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Restoration of defective L-type Ca^{2+} current in cardiac myocytes of type 2 diabetic *db/db* mice by Akt and PKC-I

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Abstract

Diabetes is associated with increased risk of heart failure and development of a cardiomyopathy whose etiology is only partially understood. Ca^{2+} entry through the voltage-dependent L-type Ca^{2+} channel $\text{Ca}_v1.2$ initiates the contractile cycle in cardiac myocytes. Decreased cardiac contractility and depressed $\text{Ca}_v1.2$ function have been reported in obese type 2 diabetic *db/db* mice. Here we demonstrate that a reduction in phosphoinositide 3-kinase (PI3K) signaling is a major contributor to the altered function of $\text{Ca}_v1.2$ in *db/db* cardiac myocytes. Using the whole-cell patch clamp technique, we determined that intracellular infusion of cardiac myocytes from *db/db* mice with phosphatidylinositol 3,4,5-trisphosphate (PIP3), the second messenger produced by PI3K, increased the L-type Ca^{2+} current ($I_{\text{Ca,L}}$) density nearly to the level seen in wildtype cells. PIP3 also reversed the positive shift in the voltage dependence of steady-state current activation observed in *db/db* myocytes. Infusion of protein kinases that act downstream of PI3K also affected $I_{\text{Ca,L}}$. Akt1 and Akt2 were as effective as PIP3 in restoring $I_{\text{Ca,L}}$ density in *db/db* myocytes, but did not affect the voltage dependence of current activation. Infusion of atypical PKC-I (the human homolog of mouse PKC- λ) caused a small but significant increase in $I_{\text{Ca,L}}$ density and completely reversed the shift in voltage dependence of steady-state current activation. These results indicate that a defect in PI3K/PIP3/Akt/PKC- λ signaling is mainly responsible for the depressed $\text{Ca}_v1.2$ function in the heart of type 2 diabetic *db/db* mice.

Keywords

Diabetes; L-type Ca^{2+} current; cardiac myocytes; phosphoinositide 3-kinase (PI3K); Akt; PKC

INTRODUCTION

Cardiovascular complications such as coronary artery disease, stroke, and peripheral arterial disease are responsible for most of the morbidity and mortality suffered by diabetic patients.¹ In addition, diabetic patients have a greater risk of developing heart failure than

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non-diabetic patients, even after controlling for factors such as coronary artery disease and hypertension. The underlying pathogenesis of this diabetic cardiomyopathy is only partially understood, and is thought to include changes in myocardial structure, metabolism and Ca^{2+} cycling.^{2,3} The voltage-dependent L-type Ca^{2+} channel $\text{Ca}_v1.2$ is the critical initiator of the contractile cycle in ventricular cardiac myocytes, and inhibition of $\text{Ca}_v1.2$ function diminishes Ca^{2+} entry and contractile force. Studies have shown that Ca^{2+} current through $\text{Ca}_v1.2$ ($\text{I}_{\text{Ca,L}}$) is reduced in cardiac myocytes from type 1 diabetic *Ins2^{Akita}* and type 2 diabetic *db/db* mice.^{4, 5} Cardiac contractility in both types of diabetic mice has also been shown to be impaired.^{4, 5}

