

# Diabetic cardiomyocyte dysfunction and myocyte insulin resistance: Role of glucose-induced PKC activity

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## Abstract

Increased protein kinase C (PKC) activity has been implicated in the pathogenesis of a number of diabetic complications, and high concentrations of glucose have been shown to increase PKC activity. The present study was designed to examine the role of PKC in diabetes-induced (and glucose-induced) cardiomyocyte dysfunction and insulin resistance (measured by glucose uptake). Adult rat ventricular myocytes were isolated from nondiabetic and type 1 diabetic animals (4–5 days post-streptozotocin treatment), and maintained overnight, with/without the nonspecific PKC inhibitor chelerythrine (CHEL = 1  $\mu$ M). Myocyte mechanical properties were evaluated using a video edge-detection system. Basal and insulin-stimulated glucose uptake was measured with [<sup>3</sup>H]-2-deoxyglucose. Blunted insulin-stimulated glucose uptake was apparent in diabetic myocytes, and both mechanical dysfunctions (e.g., slowed shortening/relengthening) and insulin resistance were maintained in culture, and normalized by CHEL. Cardiomyocytes isolated from nondiabetic animals were cultured in a high concentration of glucose (HG = 25.5 mM) medium, with/without CHEL. HG myocytes exhibited slowed shortening/relengthening and impaired insulin-stimulated glucose uptake compared to myocytes cultured in normal glucose (5.5 mM), and both impairments were prevented by culturing cells in CHEL. Our data support the view that PKC activation contributes to both diabetes-induced abnormal cardiomyocyte mechanics and insulin resistance, and that elevated glucose is sufficient to induce these effects. (*Mol Cell Biochem* **262**: 155–163, 2004)

*Key words*: contractile function, diabetes, myocyte insulin resistance, protein kinase C

## Introduction

Both clinical and experimental lines of evidence have indicated the existence of a specific diabetic cardiomyopathy independent of macrovascular (and perhaps microvascular) complications [1]. This cardiomyopathy is characterized by structural, metabolic and functional damage in the heart and may be responsible for the high incidence of cardiac dys-

function and mortality in both types 1 and 2 diabetes mellitus [2]. Diastolic dysfunction is the most prominent defect associated with diabetic cardiomyopathy and is manifested, in part, by prolonged action potential (AP) duration, slowed cytosolic Ca<sup>2+</sup> removal and slowed myocyte relengthening [3, 4]. The cellular changes associated with end-stage diabetic cardiomyopathy are well established [5]. Changes in intracellular Ca<sup>2+</sup> regulation are prominent (e.g., depressed

sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) and  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger (NCX) [6–9]), in addition to altered regulatory proteins (e.g., decreased PKA-dependent phosphorylations (reviewed by [10, 11]).

Although the cellular consequences in hearts of animal models of long-term diabetes (i.e., months) have been well characterized, only a few studies have been designed to investigate the early stages of this disease. We, and others, have shown that abnormal myocyte E–C coupling occurs within days after chemically inducing type 1 diabetes with streptozotocin (STZ) [4, 12]. The effects of short-term (*in vivo*) diabetes (i.e., cardiomyocyte dysfunction) can be recapitulated by maintaining adult rat ventricular myocytes overnight in a medium containing a high concentration of glucose (HG = 25.5 mM). HG prolongs the AP, slows cytosolic clearing (by depressed SERCA  $\text{Ca}^{2+}$  uptake), and slows contraction and relaxation [13–15]. We are utilizing both of these models as tools to investigate the pathogenesis of diabetic cardiomyopathy.

There is a significant amount of evidence demonstrating that increased protein kinase C (PKC) activity contributes to cardiovascular complications associated with diabetes (reviewed in [10]). Elevated PKC activity has been found in diabetic hearts [16] and appears to be associated with changes in expression or translocation of specific PKC isoforms [10]. There is still controversy as to which isoforms are activated [17–19]. These discrepancies in PKC isoform activation may be related to the duration or method of induction of diabetes (e.g., type 1 or type 2 diabetes), or the type of preparation evaluated (i.e., whole heart homogenates or isolated myocytes). Nevertheless, it is well established that increased myocardial PKC activity exists after long-term diabetes; whether it contributes to the development of diabetic cardiomyopathy is not known.

PKC signaling has been extensively studied in heart in the context of myocardial function, hypertrophy and preconditioning (reviewed by [20, 21]). The protein targets of PKC isoforms are not well characterized [21]. However, PKC is known to phosphorylate a number of proteins which are directly involved in cardiac excitation-contraction E–C coupling (e.g., troponin I, L-type  $\text{Ca}^{2+}$  channel, SERCA, phospholamban and the transient outward  $\text{K}^+$  channel, reviewed in [20, 21]). Such changes may contribute to the known effects of diabetes on E–C coupling (e.g., prolonged action potential duration, slower cytosolic  $\text{Ca}^{2+}$  removal and slower myocyte relengthening).

