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EFFECT OF FORMYCIN A AND PROTEIN INHIBITORS ON ADENOSINE TRANSPORT IN *Trypanosoma vivax*

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ABSTRACT: Formycin A (50 µM - 2.5 mM), a nucleoside analogue of adenosine, inhibited adenosine transport by 50%. Some sulphydryl reagents have been identified as inhibitors of the transport mechanism. For example, iodoacetate (5 mM) inhibited the transport process by 33% and 17% at 0.5 mM and 0.1 mM adenosine respectively. In contrast, the transport of 1 µM adenosine was stimulated by 5 mM iodoacetate. 2.5 mM p-chloromercuribenzoate inhibited the same process by 15%, 29% and 33% at 1 µM, 0.1 mM and 0.5 mM concentrations of adenosine respectively. 2-Mercaptoethanol stimulated adenosine transport in all cases. The differential effect of the different concentrations of these inhibitors at varied concentrations of adenosine confirms our earlier report that multiple modes of adenosine transport exists in *Trypanosoma vivax.* Protection by 2-mercaptoethanol and inhibition by p-chloromercuribenzoate indicate the presence of a carrier system for the transport process. Inhibition by iodoacetate shows that energy is required for transport. Thus, facilitated diffusion and active transport processes are both implicated in the mechanism of uptake of adenosine into the cells. these results also suggest that SH-groups are involved in the transport process because most of the reagents mentioned above are classical SH-group function effectors. However, the nature of the involvement of SH-group in the transport process is not defined by the present results.

Key Words: Adenosine transport; Protein inhibitors, Formycin A; Sulfhydryl reagents; *Trypanosoma vivax.*

INTRODUCTION

Adenosine is believed to be an important source of purines for the bloodstream forms of trypanosomes, probably because the NH_2 group on carbon 6 cannot be synthesized by the cells (1). This information stimulated our interest in the study of adenosine transport in *Trypanosoma vivax*. A good understanding of this process is necessary for a wider search into the use of antimetabolites and adenosine analogues as antitransport agents, especially when the cytotoxic effects of many purine nucleoside analogues are well documented (2-6).

Studies carried out so far on the transport of purines in trypanosomes have suggested the existence of more than one transport mechanism for adenosine (1,7). In other studies, Okochi *et al*. (7,8) have reported that iodoacetate (an inhibitor of the energy-yielding glycolytic pathway), inhibited adenosine transport at 10 mM adenosine, indicating the presence of active transport mechanisms. Also, the effects of adenosine concentration on its transport showed saturation kinetics, suggesting that a carrier system for adenosine transport exists in this microorganism.

In the present study, we aim at gaining further insight into adenosine transport mechanism(s) in *T. vivax*. Thiol reagents (iodoacetate, p-chloromercuribenzoate and 2-mercaptoethanol) have been used to probe the nature of the carrier system.

The inhibition of transport by formycin A (an analogue of adenosine) has been studied in this parasite.

MATERIALS AND METHODS

Iodoacetate, p-chloromercuribenzoate and 2-mercaptoethanol were purchased from E. Merck. Trimethylbenzene (Ready-Solve TM HP) was a product of Beckman. Formycin A was purchased from Sigma Chemical Company. Labelled aenosine was from Radiochemical Center, Amersham. *T. vivax* was obtained from the Faculty of Veterinary Medicine, University of Ibadan, and maintained in our laboratory by subcutaneous passage.

Isolation of trypanosomes from the blood of infected mice.

Heavily parasitised mice were sacrificed by ether anaesthesia and blood was collected with heparin as an anticoagulant. The blood was kept on ice and diluted 1:3 with cold phosphate-saline-glucose (PSG) buffer, pH 8.0. It was then applied on a DEAE-cellulose (type DE-52) column which has been equilibrated with PSG buffer, pH 8.0 and *T. vivax* cells were collected in the filtrate following the method of Lanham (9). The isolated trypanosomes were concentrated from the filtrate by centrifugation, washed twice with PSG buffer and finally suspended in Krebs Ringer's buffer, pH 8.5, containing 3% glucose.

Transport Assays.

The general methodology for the transport experiments was as previously reported by Okochi *et al*. (7). The incubation medium consisted of 1 ml of Krebs Ringer's buffer, pH 8.5, 0.1 ml of trypanosome suspension (23 mg/ml of suspension). Desired concentrations of unlabelled adenosine were added in a total volume of 1 ml. At the end of the required pre-incubation period, transport assay was carried out for one minute, following the introduction of 4.5 µCi 2,5,8- [^{3}H] adenosine (specific activity 41 Ci/mmol). The reaction was stopped by the addition of 8 ml of ice-cold Krebs Ringer's buffer, pH 8.5 followed by immediate centrifugation at 8,000 x g for 2 min. The wash was repeated once. The supernatant was decanted and the tubes were carefully blotted dry with strips of filter paper. The pellet was re-suspended in 2 ml of 2.5% trichloroacetic acid and heated at 90^{o}C for 30 min. 0.5 ml of the supernatant was transferred into scintillating vials to which 10 ml of trimethylbenzene (scintillating liquid) had been added. The radioactive content of the samples was determined in a Packard Tri-Carb liquid scintillating spectrometer, Model 3320.

