GLOBAL JOURNAL OF PURE AND APPLIED SCIENCES VOL. 6 NO. 3, JUNE 2000:443-448
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ANTISICKLING ACTIVITY OF PHENYLALANINE: EFFECT ON ERYTHROCYTE MEMBRANE ATPASES

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(Received 21 November 1997; Revision accepted 16 June 1998)

ABSTRACT

The sickling of homozygous sickle cells upon deoxygenation with 2% sodium metabisulphite is inhibited in the presence of phenylalanine (Phe). In the presence of 200 μm Phe there was a rapid reversion of already sickled cells. More than forty percent (46.4 ± 3.2, range 42.4 – 56.2%) sickling reversion could be achieved within 30 min after the introduction of Phe. ATPase activities were studied in erythrocyte membranes prepared from blood of normal, sickle and heterozygous individuals. Phe stimulated Na^{+}, K^{+}-ATPase activity in both normal and sickle cell ghosts in a concentration dependent manner. Membrane bound Ca^{2+}-ATPase activity in normal and sickle cell ghosts was also stimulated by Phe. Phe inhibited Mg^{2+}-ATPase activity. Inhibition was found to be non-competitive with a ki of 1.9 ± 0.08mM. The antisickling effect of Phe may be related to its effect on the erythrocyte membrane.

KEY WORDS: Phenylalanine; sickled cells, erythrocyte membrane, ATPases.

INTRODUCTION

Increasing interest in the management of sickle cell disease has led to the discovery of many antisickling agents. Certain amino acids (Noguchi and Schechter, 1977) and small peptides (Gorecki *et al*, 1980) have been shown to produce varying degrees of in vitro inhibition of deoxy-HbS gelation. Effective compounds have been shown to have aromatic aids chains which have a low solubility in aqueous medium. This activity is directly related to the hydrophobicity of the molecules (Gorecki, *et al*, 1980).

Since the mutation of the β-globin chain of HbA causes a change B_6 (glu) to B_6 (val), the importance of the hydrophobic binding is not unexpected. Non-polar substances could alter intermolecular interactions involved with a complementary hydrophobic site for B_6 (val) thereby preventing or delaying gelation of deoxy-HbS (Gorecki *et al*, 1980).

Ekeke and Shode (1990) reported that phenylalanine is the predominant antisickling agent in *Ca janus cajan* extract. Phenylalanine and *cajanus cajan* extract may be similar in their actions although the extract contains some other amino acids other than phenylalanine.

Although various antisickling agents have been proposed for the management of sickle cell disease, the mechanism of their antisickling effect is not yet clear. This work tends to investigate the effect of phenylalanine on sickling and its possible mechanism of action. The stability of the erythrocyte membrane is achieved by the unidirectional activated transport of ions across the membrane (Kyte, 1971). These vectorial transports are accomplished by the activities of the erythrocyte membrane ATPases.

MATERIALS AND METHODS

Blood Collection

Venous blood samples (5-10ml) were collected into heparinized tubes from both normal and sickle cell anaemia individuals. The samples were then separated into 36 AA, 32 AS and 24 SS using cellulose acetate paper electrophoresis.

Ghost Preparation

The erythrocyte membrane ghosts were prepared based on the method of Hamlyn and Duffy (1978). Red cells from fresh human heparinized venous blood were washed three times with 0.5M NaCl (pH 7.4). Membrane was prepared by hypotonic haemolysis in swirling ice-cold 5mM NaH_2PO_4, pH 7.7.
The mixture was centrifuged at 5,000g for 1h. The supernatant was removed and the ghost washed once in 10mM Tris-HCl pH 7.7.

The ghosts were later suspended in 3.0ml distilled water. The membranes were stored at 4°C for not more than 12h before being assayed for enzyme activity.

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Assay of ATPase Activity

Membrane ATPase activities were estimated by the procedure described by Heesketh et al (1978). ATPase activity was assayed by measuring the inorganic phosphate released from ATP hydrolysis. Inorganic phosphate released in the ATPase assay was determined by the method of Fiske and Subbarow (1925). The activity of the enzyme was expressed as µmole Pi/mg protein/h.

Na^+,K^+-ATPase ACTIVITY

The enzyme-catalysed reaction was followed spectrophotometrically in a mixture containing 0.5ml of 350mM NaCl, 0.5ml of 17.5mM KCl, 10mM Tris-HCl buffer pH 7.4 and 0.5ml of 2mM ATP-Na_2. Reaction was started by adding 0.2ml ghost cells. The mixture was incubated for 1h at 37°C. Reaction was terminated by adding 0.8ml ice-cold 10% TCA. Tubes were then allowed to stand at 4°C for 5 min. after which were centrifuged at 4,000g for 5min. To the supernatant was added 1ml of 2.5% ammonium molybdate and after 10min. 1ml of 2.0% ascorbic acid. The tubes were then allowed to stand for 20min. for colour to develop. Absorbance was read at 725 nm using spectronic 21 spectrophotometer.

