Insulin stimulation of intracellular free Ca^{2+} recovery and Ca^{2+} -ATPase gene expression in cultured vascular smooth-muscle cells: role of glucose 6-phosphate

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We have previously reported that insulin accelerates recovery of intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) from pressor agonist-induced Ca^{2+} loads and stimulates both plasmalemmal and sarcoplasmic-reticulum Ca^{2+} -ATPase gene expression in cultured and freshly isolated vascular smooth-muscle cells (VSMCs), suggesting that insulin attenuation of vascular tone may result from modulation of $[\text{Ca}^{2+}]_i$. Accordingly, we have now evaluated the linkage between this insulin-regulation of VSMC $[\text{Ca}^{2+}]_i$ and classical actions of insulin (i.e. glucose transport and metabolism). Cultured VSMCs were incubated in the presence or absence of insulin in a medium containing either pyruvate, glucose, 3-O-methylglucose or 2-deoxyglucose. Insulin caused an

87% increase in $[\text{Ca}^{2+}]_i$ recovery rate after stimulation with arginine-vasopressin ($P < 0.01$) and caused a marked increase in Ca^{2+} -ATPase mRNA and protein levels in the presence of glucose. Comparable increases in both $[\text{Ca}^{2+}]_i$ recovery and Ca^{2+} -ATPase expression were found when glucose was replaced by 2-deoxyglucose. In contrast, no stimulation was found in either the glucose-free or 3-O-methylglucose-containing medium. As both glucose analogues are transported, but only 2-deoxyglucose is phosphorylated, this indicates that glucose transport and metabolism to glucose 6-phosphate is essential for insulin regulation of VSMC $[\text{Ca}^{2+}]_i$, possibly via a glucose-6-phosphate-dependent carbohydrate-response element in the Ca^{2+} -ATPase gene.

INTRODUCTION

Insulin resistance and compensatory hyperinsulinaemia have been implicated in the pathogenesis of hypertension, and there is a significant association between hypertension and insulin resistance in both obese and non-obese individuals [1,2]. Further, both insulin resistance and hyperinsulinaemia are associated with hypertension even in the absence of obesity or overt glucose intolerance [2,3], indicating a direct relationship between insulin resistance and hypertension. However, the underlying cellular link to hypertension remains unclear.

Although hyperinsulinaemia may contribute to hypertension via stimulation of sympathetic neural output [4] and renal sodium retention [5], recent evidence from our laboratory and others strongly suggests that insulin exerts direct vasodilatory effects which are impaired in insulin-resistant states. We have shown that insulin attenuates vascular reactivity responses to pressor agonists *in vitro* [6,7], accelerates vascular smooth-muscle relaxation and Ca^{2+} -ATPase-mediated Ca^{2+} efflux [8] and inhibits responses of vascular smooth-muscle cell (VSMC) intracellular free Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) to arginine-vasopressin (AVP) [9]. Further, we have recently found insulin to accelerate $[\text{Ca}^{2+}]_i$ recovery from agonist-induced Ca^{2+} loads in both cultured rat VSMC and human pulmonary artery VSMC [10] and to stimulate gene expression for both plasmalemmal and sarcoplasmic-reticulum Ca^{2+} -ATPases [11]. Moreover, all these insulin effects were blunted in VSMCs freshly isolated from an animal model of insulin resistance and hypertension, the Zucker obese rat, and from insulinopenic rats [7,12,13]. Insulin has also been shown to exert vasodilatory effects on VSMC from canine femoral artery [14] and rabbit efferent arterioles [15].

Accordingly, we have proposed that insulin-induced modulation of Ca^{2+} transport results in vascular relaxation and reduced

vascular tone. Further, blunting of this modulation may be responsible for hypertension in insulin-resistant states. However, it is unclear whether these effects of insulin are related to or dependent on the classical actions of insulin, such as glucose transport. Therefore, to investigate further whether insulin regulation of VSMC $[\text{Ca}^{2+}]_i$ and attenuation of vasoconstriction are linked to the insulin regulation of glucose metabolism, we studied the effects of glucose transport and metabolism on insulin regulation of VSMC $[\text{Ca}^{2+}]_i$ responses to a pressor agonist (AVP) and of Ca^{2+} -ATPase gene expression. We report here that insulin regulation of VSMC $[\text{Ca}^{2+}]_i$ and attenuation of vasoconstriction appear to depend on insulin-induced glucose transport and phosphorylation to glucose 6-phosphate.

