Modulation of Erythrocyte Ghost Membrane Ca^{2+}-Pumping ATPase of Healthy and Non-Insulin Dependent Diabetic Humans by alpha, alpha Trehalose-6- Phosphate and Extracts of *Carica papaya* Linn.

Adisa RA[1], Oke JM[3] and Olorunsogo OO[2]

[1]Department of Biochemistry, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos,
[2]Laboratories for Biomembrane Research and Biotechnology,
Department of Biochemistry, College of Medicine,
[3]Department of Pharmaceutical Chemistry, University Of Ibadan, Ibadan, Nigeria.

Address correspondence to **Adisa RA,** E-mail: radisa22@yahoo.com; radisa@unilag.edu.ng

ABSTRACT

Background: Although glycosylated Ca^{2+}- ATPase has not been demonstrated in diabetes mellitus, but it is evident that the enzyme can be significantly glycated under experimental conditions in the presence of appreciable high levels of glucose.
Objective: In this study, the hypoglycaemic agents Trehalose-6-phosphate derivative (Tp) and mature but unripe fruits of *Carica papaya* L (Cp) were used to modulate the activity of the erythrocyte ghost membrane (EGM) Ca^{2+} pumping ATPase of healthy and non-insulin dependent diabetes mellitus (NIDDM) individuals.
Results: The Ca^{2+}- pumping ATPase in membrane of healthy and NIDDM individuals was activated maximally by 2.5mg/ml (2.6 folds) and 4mg/ml (1.7 folds) of the crude extracts of Cp respectively, while 3mg/ml of Tp optimally stimulated the membrane-bound enzyme in both healthy (2.1 folds) and NIDDM (1.5 folds) individuals, respectively. The NIDDM enzyme is less active with a lower affinity for the substrate (30.6±2.52 µMol ATP) compared to healthy individuals (21.5 ± 2.15 µMol ATP), whereas, exposure of the NIDDM Ca^{2+} - pumping ATPase to Tp (3mg/ml) or Cp (4mg/ml), increased the affinity of the enzyme for ATP to 22.4±2.32µMol ATP and 22.1±2.22µMol ATP, respectively.
Conclusion: These findings suggest that Cp and Tp could therapeutically not only lower blood glucose levels as hypoglycaemic agents but could also attenuate the elevated Ca^{2+} concentration implicated in diabetes mellitus.
Keywords: Diabetes mellitus, Calcium-ATPase, *Carica papaya*, Trehalose-6-phosphate

INTRODUCTION

An increase in concentration of intracellular free Ca^{2+} could initiate a myriad of physiological events, such as muscle contraction [1], hormone-release [2] and cell growth [3]. Thus, the reduction of elevated intracellular Ca^{2+} and maintenance of a low resting Ca^{2+} concentration 10^{-7} to 10^{-8} M [4] is crucial for cell homeostasis. Ca^{2+} in cells can be reduced by either sequestration of Ca^{2+} into intracellular stores or Ca^{2+} efflux across the plasma membrane.

The plasma membrane Ca^{2+}- ATPase (PCMA) is responsible for the ATP-dependent extrusion of Ca^{2+} from erythrocytes [5]. The PCMA is a multi-regulated transporter [6] and the activity of this enzyme can be modulated by calmodulin [7], protein kinases [8], proteases-calpain [9], acidic phospholipids [10], oligomerization and possibly agonists and G proteins. Erythrocytes earlier regarded as "non target" cell for insulin, have been shown to possess specific receptors for insulin with characteristic similar to typical target cells [11].

Abnormal cellular Ca^{2+} $\{Ca^{2+}\}_i$ metabolism have been proposed as the cause of insulin resistance and insulin secretion impairment in the pathogenesis of both insulin dependent and non-insulin dependent diabetes mellitus syndrome [12-15]. A striking feature of this impaired $\{Ca^{2+}\}_i$ metabolism in diabetes is the wide spectrum of abnormalities observed. However, this heterogeneity may be explained by the complexity in the interaction between the varied mechanisms involved in $\{Ca^{2+}\}_i$ homeostasis. A defect in any mechanism may cause an alteration in the function of other $\{Ca^{2+}\}_i$ regulating mechanism leading to the development of cardiomyopathy as characterized by reduced contractibility, relaxation, cardiac work and diastolic complications [16]. Other mechanisms by which hyperglycemia may lead to complications of long –term diabetics include polyol activation, formation of glycated proteins resulting in advanced glycation end products (AGEs), and an increase in oxidative stress (Horakova et al., 2013)[17]