Effect of temperature on adenosine transport.

The assay procedure described above were used to study the effect of temperature on the transport process in *T. vivax*. The temperature was varied from 4^{o}C to 50^{o}C. The reaction mixture contained 1 mM unlabelled adenosine. The uptake experiment was performed for 1 min.

Effect of sulphydryl reagents and formycin A on adenosine transport.

Cells (0.1 ml) were introduced into the reaction mixture. Iodoacetate (5 mM), p-chloromercuribenzoate (2.5 mM) and 2-mercaptoethanol (5 mM) were used in the presence of 1 µM, 0.1 mM and 0.5 mM unlabelled adenosine, respectively. The cells were preincubated for 5 min in the presence of these antimetabolites before the one-minute transport assay was carried out.

For the studies of the effect of formycin A, the first set of experiments examined the effect of a constant concentration of formycin A (2.5 mM) on adenosine transport. The cells were pre-incubated at various time intervals (0 - 10 min) in the presence of 0.1 mM unlabelled adenosine.

The second set of experiments studied the effect of varying concentrations of formycin A (0 - 1000 µM) on adenosine transport. The cells were pre-incubated for 10 min in the presence of formycin A before the one-minute transport assay was carried out.

RESULTS

Effect of temperature

The results presented in Fig. 1 shows that there was a gradual increase in the radioactive uptake as the temperature was increased, with a maximum uptake between 35^{o}C and 40^{o}C. When the temperature was increased to 50^{o}C, there was some drop in the amount of adenosine being taken up.

Effect of sulphydryl reagents and formycin A

The data presented in Table 1 are the results obtained when 5 mM iodoacetate, 2.5 mM p-chloromercuribenzoate and 5 mM 2-mercaptoethanol were used in the presence of 1 µM and 0.5 mM unlabelled adenosine respectively.

Table 1: Effect of some sulphydryl reagents on the transport of varying concentrations of adenosine.

Adenosine concentration	Inhibitor concentration	CPM/mg protein	Inhibition/ Stimulation (+/-)
1 µM	5 mM iodoacetate	6774 ± 259	(+)
	2.5 mM p-mercuribenzoate	4880 ± 221	(-)
	5 mM β-mercaptoethanol	6836 ± 260	(+)
	None (Control)	5722 ± 239	(+)
0.1 mM	5 mM iodoacetate	5865 ± 242	(-)
	2.5 mM p-mercuribenzoate	5018 ± 224	(-)
	5 mM β-mercaptoethanol	8812 ± 200	(+)
	None (Control)	7078 ± 265	(+)
0.5 mM	5 mM iodoacetate	4300 ± 207	(-)
	2.5 mM p-mercuribenzoate	4260 ± 205	(-)
	5 mM β-mercaptoethanol	8286 ± 304	(+)
	None (Control)	6374 ± 252	(+)

(+) indicates stimulation when compared to control.
(-) indicates inhibition when compared to control.

A stimulatory effect on adenosine uptake (18%) in the presence of 1 uM adenosine was observed in the presence of 5 mM iodoacetate. At 0.1 mM adenosine, the same concentration of iodoacetate produced 19% inhibition, while it produced 33% inhibition at 0.5 mM adenosine. 2-Mercaptoethenol (2.5 mM), on the other hand, stimulated adenosine transport at 1 µM, 0.1 mM and 0.5 mM adenosine by 19, 25 and 30 per cent respectively.

In the experiments with formycin A (Fig. 2), it was shown that the rate of uptake in the presence of 2.5 mM formycin A decreased by 20 - 23% without pre-incubation, based on the radioactive content of the control. After 20 min of pre-incubation, the rate of transport was further inhibited by 30%, making a total of 50% inhibition at the end of the pre-incubation period.

When the concentration of formycin A was varied (0 - 1000 µM), it was observed that 50 µM formycin A inhibited uptake by 50%. This level of inhibition did not change much on further increase to 1000 µM (Fig. 3).

