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Effect of starvation, diabetes and insulin on the casein kinase 2 from rat liver cytosol

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Starvation, diabetes and insulin did not alter the concentration of casein kinases in rat liver cytosol. However, the K_m for casein of casein kinase 2 from diabetic rats was about 2-fold lower than that from control animals. Administration of insulin to control rats did not alter this parameter, but increased the K_m for casein of casein kinase 2 in diabetic rats. Starvation did not affect the kinetic constants of casein kinases. The effect of diabetes on casein kinase 2 persisted after partial purification of the enzyme by glycerol-density-gradient centrifugation and affected also its activity on other protein substrates such as phosvitin, high-mobility-group protein 14 and glycogen synthase. The results indicate that rat liver cytosol casein kinase 2 is under physiological control.

Previous studies in rooster liver (Goldstein & Hasty, 1973), hen oviduct (Keller *et al.*, 1976) and rabbit endometrium (Miyazaki *et al.*, 1980) have demonstrated that the casein kinase activity in these tissues was altered by steroid hormones. On the other hand, Rose *et al.* (1981) have shown that hepatoma cells contain more nuclear casein kinase than normal adult liver, and we have recently shown that cytosolic casein kinase activity is increased in leukaemia (Pena *et al.*, 1983). These data suggest that casein kinase activity in the cells is under physiological control.

Two types of casein kinases are present in mammalian tissues, including rat liver (Itarte *et al.*, 1981; Hathaway & Traugh, 1982). The possible involvement of these enzymes in the control of cellular events is suggested by the increasing number of enzymes and physiologically active proteins that serve as substrates for each type of casein kinase *in vitro* (Itarte *et al.*, 1977, 1981; Singh *et al.*, 1982; Carmichael *et al.*, 1982; Hemmings *et al.*, 1982).

It is well known that starvation and diabetes greatly impair metabolism in liver. Thus it was of interest to study if there was any variation in casein kinase activity associated with the above conditions. Here we describe the quantification of each type of casein kinase in normal rat liver cytosol and their response to starvation, diabetes and insulin administration.

Abbreviations used: HMG 14, high-mobility-group protein 14.

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Experimental

Materials

HMG 14 purified from calf thymus as described by Goodwin *et al.* (1977) was kindly donated by Mr. E. Espel from the Institut de Biologia Fonamental of this University. Purified casein kinase 2 was obtained from liver cytosol of control rats. The enzyme preparation eluted from the second phosphocellulose chromatography (Itarte *et al.*, 1981) was further purified as follows. A sample (1 ml) containing 60 units of casein kinase 2 was applied to a column (1.0 cm × 2.0 cm) of DEAE-Sepharose CL-6B equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-dithiothreitol, 0.1 mM-phenylmethanesulphonyl fluoride, leupeptin (60 μg/litre) and 5% (v/v) glycerol (buffer A). After washing the column with 20 ml of 0.1 M-KCl in buffer A, a linear gradient of 0.1–0.6 M-KCl was applied to elute the enzyme. Samples containing casein kinase activity were pooled, concentrated to 1 ml, dialysed against buffer A, and then applied to a column (0.5 cm × 3.0 cm) of heparin–agarose equilibrated with buffer A. The column was washed with 4 ml of buffer A plus 0.2 M-KCl and the enzyme was eluted with buffer A plus 0.2 M-KCl. Samples containing casein kinase

activity were pooled, concentrated, dialysed against buffer A and stored at −20°C until used. The source of all other materials was as previously indicated (Itarte *et al.*, 1981).

Preparation of samples

Male Sprague–Dawley rats, weighing 150–200g, were used in all experiments. Starved rats were deprived of food 48h before being killed. Diabetes was induced by intravenous injection of 70mg of streptozotocin/kg and the rats were fed normally for 8–10 days. Insulin (3i.u./kg) was administered to each rat by intravenous injection at 24h before, and then 45min before, the rats were killed.

