Cyclic AMP level in red blood cells of *Plasmodium berghei*-infected *Mastomys natalensis*

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Summary. The present report describes the changes in cyclic AMP level which occur upon parasitization of red cells by *Plasmodium berghei.* Parasitized erythrocytes were separated from the non-parasitized population by percoll density-gradient centrifugation. An increase in the cyclic AMP content of both non-parasitized and parasitized erythrocytes of infected animals compared with that of uninfected animals was observed. The patterns of physiological response to isoproterenol in normal, parasitized and non-parasitized erythrocytes were identical.
Key words. Mastomys natalensis; *Plasmodium berghei*; cyclic AMP.

Cyclic AMP, a well-known intracellular messenger, controls metabolic processes occurring inside the cell. Parasites which depend upon the host-cell energy supply for their survival might affect cyclic AMP metabolism. It has been shown that viruses and bacteria directly derange the cyclic AMP metabolism of the host[1-3]. The metabolic alterations occurring inside the host erythrocyte during the course of malarial infection have also been well documented. Furthermore, it has been shown that non-parasitized red cells from infected animals also exhibit dramatic changes when compared to normal erythrocytes of uninfected animals[4, 5]. In the present communication we report the results of measurements of the cyclic AMP levels of parasitized and non-parasitized erythrocytes of *Mastomys natalensis* infected with *Plasmodium berghei.*

Materials and methods

Eight-week-old male *M. natalensis* were infected with *P. berghei* as reported earlier[6].

Preparation of RBC suspension. Heparinized blood from 5–6 *Mastomys* per group was pooled and centrifuged to remove plasma and buffy coat. Erythrocytes were washed four times in Krebs' Ringer bicarbonate (KRB) containing 0.1% glucose[7]. Each wash consisted of resuspending the erythrocytes in 20 volumes of the medium, then centrifuging and removing the supernatant fluid. Parasitized and non-parasitized red blood cells were separated using percoll density-gradient centrifugation[8]. Non-parasitized erythrocytes could be separated from parasitized ones. Up to 96–98% of the former could be recovered from the bottom layer after centrifugation through percoll.

Cyclic AMP assay. A set of four tubes in triplicate was used. Tube No. 1 contained only erythrocyte suspension, while in tubes 2,3 and 4 erythrocyte suspensions were incubated with theophylline (10^{-3} M), theophylline (10^{-3} M) + isoproterenol (10^{-7} M), and theophylline (10^{-3} M) + isoproterenol (10^{-7} M) + propranolol (10^{-6} M), respectively. The final volume of incubation mixture was made up to 1 ml with KRB. The incubation period was 10 min at 37 °C. Reaction was terminated by the addition of 1 ml of cold 12% TCA with vigorous mixing. Precipitated proteins were discarded by centrifugation. The supernatant was extracted 3–4 times with water-saturated diethylether to remove TCA, and then lyophilized. The contents were reconstituted in cyclic AMP assay buffer (Tris-EDTA buffer, 0.05 M, pH 7.5 containing 4 mM EDTA) in such a manner that a 50-µl sample could be expected to contain cyclic AMP in the range of 1–16 pmol. The assay of cyclic AMP was carried out using an assay kit (Amersham, U.K.). The instructions supplied with the kit were strictly followed[9, 10]. A calibration curve was drawn using standard cyclic AMP supplied with the kit, in the range of 1–16 pmol, and used to determine the cyclic AMP content of reconstituted samples.

Normal, non-parasitized and parasitized cells were also exposed to increasing concentrations of isoproterenol (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) in the presence of theophylline (10^{-3} M), and cyclic AMP levels were measured in a similar manner.

Results

The effects of theophylline, isoproterenol and propranolol are well known. Theophylline inhibits the degradation of cyclic AMP by the enzyme phosphodiesterase, thereby making it possible to measure the full cyclic AMP content of the cell; isoproterenol (β-activator) enhances cyclic AMP production, an action mediated through β-adrenergic receptors, whereas propranolol (β-blocker) blocks the activation caused by isoproterenol.