Many of the metabolic effects of insulin are thought to be mediated by phosphoinositide 3-kinase (PI3K) signaling. PI3Ks are activated in response to insulin or other growth factors and phosphorylate phosphatidylinositol 4,5-bisphosphate ($\text{PI}(4,5)\text{P}_2$) to form the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3)⁶. As a consequence of increased PIP3 production, downstream effectors including members of the protein kinase Akt family (also known as PKB) and atypical PKC- λ become phosphorylated and activated.^{7, 8} Experiments in knockout mice have shown that both Akt2 and PKC- λ (the mouse homolog of human PKC-I) contribute to insulin-stimulated glucose uptake in the heart.^{9, 10} The PI3K signaling pathway also modulates cardiac contractile function.¹¹ Ablation of the p110 γ PI3K isoform in mice resulted in enhanced cardiac contractility due to an increase in cAMP levels.¹² By contrast, we found that mice lacking the p110 α PI3K isoform in the heart exhibited decreased cardiac contractility *in vivo* and reduced $\text{I}_{\text{Ca,L}}$ in isolated myocytes.¹³ We used a number of methods to manipulate PI3K/PIP3/Akt signaling to demonstrate that activation of this pathway is needed to maintain normal $\text{I}_{\text{Ca,L}}$ and myocyte contractility.^{13, 14} Other investigators have shown that transgenic mice overexpressing constitutively active forms of p110 α or Akt in cardiac myocytes exhibited enhanced cardiac contractility and increases in $\text{Ca}_v1.2$ expression or $\text{I}_{\text{Ca,L}}$.^{15, 16} To our knowledge, the effect of PKC-I/ λ on cardiac contractility or $\text{I}_{\text{Ca,L}}$ has not been examined.

Reduced insulin activation of PI3K signaling due to either lack of insulin (type 1) or insulin resistance (type 2) is a well-studied aspect of diabetes, especially in the context of glucose uptake. In a previous study, we determined that decreased $\text{I}_{\text{Ca,L}}$ in cardiac myocytes from non-obese type 1 diabetic *Ins2^{Akita}* mice is due mainly to a reduction in PI3K activity.⁵ These results were surprising, considering the long-term hyperglycemia, multiple metabolic disturbances and other physiological changes in these mice that could have affected channel function. This led us to speculate that low PI3K signaling is also responsible for the reduction of $\text{I}_{\text{Ca,L}}$ in cardiac myocytes from obese type 2 diabetic *db/db* mice.⁴ In this study, we show that decreased $\text{I}_{\text{Ca,L}}$ in cardiac myocytes from *db/db* mice is due in large part to decreased PI3K signaling. In addition, we demonstrate that Akt and PKC-I differentially regulate $\text{Ca}_v1.2$ function.

MATERIALS & METHODS

Materials

Recombinant human PKC-I was from Invitrogen (Carlsbad, CA, USA). The active Akt1, active Akt2 and inactive Akt1 were from Millipore (Billerica, MA, USA). Phosphoinositides (all di-C8) were from Echelon Biosciences (Salt Lake City, UT, USA). *Ventricular myocyte isolation*—Male *db/db* (B6.BKS(D)-*Lepr^{db/J}*) and WT control (C57BL/6J) mice were purchased from Jackson Laboratories and used at 4-5 months of age. Hyperglycemic status of the *db/db* animals was confirmed by measuring tail vein glucose levels using a glucometer (OneTouch UltraMini, LifeScan, Inc., Milpitas, CA, USA). Mice were euthanized by intraperitoneal injection of 100 mg sodium pentobarbital per kg of body weight and ventricular myocytes were isolated as previously described¹⁴. All animal-related

experimental protocols were approved by the Institutional Animal Care and Use Committee of Stony Brook University.

Electrophysiology

Whole-cell patch clamping of isolated cardiac myocytes was performed as previously described.¹⁴ In brief, only clearly rod-shaped myocytes were studied. Whole-cell patch clamp recordings used 2–3 M borosilicate glass pipettes measured prior to sealing (Sutter Instrument), pCLAMP 8 software, the DigiData 1350 interface, and the Axopatch 1D amplifier (Axon Instruments). For the recording of $I_{Ca,L}$, pipettes contained an internal solution (111 mM CsCl, 20 mM tetraethylammonium chloride, 10 mM glucose, 14 mM EGTA, 10 mM HEPES, and 5 mM MgATP, pH 7.2, adjusted with CsOH) and cells were perfused in a Na^+ -free bath solution (137 mM tetraethylammonium chloride, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4, adjusted with tetraethylammonium hydroxide).

Data Analysis

Student's *t* tests were performed to evaluate the significance of the differences between mean values. A value of $P < 0.05$ was considered significant.

