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Ca^{++}. Mg^{++} - ATPase activity in insulin-dependent and non-insulin dependent diabetic Nigerians

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Summary
A study of Ca^{++}, Mg^{++} - ATPase activity was carried out in normal (HHm) and diabetic Nigerians of both sexes with insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM). The results showed that protein concentration of erythrocyte ghost membranes of healthy humans (HHm) was the highest when compared with protein concentrations of IDDM and NIDDM patients. The protein concentration was lowest in IDDM, while the value in NIDDM was between those of HHm and IDDM. The basal activities of erythrocyte Ca^{++} - ATPase from IDDM and NIDDM were determined and were found to be significantly lower than that of HHm. The addition of calmodulin (CaM) 2 µg/ml stimulated the activity of the calcium pump in all the groups (IDDM, NIDDM and HHm). The effects of calcium (Ca^{++}) and adenosine triphosphate (ATP) on the activity of the pump from each group were determined. Enzyme kinetics (K_m and V_{max}) revealed that the activity of Ca^{++}, Mg^{++} - ATPase was initiated by ATP in the presence of Ca^{++} in a dose-dependent manner. Calmodulin also enhanced the activity of the enzyme in the presence of Ca^{++} in all the groups, though activities in IDDM and NIDDM were significantly lower than in HHm. There was no significant difference in the activities between IDDM and NIDDM. These results suggest a defective calcium translocating mechanism in diabetic Nigerians.

Keywords: *Ca^{++}, Mg^{++} - ATPase Erythrocyte membrane, insulin-dependent diabetes mellitus, non-insulin dependent diabetes mellitus, Nigerians*

Résumé
Une étude de l'actirité de Ca^{++}, Mg^{++} - ATPase a été faite cheq les Nigérians normanx et diabitiques des denx sexes ayant le diabétes mellitus insuline dépendent (DMID). Les résultats ontmontré que la concentration proteine des erythrocytes des humains en bonne santé (HHM) était le plus élevé comparé á la concentration des proteines chez les DMLD et NIDDM. La concentration était moindre chez les DMID, alors la taux chez les NIDDM était entre ceux des HHM et DMID. Les autirites basales des erytocytes Ca^{++} -ATPase provenant des DMID et NIDDM ont été déterminés et étaient significativement bas comparé á ceux des HHM. L'addition du calmodium (CaM) 2 µg/ml a stimulé l'activité de la pompe du calcium chez tous les groups (DMID, NIDDM et HHm). Les effets du calcium (Ca^{++}), et de l'Adénosine Triphosphate (ATP) sur L'aitinité de la pompe de chaque groupe ont été déterminés. La cinétique des enzymes (K_m and V_{max}) ont montré que l'activite de Ca^{++}, Mg^{++} - ATPase était initié par l'ATP en présence du Ca^{++} dans une maniére de dosage dépendent. Le calmodulin augmente aussi l'activite des enzymes en présence du Ca^{++}, chez les DMID et NIDDM étaient significatoement bas comparé á ceux de HHM. Il n'yavait pas de difference significative dans less activities entre DMID et NIDDM. Ces résultats suggérent un méchanisme de translocation défective du calcium chez les diabétiques Nigérians.

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Introduction

The fundamental roles of calcium ion (Ca^{++}) in cell signalling and cell regulation require that its cytosolic concentration be maintained very low [1,2,3]. The erythrocyte plasma membrane Ca^{++}, Mg^{++} - ATPase is the major mechanism responsible for moving calcium from the cytosol of the red blood cell in order to maintain an intracellular calcium concentration as low as 0.1 µm [4,2]. This pump, an ATPase of P-Class, forms a phosphorylated intermediate during the reaction. It is an integral protein with a molecular weight of about 140,000 and it is activated by calmodulin (CaM) [5,6,7]. It is also stimulated by acidic phospholipids [8] and calpain [9]. This enzyme is sensitive to calcium and the sensitivity is regulated by calmodulin, a ubiquitous calcium binding protein [10] which also requires calcium for potency.