An increase of about two- and threefold was observed in the cyclic AMP content of non-parasitized and parasitized erythrocytes respectively, when compared to normal erythrocytes (table 1). The inclusion of theophylline (10^{-3} M) in the incubation mixture increased the cyclic AMP level of the erythrocytes of both normal and infected animals, indicating inhibition of enzyme phosphodiesterase. Inclusion of isoproterenol (10^{-7} M) in the incubation mixture resulted in a threefold elevation of the cyclic AMP level in erythrocytes when compared to val-

Table 1. Cyclic AMP levels of parasitized and non-parasitized erythrocytes of *P. berghei*-infected *M. natalensis*

Additions	Cyclic AMP (pmol/10^8 erythrocytes/10 min)		
	Erythrocytes of normal animals	Erythrocytes of infected animals*	
		Parasitized	Non-parasitized
None	9.7 ± 0.7	28.7 ± 2.2	18.2 ± 1.1
Theophylline (10^{-3} M)	20.1 ± 1.5	61.0 ± 4.3	37.9 ± 2.9
Theophylline (10^{-3} M) + isoproterenol (10^{-7} M)	60.1 ± 4.1	170.0 ± 17.3	100.3 ± 7.3
Theophylline (10^{-3} M) + isoproterenol (10^{-7} M) + propranolol (10^{-6} M)	21.2 ± 2.1	63.7 ± 4.9	40.7 ± 3.0

Results represent mean ± SE of twelve determinations. *Parasitemia of infected animals was 38–42%.

Table 2. Cyclic AMP production in response to isoproterenol

Additions	Cyclic AMP (pmol/10^8 erythrocytes/10 min)		
	Erythrocytes of normal animals	Erythrocytes of infected animals*	
		Parasitized	Non-parasitized
Theophylline (10^{-3} M) + isoproterenol (10^{-9} M)	30.7 ± 2.0	83.5 ± 6.7	52.6 ± 3.9
Theophylline (10^{-3} M) + isoproterenol (10^{-8} M)	40.0 ± 2.8	110.9 ± 8.1	68.1 ± 4.7
Theophylline (10^{-3} M) + isoproterenol (10^{-7} M)	60.1 ± 4.1	170.0 ± 17.3	100.3 ± 7.3
Theophylline (10^{-3} M) + isoproterenol (10^{-6} M)	68.9 ± 4.5	185.0 ± 15.6	110.2 ± 8.0
Theophylline (10^{-3} M) + isoproterenol (10^{-5} M)	75.3 ± 6.0	215.4 ± 19.7	140.0 ± 10.6

Results represent mean ± SE of twelve determinations. *Parasitemia of infected animals was 38–42%.

ues with theophylline alone. Addition of propranolol (10^{-6} M) nullified the stimulation caused by isoproterenol (table 1). These results indicated that the action of theophylline, isoproterenol and propranolol on the erythrocytes of infected animals was very similar to that on erythrocytes of normal animals.

Many hormonal activities of catecholamines are mediated by cyclic AMP. The hormone binds to the recognition site (β-adrenergic receptor) on the plasma membrane, which in turn affects the activity of the membrane-bound enzyme adenylate cyclase, which is responsible for the synthesis of cyclic AMP. In order to check whether malaria infection produces any significant change in this receptor function, cyclic AMP levels were estimated in parasitized and non-parasitized erythrocytes at different concentrations of isoproterenol.

Normal, non-parasitized and parasitized red cells all responded to isoproterenol by an increased production of cyclic AMP. Although a difference in the absolute levels of cyclic AMP of normal, non-parasitized and parasitized erythrocytes was observed, the pattern of the physiological response to isoproterenol, in terms the number of times the level was increased, was more or less identical (table 2).

These results indicate that malaria infection did not produce any major change in β-adrenergic receptor density or function of the erythrocytes.

We were unable to detect the presence of cyclic AMP in the parasites isolated from parasitized erythrocytes by saponin lysis.

Discussion

Our earlier report clearly demonstrated that during the course of malaria infection the activity of adenylate cyclase was significantly increased while phosphodiesterase remained unchanged[5]. The present report on the increased cyclic AMP content in erythrocytes of infected animals confirms this observation.