RESULTS

PIP3 Increases $I_{Ca,L}$ Density in Diabetic *db/db* Myocytes

Diabetic *db/db* mice exhibit a cardiac contractility defect and reduced $I_{Ca,L}$ density in myocytes⁴. We confirmed that $I_{Ca,L}$ density is reduced in *db/db* cardiac myocytes as compared to WT cells. Fig. 1A (upper panels) shows typical recordings of $I_{Ca,L}$ activation in myocytes isolated from WT and diabetic *db/db* mice. $I_{Ca,L}$ was elicited by depolarizing voltage pulses from -50 mV to $+50$ mV in 10 mV increments (300 ms duration) from a holding potential of -50 mV. The membrane capacitance was measured by applying a hyperpolarizing voltage step from a holding potential of -50 mV, and the current amplitudes were normalized to cell capacitance to obtain current density in pA/pF. Membrane capacitance, which is proportional to surface area, was not significantly different between the two groups of myocytes (148.0 ± 9.8 pF in WT ($n = 31$) vs. 145.0 ± 8.5 pF in *db/db* ($n = 45$)). The peak values from these recordings were used to construct current density-voltage (*I-V*) relationships that show a substantial reduction in $I_{Ca,L}$ density in *db/db* myocytes as compared to WT cells at most of the voltages tested (lower panel of Fig. 1A). To determine if decreased PI3K signaling in diabetic hearts might be responsible for this difference, we infused the cells with PIP3 through the patch pipette. PIP3 had little effect on WT cells but significantly enhanced $I_{Ca,L}$ density in the *db/db* myocytes at most of the voltages tested (Fig. 1B).

We performed additional experiments using control phosphoinositides to determine if the stimulatory effect was specific to PIP3. $I_{Ca,L}$ density was measured at $+10$ mV following a single depolarizing step of 300 ms duration from a holding potential of -50 mV. The peak $I_{Ca,L}$ density at $+10$ mV in *db/db* myocytes in the absence of PIP3 was 53% lower than in WT cells (Fig. 2). Infusion of PIP3 increased $I_{Ca,L}$ density in the *db/db* cells to nearly the wildtype level, but the control phosphoinositides $PI(3,5)P_2$ or $PI(4,5)P_2$ did not have a stimulatory effect (Fig. 2). These results are consistent with the hypothesis that decreased $I_{Ca,L}$ density in *db/db* diabetic myocytes is due mostly to reduced PI3K activity.

Next we examined the time-dependent effect of PIP3 on $I_{Ca,L}$ activation. In this protocol, the cells were repeatedly depolarized every 15 s with voltage steps 300 ms in duration to $+10$ mV from a holding potential of -50 mV. The typical “run-down” of $I_{Ca,L}$ density was

observed in both WT and diabetic myocytes under the control conditions, and current density decreased by 20% and 24%, respectively, within 240 s (Fig. 3A). Infusion of WT myocytes with PIP3 prevented the “run-down” and caused $I_{Ca,L}$ density to increase slightly over time (left panel of Fig. 3A). A much larger “run-up” was seen in *db/db* myocytes in the presence of PIP3, and a plateau was reached after about 150 s (right panel of Fig. 3A). Plateau values for $I_{Ca,L}$ density in the presence of PIP3 were approximately 11 pA/pF (WT) and 9 pA/pF (*db/db*). These results indicate that $I_{Ca,L}$ density in diabetic myocytes is increased to its maximum value within minutes after PI3K signaling is artificially enhanced by intracellular infusion of PIP3.

Independent Effects of Isoproterenol and PIP3 on $I_{Ca,L}$ Stimulation in *db/db* Myocytes