Intracellular Ca^{2+} levels could be pharmacologically manipulated by various mechanisms. For instance, many anti-diabetic drugs in the class of sulphonyl-ureas act as specific blockers of a particular type of K^+ channels in pancreatic β-cells, known as ATP-sensitive K^+ channels [18,19] and ultimately lead to Ca^{2+} influx which triggers insulin granule exocytosis in the β-cells [20].

Over half a decade years ago, oral therapy for type 2 NIDDM has focused on sulphonyl-ureas and biguanides. While the beneficial effects of these drugs on glycaemic levels are well documented [21-23], the preventive activity of these drugs against progressive nature of diabetes and its micro-macro-vascular complications was modest and not always effective.

In this regard, the search for novel hypoglycaemic agents especially from dietary sources is fast gaining recognition and is being encouraged and supported [24], however, in a long while, diabetics have been treated orally with a myriad of medicinal plants or extracts based on folk medicine [25].

In drug discovery, target specificity is critical to limit toxicity, although absolute specificity (one drug – one target) is difficult to achieve and more difficult to prove. Clearly, improvement about the structure, function and physiological role of specific targets will increase the chance for the discovery process to be transformed into a successful end-product. One such target class is the P-type ion translocating ATPases and in this case, our target is the PM Ca^{2+}-ATPase in NIDDM human subjects.

Carica papaya L belongs to the family of *caricaceae*. The plant is widely cultivated for consumption as a fresh fruit, for use in drinks, candies, jam and as dried and crystallized fruits [26] Nutritionally, the fruit is a good source of vitamin A and C [27] and biochemically, its leaves and fruits are complex producing several proteins (cysteine proteases used for protein digestion) and alkaloids with important pharmaceutical and industrial applications [28].

The leaf, fruits and root extracts of papaya are also used as traditional medicines [29,30] and the complex uncharacterized chemical composition of papaya latex is suggestive of the potential beneficial effects on the health of human or other organisms. The papaya leaves are useful in subsiding signs and symptoms of dysentery, worming, asthma [31,32] and was demonstrated to possess *in vitro* antidiabetic activity [33], nephroprotective effects in streptozotocin-induced diabetic rats [34] as well as antisickling property [35,36]. Also, the unripe mature fruit of papaya has been reported for the treatment of diabetes [37] while the roots have been suggested[38] to have possible purgative effects and antihypertensive activity [30]. The presence of Benzyl isothiocyanate (BITC) in the seeds of papaya has been demonstrated to have an effect on vascular contraction in canine carotid artery *in vitro* model [39].

Similarly, α,α-trehalose-6-phosphate, a glucose analogue synthesized by Oke and Watt [40] was demonstrated to be a potent anti-glycaemia agent in alloxan-induced diabetic rats and fasted rabbits. It was also reported that this synthetic compound is an effective micro and macro filaricidal agent [41].

In this study, we have evaluated the modulatory effects of extracts of *carica papaya* Linn compared to a synthetic hypoglycaemic agent α-α-trehalose-6-phosphate on the erythrocyte ghost membrane Ca^{2+} pumping ATPase in health and non-insulin dependent diabetic human subjects.

MATERIALS AND METHODS.

Calmodulin was purchased from Calbiochemical, La jolla, California. All other reagents used were of analytical grade and were purchased from Sigma Chemical Co. Ltd, London and British Drug Houses, Poole, England. α-α-trehalose-6-phosphate was a generous gift from Dr J.M Oke, Department of Pharmaceutical Chemistry, University of Ibadan, Nigeria.

Subjects

Venous blood samples were obtained from newly identified non-insulin dependent diabetic individuals (NIDDM) of age 45 years and above on overnight fasting at the diabetic Clinic of the Department of Medicine, University College Hospital, Ibadan, Nigeria after informed verbal consent. Thirty (30) NIDDM patients were recruited as they visit the clinic and none of them had received any medication or dietary therapy prior to the time blood was collected. Healthy human blood was collected from thirty (30) healthy age matched adult donors and were used as control. All blood samples, 10 mL each were collected in acid-citrate-dextrose buffer, stored at 4° C and used within 12hr.

