoebae have a mechanism to concentrate epinephrine intracellularly with a K_m value of 6·5 mM. More than 50 per cent of the amine accumulated by the cells was localized in the particulate membraneous fraction.

TABLE 2. UPTAKE OF EPINEPHRINE AND ITS BINDING TO MEMBRANE OF AMOEBA

External concentration of epinephrine (mM)	Uptake* (counts/min/1·62 × 10^7 cells)	Binding to membrane fraction† (counts/min/1·62 × 10^7 cells)
0·2 (10 × 10^3)	150	105
0·5 (25 × 10^3)	313	200
3·0 (150 × 10^3)	1666	—
5·0 (250 × 10^3)	1730	—
7·0 (350 × 10^3)	2357	755
10·0 (500 × 10^3)	2940	1070
13·0 (650 × 10^3)	3430	2925
15·0 (750 × 10^3)	3386	2885

Six day-old amoebae (1·62 × 10^7 cells) dispersed in 2·5 ml medium containing 150 mM NaCl, 15 mM $MgCl_2$ and epinephrine containing same sp. act. of epinephrine-[^{14}C] (Radiochemicals, Amersham, U.K. sp. act. 50 mCi/m-mole) were incubated at 37 ± 1° in a metabolic shaker (80 horizontal strokes/min 1·5 cm amplitude) for 20 min. Cells were washed with 150 mM NaCl.

* Represents counts/min accumulated by cells and

† Localized in the 100,000 *g* particulate fraction.

Figures in parentheses are the counts present in 2·5 ml medium at the start of the experiment.

Synthesis of cyclic AMP induced by amines

Results summarized in Table 3 show that 5, 2, 2 and 1 mM epinephrine, 5-hydroxy-tryptamine, tyramine and dopamine, respectively, were optimal for activating the adenyl-cyclase system as evident from increased synthesis of cAMP from adenine-8[^{14}C] incorporated into ATP.

Phosphorylation of cellular proteins

Evidence for the presence of cAMP stimulated proteinkinase activity in *H. culbertsoni* is presented in Table 4. Stimulation of the phosphorylation of proteins as judged by an increased incorporation of ^{32}P into cellular proteins by prior exposure of amoebae to amines is also evident from the results summarized in Table 4.

TABLE 3. EFFECT OF AMINES ON THE SYNTHESIS OF cAMP IN AMOEBA

	Addition	pmoles adenine-8[^{14}C] incorporated into cAMP/g (fresh wt of amoeba)
Expt. I	None	346
	0·1 mM Epinephrine	445
	0·2 mM Epinephrine	590
	2·0 mM Epinephrine	538
	5·0 mM Epinephrine	802
	8·0 mM Epinephrine	638
	10·0 mM Epinephrine	628
Expt. II	None	290
	0·1 mM 5-hydroxytryptamine	495
	0·2 mM 5-hydroxytryptamine	591
	2·0 mM 5-hydroxytryptamine	1149
	3·0 mM 5-hydroxytryptamine	1043
Expt. III	None	409
	0·1 mM Tyramine	735
	1·0 mM Tyramine	882
	2·0 mM Tyramine	1104
	4·0 mM Tyramine	1069
Expt. IV	None	700
	0·1 mM Dopamine	819
	0·2 mM Dopamine	1096
	1·0 mM Dopamine	1424
	2·0 mM Dopamine	1254

Cells (2×10^8) dispersed in 9 ml medium containing 6 mM glucose, 90 mM NaCl, 15 mM $MgCl_2$, 5 μCi adenine-8[^{14}C] (sp. act. 12·38 mCi/mM, Isotope Division, Bhabha Atomic Research Centre, Trombay), 2·25 mg streptomycin sulfate and 2200 units of Penicillin G and indicated amount of amine were incubated at 37 $\pm$ 1° for 2·5 hr. Theophylline was added to 5 mM final concentration and incubation was continued up to 5 hr. The cells were then recovered from the medium and processed for recovery and identification of cAMP.

TABLE 4. EFFECT OF cAMP AND BIOAMINES ON PHOSPHORYLATION OF CELLULAR PROTEIN IN AMOEBA

	Addition	^{32}P incorporated (counts/min/mg protein)	Per cent stimulation
Expt. I	None	567	—
	0·001 mM cAMP	550	—
	0·010 mM cAMP	650	15
	0·100 mM cAMP	870	54
Expt. II	None	583	—
	5 mM Epinephrine	1059	83
	4 mM Tyramine	904	54
	4 mM Dopamine	1308	126

Conditions. Amoebae (2×10^8 cells) suspended in 8·8 ml of medium containing 6 mM glucose, 90 mM NaCl, 2·25 mg streptomycin sulfate, 2250 units of Penicillin G, 1 mM NaF, 25 μCi carrier-free orthophosphoric acid P^{32} (Activity = 5·2 mCi—Isotope Division, Bhabha Atomic Research Centre, Trombay) were incubated for 60 min at 37 $\pm$ 1°. 0·1 mM EDTA, 0·4 mM theophylline and/or different concentrations of cAMP or amines were added and after 30 min incubation, the cells were processed for determination of protein phosphorylation.

DISCUSSION

Encystation involves the building around a dormant amoeba of a double layered cyst wall, the constructional material for which must come from stored food.[12,13] Changes in the relative concentration of glycogen and other reserve polysaccharides, nucleic acids and proteins have been mapped during encystment of *H. culbertsoni* and extensive degradation of reserve polymers has been demonstrated.[14] There is also evidence for the induction of enzymes such as cellulose synthetase and mucopolysaccharide synthetase during encystment triggered by taurine.[5,15] These changes are obviously under metabolic control and previous evidence suggests that cAMP is involved in the regulation of early molecular events that lead to encystation.

The bioamines studied induce encystation in a manner analogous to that of taurine. The amines were bound to the membrane fraction of the amoebae as evident from the data on the uptake of epinephrine[^{14}C] by the amoeba and its localization in the particulate membraneous fraction. This step is perhaps obligatory for the activation of adenylcyclase and the accumulation of intracellular cAMP of adequate concentration for exercising the subsequent regulatory effects. In agreement with the concept that cyclic AMP mediates phosphorylation of cellular proteins,[16] evidence has been adduced that on exposure of the cells to amines there is enhanced incorporation of ^{32}P into cellular proteins. It has already been shown that during encystation there is a significant fall in the activity of phosphodiesterase acting on cAMP.[7]

Parallel to the depletion of glycogen reserves during encystation by exposure to amines, the glycogen phosphorylase activity of amoebae shows significant stimulation (A. K. Verma *et al.*, unpublished observation). The glucose arising from such degradation is apparently diverted for biosynthesis of cellulose and mucopolysaccharide. At the biochemical regulatory level, the activation of adenyl-cyclase of *H. culbertsoni* by bioamines leading to degradation of polymers resembles the behaviour of mammalian liver or muscle in its response to epinephrine.[17]

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