EXPERIMENTAL

Materials

Cells from the A7r5 VSMC line were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and fetal calf serum (FCS) were obtained from Gibco-BRL (Gaithersburg, MD, U.S.A.). Insulin, mannitol, pyruvate, 2-deoxy-D-glucose, 3-O-methyl-D-glucose, the fluorescent dye fura 2 acetoxymethyl ester (fura 2/AM) and cytochalasin B were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Insulin and digitonin were prepared in 10 mM acetic acid and distilled water respectively. AVP was dissolved in serum-free Hepes-buffered salt solution (HBSS) containing 138 mM NaCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , 0.9 mM NaH_2PO_4 , 4.0 mM NaHCO_3 , 23 mM D-glucose, 6.0 mM glutamine, 20 mM Hepes and 5% BSA, pH 7.4.

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; VSMC, vascular smooth-muscle cell; AVP, arginine-vasopressin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FCS, fetal calf serum; HBSS, Hepes buffered salt solution.

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Glucose-depleted HBSS was prepared with either glucose or pyruvate as energy source and mannitol for osmotic adjustment with or without 3-*O*-methylglucose or 2-deoxyglucose, as detailed below. Guanidine isothiocyanate and CsCl_2 were purchased from Gibco-BRL.

Cell culture

A7r5 VSMCs were maintained under 95% O_2 /5% CO_2 and 90–95% humidity at 37 °C in growth medium (DMEM supplemented with 5% FBS, 5% FCS, 5000 units/ml penicillin G sodium and 5000 $\mu\text{g}/\text{ml}$ streptomycin sulphate). On reaching confluence, cells were incubated for 24 h in a 0.2% FBS medium and antibiotics as above. Quiescent cell monolayers were then rinsed with Ca^{2+} / Mg^{2+} -free Hanks balanced-salt solution (Sigma Chemicals) and released by treatment with 0.05% trypsin/0.53 mM Na_2EDTA . For fluorimetric Ca^{2+} measurement, the released cells were pelleted by centrifugation at 200 g for 5 min and the pellet was resuspended in glucose-depleted HBSS with substitutions as follows: (1) 4 mM pyruvate and 17 mM mannitol; (2) 4 mM pyruvate and 17 mM 3-*O*-methylglucose; (3) 4 mM pyruvate, 16 mM mannitol and 1 mM 2-deoxyglucose; (4) 4 mM pyruvate and 17 mM 2-deoxyglucose; (5) 25 mM D-glucose; (6) 25 mM D-glucose and 25 μM cytochalasin B; (7) 25 mM fructose.

Measurement of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ responses to and rates of recovery from AVP were assessed spectrofluorimetrically as previously described [9,10,12]. Cells resuspended in each glucose-depleted HBSS were prechilled for at least 10 min before fura 2/AM loading. Fura-2-loaded cells were incubated in the dark for a further 60 min in the presence or absence of 0.1 μM insulin in a 37 °C shaking water bath. After 1 h incubation, cells were centrifuged and resuspended in the indicated buffer to remove extracellular fura 2 dye before determination of $[\text{Ca}^{2+}]_i$ with a dual-excitation (340 and 380 nm) single-emission (510 nm) spectrofluorimeter (Hitachi F-2000; Naperville, IL, U.S.A.). The intracellular Ca^{2+} signal was calibrated using a maximum (F_{max}) and minimum (F_{min}) fluorescence ratio signal obtained by 40 μM digitonin and 100 mM Tris/100 mM EGTA, pH 8.7, respectively. $[\text{Ca}^{2+}]_i$ was then calculated using $K_d = 224$ nM [16].

After a stable baseline had been established, $[\text{Ca}^{2+}]_i$ response to and rates of recovery from AVP treatment were evaluated. The rate of $[\text{Ca}^{2+}]_i$ recovery was determined for 10 s from the peak by AVP stimulation, as previously described [10].