Rats were decapitated and, after bleeding, the livers were removed, washed in 50mM-Tris/HCl buffer, pH7.5, containing 1mM-dithiothreitol, 5% (v/v) glycerol, 0.1mM-phenylmethanesulphonyl fluoride, leupeptin (60μg/litre) and 0.25M-sucrose, and homogenized with 2vol. of the same buffer. The homogenate was centrifuged at 30000*g* for 30min and the supernatant was filtered through glass wool and then centrifuged at 100000*g* for 60min. A 1ml portion of the high-speed supernatant was applied to a column (0.4cm × 0.9cm) of phosphocellulose (Whatman P11) equilibrated with a buffer similar to the homogenizing buffer except that sucrose was absent (buffer A). The column was washed with 4ml of buffer A, followed by 4ml of 0.35M-KCl and 4ml of 1.2M-KCl, all in buffer A. Fractions (0.5ml each) were collected throughout. In all cases a peak of casein kinase devoid of histone kinase activity was eluted with the 1.2M-KCl wash, and the fractions with such activity were pooled and used in the quantification of casein kinases. The recovery of casein kinase activity in the 1.2M-KCl wash was directly proportional to the amount of sample applied to the column.

In some experiments, separation between casein kinases 1 and 2 was achieved by glycerol-density-gradient centrifugation. For this purpose, samples from phosphocellulose corresponding to four rats were pooled and concentrated with Sephadex G-25. Portions (0.1ml) of the samples was layered over an 8–30% (v/v)-glycerol gradient (4.3ml) and centrifuged at 55000rev./min for 16h at 4°C with an SW 60 rotor in a Beckman L8-70 centrifuge.

Assays

Casein kinase activity was determined essentially as described previously (Itarte *et al.*, 1981). In some cases [γ-^{32}P]GTP was used instead of [γ-^{32}P]-ATP at the same final concentration. Other modifications of the assay mixture are indicated opportunely in the text. One unit of casein kinase activity is defined as the amount catalysing the transfer of 1nmol of phosphate from [γ-^{32}P]ATP to casein/min at 30°C. The concentration of blood glucose was determined as described by Dubowski (1962). Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard.

Results

Quantification of each type of casein kinase in control livers

The accurate determination of the amount of each individual casein kinase in liver homogenates is made difficult by the presence of different protein kinases, protein phosphatases and inhibitors. This interference can be easily overcome by

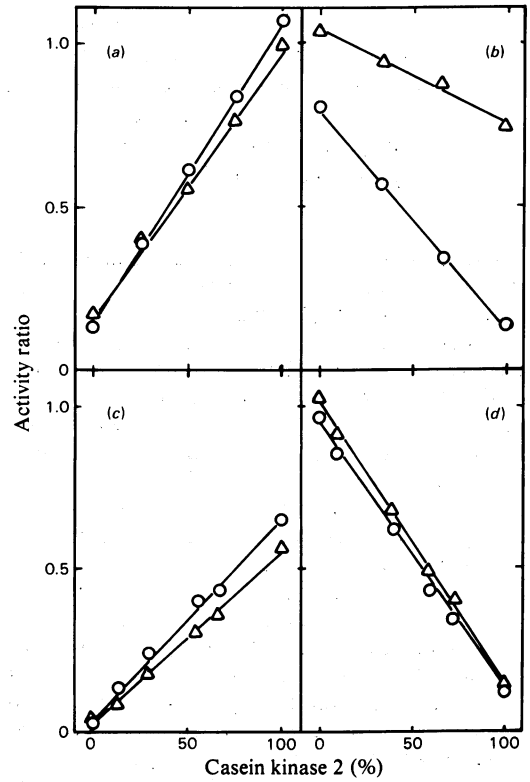

Fig. 1. *Effects of protamine, heparin and GTP on casein kinase activity as a function of the relative concentrations of casein kinase 1 and casein kinase 2*

Activity ratios are defined as follows: activity in the presence of (*a*) 1mg of protamine/ml, (*b*) 3.3μg of heparin/ml, or (*d*) 1mM-unlabelled GTP with respect to control without additions and using [γ-^{32}P]ATP as substrate, and (*c*) activity with [γ-^{32}P]GTP relative to that with [γ-^{32}P]ATP as substrate. The concentration of casein in (*a*) and (*b*) was 0.4mg/ml, whereas in (*c*) and (*d*) it was 4mg/ml. In all cases, assays were performed both in the absence (○) and in the presence (△) of 0.1M-KCl.