Insulin sensitivity is significantly reduced in hearts from both types 1 and 2 diabetic animals [22]. It has been suggested that PKC attenuates insulin-stimulated glucose uptake, perhaps by phosphorylating serine/threonine residues on the insulin receptor or its substrates [23]. In general, activation of atypical PKC isoforms appears to play a role in

insulin-stimulated glucose uptake, while increases in diacylglycerol-dependent isoforms (i.e., conventional and novel PKCs) inhibit insulin receptor signaling. High concentrations of glucose (*in vitro*) can activate PKC in vascular smooth muscle cells [24] and in ventricular myocytes [25], and attenuate insulin-stimulated glucose uptake in vascular smooth muscle cells [26]. Elevated glucose leads to tissue insulin resistance in skeletal muscle through enhanced hexosamine metabolism [27], perhaps through PKC activation [28]. Thus, it is a logical extension to anticipate that glucose-induced PKC activity leads to myocardial insulin resistance and cardiac dysfunction, but to our knowledge, there have been no reports describing both effects in isolated cardiomyocytes.

This investigation was designed to determine whether myocyte insulin resistance and abnormal myocyte mechanics coincide, and are affected by PKC activation. Our data indicate that the diabetic phenotype (i.e., blunted insulin-stimulated glucose uptake and slowed myocyte mechanics) is maintained in culture and normalized by a nonspecific PKC inhibitor, chelerythrine. Furthermore, culturing normal cardiomyocytes in high glucose is sufficient to induce the diabetic phenotype, and this can be prevented by PKC inhibition.

## Materials and methods

### *Induction of short-term diabetes*

All experiments were approved by the Institutional Animal Care and Use Committee at each institution where experiments were performed. Type 1 diabetes was induced in adult male rats (Sprague Dawley or Wistar rats purchased from Charles River Laboratories, Wilmington, MA) by a single tail vein injection of streptozotocin (STZ = 100 mg/kg/body weight dissolved in citrate buffer, pH 4.5) in anesthetized rats (80 mg/kg ketamine and 12 mg/kg xylazine i.p.). Successful induction of diabetes was initially determined by urine glucose, then later confirmed by serum glucose using a Beckman glucose analyzer at time of sacrifice (nonfasted, nondiabetic blood glucose =  $14.2 \pm 0.9$  mM,  $n = 4$ , and diabetic blood glucose =  $32.5 \pm 1.2$  mM,  $n = 9$ ). Animals were sacrificed after 4–5 days post-STZ injection by cardioexcision of anesthetized animals (pentobarbitol = 70 mg/kg i.p.). Pentobarbitol was used at the time of sacrifice because it does not interfere with serum insulin (unlike ketamine/xylazine). This model is an acute, severe form of type 1 diabetes, which has previously been used to investigate the short-term consequences of diabetes-induced, insulinopenia, dyslipidemia and hyperglycemia [4, 12]. All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

### Isolation and culture of ventricular myocytes

Ventricular myocytes were isolated from normal and diabetic rats by collagenase (Worthington Biochemical Corporation, Lakewood, NJ) and hyaluronidase perfused through the coronaries, and further digested by trypsin after the tissue was minced (as previously described [4]). Isolated myocytes were plated on either glass coverslips (for mechanical studies) or 35 mm culture dishes (for glucose uptake studies) that were precoated with laminin (10  $\mu\text{g}/\text{ml}$ ), and maintained overnight in a defined medium consisting of Medium 199 with Earle's salts containing 25 mM HEPES and  $\text{NaHCO}_3$  supplemented with albumin (2 mg/ml), L-carnitine (2 mM), creatine (5 mM), taurine (5 mM), insulin (0.1  $\mu\text{M}$ ), penicillin (100 U/ml), streptomycin (100  $\mu\text{g}/\text{ml}$ ), and gentamicin (100  $\mu\text{g}/\text{ml}$ ). This medium also contained either normal glucose (N = 5.5 mM) or high glucose (HG = 25.5 mM) concentrations. The high glucose concentration is comparable to serum glucose levels in severely diabetic rats. Subsets of each medium were also supplemented with the nonspecific PKC inhibitor chelerythrine (CHEL, 1  $\mu\text{M}$  (Calbiochem, La-Jolla, CA)). The PKC inhibitor was added at the same time the myocytes were placed in medium, and cells were incubated overnight at 37 °C under 100% humidity and 5%  $\text{CO}_2$ .