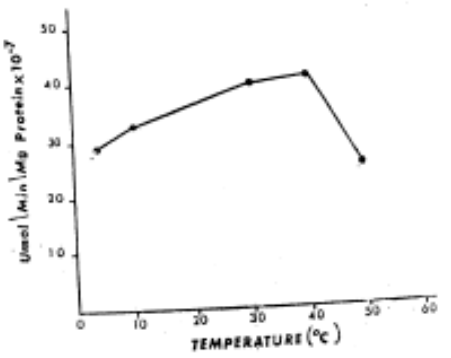

Fig 1: The effect of temperature on adenosine transport. Transport expenments were carried out at different temperatures (4 - 50°C). The reaction mixture contained ^{14}C-adenosine and 1mM cold adenosine in 1ml Kreb's buffer, pH 8.5

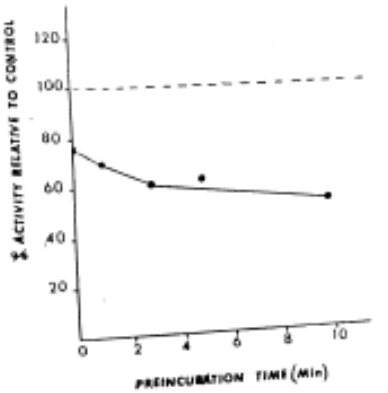

Fig.2: Adenosine transport in the presence of different concentrations of formycin A (0 - 1000 µM). The cells were preincubated in formycin A for 10 minutes before transport experiment was carried out for one minute in each case. The assay conditions are as described in the text.

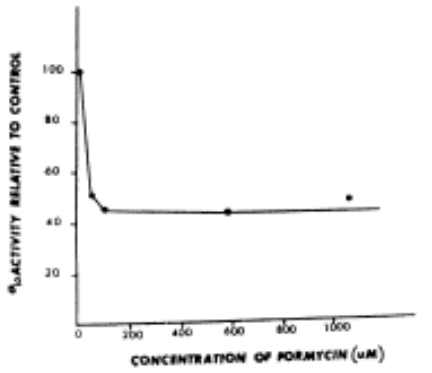

Fig. 3: The transport of adenosine in the presence of formycin A. The reaction medium contained 0.1M of cold adenosine. Other conditions are described in the text. The cells were preincubated for the time intervals specified in the plot before transport experiments were carried out in the range reported in Table 1.

DISCUSSION

The results presented here have shown that adenosine transport is sensitive to temperature changes in *T. vivax*. The effect of temperature on the transport process was examined to confirm our earlier report (8) that a carrier mediated transport system for adenosine exists in this microorganism. Membrane carriers are principally protein in nature and are, therefore, sensitive to variations in temperature. Sensitivity to temperature changes has been used as one of the criteria for establishing the presence of membrane carrier systems in many studies (10-14).

Iodoacetate (5 mM) has been found to inhibit the transport process in this microorganism at 0.1 mM and 0.5 mM adenosine respectively (Table 1), while the same concentration failed to inhibit uptake at 1 µM adenosine. This indicates that the mode of transport operating at 1 µM adenosine is different from the ones operating at 0.1 mM and 0.5 mM adenosine. The reason for the stimulation cannot be explained by the present data. It appears that at a lower level of adenosine (1 µM) facilitated transport becomes operative (8). Facilitated diffusion mechanisms are not affected by classical inhibitors of energy metabolism such as iodoacetate, but can be modulated by protein effectors, e.g. p-chloromercuribenzoate (15). It is, therefore, not surprising that in this study, p-chloromercuribenzoate inhibited adenosine uptake at 1 µM adenosine by 15% (Table 1), and also inhibited the transport process by 30% and 33% at 0.1 mM and 0.5 mM adenosine, respectively. p-Chloromercuribenzoate inhibits the transport process by reacting with the SH-groups on the carrier protein (16,17).

The fact that there is an increase in the degree of inhibition as the substrate is increased at constant inhibitor concentration can be interpreted to mean that the substrate induces a conformational change in the receptor to produce an intermediate which is inactivated by PMB. With a fixed number of receptors, this will reduce the number of available receptors as the concentration of the substrate increases. Cass and Paterson (10) have suggested similar conformational changes in other systems. This conclusion is consistent with the observed effect of 2-mercaptoethanol which stimulated the transport in all cases (Table 1). Again, as in the case of p-chloromercuribenzoate, the target seems to be the carrier protein itself, irrespective of whether the transport process operates actively or by facilitated diffusion. It is well known that 2-mercaptoethanol is a reducing agent that cleaves disulphide bridges reversibly or protects SH-groups where they are already present. Crane (17) has suggested that 2-mercaptoethanol reacts with SH-functions in such a way that the substrate is fitted more readily into the active site. Therefore, 2-mercaptoethanol must have stabilized the SH-groups exposed following the proposed conformational changes that accompany each increase in adenosine concentration. This is also in agreement with the reported changes in the transport mode observed with the variation of adenosine concentration (7).

In the present study, the observed partial inhibition of adenosine transport by formycin A confirms our earlier suggestions that adenosine has multiple routes of uptake (7,8). Our results indicate that formycin A does not use all the transport routes available to adenosine. In spite of their structural similarities, formycin A can limit adenosine transport only on the route available to both of them.

In conclusion, the differential effects of the antimetabolites used in this study, coupled with the failure of any of them, by itself, to inhibit the transport process completely, clearly indicates that adenosine is taken up by different routes in *T. vivax*. The results have also suggested that the SH functions of the carrier protein are involved in the transport process.

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