Preparation of total cellular RNA

To evaluate the expression of Ca^{2+} -ATPase, quiescent VSMC monolayers (250 cm^2 flask per determination) were incubated in the buffers indicated above in the presence or absence of 0.1 μM insulin for 4 h, and the cells were then immediately harvested by treatment with guanidine thiocyanate. The released cells were briefly sonicated and centrifuged at 140 000 g for 18 h (Sorvall Ultrasppeed Centrifuge; DuPont). Total cellular RNA was isolated from cells (using a CsCl_2 density gradient) by the guanidine thiocyanate/ CsCl_2 procedure [17]. RNA concentration was measured spectrophotometrically (A_{260}/A_{280}).

Northern-blot analysis

Total RNA (20 μg) was denatured with 6.5% formaldehyde/0.05% formamide and its integrity assessed by running on a 1%

(w/v) agarose gel containing formaldehyde (0.66 M) in a 20 mM Mops/5 mM sodium acetate/1 mM EDTA, pH 7.0, buffer at 75 V. The gels were rinsed, visualized under UV light and photographed. The separated RNA was then transferred to nylon filters (Genescreen Plus; DuPont) by Northern blot for approx. 16 h.

Hybridization and analysis

The filters were prehybridized in prehybridization solution (Gibco-BRL) containing 6 $\times$ SSC (where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate), 5 $\times$ Denhardt's solution [where 1 $\times$ Denhardt's solution is 0.02% (w/v) Ficoll 400/0.02% polyvinylpyrrolidone/0.02% (w/v) BSA], 0.5% (w/v) SDS and 100 $\mu\text{g}/\text{ml}$ sheared denatured salmon sperm DNA for 3 h at 42 °C, followed by hybridization for 20 h in hybridization solution (Gibco-BRL) containing 6 $\times$ SSC, 0.01 M EDTA (pH 8.0), 5 $\times$ Denhardt's solution, 0.5% (w/v) SDS and 100 $\mu\text{g}/\text{ml}$ salmon sperm with ^{32}P -labelled oligonucleotide probes (described below), and washed twice with 2 $\times$ SSC, and then with 0.1 $\times$ SSC/0.1% SDS at 65 °C. Autoradiography was then performed with Kodak XAR-5 film and intensifying screen at –80 °C. The resulting autoradiographs were quantified densitometrically.

Labelling of cDNA probes of Ca^{2+} -ATPases

The probes used were a 40-base, a 45-base and a 40-base oligonucleotide derived from human plasma-membrane Ca^{2+} -ATPase (Oncogene Science), rat sarcoplasmic-reticulum Ca^{2+} -ATPase and β -actin (DuPont-NEN, Wilmington, DE, U.S.A.) respectively. Hybridization probes were labelled with [α - ^{32}P]dATP using an oligonucleotide 3'-end-labelling kit (DuPont-NEN) and were used for hybridization as described above.

Western-blot analysis

To evaluate the effects of insulin on VSMC Ca^{2+} -ATPase protein content, quiescent cell monolayers were incubated in the presence or absence of 0.1 μM insulin for 4 h, as indicated above. Protein was extracted by solubilizing it in a 4% (w/v) SDS/200 mM dithiothreitol/100 mM Tris buffer, heating for 5 min at 100 °C, and sonicating. Protein was then loaded and electrophoresed through an SDS/10% polyacrylamide gel. Proteins were transferred to poly(vinylidene difluoride) membranes by electroblot for 1 h using transfer buffer containing 48 mM Tris, 39 mM glycine, 20% methanol and 0.0375% SDS. Blots were then incubated in blocking solution containing a 1:1000 dilution of the mouse monoclonal antibody to the plasmalemmal Ca^{2+} -ATPase (Affinity Bioreagents, Neshanic Station, NJ, U.S.A.) in ascites fluid. After two washes in Tris-buffered saline containing Tween 20 (TBST), the blots were incubated in 5 μCi of ^{125}I -labelled goat-anti-mouse IgG (ICN; Irvine, CA, U.S.A.). The blots were then washed three times with TBST, dried briefly, exposed to film (DuPont-NEN) and the resulting autoradiographs were quantified by laser densitometry.