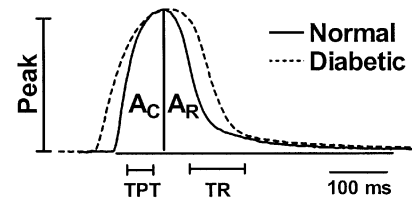
### Cell shortening/relengthening

Mechanical properties of ventricular myocytes were assessed using a video-based edge-detection system (IonOptix Corporation, Milton, MA [15]). In brief, cells were superfused at  $31 \pm 1$  °C with a buffer containing 131 mM NaCl, 4 mM KCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM glucose, 10 mM HEPES, at pH 7.4. Myocytes were displayed on the computer monitor using an IonOptix MyoCam camera, which rapidly scans the image area every 4.2 ms such that the amplitude and velocity of shortening/relengthening are recorded with good fidelity. Cells were field stimulated to contract at 0.5 Hz and approximately 10 steady-state twitches were averaged for each myocyte and analyzed offline with Clampfit (Axon Instruments, Foster City, CA). The indices used to evaluate myocyte mechanics are described in the Results section (see Fig. 1A). Subsets of N and HG cells were also exposed to CHEL (2  $\mu\text{M}$ ) for 5–45 min in the recording buffer (15 min was found to be sufficient for maximum effects on mechanical indices).

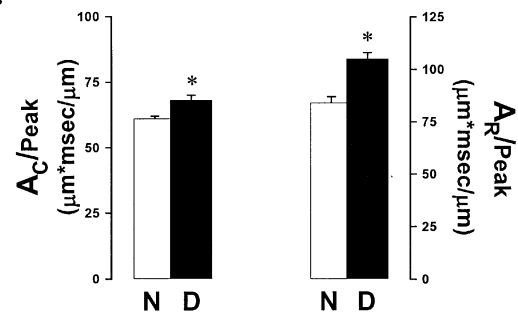
### Glucose uptake assay

Basal and insulin-stimulated [ $^3\text{H}$ ]-2-deoxyglucose (Perkin-Elmer, Boston, MA) uptake in cardiomyocytes was measured as previously described [29]. Cardiomyocytes isolated from

A.



B.



C.

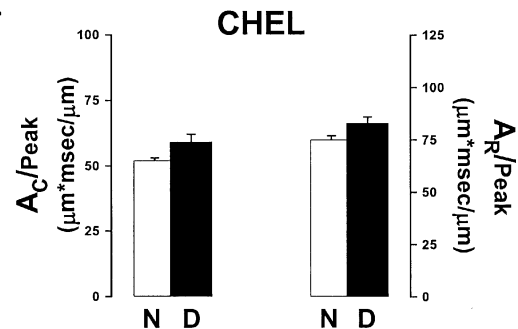


Fig. 1. Mechanical properties of myocytes isolated from normal (N) and diabetic (D) animals (4–5 days post-STZ injection) cultured with and without chelerythrine (CHEL). (A) Representative traces of myocyte twitches. Contraction is described by the area under the shortening phase normalized to peak twitch amplitude ( $A_C/\text{Peak}$ ) and time-to-peak-twitch (TPT), and relaxation is described by the area under the relengthening phase normalized to peak twitch amplitude ( $A_R/\text{Peak}$ ) and time-to-relaxation (TR). (B)  $A_C/\text{Peak}$  and  $A_R/\text{Peak}$  in myocytes isolated from N and D animals cultured overnight. Data represent mean  $\pm$  S.E.M.,  $n = 104$ – $105$  cells per group cultured overnight in normal medium. (C)  $A_C/\text{Peak}$  and  $A_R/\text{Peak}$  in myocytes isolated from N and D animals cultured overnight with CHEL (1  $\mu\text{M}$ ). Data represent mean  $\pm$  S.E.M.,  $n = 37$ – $41$  cells per group. \*  $p < 0.05$  with respect to N myocytes.

normal and diabetic animals were maintained overnight in either N or HG medium (without insulin), and subsets supplemented with CHEL (1  $\mu\text{M}$ ). We chose to omit insulin from the culture medium [29] because we recorded a more robust insulin-stimulated glucose uptake in both normal and diabetic myocytes (data not shown). We have previously shown that HG-induced myocyte dysfunction is independent of insulin in the culture medium [13, 30], and have

evidence that myocyte dysfunction is maintained in diabetic cells cultured independent of insulin (data not shown). Prior to uptake measurements, cells were washed three times with the HEPES buffer described above (but without glucose and containing 0.1% BSA), and incubated for 1 h at 37 °C in buffer  $\pm$  insulin (see Figs. 2 and 5 for concentrations). [ $^3\text{H}$ ]-2-Deoxyglucose (8  $\mu\text{Ci}$ ) was added to each plate and incubated for an additional 1.5 h. Cells were washed three times with cold HEPES buffer and solubilized in 5N NaOH. Adequate washing of cells was confirmed by preliminary studies using a second (nonpermeant isotope, [ $^{14}\text{C}$ ]-L-glucose). In addition, preliminary studies demonstrated that insulin-stimulated glucose uptake could be prevented by treating cells with cytoctasin B (40  $\mu\text{M}$ ) during the incubation pe-

riod [29]. An aliquot of the cell lysate was removed for protein quantitation by the method of Lowry. The amount of [ $^3\text{H}$ ]-2-deoxyglucose uptake was determined (in triplicate) by scintillation counting and expressed in terms of DPM/mg protein per plate.