The existence and the activities of calcium pump in the nephron, blood platelets, adipocyte plasma membrane and islet cell plasma membrane in caucasians have been reported [11, 12, 13, 14]. Alterations in the activity of Ca^{++}, Mg^{++} - ATPase and cellular calcium concentration have also been observed in some pathological conditions like sickle cell anaemia in American blacks [10,15,16], hypertension in rats [17], hypertension and atherosclerosis in caucasians [18] and protein energy malnutrition in black African children [19].

Some authors have reported reduced Ca^{++}, Mg^{++} - ATPase activity in experimental diabetes mellitus [20, 21] while some reported an increase in activity [22,23]. Schaefer *et al.*, [24] and Spieker *et al.* [25] reported a decrease in Ca^{++} - ATPase activity in diabetic caucasians whereas Mazzanti *et al.* [26] found an increase in the activity of this enzyme. These reports on the activity of cellular Ca^{++} - ATPase in diabetes mellitus in caucasians are conflicting. And extensive search of the literature shows that there is information on the Ca++, Mg++ - ATPase activity in diabetic Africans.

This study was therefore desinged to investigate the Ca++, Mg++ - ATPase activity om erythrocyte membranes of diabetic Nigerians. Two classes of diabetics

were investigated; patients with insulin dependent diabetes mellitus (IDDM) and those with non-insulin dependent diabetes mellitus (NIDDM).

Subjects, materials and methods

The study was carried out on three different groups of human subjects (i) healthy humans (HHm) who served as control, (ii) patients suffering from insulin-dependent diabetes mellitus (IDDM or Type 1) and (iii) patients suffering from non-insulin dependent diabetes mellitus (NIDDM or Type 2).

The approval of the Ethical Committee of the College of Medicine, University of Ibadan, was obtained before commencing the study. Consent was obtained from the subjects after the nature of the procedure involved in the study was explained to them. There were 12 subjects in each of the IDDM and NIDDM groups and 18 subjects in the HHm group. Subjects in these three groups are within the same age bracket (30 – 65 years). Random blood samples (20 ml per sample) were obtained from healthy human volunteers who served as control and patients who have been newly identified as insulin-dependent diabetic patients and non-insulin dependent diabetic patients at the Diabetic Clinic of the University College Hospital, Ibadan. None of the subjects had received any dietary therapy or medication prior to the time blood was collected. All blood samples were collected in acid-citrate-dextrose buffer and stored at 4°C. Erythrocyte membranes were isolated from each blood sample within 24 hours of collection.

All reagents were of the highest purity available and were purchased from Sigma Chemical Co. Ltd., London, Calbiochem La Jolla, Calif. USA, British Drug House, U.K.

Calmodulin-deficient membranes were prepared by the method of Niggli *et al.* [27], based on the principle of hypotonic lysis, developed by Dodge *et al.* [28]. All the stages of the erythrocyte ghost membrane (EGM) preparation were carried out at 4 °C. The membranes obtained were finally resuspended in 130 mM potassium chloride (KCl), 20 mM N-2-hydroxyethyl piperazine –N'-ethane sulphonic acid (HEPES), pH 7.4, 500 µM magnesium chloride (MgCl2), 50 µM calcium chloride (CaCl2) and stored at –80 °C.

Protein concentration of erythrocyte membrane preparations was determined essentially according to Lowry *et al.* [29] using fatty acid-free bovine serum albumin as the standard. Membrane fractions were treated with 0.05% w/v deoxycholic acid and then precipitated with 10% w/v trichloroacetic acid at room temperature.

Ca^{++}, Mg^{++} - ATPase activity was assayed with calmodulin (120 nM) and in the absence of calmodulin by measuring the rate of release of inorganic phosphate from the gamma (γ) - position of ATP based on the procedure of Stewart [30]. The reaction medium contained 120 mM KCl, 50 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM ethylene diamine tetracetic acid (EDTA) and 50-100 µg of erythrocyte membrane protein in a total volume of 0.8 ml. The mixture was preincubated for 5 minutes at 37 °C with constant shaking prior to the addition of 2 mM adenosine triphosphate (ATP) which initiated the reaction. The assays were run in triplicates while each experiment was repeated twice. Blanks were run to correct for non-enzymic hydrolysis of ATP. Mg^{++} - ATPase was assayed using the same procedure. The inorganic phosphate liberated was determined spectrophotometrically using Pye Unicam UV/Visible SP8-400 spectrophotometer at wavelength of 660 nm. Ca^{++} - ATPase activity was obtained by subtracting Mg^{++} - ATPase activity (in the absence of calcium) from Ca^{++}, Mg^{++} - ATPase activity assayed in the presence of calcium.