Ca^{2+} ATPase

The assay medium contained 0,5ml of 21mM $MgCl_2$, 0.5 ml of 17mM $CaCl_2$, 0.5ml of 10mM Tris-HCl buffer pH 7.4 and 0.5ml of 20mM ATP-Na_2. The reaction was started and terminated as already described for Na^+, K^+-ATPase. All assays were run in duplicate.

Mg^{2+}-ATPase

Mg^{2+}-ATPase activity was assayed spectrophotometrically in a medium containing 0.5ml of 21m $MgCl_2$, 0.5ml of 2mM ATP-Na_2, 0.5ml of 10mM Tris-HCl buffer pH 7.4 and 1.0ml distilled water and usually 0.2ml ghost cells. The inorganic phosphate liberated was measured by the method of Fiske and Subbarow (1925).

Determination of Protein

The protein contents of erythrocyte membrane preparation were determined by the method of Lowry *et. al* (1951) with bovine serum albumin (BSA) as standard.

In Vitro Sickling Experiments

In Vitro Sickling experiments were carried out according to the method described by Ekeke and Shode (1985).

5ml blood was collected by venupuncture from confirmed sicklers. The whole blood was diluted with 0.15M phosphate buffer pH 6.5. Deoxygenation occurred after incubation with 2% sodium metabisulphite for about 1h. Sickling on microscope slides was achieved by mixing equal volumes of the HbSS blood and 2% sodium metabisulphite solution in a test tube. A drop of the mixture was spotted on a microscope slide, monitored microscopically. About 300 red blood cells were always counted from which the percentage sickling was easily estimated.

Reversion of Sickling

Artificially sickled erythrocytes (prepared as already described) were used for the reversion experiment. Only blood samples with more than 40% of sickled cell at the baseline level were used for sickling reversion experiments. The method of Ekeke and Shode (1985) was employed. A 1:20 dilution of the blood samples was made. Equal volumes of the diluted blood, saline and Phe were mixed together and a drop put on microscope slide. The preparation was mounted on a microscope and the percentage of sickled cells was determined on a time-dependent basis.

EFFECT OF DIFFERENT CONCENTRATIONS OF PHE ON HUMAN ERYTHROCYTE-MEMBRANE ATPASE ACTIVITY

Na^+,K^+-ATPase Activity in Vitro

Assay conditions were as already described except that 0.5ml (0-800 µmole) Phe solution was added before incubation for 1h.

Ca^{2+}-ATPase Activity

Assay conditions were as already described. 0.5ml (0-800 µmoles) Phe solution was added before incubation.

Mg^{2+}-ATPase Activity

Assay conditions were also as already described. The solution was varied between 0-800 µmoles.

Table 1: Activities of the Membrane-bound Normal and Sickle Cell ATPases

Parameter	AA (n=36)	AS (n=32)	SS (n=24)
ATPases (µmole Pi/mg Protein/h x10^{-3})			
Na^+,K^+-ATPases	284.7± 20.2	272.4±16.4	118.0±2.8
Ca^{2+}-ATPase	314.2± 4.8	302.6± 4.8	218.4±8.2
Mg^{2+}-ATPase	144.8± 1.3	108.2± 2.8	234.4±2.0

Values are means ± Standard deviations. The number of samples analysed (n) is given in parentheses.

RESULTS

ATPase activities of sickle and normal RBC membranes are shown in Table 1. Ghosts from AA cells exhibited a higher Na^+,K^+-ATPase (284.7 ± 20.2 x 10^{-3}µmole pi/Mg protein/h). The Na^+,K^+-ATPase activities of AA and AS (272.4 ± 16.4 µmole pi/mg protein/h) cells were significantly different from that of SS (118.0 ± 2.8 µmole pi/mg protein/h).

The Mg^{2+} - ATPase activity of sickle red blood cell membranes was found to be higher than normal while the Ca^{2+} - ATPase activity of sickle cell membranes was however, lower than normal. The activity of the Ca^{2+}-ATPase in sickle erythrocytes was about 71.0% of the normal. There was no significant difference between Ca^{2+} -ATPase and Mg^{2+} -ATPase activities in AA and AS cells.