### Statistical analyses

For each experimental series, data are presented as mean  $\pm$  S.E.M., and statistical significance was considered when  $p < 0.05$ . For each mechanical variable differences were estimated by using a two-way analysis of variance (ANOVA) followed by a Bonferroni multi-comparison test when a significant interaction term was indicated (SYSTAT, Richmond, CA). The ANOVAs were constructed to analyze the differences between groups (diabetic (or high glucose) vs. non-diabetic (or normal) and treatment ( $\pm$  CHEL)). Glucose uptake measurements for the diabetic myocytes were analyzed with a nonparametric test for unpaired samples (Wilcoxon Ranked Sum). When comparing glucose uptake between N and HG myocytes, a parametric two-way ANOVA was used to determine significance between groups at different insulin concentrations (data from cells exposed to  $10^{-11}$  M insulin were excluded from the analyses because they were collected from only two cultures per group).

## Results

### *Mechanical dysfunctions in short-term diabetes are maintained in culture and normalized by inhibiting PKC*

We have previously shown that ventricular myocyte dysfunction is evident in cells isolated after only a few days of *in vivo* diabetes [4]. This dysfunction includes prolonged shortening and relengthening, and slowed cytosolic  $\text{Ca}^{2+}$  removal. Our present study shows that this dysfunction is maintained in culture. Culturing myocytes overnight ( $\pm$  CHEL, see below) had no overt effect on cell morphology. For example, cell shape, resting cell length, and presence of distinct striations were similar among all groups of cells, and no gross changes in cell viability were evident (data not shown). Fig. 1A illustrates some of the indices used to describe the mechanical properties of isolated myocytes, including; peak shortening amplitude (normalized to resting cell length (PS)), time-to-peak twitch (TPT) and time-to-relengthening (TR). We also evaluated contraction and relaxation by integrating the shortening (or contraction) phase ( $A_C$ ) and relengthening (or relaxation) phase ( $A_R$ ), each normalized to peak twitch amplitude ( $A_C/\text{Peak}$  and  $A_R/\text{Peak}$ , respectively). In culture, diabetic myocytes maintained prolonged duration of shortening and relengthening as indicated by larger  $A_C/\text{Peak}$  and  $A_R/\text{Peak}$ ,

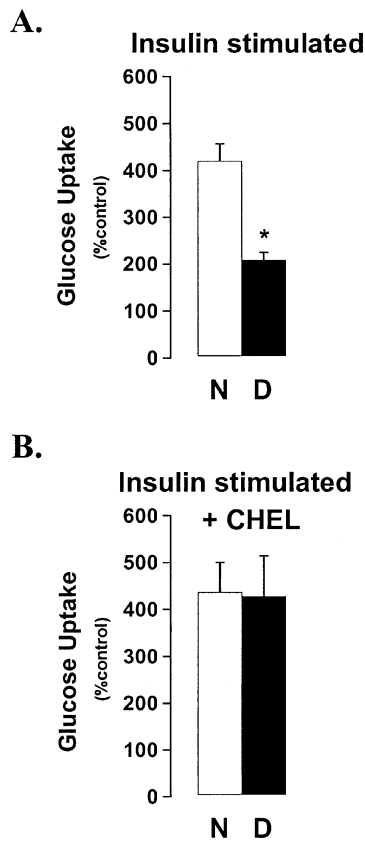


Fig. 2. [ $^3\text{H}$ ]-2-Deoxyglucose (2DG) uptake in ventricular myocytes isolated from N and D animals and cultured overnight with or without CHEL. Basal glucose uptake was determined for each group. Insulin-stimulated glucose uptake was determined after incubating cells in insulin ( $10^{-7}$  M) for 1 h prior to adding 2DG (8  $\mu\text{Ci}$ ), then allowing uptake of isotope for an additional 1.5 h (at 37 °C). (A) N and D myocytes were cultured overnight and insulin-stimulated 2DG uptake was expressed as a percentage increase above basal (control) uptake. (B) Subsets of the same cells from N and D myocytes were cultured overnight in CHEL (1  $\mu\text{M}$ ), and insulin-stimulated 2DG uptake was expressed as a percentage increase above CHEL alone. Data represent mean  $\pm$  S.E.M. of triplicates from three to four cultures per group, \* $p < 0.05$  with respect to N myocytes.

respectively (Fig. 1B), and long TPT and TR (data not shown). PS was similar in normal ( $8.17 \pm 0.29\%$  resting cell length) and diabetic animals ( $7.46 \pm 0.33\%$  resting cell length,  $n = 104$ – $105$  isolated from 5 animals per group).

Mechanical indices of diabetic myocytes were normalized by culturing cells in CHEL ( $1 \mu\text{M}$ ). Shortening and relengthening were significantly faster in CHEL-treated diabetic cells than in untreated diabetic myocytes (Figs. 1B and 1C), and were comparable to the nondiabetic cells (Fig. 1C). Similar effects were seen with TPT and TR (data not shown). PS was unaffected by CHEL in either diabetic ( $7.00 \pm 0.49\%$ ,  $n = 37$ ) or nondiabetic cells ( $7.51 \pm 0.38\%$ ,  $n = 41$ ). Thus, maintaining myocytes in CHEL overnight reversed the *in vivo* diabetes-induced dysfunctions.