Isolation of Erythrocyte Ghost Membrane

Calmodulin (CAM)-deficient haemoglobin-free ghost membranes were prepared from these samples after haemolysis in hypotonic buffer (1mM Na-EDTA, 10mM Tris pH 7.4) containing 1μM soybean trypsin inhibitor and 200μM phenylmethylsulfonyl fluoride (PMSF) as previously reported [42]. Finally, the calmodulin-depleted, haemoglobin-free ghost membranes were resuspended and centrifuged at 18000rpm for 20mins in storage buffer containing 130mM KCl, 20mM HEPES, pH 7.4, 500μM $MgCl_2$ and 50μM $CaCl_2$. The membrane pellet was suspended and stored in the same buffer at -60° C. All buffers contained 200μM PMSF and membrane preparations were done at 4° C.

Membrane protein concentration was estimated according to the procedure of Lowry *et al* [43]. Fatty acid free bovine serum albumin was used as standard.

Assay of human erythrocyte plasma membrane Ca^{2+}-ATPase activity

Erythrocyte ghost membrane (EGM) Ca^{2+} activity was assayed by monitoring the rate of release of inorganic phosphate from the γ- position of the ATPase as previously described [44]. CAM-depleted membranes (100-200μg) were incubated at 37° C for 5mins in a final volume of 1ml. The assay medium contained 120mM KCl, 50mM HEPES, pH 7.4, 2mM $MgCl_2$, 20μM $CaCl_2$, 2mM EGTA (when added), 2μg/ml calmodulin, or 2.5 – 4 mg/ml of α,α-trehalose-6-phosphate (Tp) or extracts of *carica papaya* L (Cp) (added when necessary). The reaction commenced upon addition of 2mM ATP (final concentration). After 30mins of incubation at 37° C with constant shaking, the reaction was stopped with 10% sodium dodecyl sulphate (1ml). The inorganic phosphate (pi) released into the medium was estimated spectrophotometrically using a modification of the method described by Fiske and Subarrow [45]. The difference in the activity between the Ca^{2+} Mg^{2+} -ATPase activity and Mg^{2+} -ATPase activity was used to obtain the basal Ca^{2+} -ATPase activity. All assays were run in triplicates and blanks were run to correct for non-enzymic hydrolysis of ATP. Specific activity is expressed as μmol pi released per mg protein per hour.

Preparation of crude extracts of *Caraica papaya* L

Mature unripe fruits of *carica papaya L* were commercially purchased at Agbowo area, opposite University of Ibadan, Oyo state Nigeria. The epicarp of these fruits was pulled off, sliced open and decongested of the unripe seeds. The mesocarp was diced into small pieces and washed thoroughly with distilled water. The prepared fruits (200g) was soaked with 60% methanol and kept in the dark at room temperature. After one week, the solvent was collected, filtered and concentrated using a rotary evaporator at 40° C and later lyophilized. The extracts of *Carica papaya* obtained – a yellowish white powder was stored at -20° C until required for use.

Statistical analysis

Data were analyzed using SPSS version (Statistical Package for Social Sciences) Version 16.0 (SSS Inc, Chicago, Il) and expressed as mean ± standard error. One way Analysis of Variance was used to analyze the mean differences between treatments followed by Duncan's multiple comparison test. A significant difference was considered to be $p<0.05$

RESULTS

Table 1 shows the results of an experiment designed to measure the activity of two EGM enzymes -Ca^{2+} - ATPase and Mg^{2+} - ATPase of healthy and NIDDM individuals in the presence of Tp and Cp. The specific activities of these enzymes were significantly lowered in erythrocyte membranes of NIDDM individuals than were significantly lowered ($p<0.05$) in the erythrocytes of healthy individuals.