In Vitro Sickling Experiments

2% sodium metabisulphite was able to induce sickling. The mean values of relative percentage sickling with time (min) were plotted (figure 1). In the presence of 2% sodium metabisulphite, there was an initially slow increase in sickling, then followed by a rapid increase. In 30min. over 30.5 ± 0.4% increase above the intial level of sickled cells was observed.

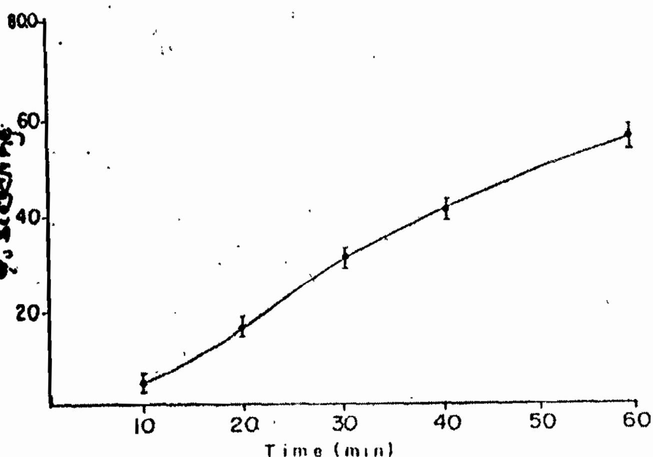

Fig 1: Sickling In the Presence of 20% Sodium Metabisulphite (n = 10) ± SD, n = Number of Samples

Effect of Phenylalanine on Sickling in Vitro

The sickling inhibition of Phe is shown in figure 2. From the graph it could be seen that if sodium metabisulphite was introduced the HbSS were capable of sickling. However, in the presence of 200 µmoles Phe there was reversion of already sickled cells. Results show that 48.4 ±3.2% (range 42.4-56.2%) sickling reversion could be achieved within 30 min in the presence of Phe.

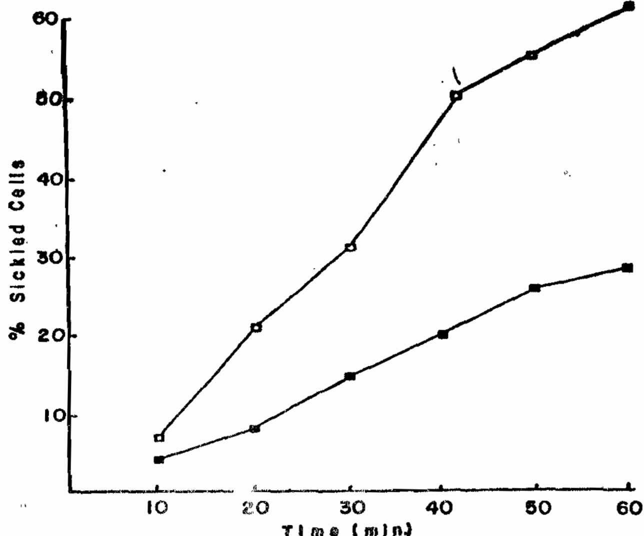

Fig. 2: Sickling inhibition of Phenylalanine at 200 µmole Concentration.

Control — Phe

Effect of Phe on Human Erythrocyte Membrane ATPase Na^+,K^+-ATPase

Figure 3 shows the effect of Phe on the activities of the membrane-bound Na^+,K^+-ATPase in normal human red cells and sickle cell ghosts. The activity of the enzyme was stimulated by Phe in both normal (AA and AS) and SS ghosts and stimulation of the enzyme by Phe was concentration-dependent. The stimulation was observed at concentrations between 0-800 µmole. A maximal stimulation (57.2%) was obtained with AA human erythrocyte Na^+,K^+-ATPase at 400 µmole Phe compared with a maximal stimulation of 65.7% obtained with the sickle cell membrane Atpase at the same Phe concentration. At 400 µmole Phe, the maximal stimulation obtained with AS ghost Na^+,K^+-ATPase was 22.6%.

Ca^{2+}-ATPase:

The effect of Phe on the activity of normal and sickle ghosts Ca^{2+}-ATPase activity is shown in figure 4. Phe stimulated membrane-bound Ca^{2+}-ATPase activity in normal and sickle cell ghosts. The effect of Phe on Ca^{2+}-ATPase activity depended on its concentration. Maximum stimulations of 20.0%, 19.3% and 37.2% were obtained with AA, AS and SS human erythrocyte Ca^{2+}-ATPase respectively at 400 µmole. At concentrations above 400µmole, activity greatly decreased.