#### *Blunted insulin-stimulated glucose uptake occurs after short-term diabetes and normalized by PKC inhibition*

[ $^3\text{H}$ ]-2-deoxyglucose uptake was used as a measure of glucose uptake in isolated ventricular myocytes. Insulin-stimulated glucose uptake was normalized to protein content (in order to account for potential differences in cell number per culture dish) and expressed as a percentage of basal uptake. Glucose uptake was measured in both freshly isolated and cultured myocytes. In freshly isolated cells, insulin sensitivity was significantly depressed in diabetic myocytes ( $116 \pm 6\%$  above basal,  $n = 3$ ) compared to nondiabetic cells ( $395 \pm 7\%$  above basal,  $n = 3$ ). Insulin-stimulated glucose uptake in cultured myocytes from diabetic animals remained significantly blunted compared to nondiabetic myocytes (Fig. 2A), while basal glucose uptake was normal (data not shown). Thus, insulin resistance develops after only a few days of *in vivo* diabetes, and is maintained in culture. Culturing myocytes in CHEL completely reversed insulin insensitivity in diabetic myocytes without affecting glucose uptake in nondiabetic cells (Fig. 2B).

#### *PKC inhibition prevents glucose-induced mechanical dysfunctions*

Each culture consisted of myocytes isolated from nondiabetic animals, maintained in either normal glucose (N = 5.5 mM) or high glucose (HG = 25.5 mM) medium  $\pm$  CHEL ( $1 \mu\text{M}$ ). Consistent with our previous reports [13, 14], culturing myocytes in HG medium prolonged the duration of shortening ( $A_C/\text{Peak}$ ) and relengthening ( $A_R/\text{Peak}$ , Fig. 3). Myocytes cultured in HG medium also exhibited reduced PS compared to those of N (N =  $7.50 \pm 0.35\%$ ,  $n = 51$  and HG =  $6.26 \pm 0.34\%$ ,  $n = 52$ ,  $p < 0.05$ ).

Culturing N cells with CHEL had no effect on any mechanical index measured, including PS, TPT and TR (data not shown), and  $A_C/\text{Peak}$  and  $A_R/\text{Peak}$  (Figs. 3A and 3B).

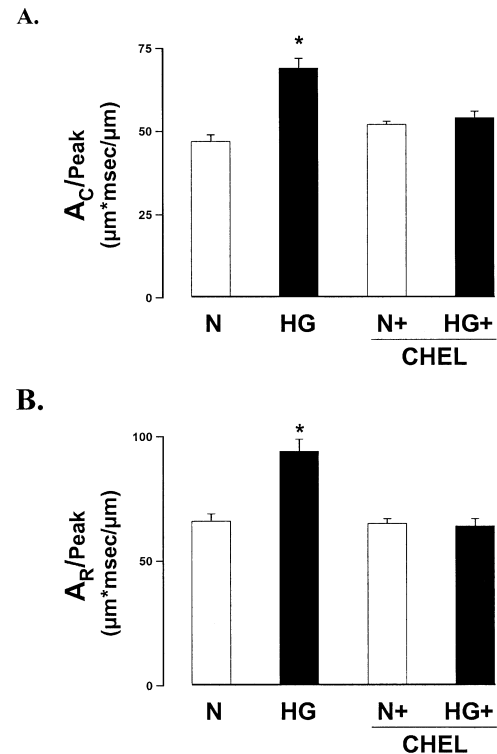


Fig. 3. Effects of the PKC inhibitor CHEL on glucose toxicity-induced mechanical dysfunction. Ventricular myocytes were cultured overnight in either normal glucose (N: 5.5 mM) or high glucose (HG: 25.5 mM) medium, with and without CHEL ( $1 \mu\text{M}$ ). (A) Myocyte shortening ( $A_C/\text{Peak}$ ) and (B) relengthening ( $A_R/\text{Peak}$ ) are indicated for N and HG cells. Data represent mean  $\pm$  S.E.M.,  $n = 51$ – $54$  cells per group, \* $p < 0.05$  with respect to N myocytes without CHEL.

High concentrations of CHEL have been reported to induce apoptosis. We also found that cell viability was significantly reduced when myocytes were cultured in  $10 \mu\text{M}$  CHEL (data not shown) but  $1$ – $2 \mu\text{M}$  CHEL did not have any appreciable effects of cell survival. All mechanical properties in HG myocytes were found to be normal after overnight culture in CHEL (e.g.,  $A_C/\text{Peak}$  and  $A_R/\text{Peak}$ , Figs. 3A and 3B). It should be noted that myocyte mechanics were evaluated without CHEL in the recording buffer, therefore this nonselective PKC inhibitor was efficacious in preventing glucose-induced toxicity.