Specifically, the basal activity (0.55 ± 0.04 µmol pi released mg protein^{-1} hr^{-1}) of the Ca^{2+}-pumping ATPase of the erythrocyte membranes of NIDDM was about 39% lower than that (0.90 ± 0.05 µmol pi released mg protein^{-1} hr^{-1}) observed in the erythrocyte membranes of healthy individuals. The results further revealed that calmodulin (CAM) and the hypoglycaemic agents enhanced the basal activities of the erythrocyte membrane enzymes of NIDDM and healthy individuals. In particular, 2.5mg/ml and 4.5mg/ml of Cp activated the erythrocyte Ca^{2+} -pumping ATPase in membranes of healthy and NIDDM individuals maximally by 2.6 and 1.7 folds respectively whereas 3mg/ml of Tp maximally stimulated the membrane bound enzymes by 2.1 and 1.5folds, respectively. Indeed, there was no significant difference ($P \geqslant 0.05$) between the CAM-stimulated and Cp-stimulated erythrocyte membrane Ca^{2+}-ATPase activities in NIDDM individuals.

Effect of varying concentrations of hypoglycaemic agents on Ca^{2+}- ATPase activity in NIDDM and healthy humans

Fig 1 shows the pattern of the specific activity of the erythrocyte Ca^{2+}- pumping ATPase from healthy and NIDDM humans on incubation of the membrane with varying concentrations of the hypoglycaemic agents – Tp and Cp. It is clear from the data that Tp and Cp stimulated the Ca^{2+}- pump from both sources in a concentration-dependent manner. The activity of these enzymes in both groups progressively increased with increasing concentration of the hypoglycaemic agents until a peak was reached after which no further increase in activity occurred with increased concentration of agents. The results indicated that the EGM Ca^{2+}-ATPase in healthy humans showed maximum stimulation (2.1 and 2.6 folds, respectively) following incubation with concentrations of Tp and Cp above 3mg/ml and 2.5mg/ml, respectively. On the other hand, the enzyme protein in the NIDDM human subjects showed maximum activity of 1.7 and 1.5 folds in the presence of concentrations of Tp and Cp above 4mg/ml and 3mg/ml, respectively.

Assessment of ATP-dependence of EGM Ca^{2+} - ATPase in healthy and NIDDM human subjects in the presence of hypoglycaemic agents.

The results obtained by a determination of the dependence of Ca^{2+} ATPase activity of EGM of healthy and NIDDM human subjects on ATP in the presence of Tp and Cp are as presented in Table 2. The result clearly indicates that the Ca^{2+}- pumping enzyme in NIDDM exhibited a lower affinity for its substrate ATP (30.6 ± 2.52 µmol ATP). Indeed, the inclusion of Tp and Cp in the reaction medium increased the affinity of the Ca^{2+}- pump to 22.40 µmol ATP and 22.70 ± 2.20 µmol ATP, respectively. Whereas, the presence of either Tp or Cp in the reaction medium did not have any significant effect on the Ca^{2+} ATPase activities in membranes from healthy subjects ($P > 0.05$) compared to Ca^{2+} - ATPase activity of healthy untreated EGM.

Assessment of Ca^{2+} dependence of Erythrocyte Plasma Membrane Ca^{2+}- ATPase in healthy and NIDDM humans in the presence of hypoglycaemic agents.

The sensitivity of the erythrocyte plasma membrane Ca^{2+}-ATPase of diabetic and healthy humans Ca^{2+} in the presence of Tp (3mg/ml) and (4mg/ml) is as shown in Figure 2. The results indicate that the inclusion of the hypoglycaemic agents in the reaction medium increased the affinity of the pump for Ca^{2+} in the presence of concentrations of calcium as low as 5µM calcium. The maximum ATPase activity was obtained between 20-30µM of the calcium with the V_{max} being increased 2 folds by Tp and Cp, respectively in healthy humans. Similarly, the V_{max} of the erythrocyte membrane Ca^{2+} ATPase of diabetic humans was increased up to 1.3 and 1.5 folds by Tp and Cp, respectively. However, there was no significant difference ($P > 0.05$) between the enzyme activity obtained in the presence of Tp and Cp in NIDDM individuals except in erythrocyte membrane enzyme of healthy humans.

