The Effect of In Vivo Glucose Administration on Human Erythrocyte Ca^{2+}-ATPase Activity and on Enzyme Responsiveness In Vitro to Thyroid Hormone and Calmodulin

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SUMMARY

To characterize endogenous control mechanisms for human erythrocyte membrane Ca^{2+}-ATPase ("calcium pump") activity, we studied the effect of changes in blood glucose concentration in vivo within the physiologic range on Ca^{2+}-ATPase activity in red cells. Red cells obtained in the course of induced hyperglycemia were also studied to determine susceptibility of membrane Ca^{2+}-ATPase to stimulation in vitro by thyroid hormone and calmodulin, both of which have been shown previously to enhance Ca^{2+}-ATPase activity.

Oral glucose administration (75 g) to eight healthy, adult subjects induced predictable increases in concentrations of blood glucose and immunoreactive insulin. Basal levels of activity of Ca^{2+}-ATPase in red cells obtained after glucose ingestion fell 55% ($P < 0.025$) by 30 min after glucose with recovery of enzyme activity to levels not significantly different from basal by 60 min. Activity of red cell Ca^{2+}-ATPase at time zero was significantly stimulated in vitro by thyroxine (T_4, 10^{-10} M), triiodo-L-thyronine (T_3, 10^{-10} M), and calmodulin (100 ng/mg membrane protein). In vivo glucose administration led to depression of red cell enzyme responsiveness in vitro to T_4 and T_3; recovery from this effect did not occur by 120 min after oral administration of glucose. Calmodulin responsiveness of the enzyme in vitro was less significantly reduced in red cells obtained after glucose ingestion.

Intravenous (i.v.) glucose administration (20 g) to five subjects also led to decreased basal enzyme activity (61% of fasting level at 20 min). A significant decrease in response of enzyme to T_4 was achieved by 8 min after glucose administration ($P < 0.02$), with recovery by 60 min. Calmodulin responsiveness showed an insignificant downward trend.

Exposure of red cell membranes to glucose (200 mg/dl) or insulin (100 μU/ml) in vitro resulted in 26% and 17% reductions, respectively, in basal Ca^{2+}-ATPase activity ($P < 0.001$), together with a reduction in thyroid hormone stimulation of the enzyme. Tunicamycin, an inhibitor of enzymatic protein glycosylation, reversed these in vitro effects of glucose, but not of insulin, on erythrocyte Ca^{2+}-ATPase activity.

These observations are relevant to the interpretation of clinical studies of Ca^{2+}-ATPase activity and to the physiology of the enzyme. Glucose administration predictably decreases Ca^{2+}-ATPase in the human red cell, an alteration in calcium pump activity that would allow pulsing of Ca^{2+} into the cell. DIABETES 1985; 34:639–46.

The activity of red cell membrane Ca^{2+}-stimulable, Mg^{2+}-dependent adenosine triphosphatase (Ca^{2+}-ATPase) is regulated by intracellular free Ca^{2+} through the formation of complexes with cytoplasmic calmodulin.[1,2] The calmodulin · Ca^{2+} complex activates Ca^{2+}-ATPase ("calcium pump"). The responsiveness of this enzyme to extracellular factors is less well-understood. It is clear that physiologic concentrations of thyroid hormone added in vitro to human[3] and certain animal[4] red cells significantly enhance Ca^{2+}-ATPase activity. We have shown this hormonal effect to be analogue-specific[5] and calmodulin-dependent.[6]

We have also found that hyperthyroidism and hypothyroidism result, respectively, in increased and decreased levels of red cell Ca^{2+}-ATPase activity.[7] Chronic illnesses, such as end-stage renal disease (ESRD),[8] importantly decrease enzyme activity and the ability of Ca^{2+}-ATPase to respond to calmodulin and to L-thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3). Sickle cell disease[9] and cystic fibrosis[10] may also alter endogenous levels of calcium pump activity.

As part of a study of physiologic control of human red cell Ca^{2+}-ATPase activity, we have investigated the effect of glucose administration in vivo on this enzyme. Eating has been reported to increase[11] or to have no effect[12] on human eryth-

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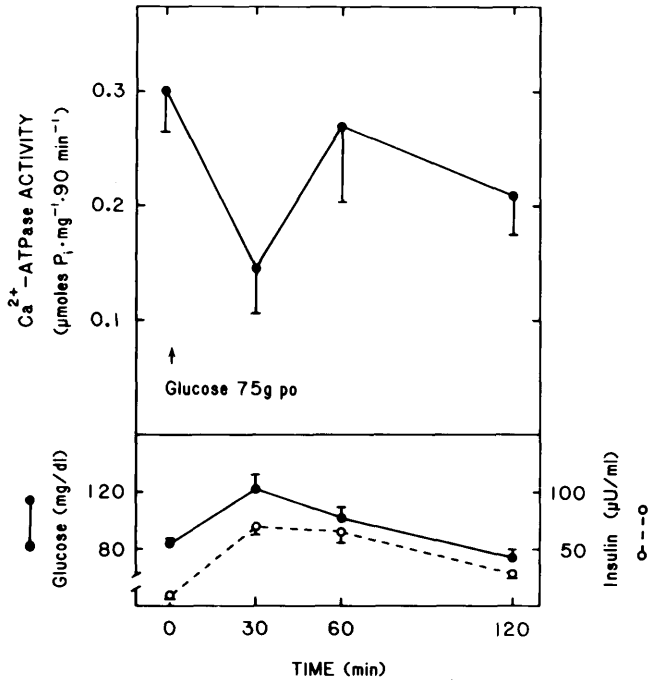