Mg^{2+}-ATPase

The effect of different concentrations of Phe on erythrocyte membrane-bound Mg^{2+}-ATPase is

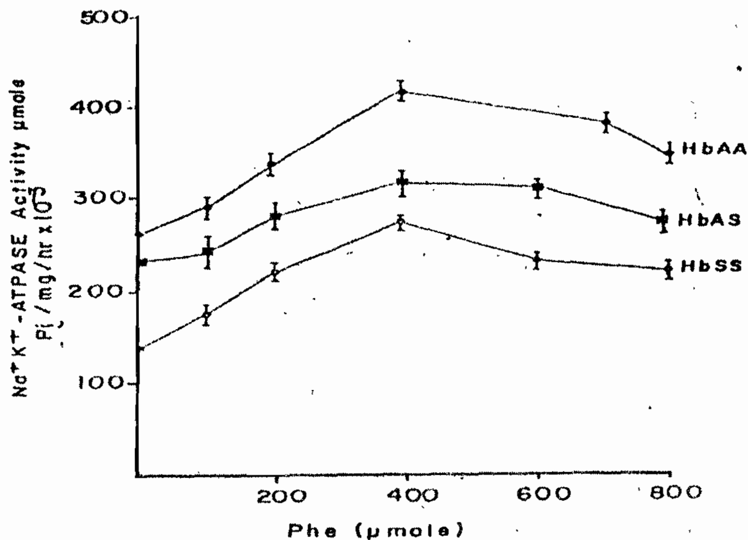

Fig. 3: Activation of the Basal Rate of Sickle and Normal Ghost Na^+K^+-ATPase By Different Concentrations of Phenylalanine. ATPase Activity was Determined as Described Under "Materials and Method"

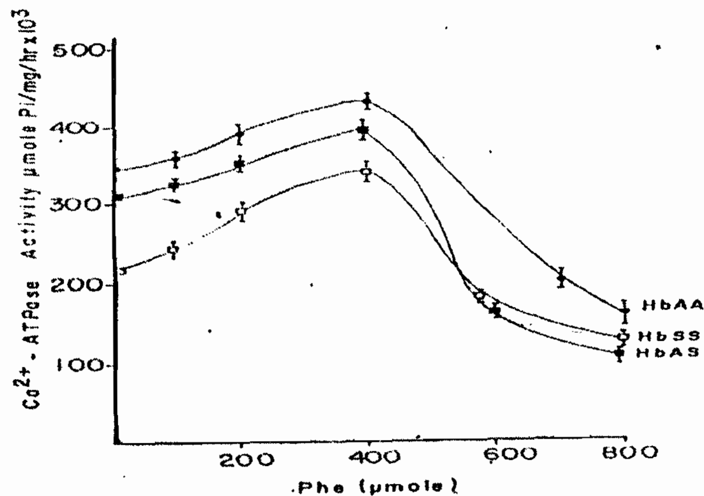

Fig. 4: Effect of Phenylalanine on the Basal Activity of Sickle and Normal Ghost Ca^{2+}ATPase Activity.

summarized in figure 5. In the presence of Phe, Mg^{2+}-ATPase activity markedly decreased. Inhibition of Mg^{2+}-ATPase by Phe was observed in AA, AS and SS ghost at all the concentrations studied. The inhibition by Phe was found to be non-competitive (figure 6). The inhibitor constant Ki was calculated to be 1.9 ± 0.0mM using Dixon's plot (figure 7).

DISCUSSION

Phenylalanine is an essential aromatic amino acid. It has significant antisickling activity as demonstrated by visual assays. The reasons why it has this activity are not fully understood. Its amphipathic nature together with its molecular weight are probably responsible for its easy passage through the mosaic lipid protein bilayer of the erythrocyte membrane (Naccache and Shalafi, 1973). However, permeability by itself does not appear to be the only factor in the antisickling activity of phenylanine. The antisickling effect of phenylalanine suggests that this amino acid might be penetrating the erythrocyte cell membrane. This might explain its effect on the activities of the erythrocyte membrane ATPases. Phenylalanine stimulated Na^+,K^+-ATPase and Ca^{2+}-ATPase and inhibited Mg^{2+}-ATPase. This suggests an additional role outside that of the antigelling activity of phynylalanine at low concentrations. Gorecki *et al* (1980) reported that many amphipathic molecules bind in a biphasic manner to intact erythrocytes. At low concentrations they give linear binding and at higher concentrations they induce sphericity followed by haemolysis. This morphological feature may suggest an interaction between the molecule and