submitting the sample to chromatography on phosphocellulose, to which the casein kinases bind strongly and can be separated from interfering enzymes and inhibitors (Itarte *et al.*, 1981; Bertomeu *et al.*, 1981). The method used for the preparation of the 1.2M-KCl eluate, which contains both casein kinases, is described in the Experimental section. The recovery of casein kinase activity in this fraction was about 80% of the total eluted from the column. It is known that other protein kinases also bind to phosphocellulose, but they would be eluted by the 0.35M-KCl wash (Itarte *et al.*, 1981; Cohen *et al.*, 1982; Ahmad *et al.*, 1982), would not phosphorylate casein (Cohen *et al.*, 1982) or would be inactive towards this substrate under the standard assay conditions used herein where no Ca^{2+} or calmodulin are present (Woodgett *et al.*, 1982).

As with other mammalian casein kinases, (Hathaway & Traugh, 1982), rat liver cytosol casein kinase 2 used GTP as phospho donor ($K_m \simeq 50\,\mu M$), whereas casein kinase 1 did not. This property, together with the differential effects of protamine and heparin on both enzymes (Plana *et al.*, 1982), was used to develop a method for the determination of each casein kinase in the presence of them both. For this purpose, a series of mixtures containing similar total casein kinase activities, but different percentages of purified casein kinase 1 and casein kinase 2, were used as controls. As Fig. 1 shows, there was a linear relationship between the percentage of casein kinase 2 in the sample and (*a*) the effect of protamine, (*b*) the effect of heparin, (*c*) the activity with [γ-^{32}P]GTP as substrate and (*d*) the apparent inhibition by unlabelled GTP. The concentration of casein in the assay for determining the activity ratio in the presence of heparin or protamine was 0.4mg/ml, since the effect of these compounds was reversed by high concentrations of casein (Plana *et al.*, 1982). Since the samples to be analysed contained 1.2M-KCl, the effect of this salt on these parameters was studied. The presence of 0.1M-KCl in the assay markedly reversed the inhibitory effect of heparin, as indicated for rabbit muscle PC 0.7 (DePaoli-Roach *et al.*, 1981) casein kinase (DePaoli-Roach & Roach, 1982), whereas the other parameters were almost unaffected by the salt. Furthermore, we have shown previously that, although the activities of casein kinase 1 and casein kinase 2 on casein are differently affected by high concentrations of KCl (Itarte *et al.*, 1981), they are similarly stimulated (1.5-fold) by 0.1M-KCl in the assay. On the other hand, extensive dialysis of the phosphocellulose eluate to completely remove the KCl led to the appearance of a precipitate and a significant decrease of the casein kinase activity of the sample. In consequence, the inhibition by protamine and unlabelled GTP and the use of [γ-^{32}P]GTP in the presence of 0.1M-KCl were the criteria employed to calculate the percentage of each casein kinase in the samples, diluted with 1.5vol. of buffer A before assay.

The results obtained by the three criteria were similar and, as shown in Table 1, about 60% of the total casein kinase activity in the 1.2M-KCl phosphocellulose eluate from normal rat livers corresponded to an enzyme that behaved as casein kinase 2, and the remaining 40% corresponded to casein kinase 1. Diluting the samples before assays did not alter the results. Furthermore, when a control sample containing 0.5unit of total casein kinase activity/ml was mixed with an equal volume of a solution containing either 0.6unit of casein

Table 1. *Blood glucose concentration, total casein kinase activity and percentage of casein kinase 2 in liver cytosol from control, starved, diabetic and insulin administered rats*

Total casein kinase activity was determined by using 4mg of casein/ml and 0.125mM-[γ-^{32}P]ATP in the presence of 0.1M-KCl. The percentage of casein kinase 2 was determined by the criteria based on the activity ratio in the presence of (*a*) 1mg of protamine/ml, or (*d*) 1mM-unlabelled GTP with respect to control without additions, with 0.125mM-[γ-^{32}P]ATP as phospho donor and (*c*) the activity with 0.125mM-[γ-^{32}P]GTP relative to that with 0.125mM-[γ-^{32}P]ATP as substrate. The concentration of casein in (*a*) was 0.4mg/ml, whereas in (*c*) and (*d*) it was 4mg/ml. Samples containing known concentrations of both purified casein kinase 1 and casein kinase 2 were used as standards. In all cases the assays were performed in the presence of 0.1M-KCl. Data are means ± S.D. The numbers of samples analysed is given (*n*).