β -adrenergic stimulation of $I_{Ca,L}$ is a well-known mechanism that regulates cardiac contraction. To determine if a defect in β -adrenergic regulation is responsible for the suppression of $I_{Ca,L}$ in *db/db* myocytes, we treated cells with the β -adrenergic agonist isoproterenol (ISO). ISO stimulated $I_{Ca,L}$ in a time-dependent manner in both WT and *db/db* myocytes (Fig. 3B). Infusion of WT myocytes with PIP3 caused a small increase in $I_{Ca,L}$ density that was further enhanced to a much larger extent by subsequent application of ISO (left panel of Fig. 3C). PIP3 caused a comparatively larger increase in current density in diabetic cells that was also further increased in the presence of ISO (right panel of Fig. 3C). Summary data for current density at +10 mV show that ISO increased $I_{Ca,L}$ by 1.4-fold in both WT and *db/db* cells (Fig. 2). However, total current density in the presence of ISO plus PIP3 was much lower in *db/db* cells than in WT myocytes (Fig. 2). These results indicate that β -adrenergic regulation of channel function is intact in the diabetic cells and suggest that modulation of $I_{Ca,L}$ by PIP3 is independent of PKA.

Akt and PKC-I Increase $I_{Ca,L}$ Density in *db/db* Myocytes

The protein kinases Akt1, Akt2 and PKC- λ /I are activated downstream of PI3K. We next asked if either Akt or PKC- λ /I would mimic the stimulatory effect of PIP3 on $I_{Ca,L}$ density in diabetic myocytes. Indeed, infusion of *db/db* myocytes with Akt1 or Akt2 caused a significant enhancement of $I_{Ca,L}$ density at most of the voltages tested, whereas unactivated Akt1 had little or no effect (Fig. 4). Because mouse PKC- λ is not commercially available, we used the human homolog PKC-I in infusion experiments. PKC-I also caused an increase in $I_{Ca,L}$ density at most of the voltages tested (Fig. 4). Summary data for current density at +10 mV show that infusion of *db/db* myocytes with either Akt1 or Akt2 enhanced $I_{Ca,L}$ density to nearly the level in WT cells, and the effects were comparable to those obtained with PIP3 (Fig. 2). Although the increase in $I_{Ca,L}$ with PKC-I was significant ($P < 0.05$ as compared to *db/db* control), the average $I_{Ca,L}$ density was less than that seen with PIP3, Akt1 or Akt2 (Fig. 2). Thus, Akt and, to a lesser extent, PKC-I, partially reversed the defect in $I_{Ca,L}$ density in myocytes from *db/db* mice.

Effect of PIP3 on Steady-state Activation, Inactivation and Recovery Kinetics of $I_{Ca,L}$

The decrease in $I_{Ca,L}$ density in *db/db* diabetic myocytes has been shown to be due in part to altered voltage dependence that affects the steady-state activation of $I_{Ca,L}$.⁴ To determine the effect of PIP3 on steady-state activation, cells were exposed to depolarizing voltage steps from -50 mV to +10 mV (300 ms duration) from a holding potential of -50 mV and the data were fit to a Boltzmann 2-state model. We confirmed the earlier report⁴ that the steady-state activation curve for *db/db* myocytes was shifted toward more positive potentials as compared to the WT curve (Fig. 5A) and there was a significant difference between the voltages at 50% activation (V_h) for WT and *db/db* cells (Table 1). Infusion of WT cells with PIP3 did not alter the steady state activation of $I_{Ca,L}$, whereas infusion of *db/db* cells caused the curve to shift to the left so that it was nearly superimposed on the WT curves (Fig. 5A). V_h was essentially the same for WT cells (plus or minus PIP3) and *db/db* cells in the

presence of PIP3 (Table 1). There were no differences in the corresponding slope factors (K) among the four groups ($P>0.05$ when compared to WT control) (Table 1). These results indicate that the change in steady-state activation of $I_{Ca,L}$ in *db/db* myocytes is completely reversed by PIP3.

