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Action of imidazole and metronidazole on the differentiation of *Hartmannella culbertsoni**

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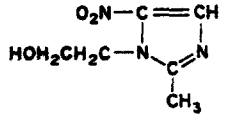
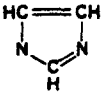
Metronidazole (I) (1-β-hydroxyethyl-2-methyl-5-nitroimidazole) is a potent amoebicidal agent [1–3] and is structurally related to imidazole (II) which is known to possess antilipolytic activity [4–6], to stimulate glycolysis in rat diaphragm [7] and to activate cyclic 3',5'-adenosine monophosphate phosphodiesterase [8–13].



(I)



(II)

Taurine and biogenic amines induce the differentiation of *Hartmannella culbertsoni* into viable cysts [14, 15]. Besides the extensive degradation of lipids and glycogen [16, 17] that occurs in the amoebae committed to encyst under the triggering action of taurine or epinephrine, a membrane localized adenylate cyclase is also activated [14, 15, 18, 19]. It was of interest, therefore, to examine the effect of imidazole and metronidazole on the differentiation of this free living amoeba.

MATERIALS AND METHODS

Hartmannella culbertsoni [20], from the collection of Dr. B. N. Singh of this Institute, was grown axenically in a medium containing 2% (w/v) peptone (British Drug Houses, India), 0·5% (w/v) NaCl and 10 mg thiamine HCl/l. and 5 μg canocobalamin/l. (D. C. Kaushal and O. P. Shukla, unpublished observations from this laboratory). One-litre Erlenmeyer flasks containing 250 ml of the above medium (pH 6·8) were autoclaved at 15 lb/in.^{2} for 20 min and inoculated with 50 ml of 4-day-old culture (10^8 cells). The flasks were shaken in a rotary shaker (Emenvee Engineering Co., Poona, India) at 300 rev/min and maintained at 34 ± 2°. During 4 days growth 2 × 10^6 cells/ml was obtained. The cells were harvested by centrifugation at 800 *g* for 10 min and washed twice by dispersal in sterile 150 mM NaCl followed by centrifugation at 800 *g* for 10 min and finally suspended in the required media. All the operations were carried out aseptically.

The cells suspended in 150 mM NaCl were homogenized with a Potter Elvehjem type of tissue grinder using a Teflon pestle by eight up and down strokes and a rotor speed of 2000 rev/min. The homogenizer and contents were chilled during homogenization. Microscopic examination showed that all the cells were ruptured by this procedure.

Imidazole or metronidazole were dissolved in 150 mM NaCl to the required concentration and the pH was adjusted to 7·0 and the solutions seitz filtered. Freshly harvested amoebae suspended in 150 mM NaCl were exposed to a sterile solution of the drug under test for the required period. The cells were then recovered by centrifugation, washed by dispersal in sterile 150 mM NaCl followed by centrifugation and finally inoculated into axenic medium. Growth was followed by counting with the aid of a haemocytometer.

Freshly harvested cells (3 × 10^6) were suspended in 10 ml medium containing 80 mM NaCl, 20 mM taurine, 15 mM $MgCl_2$ and imidazole or metronidazole in desired concentrations. The cells were shaken at 37 ± 1° for 6 hr in a metabolic shaker (80 horizontal strokes/min, 1·5 cm amplitude). The cells were recovered by centrifugation and washed twice in 150 mM NaCl. The cells were then spread over plain non-nutrient agar plates and incubated at 27 ± 1°. Samples were examined microscopically for morphological changes. Counts of trophozoites and cysts were made in triplicate samples in a haemocytometer.

Cyclic AMP synthesis was followed by prelabelling ATP *in situ* with adenine-8-^{14}C and measuring the amount of cyclic AMP-8-^{14}C formed in the presence of theophylline [14]. The identity of cyclic AMP was established by chromatography and chemical and enzymic degradation using an authentic sample of cyclic AMP.

Cyclic AMP phosphodiesterase (EC 3.1.4.1) was assayed using the method of Butcher and Sutherland [8].

Table 1. Effect of imidazole and metronidazole on growth of *H. culbertsoni*

Concentration of drug (mM)	Imidazole Time of exposure (hr) 6	72	Metronidazole Time of exposure (hr) 6	72
0	20·0	20·5	21·0	17·0
1	—	—	21·5	11·5
2	—	—	23·0	9·7
5	—	—	22·0	0
10	—	—	20·0	0
20	19·9	17·0	20·5	0
40	20·2	0	18·0	0
100	19·7	0	17·5	0

2 × 10^5 freshly harvested cells were incubated with 0–100 mM imidazole or metronidazole in 150 mM NaCl for 6 and 72 hr at 34 ± 2°, on a rotary shaker (300 rev/min). At intervals cells were harvested, washed free from drug and inoculated into growth medium in small test tubes. Haemocytometric counts were made each day. The results show cells × 10^5/ml on the fourth day of growth.