FIGURE 1. Red blood cell membrane Ca^{2+}-ATPase activity (upper panel) in eight normal, fasting subjects before and after ingestion of 75 g glucose. Activity levels are mean ± SEM of all determinations at each time point. Serum glucose and plasma immunoreactive insulin levels (mean ± SEM) are shown in the lower panel.

rocyte membrane Na,K-ATPase activity, but the possible effect of feeding on Ca^{2+}-ATPase activity has not been studied. In the current report, glucose administration orally or i.v. is shown to decrease red cell Ca^{2+}-ATPase activity and to alter enzyme response in vitro to thyroid hormone and calmodulin. In vitro studies suggest that this effect of in vivo glucose administration may be mediated by erythrocyte membrane glycosylation.

MATERIALS AND METHODS

In vivo oral and i.v. glucose loading. Four male and four female subjects in good health (ages 23–46 yr) underwent oral glucose loading after an 8-h fast. After insertion of an indwelling i.v. line and withdrawal of 20 ml of heparinized blood, the subjects ingested 75 g glucose as Glucola (Ames, Elkhart, Indiana); additional heparinized 20-ml samples were then obtained at 30, 60, and 120 min after oral glucose administration. In separate studies, three male and two female healthy volunteers between the ages of 30 and 48 yr were given 20 g glucose i.v. over a 1-min period. Twenty-milliliter samples of heparinized blood were drawn before glucose, and at 8, 20, 40, and 60 min after glucose.

Three fasting subjects underwent control studies, receiving no glucose, with blood samples drawn at time zero and at 8, 20, 40, and 60 min. Red cell membranes were prepared and enzyme assays conducted as with membranes from glucose-loading studies (see below). All subjects provided informed, written consent to this study, which was approved by the institutional Human Research Committee.

Preparation of erythrocyte membranes. Red cells from the timed samples were pelleted by centrifugation and, after removal of the plasma and buffy coat, were lysed hypotonically in 10 vol of 5 mM Tris buffer, pH 7.4, in the presence of 0.1 mM EDTA, as previously reported.[3] Resultant membranes were then washed twice in 17 mM NaCl, twice in 10 mM Tris buffer, and suspended in 10 mM Tris, pH 7.4, to a protein concentration of 1 mg/ml, as determined by the Lowry method.[13] We have previously shown that these steps remove virtually all endogenous thyroid hormone from the membranes.[3] Membrane suspensions from each study were prepared on the day of glucose administration, and were stored at −70°C for 1–3 days until assayed for enzyme activity.

Hormones and reagents. T_4, T_3, Na_2ATP, and tunicamycin were purchased from Sigma Chemical Co. (St. Louis, Missouri). Purified rat testis calmodulin was obtained from CAABCO (Houston, Texas), and porcine crystalline zinc insulin from Eli Lilly and Company (Indianapolis, Indiana).

TABLE 1
Effect of in vivo oral glucose administration on stimulation by T_4, T_3, and calmodulin of human red cell membrane Ca^{2+}-ATPase activity

		Ca^{2+}-ATPase activity* (μmol P_i/mg/90 min)			
		ΔCa^{2+}-ATPase-T_4†			
Min	Basal	Without CaM	With CaM	ΔCa^{2+}-ATPase-T_3	ΔCa^{2+}-ATPase-CaM
0	0.301 ± 0.039	+0.078 ± 0.005 (<0.001)‡	+0.082 ± 0.007 (<0.001)	+0.064 ± 0.005 (<0.001)	+0.069 ± 0.011 (<0.001)
30	0.146 ± 0.041 (<0.025)	+0.014 ± 0.006 (<0.001)	+0.004 ± 0.013 (<0.001)	+0.018 ± 0.011 (<0.002)	+0.028 ± 0.014 (<0.06)
60	0.269 ± 0.063 (<0.63)	+0.030 ± 0.008 (<0.001)	+0.038 ± 0.007 (<0.002)	+0.029 ± 0.009 (<0.02)	+0.016 ± 0.014 (<0.02)
120	0.210 ± 0.036 (<0.18)	+0.027 ± 0.012 (<0.001)	+0.034 ± 0.008 (<0.001)	+0.022 ± 0.011 (<0.004)	+0.041 ± 0.020 (<0.2)