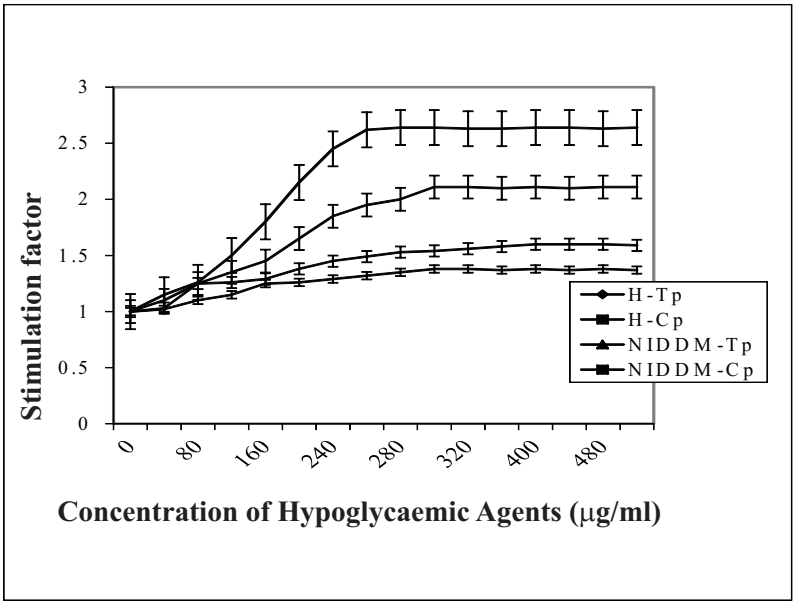

Figure 1: Dose-dependent stimulation of the erythrocyte membrane Ca^{2+} - ATPase activity in healthy and NIDDM diabetic patients

Data are mean ±S.E of 10 different experiments. Tp – α, α Trehalose -6- phosphate; Cp, *Carica papaya*, CAM- Camoldulin; NIDDM- Non insulin dependent diabetes mellitus, H- healthy

Table 1

Erythrocyte ghost membrane Ca^{2+}- ATPase activity of healthy and NIDDM individuals in the presence of α-α-trehalose-6-phosphate and extracts of C. *papaya* L.

	Ca^{2+} -ATPase activity of individuals (µmol pi/mgprotein/hr)	
Enzyme	**Healthy**	**NIDDM**
Mg^{2+} -ATPase	0.35 ± 0.03	00.25 ± 0.02
Ca^{2+} - ATPase	0.90 ± 0.05	0.55 ± 0.04
+CAM	2.97 ± 0.15 (3.3 fold)	0.94 ± 0.09* (1.8 fold)
+Cp	2.34 ± 0.12 (2.6 fold; 2.5mg)	0.93 ± 0.08* (1.7fold, 4mg)
+Tp	1.89 ± 0.11 (2.1 fold; 3mg)	0.80 ± 0.06 (1.5fold, 3mg)

Values are mean ± standard error of at least 10 experiments.
* Ca^{2+} ATPase activities are significantly different from each other ($P < 0.05$) compared to basal activity. Tp – α, α Trehalose -6- phosphate; Cp, Carica papaya, CAM- Camoldulin; NIDDM- Non insulin dependent diabetes mellitus