It has been reported that cyclic AMP affects the biophysical interaction of spectrin with membrane lipids, and this is responsible for altered lipid fluidity[11]. During the course of malaria infection both parasitized and non-parasitized red cells exhibited dramatic changes in spectrin, phospholipid composition and lipid fluidity[4, 12, 13]. Increased cyclic AMP content in parasitized and non-parasitized red cells, as observed in the present study, might be responsible for these membrane-associated alterations. Further, the host may synthesize an increased amount of cyclic AMP to inhibit the reproduction of the parasite, as has been observed in trypanosomes[14]. On the contrary, it is also possible that the parasite evades the host's immune reactions through a cyclic AMP-dependent mechanism, as observed in *Mycobacterium microti*[15]. Enhanced activity of glycolytic enzymes during malaria infection may possibly be linked to the increased production of cyclic AMP[16, 17].

These studies have demonstrated that changes in the level of cyclic AMP take place during malaria infection. The specific molecular mechanisms for increased synthesis of cyclic AMP remain to be elucidated.

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Insect tissues, not microorganisms, produce linoleic acid in the house cricket and the American cockroach[1]

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Summary. Biosynthesis of linoleic acid, 18:2(n−6), was unambiguously demonstrated to occur in the cockroach, *Periplaneta americana*, and the cricket, *Acheta domesticus*. Axenic tissue from both of these insect species was demonstrated by radio-gas-liquid chromatography (radio-GLC) and radio-high-performance liquid chromatography (radio-HPLC) to incorporate [1-^{14}C]acetate and [1-^{14}C]oleate into this essential fatty acid.
Key words. Linoleic acid biosynthesis; essential fatty acid; *Periplaneta americana*; *Acheta domesticus*.

The biosynthesis of linoleic acid, 18:2(n−6), ((Z,Z)-6,9-octacosadienoic acid), generally has been considered to occur only in plants[3], fungi[4], and protozoa[5]. The tenet that animals are unable to synthesize linoleic acid has been accepted despite occasional findings to the contrary, particularly among several insect species[6–8]. These early reports of linoleic acid synthesis in insects were discounted due to criticisms regarding inadequate analytical techniques and the possibility that microorganisms could contribute to linoleate production[9]. However, more recently, it has been shown by radio-GLC and radio-HPLC techniques that 12 of the 38 insect species investigated can synthesize 18:2 de novo from acetate[10–13]. In these studies, rigorous characterization of the newly-synthesized labeled 18:2 by gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), and radiotracer studies using analyses by radio-GLC, radio-HLPC, and ozonolysis followed by radio-GLC confirmed that 18:2(n−6) was formed.

The questions regarding the potential role of microorganisms have not been adequately addressed. Many insects have microorganisms in the gut tract, in specialized cells called mycetocytes or bacteriocytes, and on the cuticle. It is possible that these microorganisms are responsible for de novo synthesis of 18:2 from acetate. To determine whether insect tissue or associated microorganisms are involved in linoleate synthesis in insects, epidermal, fat body and testes tissue from *A. domesticus*, and epidermal and fat body tissue from *P. americana*, were examined under axenic conditions for their capability to produce linoleic acid. The results of these experiments reported here demonstrate unequivocally that it is insect tissue that produces linoleic acid.

Methods and materials

Cockroaches, *P. americana*, were reared in metal garbage cans and fed Purina dog chow and water ad libitum. Crickets, *A. domesticus*, were obtained from Fluker's Cricket Farm, Baton Rouge, Louisiana. Adult male cockroaches and penultimate instar male crickets were anesthetized with CO_2 and ligated at the neck with cotton thread. The insects were surface sterilized by immersion in 70% ethanol with 2 drops Tween 80/l for 5 min followed by immersion in Zephiran-Cl for 5 min, and rinsed in sterile distilled water for 5 min. Under sterile conditions, fat body, testes and epidermal-enriched tissues were dissected from the crickets, and fat body and epidermal-enriched tissues from the cockroach. Tissues were placed in 0.1 ml incubation medium in 96 well plates and incubated at 30 °C for 24 h. The incubation medium, L15B[14] contained penicillin (100 U/ml), streptomycin (100 µg/ml) and gentamycin (50 µg/ml), and either [1-^{14}C]acetate (0.75 µCi/0.1 ml) or [1-^{14}C]oleate (0.33 µCi/0.1 ml). The [1-^{14}C]oleate was suspended first in 100 µl ethanol, then added to 10 ml of the medium. The medium was then warmed (37 °C) and filtered. Following incubation, the wells were checked by microscopy for contamination and any showing evidence of either fungi or bacteria were discarded.

Tissues were obtained from six wells and pooled in Eppendorf tubes for a total of 3–4 replicates of each. Total