*Results are mean ± SEM of duplicate determinations in two assays conducted on 2 days after glucose loading and membrane preparation in eight subjects.
†ΔCa^{2+}-ATPase-T_4, ΔCa^{2+}-ATPase-T_3, and ΔCa^{2+}-ATPase-CaM indicate change in enzyme activity with in vitro addition of T_4, T_3 (both 10^{-10} M), and calmodulin (CaM, 100 ng/mg membrane protein).
‡P-values (parentheses) for zero time hormone and CaM effects were calculated by paired *t*-test; P-values to show change in basal activity and ΔCa^{2+}-ATPase-T_4, ΔCa^{2+}-ATPase-T_3, and ΔCa^{2+}-ATPase-CaM after glucose were calculated by multivariate repeated measures analysis of variance.

TABLE 2
Effect of in vivo oral glucose administration on plasma calcium, phosphorus, potassium, and free fatty acid levels

Min	Calcium (mg/dl)	Phosphorus (mg/dl)	Potassium (meq/L)	Free fatty acids (meq/L)
0	9.3 ± 0.1	3.5 ± 0.2	4.2 ± 0.1	1272 ± 129
30	9.1 ± 0.1	3.2 ± 0.2	3.8 ± 0.1	919 ± 49
60	9.0 ± 0.1	3.1 ± 0.1	4.1 ± 0.1	847 ± 51
120	9.0 ± 0.1	3.2 ± 0.1	4.1 ± 0.1	763 ± 51

Membrane incubation before Ca^{2+}-ATPase assay. Red cell membranes (1 mg protein/ml of buffer) were incubated at 37°C for 60 min before enzyme assay with T_4 (10^{-10} M), T_3 (10^{-10} M), or calmodulin (100 ng/mg membrane protein). Control samples contained diluent for hormone or calmodulin, but no added active material.

Ca^{2+}-ATPase assay. Enzyme activity was measured by our previously described method,[3,6] and was expressed as the difference in inorganic phosphate (P_i) hydrolyzed from Na_2ATP in the presence and absence of Ca^{2+}. The results are reported as μmol P_i/mg membrane protein/90-min assay period, with P_i measured by the Fiske-Subbarow method.[14] All Ca^{2+}-ATPase measurements in red cell ghosts from each *in vivo* glucose study were carried out in duplicate in two assays performed 1–4 days after glucose loading. The effect of T_4, T_3, and calmodulin on Ca^{2+}-ATPase activity was measured as the difference in enzyme activity in the presence and absence of each of those factors. The Ca^{2+}-ATPase activity in the absence of added thyroid hormone or calmodulin is described as "basal enzyme activity." The range of basal enzyme activities among normal subjects in our laboratory was from 0.124 to 0.671 μmol P_i/mg/90 min, as measured over a 12-mo period. Intrasubject variability was ± 35%.

Effect of in vitro glucose, insulin, and tunicamycin on red cell membrane Ca^{2+}-ATPase activity. Human red cell membranes were prepared from healthy, fasting donors as described above. The effect of in vitro glucose and insulin, with and without tunicamycin, on basal and T_4-stimulated Ca^{2+}-ATPase activity was determined as the difference in enzyme levels with and without these factors. Membranes from a single donor were used for each experiment.

Na,K-ATPase assay. The difference in ATPase activity determined in the presence and absence of 1 mM ouabain, without calcium, was an expression of red cell membrane Na,K-ATPase activity. Our method for measurement of membrane Na,K-ATPase has been reported.[15] These enzyme assays were conducted at the same time as the Ca^{2+}-ATPase assays.

Plasma potassium, calcium, phosphorus, glucose, insulin, glucagon, and free fatty acids. Plasma potassium, calcium, phosphorus, and glucose were determined by standard Autoanalyzer methods. Immunoreactive insulin[16] and glucagon[17] were measured by radioimmunoassay employing double-antibody techniques as previously described.[18] Free fatty acids were measured colorimetrically by the method of Pinelli.[19]

Statistical analysis. Paired *t*-tests were used to test the effect of thyroid hormone and/or calmodulin in vitro on Ca^{2+}-ATPase activity of membranes obtained before in vivo glucose administration. The same analysis was used to test significance of changes in Na,K-ATPase after in vivo glucose, to examine Ca^{2+}-ATPase activity in three control subjects who did not receive glucose, and to evaluate effects of glucose, insulin, and tunicamycin in vitro.

Multivariate repeated measures analysis of variance[20,21] was used to evaluate the effects of glucose administration on enzyme activity and the interaction between the effects of thyroid hormone and/or calmodulin in vitro (within subjects comparison) and glucose given in vivo (between subjects comparison) on Ca^{2+}-ATPase activity.