			Casein kinase activity			
				Casein kinase 2 (%)		
Conditions	*n*	[Blood glucose] (mg/100ml)	(Units/g of liver)	(*a*)	(*c*)	(*d*)
Control	18	122 ± 14	10.3 ± 1.4	62.8 ± 6.0	63.6 ± 1.7	59.8 ± 4.0
Starved	12	56 ± 9	9.4 ± 0.7	56.8 ± 8.1	62.0 ± 2.1	58.1 ± 7.3
Diabetic	18	368 ± 30	10.4 ± 2.9	57.8 ± 7.9	57.3 ± 3.3	56.4 ± 3.2
Insulin-treated	12	62 ± 15	9.4 ± 1.4	58.1 ± 4.5	56.4 ± 4.2	54.8 ± 8.9
Insulin-treated diabetic	12	196 ± 30	11.3 ± 3.6	54.5 ± 3.5	59.5 ± 3.6	58.0 ± 9.1

Table 2. *Effect of starvation, diabetes and insulin administration on the K_m for casein and GTP of rat liver cytosol casein kinase 2*

To determine the K_m for casein, casein kinase activity of the samples was assayed with concentrations of casein ranging from 0.2 to 4.0 mg/ml in the presence of 0.125 mM-[γ-^{32}P]GTP. The K_m for [γ-^{32}P]GTP was determined with concentrations of this nucleotide ranging from 5 to 200 μM in the presence of 4.0 mg of casein/ml. In both cases, K_m values were calculated by double-reciprocal plots, the lines being fitted by linear-regression analysis. Data corresponding to each experiment are means ± S.D. for six to eight samples. *$P < 0.01$ with respect to control.

	K_m for casein (mg/ml)			K_m for GTP (μM)
Conditions	Expt. 1	Expt. 2	Expt. 3	Expt. 2
Control	0.79 ± 0.08	0.89 ± 0.15	1.28 ± 0.09	53.3 ± 6.5
Starved	0.77 ± 0.07	0.82 ± 0.02	—	53.6 ± 2.5
Diabetic	0.47 ± 0.19*	0.53 ± 0.20*	0.83 ± 0.12*	54.9 ± 5.9
Insulin-treated	—	0.99 ± 0.10	1.34 ± 0.06	41.5 ± 8.7
Insulin-treated diabetic	—	0.76 ± 0.08	1.07 ± 0.09	49.8 ± 1.0

kinase 1/ml or 0.9 unit of casein kinase/ml, the percentages of casein kinase 2 determined were 25 and 85% respectively. These data confirm the validity of the assays and rule out the presence of interfering substances. Since total casein kinase activity was 8.9 units/g of wet tissue, the concentration of casein kinase 1 and casein kinase 2 in liver cells would be 3.2 and 4.8 units/g of wet tissue respectively.

Effects of starvation, diabetes and insulin

Starvation for 48 h, diabetes or insulin did not significantly change total casein kinase activity or the percentage of casein kinase 2 in rat liver cytosol, although they affected blood glucose concentration (Table 1). However, when determined with [γ-^{32}P]GTP as phospho donor, the ratio of the casein kinase activities at low (0.4 mg/ml) and high (4.0 mg/ml) concentrations of casein of the samples from diabetic rats (0.51 ± 0.04) was significantly higher than that for the samples from control rats (0.36 ± 0.02). Since only casein kinase 2 uses GTP as substrate, this result suggested that diabetes affected the affinity of this enzyme for casein. When the K_m for this protein substrate was determined in the presence of [γ-^{32}P]GTP, a significant decrease was observed in diabetic as opposed to control rats (Table 2). No changes in this parameter were observed on starvation of, or insulin administration to, control rats. On the other hand, when diabetic rats were treated with insulin, the K_m for casein of casein kinase 2 increased to values close to that of the control, which indicated that insulin reversed the effect of diabetes on this enzyme.

No significant changes in the K_m for GTP were observed under any of these conditions.