In order to ascertain whether the protective effect of CHEL could be attributed to inhibition of PKC (rather than specific to the drug) we cultured myocytes in 4- $\beta$  phorbol 12-myristate-13-acetate (PMA  $1 \mu\text{M}$ ). It is known that acute (min) exposure to PMA activates PKC, whereas long-term exposure downregulates PKC. Although PMA decreased the PS in NG cells [N =  $7.3 \pm 0.3\%$  ( $n = 53$ ), versus N+PMA =  $5.8 \pm 0.3\%$  ( $n = 53$ )] and prolonged the duration and area of cell shortening and relengthening, it abolished the

HG-induced mechanical abnormalities, similar to the effects of CHEL (data not shown).

*Acute inhibition of PKC with CHEL reverses glucose-induced mechanical abnormalities*

To address the question of whether HG-induced abnormal mechanical function involves sustained activation of PKC, myocytes were first cultured in N and HG overnight, then twitches were recorded in buffer with or without CHEL ( $2 \mu\text{M}$ ). Acute exposure (5–45 min) to CHEL in the recording buffer had no effect on myocyte function in N cells (Fig. 4). Prolonged shortening and relengthening in HG myocytes (seen in the absence of CHEL) were reversed after 15 min superfusion with CHEL. Thus, CHEL both prevents glucose toxicity in overnight cultures as measured by myocyte mechanics (Fig. 3), and reverses these effects when applied acutely in the recording buffer (Fig. 4). These data suggest that activation of PKC is sustained (i.e., not just a transient effect) and is an important component of the HG phenotype.

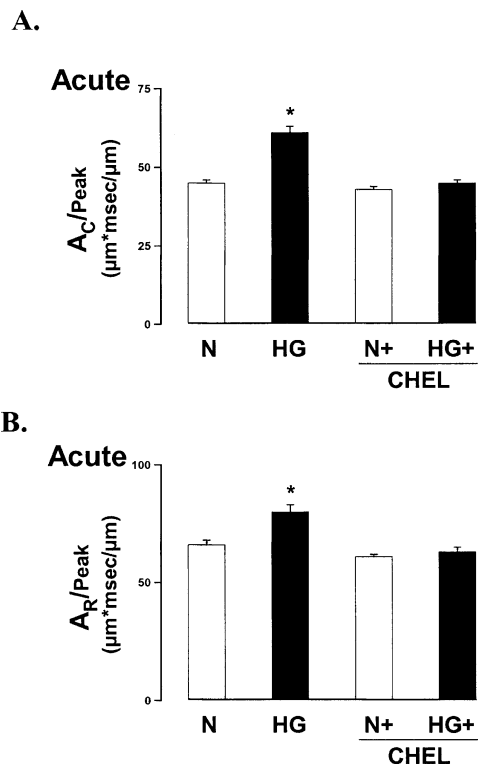


Fig. 4. Effects of acute application of the PKC inhibitor CHEL on myocyte mechanics in N and HG cells. Subsets of myocytes were superfused with recording buffer containing CHEL ( $2 \mu\text{M}$ ) for 15 min. (A) Myocyte shortening ( $A_C/\text{Peak}$ ) and (B) relengthening ( $A_R/\text{Peak}$ ) are indicated for N and HG cells. Data represent mean  $\pm$  S.E.M.,  $n = 33$ –54 cells per group. \* $p < 0.05$  with respect to N cells recorded in control buffer (i.e., without CHEL).

*Glucose toxicity involves blunted insulin-stimulated glucose uptake*

We (Fig. 2) and others [29] have shown that cardiomyocytes from diabetic rats exhibit reduced sensitivity to insulin-stimulated glucose uptake. To determine whether our HG cells also present with impaired insulin signaling, basal and insulin-stimulated [ $^3\text{H}$ ]-2-deoxyglucose uptake was measured in N and HG myocytes. Basal glucose uptake was normal in HG cells ( $\text{HG}/\text{N} = 1.00 \pm 0.09$ ,  $n = 13$  cultures). At near physiological levels of insulin ( $10^{-11}$  M), both HG and N cells exhibited slight increases in glucose uptake (Fig. 5A). Glucose uptake was significantly blunted in HG myocytes when exposed to high concentrations of insulin