RESULTS

In vivo oral glucose administration: effect on basal Ca^{2+}-ATPase activity, plasma glucose, and insulin levels. In the eight subjects who received oral glucose, basal Ca^{2+}-ATPase activity (i.e., no thyroid hormone or calmodulin added in vitro) fell significantly by 30 min ($t = 2.37$, $P < 0.025$) from 0.301 ± 0.039 (SEM) to 0.146 ± 0.041 μmol P_i/mg/90 min (55% decrease); enzyme activity was 0.269 at 60 min and 0.210 at 120 min (Figure 1); these levels were not significantly different from the fasting level. The maximum mean glucose level achieved after glucose loading was 122 ± 11 mg/dl (fasting 85 ± 2 mg/dl), and the highest mean immunoreactive insulin concentration was 70.5 ± 6.7 μU/ml at 30 min (fasting insulin concentration, 10.4 ± 1.3 μU/ml) (Figure 1).

Effect of in vivo oral glucose administration on in vitro thyroid hormone and calmodulin stimulation of Ca^{2+}-ATPase activity. T_4, T_3, and calmodulin significantly stimulated Ca^{2+}-ATPase activity in red cells obtained from the subjects before in vivo glucose loading ($t = -15.56$, $P < 0.001$; $t = -14.13$, $P < 0.001$; $t = -6.36$, $P < 0.001$, respectively) (Table 1). The percent increases were 26, 21, and 23 with

TABLE 3
Alterations in human red cell Na,K-ATPase activity after in vivo oral glucose administration

	Na,K-ATPase activity* (μmol P_i/mg/90 min)			
	0 Min	30 Min	60 Min	120 Min
Basal	0.088 ± 0.011	0.123 ± 0.013 (<0.05)†	0.106 ± 0.009 (<0.1)	0.119 ± 0.011 (<0.02)
T_4 (10^{-10} M)	0.087 ± 0.011	0.117 ± 0.019	0.114 ± 0.010	0.108 ± 0.012

*Results are mean ± SEM of duplicate determinations in two assays on membranes from each of seven subjects.
†P-values (parentheses) calculated by paired *t*-test show significance of change in enzyme activity after glucose compared with preglucose levels. There was no significant T_4 stimulation of Na,K-ATPase at any time point.

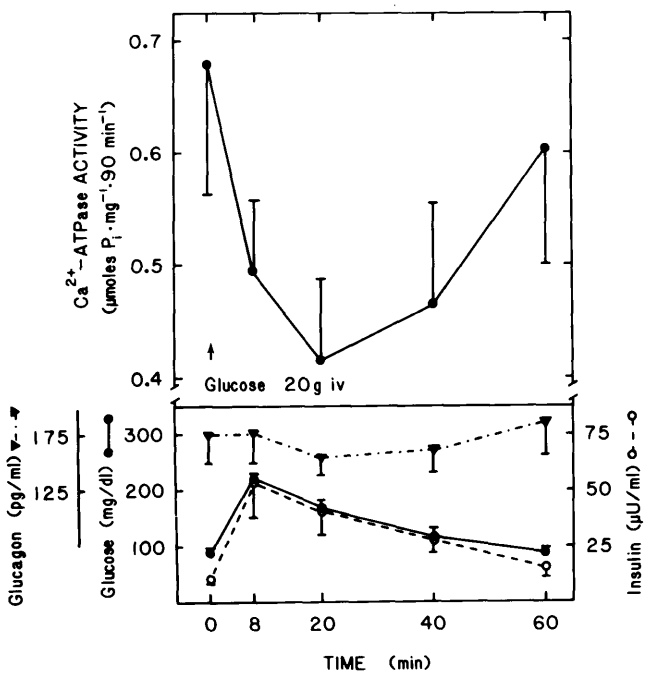

FIGURE 2. Red blood cell membrane Ca^{2+}-ATPase activity (upper panel) in five normal, fasting subjects before and after the i.v. administration of 20 g glucose. Enzyme activity levels are the mean ± SEM of all determinations at each of five time points. In the lower panel, serum glucose and plasma immunoreactive insulin and glucagon levels are shown.

T_4, T_3, and calmodulin, respectively. The increased responsiveness of Ca^{2+}-ATPase activity to T_4 compared with T_3 has been previously described.[3,5] After oral glucose, enzyme stimulation by T_4 and T_3 (ΔCa^{2+}-ATPase-T_4 and ΔCa^{2+}-ATPase-T_3, respectively) was significantly depressed by 30 min after glucose administration, as indicated by a significant interaction between the effects of hormone addition on the enzyme and time ($F[3,28] = 12.38$, $P < 0.0001$ for T_4; $F[3,28] = 5.31$, $P < 0.0051$ for T_3) and by subsequently comparing the degree of stimulation preglucose with the degree of hormone stimulation at 30 min after glucose. Enzyme stimulation by T_4 and T_3 remained depressed at 60 and 120 min compared with zero time values.