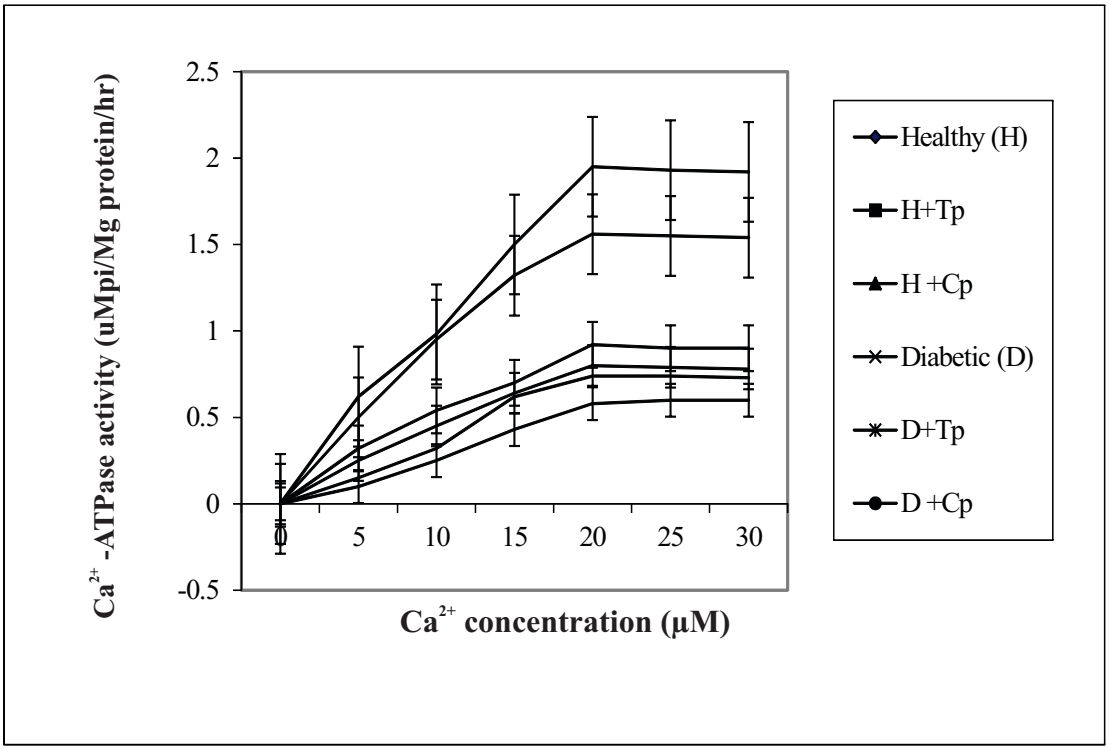

Figure 2: Ca^{2+} dependence of erythrocyte plasma membrane Ca^{2+}-ATPase of healthy and NIDDM individuals in the presence or absence of hypoglycaemic agents

Data are mean ± S.E of 10 different experiments Tp – α, α Trehalose -6- phosphate; Cp, *Carica papaya.*

Table 2
Kinetic Parameters of the erythrocyte ghost membrane Ca^{2+} - ATPase of NIDDM patients

EGM	K_mATP (µmol)	V_{max} (µmol ATP mg protein/hr)
HS	21.50 ± 2.15^{b}	3.10 ± 0.35^{c}
HS + Tp	20.20 ± 2.12^{b}	3.50 ± 0.20
HS + Cp	19.80 ± 2.01^{b}	3.80 ± 0.32
NIDDM	30.60 ± 2.52^{e}	1.80 ± 0.15^{d}
NIDDM +Tp	22.40 ± 2.32^{b}	2.50 ± 0.17^{a}
	22.40 ± 2.32^{b}	2.50 ± 0.17^{a}
NIDDM +Cp	22.10 ± 2.22^{b}	2.60 ± 0.20^{a}

Values are Mean ± standard deviation of at least 10 experiments. Values with different superscripts denote significant differences in the kinetic parameters of Ca^{2+} ATPase of NIDDM and healthy individuals ($P < 0.05$).
EGM-Erythrocyte ghost membrane, Hs-Healthy subject
CP- carica papaya, Tp-Trehalose 6-phosphat, NIDDM-Non insulin dependent diabetes mellitus