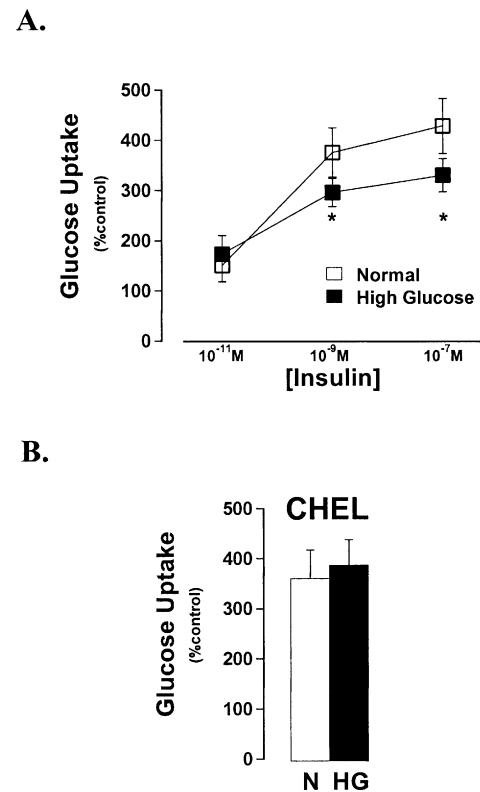


Fig. 5. [ $^3\text{H}$ ]-2-Deoxyglucose (2DG) uptake in ventricular myocytes cultured overnight in either N or HG medium. (A) Basal and insulin-stimulated glucose uptake was determined after incubating N and HG cells in various concentrations of insulin for 1 h prior to adding 2DG, then allowing uptake of isotope for an additional 1.5 h (at  $37^\circ\text{C}$ ). (B) Subsets of N and HG myocytes were cultured overnight in CHEL ( $1 \mu\text{M}$ ), and basal and insulin-stimulated ( $10^{-7}$  M) 2DG uptake was determined. The extent of glucose uptake was expressed relative to basal (control) uptake for each culture. Data for each insulin concentration are paired data (i.e., measured in cells from the same culture, exposed to either N or HG). Data represent mean  $\pm$  S.E.M.,  $n = 2$ –10 cultures per group per condition. There were only two cultures represented at  $10^{-11}$  M (and thus excluded from statistical analysis), whereas sample sizes were 8–10 cultures in (A) and 6 cultures in (B). \* $p < 0.05$  with respect to N cells for a given insulin concentration.

( $10^{-9}$  and  $10^{-7}$  M) compared to N cells (Fig. 5A). Although there was variability in the absolute value of glucose uptake among cultures, glucose uptake within each culture was consistently higher in N myocytes than in HG cells (confirmed with a Wilcoxon Signed Rank test for paired data within each insulin concentration). In order to determine the effects of PKC inhibition on insulin-stimulated glucose uptake, myocytes were cultured overnight with CHEL ( $1 \mu\text{M}$ ). CHEL prevented the blunted insulin ( $10^{-7}$  M) response in HG myocytes (Fig. 5B) and at  $10^{-9}$  M insulin (data not shown). In fact, there was a trend (though not significant) for CHEL to increase insulin-stimulated glucose uptake in HG myocytes compared to N cells (Fig. 5B). CHEL itself had no effect on basal glucose uptake in either N or HG myocytes (data not shown).

## Discussion

The significant findings of this investigation are that (1) cardiomyocyte insulin resistance coincides with abnormal myocyte mechanics and develops within days after induction of diabetes; (2) the blunted insulin response and impaired myocyte mechanics are maintained in cultured cells, and both are normalized with a nonselective PKC inhibitor; (3) exposure to high extracellular glucose (HG) overnight is sufficient to induce both insulin resistance and abnormal E–C coupling (similar to the diabetic phenotype); and (4) these effects are prevented by culturing HG myocytes in a PKC inhibitor. Thus, both myocardial insulin resistance and abnormal E–C coupling most likely involves elevated PKC activity in early stages of pathogenesis.

Diabetic cardiomyopathy has been well characterized in terms of whole heart dysfunction and changes in gene expression after prolonged (i.e., months) of diabetes. In particular, changes in substrate utilization (i.e., glucose and fatty acid metabolism) and  $\text{Ca}^{2+}$  regulation clearly contribute to the effects of long-term diabetes [5, 22, 31]. In spite of altered insulin signaling associated with either type 1 or type 2 diabetes, the whole heart may have the capacity to compensate, such that functional impairments are mild at early stages of the disease [32–34]. Belke *et al.* [35] have recently shown that whole heart function is only mildly impaired in cardiomyocyte-specific insulin receptor knockout (IR-KO) mice, even though insulin-stimulated glucose uptake is virtually nonexistent in isolated myocytes. Changes in cardiomyocyte E–C coupling appear to precede whole heart decompensation in several different models of diabetes [4, 12, 36], although this has not been systematically evaluated. For example, cardiomyocytes isolated from Zucker obese rats demonstrate decreased insulin sensitivity [23, 37], prolonged relaxation times and a blunted positive inotropic response to IGF-1 compared to their lean controls [36]. One fundamental

difference between cardiomyocyte-specific IR-KO mice and models of diabetes is that IR-KO animals do not present with systemic metabolic changes associated with diabetes (e.g., hyperglycemia and dyslipidemia). Whether myocardial insulin resistance leads to abnormal myocyte E–C coupling remains to be determined.