Calmodulin stimulation of Ca^{2+}-ATPase was not depressed overall by the administration of oral glucose (lack of interaction between calmodulin stimulation and time [$F(3,28) = 2.27$, $P < 0.10$]). However, planned comparisons revealed that while no significant depression occurred at 30 and 120 min, significant depression of calmodulin stimulation did occur at 60 min ($t = 2.47$, $P < 0.02$) (Table 1). Therefore, the in vitro effect on Ca^{2+}-ATPase of thyroid hormone is depressed by the administration of glucose in vivo for as long as 120 min, while calmodulin stimulation of enzyme activity is influenced only to a limited degree. The effect of T_4 on enzyme activity was not restored by the presence of exogenous calmodulin (Table 1).

Effect of oral glucose loading on plasma calcium, phosphorus, potassium, and free fatty acid levels. The effect of oral glucose on plasma calcium, phosphorus, and potassium levels is shown in Table 2. Small but significant ($P < 0.05$) reductions in all three measurements were seen by 30 min after glucose, and the changes in calcium and phosphorus persisted for 120 min. Free fatty acid levels fell progressively during the study.

Effect of oral glucose administration on red cell Na,K-ATPase activity. The red cell Na,K-ATPase activity of seven subjects who received oral glucose rose significantly in 30 min from 0.088 ± 0.011 to 0.123 ± 0.013 μmol P_i/mg/90 min ($P < 0.05$; Table 3). By 60 min after glucose ingestion, the Na,K-ATPase activity was not different from the preglucose level. At 120 min, a secondary rise was observed. There was no stimulation of Na,K-ATPase activity by T_4 in vitro at any time.

In vivo i.v. glucose loading: effect on basal Ca^{2+}-ATPase activity, serum glucose, and plasma insulin and glucagon levels. In five subjects receiving i.v. glucose, there was a trend toward reduction in basal red cell Ca^{2+}-ATPase activity (Figure 2) with a nadir (61% of fasting levels) achieved 20 min after glucose. Only at this point does the difference in basal enzyme activity approach significance ($t = 2.00$, $P < 0.06$, by multivariate analysis of repeated measures). Because of the range of preglucose enzyme activity values in our sub-

TABLE 4
Lack of variability with time in human erythrocyte Ca^{2+}-ATPase and in vitro response to thyroid hormone and purified calmodulin

		Ca^{2+}-ATPase activity* (μmol P_i/mg/90 min)		
		ΔCa^{2+}-ATPase-T_4†		
Min	Basal	Without CaM	With CaM	ΔCa^{2+}-ATPase-CaM
0	0.474 ± 0.060	+0.075 ± 0.007 (<0.01)‡	+0.079 ± 0.004 (<0.01)	+0.094 ± 0.016 (<0.02)
8	0.490 ± 0.052	+0.066 ± 0.010	+0.090 ± 0.012	+0.069 ± 0.010
20	0.483 ± 0.050	+0.080 ± 0.008	+0.087 ± 0.016	+0.082 ± 0.016
40	0.490 ± 0.059	+0.088 ± 0.004	+0.080 ± 0.010	+0.073 ± 0.009
60	0.487 ± 0.060	+0.071 ± 0.011	+0.085 ± 0.014	+0.066 ± 0.010

*Results represent mean ± SEM from studies in three fasting subjects; membrane enzyme activity was assayed in duplicate on 2 days after membrane preparation.
†ΔCa^{2+}-ATPase-T_4 and ΔCa^{2+}-ATPase-CaM indicate change in enzyme activity with in vitro addition of T_4 (10^{-10} M) and calmodulin (CaM, 100 ng/mg membrane protein).
‡P-values (parentheses) calculated by paired t-test showed significance of T_4 and CaM effect at time zero; there was no significant change in any values over the 1-h period.

TABLE 5
Effect of i.v. glucose administration on stimulation by T_4 and calmodulin of human red cell membrane Ca^{2+}-ATPase activity

		Ca^{2+}-ATPase activity* (μmol P_i/mg/90 min)		
		ΔCa^{2+}-ATPase-T_4†		
Min	Basal	Without CaM	With CaM	ΔCa^{2+}-ATPase-CaM
0	0.679 ± 0.120	+0.086 ± 0.012 (<0.01)‡	+0.090 ± 0.014 (<0.01)	+0.088 ± 0.027 (<0.05)
8	0.496 ± 0.064 (<0.18)	+0.023 ± 0.024 (<0.015)	+0.028 ± 0.016 (<0.007)	+0.111 ± 0.037 (<0.57)
20	0.417 ± 0.072 (<0.06)	−0.017 ± 0.013 (<0.001)	−0.020 ± 0.011 (<0.001)	+0.078 ± 0.016 (<0.82)
40	0.465 ± 0.091 (<0.12)	−0.006 ± 0.017 (<0.001)	+0.021 ± 0.008 (<0.003)	+0.115 ± 0.039 (<0.52)
60	0.604 ± 0.105 (<0.58)	+0.081 ± 0.014 (<0.86)	+0.083 ± 0.021 (<0.74)	+0.088 ± 0.015 (<0.99)