The Michaelis-Menten's constant (K_M) and the maximal velocity of enzyme reaction (V_{max}) of Ca^{++}, Mg^{++} - ATPase were obtained from the reciprocal plots of enzyme activity against substrate concentration i.e. $^1/v$ against $^1/[S]$. K_M is equal to substrate concentration at which the reaction rate is half of its maximal value while V_{max} is the maximum rate of enzyme reaction when the enzyme is fully saturated with substrate.

All values given are the mean ± standard deviation (± SD) of the parameters measured.

Level of significance was assessed using Student's t-test and P values of 0.05 or less were taken as statistically significant.

Results

The erythrocyte membrane protein concentration of HHm was highest, being 5.5 ± 0.01 µg/ml. The protein concentration in IDDM was lowest (4.5 ± 0.01), while that of NIDDM lies between the values of HHm and IDDM (5.1 ± 0.02). The protein concentrations in IDDM and NIDDM were significantly different ($P < 0.05$) from one another and also from that of HHm.

Effect of varying concentrations of calcium on Ca^{++} Mg^{++} - ATPase activity

Ca^{++}, Mg^{++} - ATPase was inactive in the absence of calcium in all the groups (Fig. 1), however, the presence of calcium initiated activity in the three groups. There was progressive increase in activity with increasing concentration of calcium until a peak was reached after which further increase in calcium concentration did not cause any further increase in activity. The same concentrations of calcium stimulated the activity of the pump differently in the three groups. The activity of calcium pump in HHm was highest being 0.42 ± 0.02 n mole Pi/mg protein/hour and lowest in IDDM (0.20 ± 0.024). The activity plateaued in the three groups at calcium concentration above 40 µm/ml (Figure 1).

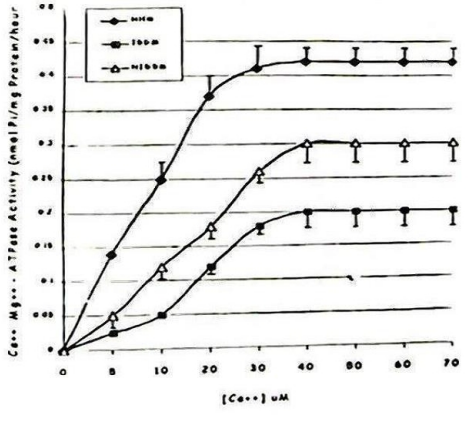

Fig. 1

Effect of variation in the concentration of ATP on the activity of Ca^{++}, Mg^{++} - ATPase

Ca^{++}, Mg^{++} - ATPase was inactive without ATP in the three groups studied. Activity was initiated by the presence of ATP and increased with increasing concentration of ATP. About 0.4 mM ATP stimulated activity in all the groups but the basal activity of calcium pump in IDDM and NIDDM were significantly different from the basal activity in HHm. Maximum activity was attained at ATP concentration of 2.4 mM ATP in all the groups (Table 1).

Table 1: Effect of varying concentrations of ATP on the activity of Ca^{++}, Mg^{++} - ATPase from EGM of HHm, IDDM and NIDDM.