DISCUSSION

In erythrocytes, the transport systems involved in the regulation of intracellular Ca^{2+} concentration include Ca^{2+} - pumping ATPase, Na^{+}/ Ca^{2+} exchanger [46], and Ca^{2+} channels [47]. Of these systems, the Ca^{2+}- pump is the most widely studied and best understood. Previous studies have shown that a defective Ca^{2+}- pumping ATPase could occur in human erythrocyte plasma membrane of certain pathological conditions such as sickle cell anaemia [48], essential hypertension [49] and Kwashiokor [50]. Evidence has also indicated the involvement of altered calcium ion metabolism in the pathogenesis of Diabetes mellitus [51]. Although opinion differs as to whether the activity of the Ca^{2+} $^{+}Mg^{2+}$ ATPase increases or decreases in caucasian diabetes [52]. The reports on the activity of Ca^{2+} + Mg^{2+} ATPase from IDDM and NIDDM diabetic black Africans is consistent with the reports of Schaffer *et al.,* [15] in Caucasian but it is at variance with reports of Mazzani *et al* ., [53] also in Caucasians. Therefore, Ca^{2+} pump function remains the target for investigation whether or not a generalized or specific membrane defect occurs in this pathological state.
In this study, an assessment of the Ca^{2+} ATPase activity of the erythrocyte plasma membrane of individuals having NIDDM showed that this ATPase activity is lowered compared to healthy individuals (Table 1). The reduction obtained in enzyme activity is a consequence of the non-enzymic glycation of the Ca^{2+} pump and other membrane proteins with glucose thus corroborating earlier reports [54, 55, 56]. It is also possible that the alteration may have distorted the conformationally active structure of this enzyme and consequently altered the enzyme activity. Another possibility is that NIDDM may have secondarily changed the membrane lipid asymmetry environment and therefore the pump activity. Our findings support earlier reports in human and rat models [12, 57, 58], interestingly, the lowered activity of this pump in NIDDM individuals was stimulated in the presence of Tp and Cp (Figure 1). The stimulation of the Ca^{2+} ATPase activity in both healthy and diabetic state by Tp and Cp is concentration dependent. The enzyme activity increased until the binding site was totally saturated such that increases in the concentration of the hypoglycaemic agents had no corresponding effects on the enzyme activity. Although there was no significant difference between the CAM-stimulated activities of this enzyme in NIDDM individuals, the mechanism by which this membrane potential is stimulated is not likely to be the same.
Moreover, our results demonstrated that the basal activities of the CAM-deficient Ca^{2+} pumping ATPases in healthy and NIDDM membranes are susceptible to activation by both hypoglycaemic agents suggesting that these agents could be binding directly to the pump protein or calmodulin binding sites rather than to calmodulin. This deduction was also corroborated by the data obtained from the Ca^{2+} dependence of the enzyme from both sources following incubation with the hypoglycaemic agents. The implications of an enhanced affinity for Ca^{2+} is that the Ca^{2+} overload experienced in diabetes, which has been linked to the secondary complications in the disease, could be reversed.
Furthermore, the data showed that Cp stimulated the Ca^{2+} ATPase activity in both healthy and NIDDM individuals to a greater extent than Tp even though at a higher concentration particularly in NIDDM. The observed higher modulatory effects of Cp on the Ca^{2+} pump activity may be due to the combined proteolytic activity [59] of the cysteine proteinases (papain, chymopapain, caricain and glycyl endopeptidase) [60, 61] present in the extracts of Cp. This deduction is further supported by the results obtained for the affinity of the pump protein for ATP in the presence of Tp and Cp. Although the mechanism by which these hypoglycaemic agents carry out their activity is not known, but our results clearly indicate that the affinity of the enzyme for its substrate, ATP was potentiated by the hypoglycaemic agents. The hypoglycaemic agents exhibited similar modulatory effects to that of calmodulin on the functional integrity of the Ca^{2+} pump in NIDDM humans. Also, it seems likely that the higher concentration of the Cp required to maximally stimulate the NIDDM enzyme may be caused by interference of other components on the extract of Cp with its potent compound or proteolytic activity of the proteases such as papain therein.
It follows therefore, that in order to gain insight into the mechanism of modulation of the functional integrity of the Ca^{2+} pump by the hypoglycaemic agents, there is need to isolate and purify bio-active hypoglycaemic compounds or proteins in the extracts of Cp using bioassay guided methods. Conversely, a plausible explanation for the modulatory activity of the Ca^{2+} ATPase of both enzymes in healthy and NIDDM humans by Tp could be that the ATPase hydrolytically cleaved the terminal phosphoryl group of Tp and becomes phosphorylated in the process and hence more active because, the data obtained also indicated an increase in enzyme activity.

Taking together, this study findings indicated that there is a defective Ca^{2+} pump activity in NIDDM individuals as was earlier reported [56]. This defect could be responsible for an elevated extracellular Ca^{2+} content of erythrocytes from NIDDM individuals resulting in greater membrane rigidity and an increase in the erythrocyte diameter secondary to the greater intracellular osmotic pressure and cell death. And that this defect in Ca^{2+} ATPase activity and the associated secondary pathological complications resulting from Ca^{2+} overload could be reversed by the dietary intake of *Carica papaya* L – which is a natural source of dietary supplements containing among other components vitamins, micronutrients and proteolytic enzymes whose pharmacological roles are known.

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