*Results are mean ± SEM from studies in five subjects, with membrane enzyme activity assayed in duplicate on 2 days after membrane preparation.
†ΔCa^{2+}-ATPase-T_4 and ΔCa^{2+}-ATPase-CaM indicate change in Ca^{2+}-ATPase activity with in vitro addition of T_4 (10^{-10} M) and calmodulin (CaM, 100 ng/mg membrane protein).
‡P-values (parentheses) for preglucose T_4 and CaM effects were calculated by paired *t*-test; P-values for change in basal activity, ΔCa^{2+}-ATPase-T_4, and ΔCa^{2+}-ATPase-CaM after glucose were calculated by multivariate repeated measures analysis of variance.

jects (0.344 to 1.084 μmol P_i), we compared time zero and 20 min values by paired *t*-test; the difference was significant at $P < 0.05$ ($t = 3.253$). Peak serum glucose (220 mg/dl) and plasma insulin (53.0 μU/ml) levels were reached at 8 min (Figure 2), and plasma immunoreactive glucagon levels showed a slight decrease 20 min after glucose injection.

Three control subjects did not receive glucose but had blood samples drawn at the same time intervals used in the i.v. glucose tests. Basal Ca^{2+}-ATPase activity did not change significantly over 1 h in the control subjects, as determined by paired *t*-test (Table 4).

The effect of i.v. glucose in vivo on thyroid hormone and calmodulin stimulation in vitro of red cell Ca^{2+}-ATPase activity. In vitro stimulation of Ca^{2+}-ATPase activity by T_4 was reduced by the i.v. administration of glucose in vivo, as determined by a significant ΔCa^{2+}-ATPase-T_4 by time interaction ($F[3,18] = 20.18$, $P < 0.0003$) followed by comparisons of degree of T_4 stimulation at time zero with the other time points. T_4 stimulation was depressed at 8, 20, and 40 min after glucose administration ($t = 2.67$, $P < 0.15$; $t = 4.40$, $P < 0.0001$; and $t = 3.93$, $P < 0.0001$, respectively) (Table 5). Sixty minutes after glucose administration, stimulation of the enzyme by T_4 was not different from that obtained at zero time. Responsiveness of the enzyme in vitro to calmodulin did not fall significantly during 60 min after i.v. glucose and addition of calmodulin to membranes did not enhance or restore T_4 stimulation at any time during the i.v. test (Table 5). Membranes from three control subjects who did not receive glucose showed no significant change during the hour in amount of enzyme stimulation by T_4, calmodulin, or both, as determined by *t*-test (Table 4).

The effect of in vitro glucose, insulin, and tunicamycin on basal and thyroid hormone-stimulated Ca^{2+}-ATPase activity. In vitro addition of glucose, 100–200 mg/dl, to red cell membrane preincubation and assay resulted in a significant 26% reduction in basal enzyme activity at a glucose concentration of 200 mg/dl ($P < 0.001$, Table 6). Stimulation by thyroid hormone of Ca^{2+}-ATPase activity was significantly diminished at 150 mg/dl of glucose, and negligible at 200 mg/dl of glucose. As shown in Table 7, insulin was also capable of reducing basal and T_4-stimulated enzyme activity. At a concentration of 100 μU/ml, insulin in vitro caused a 17% fall in basal Ca^{2+}-ATPase activity ($P < 0.05$). There was no effect of insulin at 50 μU/ml on basal enzyme activity. In contrast, loss of T_4 stimulability was seen with insulin concentration as low as 50 μU/ml ($P < 0.01$).

Table 8 shows the effect of tunicamycin on glucose-induced changes in Ca^{2+}-ATPase activity (glucose 200 mg/dl). While tunicamycin itself caused no significant changes in Ca^{2+}-ATPase activity, in a concentration of 0.001 ng/ml it reduced the effect of glucose on basal activity and ΔCa^{2+}-ATPase-T_4; at a tunicamycin concentration of 0.01 ng/ml the glucose effect was eliminated. The same tunicamycin concentrations had no effect on insulin-induced decreases in basal and hormone-stimulated Ca^{2+}-ATPase activity (results not shown).