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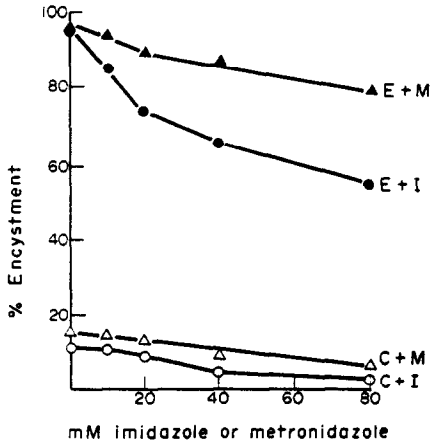

Fig. 1. Effect of imidazole and metronidazole on encystment of *H. culbertsoni*. 3×10^6 cells in 10 ml medium containing 80 mM NaCl, 20 mM taurine, 15 mM $MgCl_2$ and different concentrations of imidazole or metronidazole were incubated in a metabolic shaker (80 horizontal strokes/min, 1·5 cm amplitude) at $37 \pm 1°$ for 6 hr. Cells were washed twice with 150 mM sterile NaCl and dispersed over plain non-nutrient agar plates. The mean per cent encystation at 72 hr of three experiments is shown. (▲), encystment + metronidazole; (●), encystment + imidazole; (Δ), control + metronidazole; (○), control + imidazole.

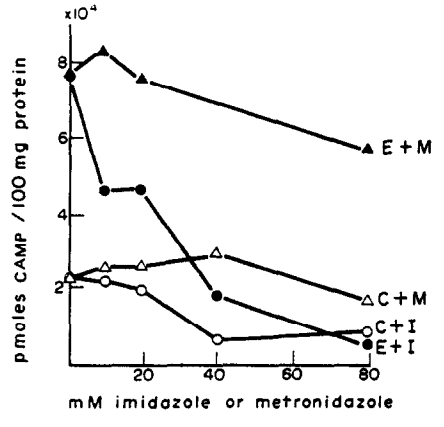

Fig. 2. Effect of imidazole and metronidazole on synthesis of cyclic AMP. Cells (2 g wet wt) were incubated in 50 ml medium containing 80 mM NaCl (control) or 80 mM NaCl plus 20 mM taurine and 15 mM $MgCl_2$ (encystment), in the presence of different concentrations of imidazole or metronidazole. At intervals 10 ml of suspensions were withdrawn aseptically, cells recovered by centrifugation and washed twice with sterile 150 mM NaCl. Cells were used for synthesis of cyclic AMP. Cells (100 mg protein) dispersed in 2 ml medium containing 6 mM glucose, 90 mM NaCl, 3 μCi adenine-8-^{14}C (sp. act. 29·47 mCi/mmole, Bhabha Atomic Research Centre, Trombay), 0·45 mg streptomycin sulfate and 440 units of penicillin G were incubated at $37 \pm 1°$ in a metabolic shaker (80 horizontal strokes/min, 1·5 cm amplitude) for 2·5 hr. Theophylline was added to 5 mM final concentration and incubation continued up to 5 hr. The cells were recovered from the medium and processed for isolation and identification of cyclic AMP [15]. (▲), encystment + metronidazole; (●), encystment + imidazole; (Δ), control + metronidazole; (○), control + imidazole.

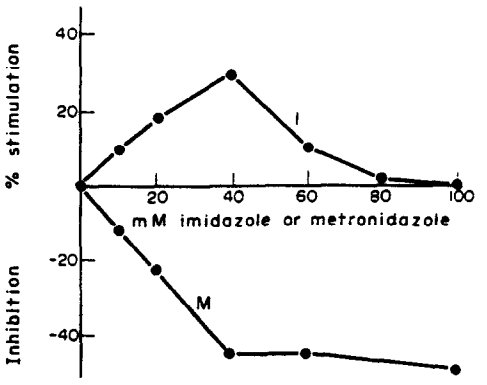

Fig. 3. Effect of imidazole and metronidazole on cyclic AMP phosphodiesterase of *H. culbertsoni*. Reaction mixture (0·8 ml) contained 360 mμmoles of cAMP, 1·8 μmoles of $MgSO_4$, 36 μmoles of acetate buffer pH 5·6 and enzyme (800 μg protein). After incubation for 1 hr at $37 \pm 1°$ the reaction was stopped by holding the tubes in boiling water for two minutes. The tubes were cooled and the pH was adjusted to 7·4. 0·1 ml of snake venom (1 mg/ml Naja-Naja, Haffkine Institute, Bombay, in 10 mM Tris–HCl buffer pH 7·4) was added and incubation continued at 37°C for 30 min. The reaction was stopped by adding 0·1 ml of 55% (w/v) trichloroacetic acid. Aliquots were treated with activated charcoal to avoid any interference in the estimation of P_i according to Fiske and Subbarow [21]. The per cent stimulation or inhibition in the presence of a drug is illustrated. I = imidazole; M = metronidazole.