Effect of diabetes on the activity of casein kinase 2 towards other protein substrates

It is known that casein kinase 2 phosphorylates, besides casein, phosvitin and glycogen synthase. In addition, we have observed that casein 2 purified from rat liver cytosol also phosphorylates purified HMG 14. The phosphorylation reaction of this substrate was not stimulated by 20 μM-cyclic AMP or 5 μM-cyclic GMP, was inhibited by heparin (30 μg/ml), but not by the cyclic AMP-dependent protein kinase inhibitor protein (0.2 mg/ml) and proceeded at a similar rate when either ATP or GTP were used as phospho donors. The maximal rate of phosphorylation of HMG 14 was about 30% that of casein and 45% that of phosvitin, but about 10-fold greater than that of glycogen synthase.

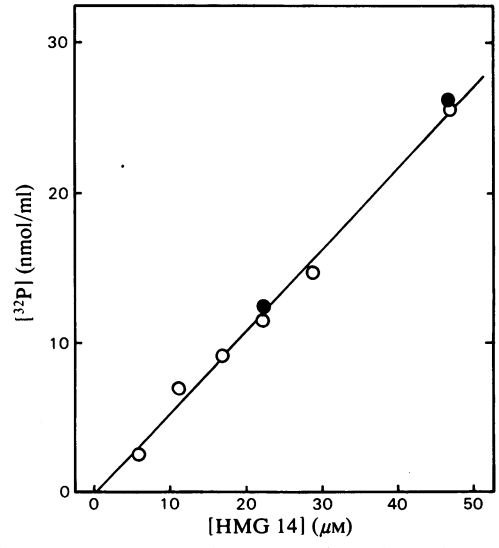

Fig. 2. *Stoichiometry of HMG phosphorylation by casein kinase 2*

The phosphorylation reaction was allowed to proceed for 3 h at different concentrations of HMG 14 (M_r 10400) with 2 units of casein kinase 2/ml and either [γ-^{32}P]ATP (○) or [γ-^{32}P]GTP (●) as phospho donor.

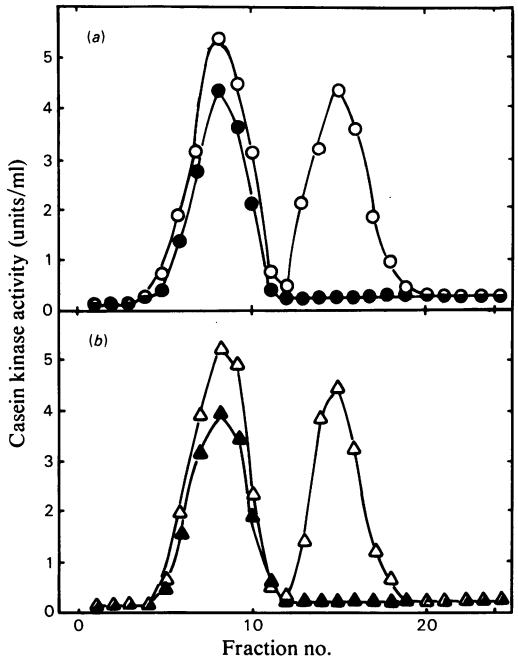

Fig. 3. *Glycerol-density-gradient centrifugation*
Samples corresponding to the 1.2M-KCl eluates from phosphocellulose of control (*a*) and diabetic rats (*b*) were submitted to glycerol-density-gradient centrifugation as indicated in the Experimental section. After centrifugation, fractions (0.18ml) were collected and analysed for the presence of casein kinase activity with ATP (○, △) and GTP (●, ▲) as phospho donors.

Table 3. *Kinetic constants for protein substrates of casein kinase 2 isolated from control- and diabetic-rat liver cytosol*
Casein kinase activity of samples from the glycerol-density-gradient centrifugation was assayed using [γ-^{32}P]ATP and concentrations of protein substrate ranging from 0.2 to 4.0mg/ml for casein, from 0.05 to 0.5mg/ml for phosvitin and from 0.1 to 1mg/ml for HMG 14 and glycogen synthase. K_m values were estimated from Hill plots by considering the $V_{max.}$ obtained by extrapolation of Eadie–Hofstee plots. Data are the means ± S.D. for four samples.