TABLE 6
Effect of in vitro glucose on basal and T_4-stimulated human red cell membrane Ca^{2+}-ATPase activity

	Ca^{2+}-ATPase activity* (μmol P_i/mg/90 min)	
Glucose (mg/dl)	Basal	ΔCa^{2+}-ATPase-T_4†
0	0.451 ± 0.054	0.096 ± 0.008
100	0.426 ± 0.045	0.082 ± 0.004
150	0.426 ± 0.051	0.060 ± 0.007 (<0.02)‡
200	0.335 ± 0.052 (<0.001)	0.027 ± 0.005 (<0.001)

*Results are mean ± SEM of duplicate determinations in four assays.
†ΔCa^{2+}-ATPase-T_4 indicates change in enzyme activity with addition of T_4 (10^{-10} M).
‡P-values (parentheses) show significance of difference in basal enzyme activity and ΔCa^{2+}-ATPase-T_4 with and without glucose, as measured by paired *t*-test.

TABLE 7
Effect of in vitro insulin on basal and T_4-stimulated human red cell membrane Ca^{2+}-ATPase activity

Insulin (μU/ml)	Ca^{2+}-ATPase activity* (μmol P_i/mg/90 min) Basal	ΔCa^{2+}-ATPase-T_4†
0	0.318 ± 0.033	0.096 ± 0.012
25	0.321 ± 0.036	0.072 ± 0.002
50	0.301 ± 0.031	0.072 ± 0.010 (<0.01)‡
100	0.263 ± 0.034 (<0.05)	0.030 ± 0.002 (<0.01)

*Results are mean ± SEM of duplicate determinations in four assays, in which indicated amounts of insulin were added to membranes before enzyme assay.
†ΔCa^{2+}-ATPase-T_4 indicates the change in enzyme activity with addition of T_4 (10^{-10} M).
‡P-values (parentheses) show significance of change in basal enzyme activity and ΔCa^{2+}-ATPase-T_4 with addition of insulin, as measured by paired *t*-test.

DISCUSSION

We have demonstrated significant depression of human red blood cell membrane Ca^{2+}-ATPase activity after oral and i.v. glucose loading in man. This effect is reversible and recovery from the acute decrease in activity of this calcium pump-associated enzyme occurs within 60 min after glucose administration. Thus, glucose administration is to be included among the extracellular factors—such as thyroid hormone[6,7] and certain pharmacologic agents[22]—that regulate red cell Ca^{2+}-ATPase. Whether glucose administration affects Ca^{2+}-ATPase in cells other than the erythrocyte awaits further study, although the red cell enzyme is thought to be biochemically representative of Ca^{2+}-ATPases in several other tissues[23] and our own preliminary studies of rabbit myocardial membrane Ca^{2+}-ATPase indicate that enzyme activity is decreased by glucose in physiologic concentrations in vitro (Davis, F. B., Davis, P. J., and Blas, S. D.: unpublished observations).

The temporal relationship between the reduction in red cell Ca^{2+}-ATPase activity and the rise in serum glucose concentration suggested that a rapidly reversible interaction of membrane and glucose may underlie the observed decrease in enzyme activity. Physicochemical properties of the red cell membrane are changed by hyperglycemia in both in vitro[24] and in vivo[25] settings, resulting, it is thought, in disorganization of the polypeptide component of the membrane. The time required to achieve such changes, however, and to obtain their reversal is many hours.[26] Glucose binding to the membranes was demonstrated in such studies by Campagnucci et al.[26] Our data indicate that glucose-induced alteration in red cell Ca^{2+}-ATPase activity in man occurs minutes after in vivo glucose administration. Our in vitro studies show that incubation of human red cell membranes with glucose (200 mg/dl) results in significant decreases in both basal enzyme activity and degree of thyroid hormone stimulation. In the presence of tunicamycin, an antibiotic that inhibits asparagine-linked N-glycosylation of proteins,[27] the glucose effect is blocked. This suggests that glycosylation of membrane proteins, perhaps of the Ca^{2+}-ATPase itself, accounts for the observations we have made in the present in vivo studies.

Two aspects of membrane Ca^{2+}-ATPase activity were studied in the present glucose-loading protocol: endogenous (basal) enzyme activity and enzyme activity inducible in vitro by exposure of red cell membranes to physiologic concentrations of thyroid hormone.[3,6] Glucose administration suppressed enzyme activity attributable to the presence of thyroid hormone, as well as endogenous activity. Both activities are dependent on the presence of calmodulin (or the calmodulin·Ca^{2+} complex), an endogenous stimulator of Ca^{2+}-ATPase.[1,2] The addition of purified calmodulin in vitro to membranes obtained from glucose-loaded subjects partially reversed the glucose-induced decrease in enzyme activity. This raises the possibility that changes in the red cell membrane associated with glucose administration result in loss of endogenous calmodulin bound to membranes. In previous studies carried out with antibody to calmodulin, we have shown that membrane-bound calmodulin contributes to "basal" levels of Ca^{2+}-ATPase activity.[6]

We also considered the possibility that metabolic responses to glucose administration, rather than change in blood glucose concentration itself, resulted in decreased red cell Ca^{2+}-ATPase activity in the present experiments. Insulin has been shown in the adipocyte to inhibit membrane Ca^{2+}-ATPase,[28] paradoxically in association with increased binding of calmodulin to the cell membrane.[29] In the present in vivo studies, the changes in blood glucose and insulin levels were predictably closely related. However, insulin is thought to have no action on the human red cell, despite the presence of insulin-binding sites on the cell membrane.[30] We have shown in in vitro studies that insulin concentrations up to 50