RESULTS

Role of insulin and glucose in VSMC $[\text{Ca}^{2+}]_i$ recovery

In the presence of glucose, insulin significantly accelerated the rate of $[\text{Ca}^{2+}]_i$ recovery after stimulation with AVP (Figure 1 and Table 1), as we have previously reported [10,12]. However, when A7r5 VSMCs were incubated in the absence of glucose, but in the

Table 1 Effects of insulin ($0.1 \mu\text{M}$) on the rate of AVP-elicited $[\text{Ca}^{2+}]_i$ recovery in fura-2-loaded A7r5 VSMCs

All data are expressed as means $\pm$ S.E.M. Rate of recovery is the rate of return to baseline after AVP stimulation. Mannitol and pyruvate were added to all glucose-free preparations for osmotic control and to provide energy respectively. * $P < 0.005$, ** $P < 0.02$ and *** $P < 0.01$, compared with control.

Treatment	Rate of recovery (nM/min)		Insulin stimulation (%)
	Control	+ Insulin	
Glucose-free	21.98 ± 5.79	18.62 ± 2.97	-15
17 mM	6.94 ± 1.89	7.35 ± 1.50	4
3-O-Methylglucose			
1 mM	5.67 ± 0.80	$9.57 \pm 1.45^*$	57*
2-Deoxyglucose			
17 mM	5.67 ± 0.90	$8.89 \pm 1.47^{**}$	69**
2-Deoxyglucose			
25 mM Glucose	5.83 ± 0.90	$10.91 \pm 1.8^{***}$	87***

presence of 4 mM pyruvate and 17 mM mannitol to provide oxidative substrate and osmotic control respectively, insulin did not significantly affect peak $[\text{Ca}^{2+}]_i$, the rate of recovery to baseline or the area under the $[\text{Ca}^{2+}]_i$ response curve to AVP (Figure 1 and Table 1).

Similarly, insulin was without effect when glucose transport was inhibited with cytochalasin B or when the non-metabolizable glucose analogue 3-O-methylglucose, which is transported into the cell but not phosphorylated, was substituted for glucose. In contrast, insulin markedly accelerated the rate of $[\text{Ca}^{2+}]_i$ recovery to basal levels after AVP stimulation in the presence of either 1

Figure 2 Representative autoradiograph of plasma-membrane Ca^{2+} -ATPase mRNA (a) and sarcoplasmic-reticulum Ca^{2+} -ATPase mRNA (b) in A7r5 VSMCs

2-DG, 2-deoxyglucose; 3-O-MG, 3-O-methylglucose; Mtl, mannitol; Cyto, cytochalasin B. A representative autoradiograph from eight experiments is shown.

or 17 mM 2-deoxyglucose (Table 1), which is phosphorylated by hexokinase to 2-deoxyglucose 6-phosphate but not further metabolized.

Figure 1 Representative fluorimetric $[\text{Ca}^{2+}]_i$ responses to 100 nM AVP in cultured A7r5 VSMCs in the presence of (a) glucose, (b) glucose and insulin, (c) 2-deoxyglucose and insulin, (d) 3-O-methylglucose and insulin, (e) mannitol, pyruvate and insulin and (f) cytochalasin B and insulin

Representative traces from eight experiments are shown.

Table 2 Effects of insulin on plasmalemmal Ca^{2+} -ATPase, sarcoplasmic-reticulum Ca^{2+} -ATPase and β -actin mRNA in A7r5 VSMCs

All data are expressed as means $\pm$ S.E.M. in arbitrary densitometric units. Mannitol and pyruvate were added to all glucose-free preparations for osmotic control and to generate energy respectively. Cytochalasin B was added in the presence of 25 mM glucose. * $P < 0.04$, ** $P < 0.005$ compared with control.