ATP (Mm)	Activity (nmol Pi/mg Protein/hr.)		
	HHm	IDDM	NIDDM
0	0	0	0
0.4	0.30 ± 0.022	0.20 ± 0.034	0.20 ± 0.024
0.8	0.40 ± 0.019	0.30 ± 0.032	0.30 ± 0.021
1.2	0.50 ± 0.033	0.40 ± 0.021	0.40 ± 0.014
1.6	0.70 ± 0.043	0.60 ± 0.031	0.60 ± 0.014
2.0	0.90 ± 0.026	0.70 ± 0.020	0.75 ± 0.021
2.4	0.92 ± 0.025	0.71 ±0.014*	0.75 ±0.021*
2.8	0.92 ± 0.025	0.71 ± 0.014	0.75 ± 0.021

n = 18 for HHm; 12 for IDDM; 12 for NIDDM
(- P < 0.05) Values are Mean ± S.D.*

Effect of varying concentration of calmodulin on the activity of Ca^{++}, Mg^{++} - ATPase

The activity in all the groups was increased in the presence of CaM in a concentration dependent manner. Maximum activity of Ca^{++}, Mg^{++} - ATPase in the presence of CaM was highest in HHm and Lowest in IDDM (Table 2).

Table 2: Effect of varying concentrations of calmodulin on the activity of Ca^{++}, Mg^{++} - ATPase from erythrocyte ghost membrane of HHm, IDDM and NIDDM

Calmodulin (ng/µg protein)	Activity (nmole Pi/mg protein/hour)		
	HHm	IDDM	NIDDM
0	0.91 ± 0.010	0.81± 0.021*	0.85 ± 0.034*
2	1.50 ± 0.027	1.00± 0.068	1.30 ± 0.023
4	2.20 ± 0.058	1.20 ± 0.035	1.50 ± 0.031
6	2.90 ± 0.024	1.55 ± 0.022	1.70 ± 0.031
8	3.00 ± 0.081	1.70 ± 0.044	1.75 ± 0.054
10	3.08 ± 0.041	1.80 ± 0.034	1.85 ± 0.030
12	3.10 ± 0.060	1.85 ± 0.032*	1.90 ± 0.030*
14	3.10 ± 0.064	1.85 ± 0.032	1.90 ± 0.030

(n = 18 for HHm; 12 for IDDM; 12 for NIDDM)
Values are Mean ± SD; (- P<0.05)*

K_M and Vmax of Ca^{++}, Mg^{++} - ATPase in EGM of HHm, IDDM and NIDDM

The K_M values for Ca^{++}, Mg^{++} - ATPase in the absence and presence of CaM was lowest in HHm (1.00 ± 0.064) and highest in NIDDM (1.35 ± 0.035) while that of IDDM was 1.31 ± 0.023. The K_M values for IDDM and NIDDM were not significantly different from each other but were both significantly higher than that of HHm (Table 3).

Table 3: K_M values of Ca^{++}, Mg^{++}-ATPase from erythrocyte ghost membranes of HHm, IDDM and NIDDM

Embrane groups	K_M (mMol ATP)	
	- CaM	+ CaM
HHm	1.00 ± 0.064	1.00 ± 0.064
IDDM	1.32 ± 0.022*	1.31 ± 0.023*
NIDDM	1.32 ± 0.026*	1.35 ± 0.035*

n =18 for HHm; 12 for IDDM; 12 for NIDDM)
values are mean ± SD (- P < 0.05)*

V_{max} value of HHm was highest with or without calmodulin (3.33 ± 0.021). The V_{max} of IDDM (2.47 ± 0.038) and V_{max} of NIDDM (2.58 ± 0.025) were significantly different from one another (P < 0.05) (Table 4).

Table 4: V_{max} of Ca^{++}, Mg^{++}-ATPase from erythrocyte ghost membranes of HHm, IDDM and NIDDM (n = 18 for HHm; 12 for IDDM; 12 for NIDDM)

Membrane groups	V_{max} (nmol Pi/mg protein/hour)	
	- CaM	+ CaM
HHm	2.50 ± 0.043	3.33 ± 0.021
IDDM	2.16 ± 0.028*	2.47 ± 0.038*
NIDDM	2.17 ± 0.035*	2.58 ± 0.025*

Values are Mean ± SD (- P < 0.05)*

Discussion

The significantly higher protein concentration observed in the EGM of HHm compared with protein concentration in IDDM and NIDDM is consistent with the observations of Weed *et al.* [31], Eaton *et al.* [15] and Olorunsogo *et al.* [32]) Previous studies on protein concentration in sickle cell disease [15] and hypertension [32] showed similar reduction in EGM protein when compared with healthy controls. The reduced cellular protein concentration observed in this study may well be due to the well known utilization of body protein for glucose production in gluconeogenesis in diabetes mellitus [33, 34 and 35]. From this study, the IDDM group had the lowest level of cellular protein which suggests a likely higher rate of wasting in this group and this is consistent with the observations of Zimmet and King that this disease is accompanied by severe symptoms including body wasting (36).