Few studies have focused on the pathogenesis of diabetic cardiomyocyte dysfunction. To gain insight into whether there is a link between the development of cellular insulin resistance and abnormal E–C coupling, we have utilized a model of short-term *in vivo* diabetes that produces cardiomyocyte mechanical dysfunction within a few days after STZ injection. As previously reported, short-term diabetes slows myocyte contraction and relaxation [4]. We now show for the first time that these myocytes are insulin resistant (as measured by blunted insulin-stimulated glucose uptake) and that this phenotype is maintained when cells are cultured overnight in a medium containing normal glucose concentrations (5.5 mM; Figs. 1B and 2A). It should be noted that the effect of short-term diabetes on  $A_C/\text{Peak}$  is not as profound as on  $A_R/\text{Peak}$ . This is consistent with observations that diastolic dysfunction precedes systolic dysfunction [38]). Thus, both insulin resistance and abnormal E–C coupling occur within days of uncontrolled type 1 diabetes. These findings are particularly useful because this culture system can now be exploited to investigate the signaling pathways associated with the pathogenesis and reversibility of diabetic cardiomyocyte dysfunction.

PKC activity is elevated in diabetic heart tissue, although there remains some controversy as to which isoforms are activated and whether there are changes in membrane/cytosolic translocations [16, 17, 19, 24]. To date, we have not measured PKC activity in this short-term diabetic model. However, our data suggest that sustained PKC activity is necessary to maintain abnormal E–C coupling, since inhibition of PKC in cultured cells can ameliorate *in vivo* diabetes-induced myocyte dysfunction (Fig. 1C). PKC $\beta$  isoform has been implicated in diabetic cardiomyopathy [24], but increases in other isoforms (e.g., PKC $\epsilon$ ) have also been measured [15]. Since hyperglycemia is a major risk factor in the development of diabetic cardiomyopathy and is known to activate PKC, we evaluated the impact of a nonselective PKC inhibitor on E–C coupling in nondiabetic myocytes cultured in a HG medium. Fig. 3 shows that culturing cells in CHEL prevents the adverse effects of HG on myocyte mechanics, without affecting N cells. Furthermore, acute (i.e., 15 min) application of CHEL can reverse HG-induced mechanical dysfunctions (Fig. 4). Coupled with the observation that myocyte mechanics can be normalized in diabetic cells cultured in medium containing CHEL (Fig. 1), these data support the hypothesis that PKC remains elevated and contributes to myocyte dysfunction in diabetic and HG cells. We have preliminary evidence indicating that the PKC $\beta$ -specific inhibitor LY379196,

both prevents and reverses glucose-induced myocyte mechanical dysfunction [39]. Whether this isoform contributes to diabetes-induced myocyte dysfunction and/or insulin sensitivity remains to be determined.

The relationship between myocyte insulin resistance and abnormal E–C coupling in diabetic myocardium likely involves glucose-induced activation of PKC. Support for this hypothesis is based on our findings that culturing myocytes in HG recapitulates changes in E–C coupling and insulin resistance seen in diabetic myocardium, and that these abnormalities are prevented (and reversed) by PKC inhibition (Figs. 3–5). We have recently shown that HG cells are resistant to both the positive inotropic effects of IGF-1 and its stimulation of Akt/PKB phosphorylation (which is downstream of IGF-1 and insulin receptors [40]), thus providing further evidence to support the view that HG induces myocyte insulin resistance.

The effects of PKC on insulin signaling are both tissue- and isoform-specific (reviewed in [41]). Elevated PKC activity has been implicated in downregulating insulin signaling, particularly in skeletal muscle, adipose tissue and liver, perhaps through abnormal phosphorylation of the insulin receptor or its substrates [10]. Antidiabetic agents known to improve insulin sensitivity may work in part by inhibiting PKC. For example, troglitazone has been shown to restore insulin sensitivity in cardiomyocytes from Zucker obese rats [42]. In addition to its ability to activate peroxisome proliferator-activating receptors, troglitazone (and other thiazolidinediones) inhibits PKC membrane translocation [43]. Troglitazone also reverses hexosamine-induced insulin resistance of skeletal muscle in transgenic mice [44].

It is known that excess intracellular glucose is shunted through the hexosamine pathway and contributes to the development of cellular insulin resistance [27]. Glucosamine induces insulin resistance in a variety of cell types (e.g., adipose and skeletal muscle tissue [27]) and activates PKC in adipose tissue [28]. We have previously shown that glucosamine mimics glucose toxicity in terms of myocyte mechanics, and inhibiting hexosamine biosynthesis prevents our HG effects [14]. Thus, our finding that insulin-stimulated glucose uptake was blunted in HG cells (Fig. 5) would be based on our glucosamine study [14] and effects seen in other tissues [27]. We have previously shown that both troglitazone and metformin (known insulin sensitizers) prevent the adverse mechanical effects of culturing cardiomyocytes in HG medium [45, 46]. Whether these interventions prevent glucose-induced myocyte insulin resistance remains to be determined.

Our study provides evidence that PKC inhibitors may be considered an effective treatment for both preventing and reversing diabetic cardiomyocyte dysfunction and insulin resistance. To our knowledge, we are the first to demonstrate an association between glucose-induced cellular insulin resistance and abnormal cardiomyocyte E–C coupling. These

findings provide a foundation for determining whether there is a direct link between these processes.

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