Treatment	Plasmalemma Ca^{2+} -ATPase mRNA		Sarcoplasmic reticulum Ca^{2+} -ATPase mRNA		β -Actin mRNA	
	Control	Insulin	Control	Insulin	Control	Insulin
25 mM Glucose	2.364 $\pm$ 0.29	5.733 $\pm$ 0.50*	2.368 $\pm$ 0.226	5.793 $\pm$ 0.51*	7.33 $\pm$ 1.77	7.69 $\pm$ 1.92
17 mM 2-Deoxyglucose	0.420 $\pm$ 0.20	2.946 $\pm$ 1.03**	0.568 $\pm$ 0.27	3.746 $\pm$ 0.93**	7.72 $\pm$ 1.72	8.27 $\pm$ 1.78
17 mM 3-O-Methylglucose	2.293 $\pm$ 0.93	2.455 $\pm$ 1.14	2.545 $\pm$ 0.90	2.376 $\pm$ 0.86	7.21 $\pm$ 2.05	7.16 $\pm$ 2.04
Glucose-free	1.867 $\pm$ 0.71	1.878 $\pm$ 0.87	2.178 $\pm$ 0.83	1.732 $\pm$ 0.67	6.45 $\pm$ 1.13	6.60 $\pm$ 0.97
25 mM Fructose	4.296 $\pm$ 0.51	3.918 $\pm$ 0.58	4.680 $\pm$ 0.24	4.391 $\pm$ 0.26	8.05 $\pm$ 1.12	7.12 $\pm$ 0.23
Cytochalasin B	3.989 $\pm$ 0.71	3.140 $\pm$ 0.41	3.892 $\pm$ 0.50	3.319 $\pm$ 0.46	6.86 $\pm$ 0.88	6.27 $\pm$ 1.08

Effects of carbohydrates on insulin stimulation of Ca^{2+} -ATPase pump

We have previously demonstrated that insulin stimulates both plasmalemmal and sarcoplasmic-reticulum Ca^{2+} -ATPase mRNA levels in cultured VSMCs [11]. This effect is evident within 1 h of incubation and plateaus after approx. 4 h (11). Moreover, this increase in mRNA resulted in acceleration of cytosolic Ca^{2+} removal after VSMC Ca^{2+} loads [10,12]. In the present study, incubation of cultured VSMCs for 4 h with 0.1 μM insulin significantly ($P < 0.05$) increased both plasmalemmal and sarcoplasmic-reticulum Ca^{2+} -ATPase expression in the presence of either glucose or 2-deoxyglucose (glucose, 243 and 244 % stimulation of plasmalemmal and sarcoplasmic-reticulum Ca^{2+} -ATPase respectively; 2-deoxyglucose, 702 and 660 % stimulation respectively) (Figure 2 and Table 2). However, when cells were incubated with 3-O-methylglucose substituted for glucose, insulin was without effect on the expression of both Ca^{2+} pumps (Figure 2 and Table 2). Furthermore, both incubation with 25 mM fructose, which is independent of insulin, and inhibition of glucose transport with cytochalasin B resulted in both a marked diminution of Ca^{2+} -ATPase expression and a lack of insulin stimulation of expression. In order to verify that increased levels of Ca^{2+} -ATPase expression leads to increased levels of translated protein, we evaluated the effects of insulin on VSMC Ca^{2+} -ATPase protein. Exposure to insulin for 1 h resulted in a 73 % increase ($P < 0.01$) in Ca^{2+} -ATPase protein, and a 4 h exposure resulted in a 206 % increase ($P < 0.01$).

DISCUSSION

Data from the present study demonstrate that VSMC Ca^{2+} transport is dependent on glucose transport and metabolism. Our data demonstrate that this regulation occurs in the presence of glucose and a glucose analogue that undergoes phosphorylation (2-deoxyglucose). Accordingly, it appears that insulin regulation of Ca^{2+} metabolism is linked to 'classical' actions of insulin. Indeed, although VSMCs are not generally thought to be responsive to insulin, we have recently found insulin regulation of glucose transport in VSMCs and that this regulation is impaired in insulin-resistant states (W. J. Banz, M. A. Maher and M. B. Zemel, unpublished work).