TABLE 8
Effect of tunicamycin on inhibition of basal and T_4-stimulated Ca^{2+}-ATPase activity by glucose

Tunicamycin (ng/ml)	Ca^{2+}-ATPase activity* (μmol P_i/mg/90 min) No glucose Basal	No glucose ΔCa^{2+}-ATPase-T_4†	Glucose (200 mg/dl) Basal	Glucose (200 mg/dl) ΔCa^{2+}-ATPase-T_4
0	0.458 ± 0.047	0.048 ± 0.007	0.355 ± 0.046 (<0.001)‡	−0.016 ± 0.002 (<0.001)
0.001	0.460 ± 0.051	0.082 ± 0.018	0.443 ± 0.045	0.035 ± 0.006
0.01	0.440 ± 0.030	0.077 ± 0.012	0.464 ± 0.044	0.107 ± 0.028

*Results show mean ± SEM of duplicate determinations in three assays.
†ΔCa^{2+}-ATPase-T_4 indicates the extent to which enzyme activity is altered with addition of T_4 (10^{-10} M).
‡P-values (parentheses) show significance (paired *t*-test) of glucose-induced inhibition of basal enzyme activity and ΔCa^{2+}-ATPase-T_4.

μU/ml—levels achieved in the i.v. glucose-loading studies—have no effect on basal activity of human erythrocyte Ca^{2+}-ATPase. In the presence of 100 μU/ml insulin in vitro, there was reduction of both basal and hormone-stimulated enzyme activity, an effect that is not reversed by tunicamycin. This effect of insulin on basal Ca^{2+}-ATPase activity is similar to that reported by Pershadsingh and McDonald in the adipocyte;[28] whether it contributes to our in vivo findings is not clear.

Free fatty acids are known to stimulate Ca^{2+}-ATPase in vitro.[31] Whether small decreases in serum free fatty acids (such as those observed in our subjects during glucose loading) may lead to a fall in red cell Ca^{2+}-ATPase activity is unknown but seems unlikely, as free fatty acid levels steadily decreased during the 2-h oral glucose challenge, while basal Ca^{2+}-ATPase activity returned toward fasting levels at 60 min.

The metabolic consequences and role of glucose-associated reductions in human red cell Ca^{2+}-ATPase activity are speculative, but it seems likely that intracellular Ca^{2+} levels are transiently raised by glucose. In a cell system in which access of Ca^{2+} to the intracellular space is usually severely restricted (by membrane impermeability to the cation and by the efficiency of the calcium pump) transient inhibition of Ca^{2+}-ATPase offers a mechanism for pulsing Ca^{2+} into the cell. Among the enzymes in the red cell (and other tissues) that are influenced by Ca^{2+} and by calmodulin are phosphodiesterases and glucose-1,6-bisphosphatase,[32] so that a teleologic argument may be advanced for the existence of glucose-related inhibition of the calcium pump. In the β-cell, of course, there is evidence that glucose-induced increases in intracellular Ca^{2+} lead to insulin release,[33] but the mechanism is not generally thought to involve Ca^{2+}-ATPase.

The current studies emphasize the importance of conducting clinical studies of red cell Ca^{2+}-ATPase activity in fasting subjects. Failure to have done so may have influenced conclusions reached about the activity of this enzyme in several disease states, including sickle cell disease,[9] cystic fibrosis,[10] and muscular dystrophy.[34] Our own studies of red cell membrane calcium transport in patients with end-stage renal disease were carried out with subjects in the fasting state.[8] Other factors, such as diet and stress, may also contribute to the inter- and intrasubject variability in Ca^{2+}-ATPase activity that we have observed.

In addition to Ca^{2+}-ATPase, the sodium pump-associated enzyme, Na,K-ATPase, may also be influenced acutely by dietary intake. The effect of a mixed meal on human erythrocyte Na,K-ATPase was found by Mir et al. to be a substantive increase in enzyme activity after 60 min.[11] DeLuise et al. found, in contrast, no change in erythrocyte ouabain binding or potassium influx in subjects studied 60–180 min after a meal.[12] Neither group administered glucose alone, and the composition of the test meal in each study differed materially, particularly in terms of fat content. These groups did not study enzyme activity at time periods <60 min after food ingestion. In our studies, glucose administration led to a small, statistically significant increase in Na,K-ATPase activity within 30 min; enzyme activity normalized by 60 min. These various results underscore the hazards of clinical studies of enzymes that are rapidly and transiently altered in activity. The timing of sampling, as well as dietary composition, may importantly affect conclusions reached with regard to "physiologic" changes in ATPases.

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