	K_m (mg/ml)	
Substrate	Control	Diabetes
Casein	1.16 ± 0.07	0.65 ± 0.06
Phosvitin	0.21 ± 0.04	0.11 ± 0.01
HMG 14	0.42 ± 0.06	0.26 ± 0.05
Glycogen synthase	0.50 ± 0.09	0.28 ± 0.12

Total ^{32}P incorporation into HMG 14 by casein kinase 2 was directly related to the amount of protein substrate present in the assay, reaching in all cases a value close to 0.5mol/mol (Fig. 2).

To confirm the effect of diabetes on casein kinase 2, we studied the activity of the enzyme from control and diabetic rats on phosvitin, glycogen synthase and HMG 14. In order to avoid the possible interference of casein kinase 1 on these assays, both casein kinases were separated from each other by glycerol-density-gradient centrifugation.

As shown in Fig. 3, the behaviour of the enzymes from control and diabetic rats in this centrifugation was similar, indicating that diabetes did not cause changes in the apparent M_r of both casein kinases. The results also confirm that no significant changes in the percentage of casein kinase 2 were induced by diabetes.

Kinetic analysis of the phosphorylation of casein by isolated casein kinase 2 showed that the effect of diabetes in decreasing its K_m value persisted after glycerol-density-gradient centrifugation (Table 3). Furthermore, diabetes also decreased the K_m value for phosvitin, HMG 14 and glycogen synthase.

Discussion

The results reported here indicate that rat liver cytosol casein kinase 2 is affected by diabetes and insulin, which alter its K_m for the protein substrate but not its total concentration in rat liver cytosol. The mechanism underlying this effect is unknown, but does not seem to be due to the presence of modulators and could represent a modification of the enzyme structure affecting the protein substrate-binding site, although the possibility of synthesis of isoenzymes cannot be ruled out.

It is known that oestrogen administration to roosters also promotes a significant decrease of the K_m for phosvitin of liver phosvitin kinase, without affecting its K_m for ATP (Goldstein & Hasty, 1973). On the other hand, the K_m values for GTP and ATP of nuclear casein kinase N II from hepatoma are approx. 2.4-fold lower than those for the enzyme from control rat livers (Rose *et al.*, 1981). The general properties of phosvitin kinases from rooster liver and casein kinase N II from rat liver nuclei are similar to those of rat liver cytosol casein kinase 2 and other type-II casein kinases (Itarte *et al.*, 1981; Hathaway & Traugh, 1982). Thus, although some of these stimuli also affect total type-II casein kinase activity, all of them alter its kinetic properties.

The decrease in the K_m value promoted by diabetes on casein kinase 2 was not restricted to casein and phosvitin, but was also observed when HMG 14 and glycogen synthase were used as substrate and thus may also affect its activity on other proteins that may serve as possible physiological substrates for this enzyme. Walton & Gill (1983) have recently shown that phosphorylation *in vitro*

of HMG 14 from HeLa cells by casein kinase 2 occurs at a site that is phosphorylated *in vivo* during the cell cycle, an observation that indicates a role of this enzyme in the control of its phosphorylation *in vivo*. Since the HMG proteins are highly conserved (Walker, 1982), it is likely that rat liver HMG 14 is also a substrate for casein kinase 2. It is accepted that HMG proteins play an important role inside the nucleus, but their synthesis occurs in the cytoplasm, and the presence of HMG proteins has been detected in the cytoplasm of various types of cells, including rat liver cells (Bustin & Niehart, 1979; Seyedin & Kistler, 1979). Thus phosphorylation of HMG 14 could take place in the cytoplasm, and changes in the activity of the protein kinases that act on this HMG protein would cause changes in the level of phosphorylation at which it enters the nucleus, which may influence its binding to the nucleosome.

Regarding the activity of casein kinase 2 on glycogen synthase, it is interesting to note that although the effects produced by this kinase on glycogen synthase inactivation are more discreet than those observed with other glycogen synthase kinases (Itarte *et al.*, 1981; Huang *et al.*, 1982; Cohen *et al.*, 1982), recent reports on rabbit muscle have shown that previous phosphorylation of glycogen synthase by casein kinase 2 greatly increases the phosphorylation of this substrate by F_A/glycogen synthase kinase 3 (Picton *et al.*, 1982; DePaoli-Roach *et al.*, 1983). These data suggest that changes in the activity of casein kinase 2, as observed in diabetes, could ultimately affect the susceptibility of glycogen synthase to phosphorylation and inactivation by other glycogen synthase kinases.

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