Consequently, it appears likely that VSMC manifestations of resistance to classical actions of insulin may be responsible for hypertension in insulin-resistant states, as these actions are essential for insulin regulation of Ca^{2+} transport. We previously demonstrated that insulin attenuates *in vitro* vasoconstrictor responses to pressor agonists [6,7], stimulates VSMC $^{45}\text{Ca}^{2+}$

efflux [8] and accelerates vascular relaxation after contraction of endothelium-denuded aortic strips [6,7]. We have also found that insulin accelerates the rate of $[\text{Ca}^{2+}]_i$ return to baseline after stimulation with either angiotensin II or AVP and increases the expression of plasmalemmal Ca^{2+} -ATPase [16]. Moreover, these effects are blunted in insulin-resistant states [6,13]. Thus it appears that insulin regulation of Ca^{2+} transport contributes to vascular relaxation after agonist stimulation and that impairment of this process may lead to hypertension. Alternatively, however, a recent report [18] indicates that insulin-mediated vasodilation may also result, in part, from stimulation of nitric oxide release.

Our present observations suggest that glucose-phosphate regulates Ca^{2+} -ATPase expression, possibly at the transcriptional level via a carbohydrate-response element. Several lines of evidence have shown that glucose increases the mRNA concentrations and the transcription of glycolytic and lipogenic enzymes such as aldolase B [19], spot S14 [20] and L-type pyruvate kinase [21]. As glucose induces mRNAs of enzymes catalysing intermediate and distal reactions in the glycolytic and lipogenic pathway, and the regulation of glucokinase transcription by insulin is independent of glucose [22,23], it can be speculated that insulin may have only a permissive role by allowing glucose phosphorylation to glucose 6-phosphate. Indeed, recent evidence [24] demonstrated that a low concentration (1 mM) of 2-deoxyglucose very efficiently stimulated the expression of lipogenic enzymes (fatty acid synthase and acetyl-CoA carboxylase) in cultured adipocytes. Moreover, there was a clear correlation between intracellular glucose 6-phosphate concentration and expression of lipogenic enzymes.

In contrast, 3-O-methylglucose was unable to induce transcription of genes for glycolytic or lipogenic enzymes. It seems therefore that activation of glycolytic and lipogenic genes by glucose requires accumulation of glucose 6-phosphate but not necessarily further entry into the glycolytic pathway. Similarly, the present study indicates that phosphorylation of glucose is necessary for insulin regulation of VSMC $[\text{Ca}^{2+}]_i$ and for specific activation of Ca^{2+} -ATPase gene transcription.

Although the mechanism by which carbohydrate induces gene transcription is not well understood, it appears to involve an intracellular signal resulting from increased glucose metabolism which can affect nuclear events involving *cis*-acting elements and *trans*-acting factors [25]. Several glucose-response elements responsible for the glucose-dependent regulation of glycolytic and lipogenic genes have been identified. The box L4 in a 183 bp minimal promoter fragment of the L-pyruvate kinase gene has been shown to function as a glucose/insulin-response element *ex vivo* by transient expression assays in hepatocytes in primary

culture [26–28] and *in vivo* in transgenic mice [29]. This element was also necessary for the inhibitory action of glucagon and cyclic AMP [26,27,30]. The imperfect E boxes CACGGG of the L-pyruvate kinase glucose-response elements were also indispensable for the glucose-response-element activity.

Further, in addition to the glucose-response elements in the regulatory regions of the L-pyruvate kinase, a similar CACGTG core consensus element has been observed in the regulatory region of the spot 14 gene [29] and in the first intron of the fatty acid synthase gene [25]. Accordingly, our data suggest that glucose 6-phosphate may act similarly either on a primary responsive gene, which mediates its effect on the Ca^{2+} -ATPase gene, or by directly activating Ca^{2+} -ATPase transcription. Such a carbohydrate-response element has not yet been identified in the Ca^{2+} -ATPase gene. However, a GenBank search of the 5'-flanking region of both human [31] and rat [32] plasmalemmal Ca^{2+} pumps demonstrates similar imperfect E-box motifs (e.g. CATGGG, CCCGGG, CAGGGG, CAAGTG, GACGTG and CGCGTG), suggesting that Ca^{2+} -ATPase may indeed contain such a glucose 6-phosphate-response element; work is currently in progress to identify such elements in the Ca^{2+} -ATPase gene.

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