The initiation of activity by the presence of Ca^{++} as observed in this study is consistent with the reports of

Carafoli [37] and Roufogalis and Wang [3] about the dependence of Ca^{++}, Mg^{++} - ATPase activity on Ca^{++} The fact that no further increase in activity occurred at calcium concentrations above 40 μm probably suggests that all the Ca^{++} domains on the pump were occupied at 40 μm calcium. Since the maximum stimulated activity of the pump by Ca^{++} in all the groups was at 40 μm Ca^{++}, this suggests that the affinity of the pump for Ca^{++} was similar in the three groups. It may not be unreasonable therefore, to conclude that the ability of the pump to bind Ca^{++} is unaffected by diabetes mellitus although the rate of activity differed. However, it is worthy of note that at the same maximum concentration of Ca^{++} (40 μm), the activity in HHm was highest and that of IDDM was lowest. Calcium pump has been reported to be sensitive to its surroundings and any alteration in its phospholipid surrounding affects the activity [37, 38]. The differences observed in the activities of the pumps even with the same maximum concentration of Ca^{++} could be a consequence of possible aberration in the phospholipid ambient of the pumps in IDDM and NIDDM.

The role of ATP in this study is consistent with earlier reports that Ca^{++}, Mg^{++} - ATPase like other active ion pumps utilize ATP as their energy substrate [39,40] The effect of CaM on Ca^{++}, Mg^{++} - ATPase is consistent with earlier reports that CaM modulates the pump and potentiate its activity [41]. The relatively constant values of K_M with and without CaM observed on the three groups suggest that affinities of these groups for substrate were not affected by the presence or absence of CaM. Since the K_M values in HHm remained constant while the activity increased in the presence of CaM, it seems likely therefore that CaM stimulated the pump by increasing the turnover rate while its affinity for substrate remained unaltered; these observations are similar to that of Scharff and Foder [42] and Caride *et al.* [43]. The significantly low affinities observed in IDDM and NIDDM suggests that the pumps were not adequately responsive to the available substrates or have lower affinity, hence a low turnover even in the presence of CaM.

It seemed likely therefore, that the defect in the affinity of Ca^{++}, Mg^{++} - ATPase from IDDM and NIDDM could possibly be one of the factors responsible for the reduced activity of the pump and the consequently high K_M values. The fact that the activity of the pump was low even in the presence of CaM shows that the binding ability of the pump to form CaM – Ca^{++} complex is defective.

The significant differences in the V_{max} within the groups suggest that the turnover rates were markedly different from one another. The activity of the pump in HHm was highest, while the activities of the pumps from IDDM and NIDDM were markedly reduced. This suggests that the binding ability of the enzymes of CaM - Ca^{++} complex in IDDM and NIDDM though defective, was not completely destroyed. This probably explains the reason for a much lower rate of potentiation than in HHm.

From the literature, there are conflicting reports on Ca^{++}, Mg^{++} - ATPase activity in diabetes mellitus. Mazzanti *et al.* [26] reported increased activity of the pump in caucasians while Schaefer *et al.* [24] reported a decrease in activity, also in caucasians.

However, observations from this study is a significant decrease in the activity of the pumps and this is consistent with the reports of Schaefer *et al.* in caucasians [24], but is at variance with the reports of Mazzanti *et al.* [26] also in caucasians. The present study is the first report on the activity of Ca^{++}, Mg^{++} - ATPase from insulin and non-insulin dependent diabetic black Africans. Activity was found to be reduced. There is need for more studies on Ca^{++}, Mg^{++} - ATPase activity in diabetics in both caucasians and black Africans before a final conclusion can be drawn as to whether Ca^{++}, Mg - ATPase activity is actually reduced or increased in diabetes mellitus.

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