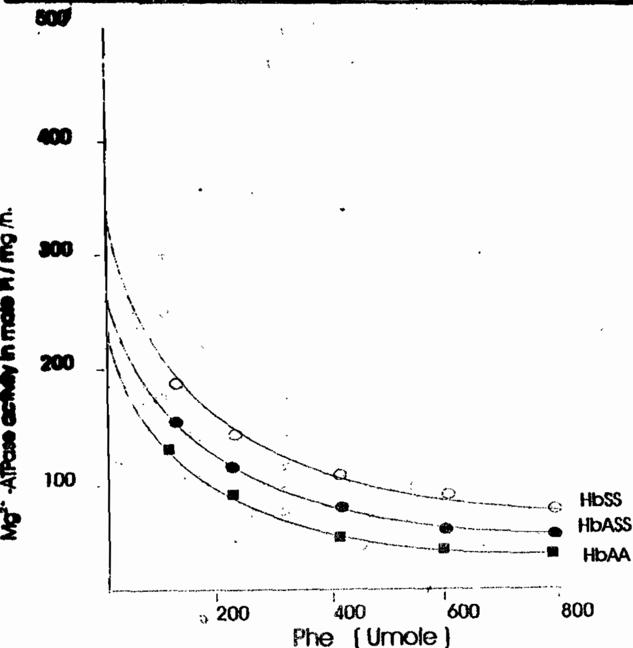

Fig. 5: Inhibition of Sickle and Normal Ghost Mg^{2+} ATPase Activity by Phenylalanine. ATPase Activity was Determined as Described Under "Materials and Method" Except that Different Concentrations of Phenylalanine was Used.

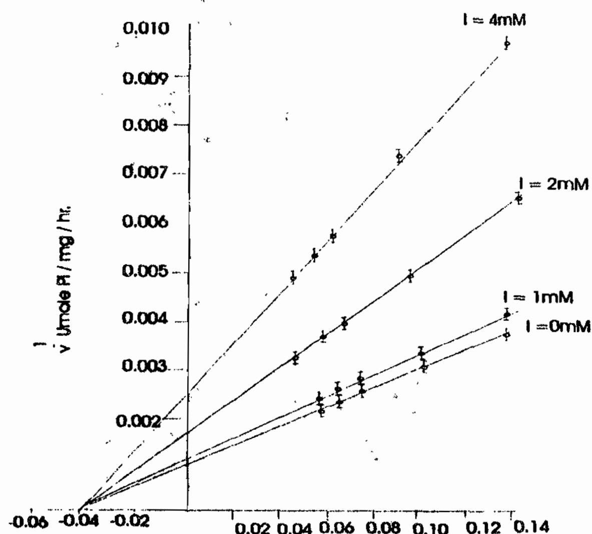

Fig.6: LINE WEAVER - BURKPLOT (1/V VS 1/[S]) AT DIFFERENT INHIBITOR CONCENTRATIONS.

erythrocyte membrane. In the case of phenylalanine this may have led to an increase or decrease in the activities of the erythrocyte membrane ATPases.

The antigelling activity of phenylalanine may be due to binding to deoxy HbS together with its effect on erythrocyte membrane ATPases caused by membrane changes. It is not clear which is the major contributor to phenylalanine's antisickling activity. It may be possible that the two may be acting

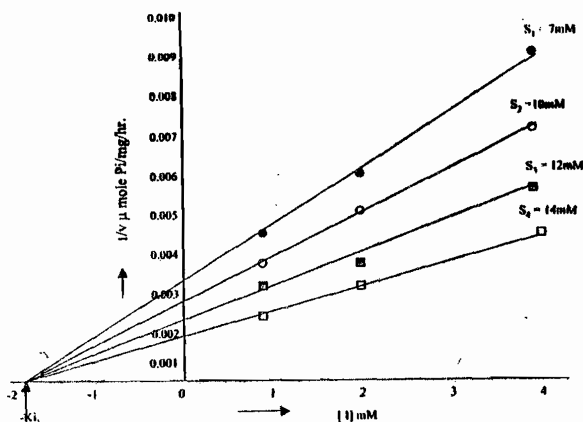

Fig. 7: Dixon's Plot of the inhibition of Erythrocyte Ghost Membrane Mg^{2+}-ATPase Activity by Phenylalanine.

synergistically. The erythrocyte membrane ATPpases (Na^+,K^+,Ca^{2+} and Mg^{2+}) are responsible for the vectoral transport of ions across the membrane and the stability of the erythrocyte membrane is achieved by the unidirectional activated transport of ions. The inhibition of sickling of HbSS erythrocytes *in vitro* could be significant, if the same effect is shown *in vivo*. If this is the case, then phenylalanine can be listed as one of the potential antisickling agents in recent times and explanation to its mode of action found.

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