RESULTS

The effect of imidazole and metronidazole on the growth of amoeba is shown in Table 1.

The effect of imidazole and metronidazole on encystment induced by taurine–$MgCl_2$ is shown in Fig. 1. Similar results were obtained when epinephrine was used as the triggering agent for inducing encystation. The small degree of inhibition of encystment by 80 mM metronidazole is due to its amoebicidal effect and the consequent reduction in the number of cells undergoing differentiation.

Since imidazole inhibits the encystment of *H. culbertsoni* at concentrations which are not toxic to cells and metronidazole seems to affect the viability of cells only, without interfering with encystment, it was of interest to examine the relative rates of synthesis of cyclic AMP during encystment in the presence of these agents. From the results shown in Fig. 2, it is apparent that previous exposure of cells to imidazole inhibits the synthesis of cyclic AMP to a significant extent. The effect of metronidazole at identical concentrations is not so pronounced as that of imidazole.

The effects of imidazole and metronidazole on cyclic AMP phosphodiesterase activity are depicted in Fig. 3. It can be seen that 40 mM imidazole stimulates the enzyme activity by about 30 per cent while the same concentration of metronidazole inhibits the enzyme activity by about 40 per cent.

DISCUSSION

Metronidazole does not inhibit encystment, activate cyclic AMP phosphodiesterase or affect the synthesis of cyclic AMP. The substitution of the imidazole ring substantially alters its effect on enzymes. The inhibitory effect on encystment and cyclic AMP synthesis exerted by metronidazole at very high concentrations can be attributed to its amoebicidal action and the consequent reduction of cell numbers. The results thus confirm observations of other workers on the amoebicidal action of the compound [22] and reveal that it does not interfere with the differentiation of trophozoites to cysts.

Previous reports [14, 15] have indicated a role for cyclic AMP in the differentiation of *H. culbertsoni* induced by biogenic amines. The present studies with imidazole suggest that the role of cyclic AMP is that of a second messenger almost comparable to its action on gluconeogenesis in liver and muscle triggered by epinephrine. Moreover, during encystment there is extensive degradation of lipids (M. K. Raizada, unpublished observations) along with glycogen [16–19].

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Characterization of acute tolerance to morphine using reserpine and cycloheximide*

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It has recently been demonstrated that morphine sulfate (MS) (25 mg/kg, 30 min) produces a significant lowering of tissue calcium in regional areas of rat brain [1]. This decrease was also shown to be antagonized by naloxone. In outlining acceptable criteria to establish an action as directly due to opiate effects, it has been necessary to demonstrate selective blockade by opiate antagonists, as well as dose levels approximating a pharmacological dose range. In addition, a major criterion imposed on previous investigations has been the production of tolerance to the parameter being examined. In continuing an investigation into the role of membrane calcium in the actions of opiate analgesics, the present study was undertaken to investigate the ability of morphine to induce tolerance to the calcium depletion effect in regional brain areas of the rat.

Rats (male, Sprague–Dawley), weighing between 175 and 250 g, were used throughout all experiments. Control or drug-treated animals were sacrificed at appropriate times (see table legends) by decapitation. The skull was opened, peeled back and the brain was removed as rapidly as possible into ice-cold isotonic saline. After successive rinses in this medium to remove any adhering blood, the brains were lightly blotted dry, and regional brain dissection was performed according to the method of Glowinski and Iverson [2]. Tissues samples in the range of 10–30 mg were placed in preweighed disposable tubes (Corning disposable culture tubes, 16 × 100 mm).

Calcium levels were determined by atomic absorption spectroscopy using a Perkin Elmer model 303 unit. Concentrated nitric acid (analytical grade) (200 μl) was added and the total contents of each tube were evaporated to a dry ash over a hot plate. After cooling, the residue was resuspended in 0·5 ml of 0·1 N HCl plus 4·5 ml of 1·0% lanthanum (as the oxide) in 0·6 N HCl.

Morphine sulfate (injectable) was purchased from Lilly Laboratories, Indianapolis, Ind.; reserpine was obtained from Ciba-Geigy, Summit, N.J. (Serpasil), and cycloheximide was purchased from Sigma Co., St. Louis, Mo. Lanthanum and calcium standards were obtained from Research Chemical Corp., Sun Valley, Calif., and Fisher Scientific, Fairlawn, N.J.

The development of tolerance to morphine's ability to deplete calcium was examined in the following manner. Animals were given saline or the appropriate drug and sacrificed at the times listed in the table legends. The initial dose of morphine was repeated, and a lesser response was usually observed to the second dose. The observation of this lesser response was used as the criterion for tolerance in this study. Table 1 illustrates the effect of morphine sulfate in producing acute tolerance to the calcium depletion effect at 4 hr. Two groups of animals

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