To investigate the steady-state inactivation of $I_{Ca,L}$, cells were subjected to conditioning pulses 2 s in duration from -80 mV to $+40$ mV in 10 mV increments from a holding potential of -50 mV followed by a test step to $+10$ mV. The data fit by a Boltzmann 2-state model showed that there was no significant difference in the membrane potential at which 50% of the channels are inactivated ($V_{1/2}$) or K between WT and *db/db* cells in the presence or absence of PIP3 (Table 1) ($P>0.05$ for all groups when compared to WT control, t-test). We then examined the effect of PIP3 on the kinetics of recovery from inactivation of $I_{Ca,L}$ using a 2-pulse protocol. First, a depolarizing pulse 300 ms in duration was applied from a holding potential of -50 mV to the test potential of $+10$ mV to activate $I_{Ca,L}$. A variable interval was allowed for recovery, then a second identical test pulse was applied. The cycle length was 8 s. The data were fit to a single exponential using the equation $I/I_{max}=1-e^{(-t/\tau)}$ to calculate the average time constant (τ) of $I_{Ca,L}$ recovery at different test voltages. There were no significant differences in τ between WT and *db/db* myocytes in the presence or absence of PIP3 ($P>0.05$ for all groups when compared to WT control) (Table 1). These results indicate that the steady-state inactivation and recovery from inactivation of $I_{Ca,L}$ are not altered in *db/db* myocytes.

PKC-I Modulates the Steady-State Activation of $I_{Ca,L}$ in *db/db* Myocytes

We also examined the effect of Akt1, Akt2 and PKC-I on the steady-state activation of $I_{Ca,L}$. The steady-state activation curves generated in *db/db* cells infused with Akt1, Akt2 or inactive Akt1 were very similar to each other (Fig. 5B) and the V_h and K values were not significantly different as compared to the *db/db* control (Table 1) ($P>0.05$). In contrast, PKC-I infusion of *db/db* myocytes caused a negative shift of the steady-state activation curve (Fig. 5B), yielding V_h and K values that were statistically indistinguishable from those for control WT cells ($P>0.05$) (Table 1). In summary, PKC-I completely reversed the alteration in steady-state activation of $I_{Ca,L}$ in *db/db* myocytes.

DISCUSSION

The number of diabetic adults worldwide in 2010 is estimated to be 285 million, and this number is expected to increase to 439 million by 2030.¹⁷ The total number of excess deaths in adults attributable to diabetes is estimated to be 3.96 million for the year 2010, accounting for 6.8% of all mortality.¹⁸ Approximately 90% of diabetic patients have type 2 diabetes. Since cardiovascular diseases are the most common cause of death in people with diabetes,¹ a better understanding of the mechanisms that cause cardiovascular complications in type 2 diabetes is urgently needed.

The *db/db* mouse is a well-studied model of obesity and type 2 diabetes. A previous study showed that decreased cardiac contractility in type 2 diabetic *db/db* mice is due to a defect in Ca^{2+} transients caused at least in part by a reduction in $I_{Ca,L}$ density. A positive shift in the voltage dependence of steady-state activation of $I_{Ca,L}$ was also noted.⁴ Here we show that these defects in $I_{Ca,L}$ are mainly the result of low PI3K signaling. Infusion of diabetic cardiac myocytes with PIP3 or Akt increased $I_{Ca,L}$ nearly to the level seen in control cells, and intracellular delivery of PKC-I corrected the alteration in voltage dependence of activation.

The relatively rapid and large increase in $I_{Ca,L}$ density upon infusion of PIP3 or protein kinases that are activated upon production of PIP3 suggests that the *db/db* cells contain a

pool of inactive channels that responds to an increase in PI3K signaling. We showed earlier that $I_{Ca,L}$ in myocytes from type 1 diabetic *Ins2^{Akita}* mice is low because of decreased expression of $Ca_v1.2$ on the cell surface, and that PIP3 increases $I_{Ca,L}$ density in these cells by promoting trafficking of the channel to the cell surface.⁵ L-type Ca^{2+} channels in the heart are composed of the pore-forming $Ca_v1.2$ subunit, which determines the principal biophysical and pharmacological properties of the channel, in a complex with accessory β_2 and $\alpha_2\delta$ subunits that are thought to play a role in trafficking.^{19, 20} Akt phosphorylation of the β_{2a} subunit in transfected cells has been shown to increase Ca^{2+} V channel activity by promoting trafficking of the channel to the plasma membrane.²¹ We assume that a similar Akt- (or, to a lesser extent, PKC- λ -) dependent trafficking mechanism is mainly responsible for the stimulatory effect of PI3K signaling on $Ca_v1.2$ function in the *db/db* diabetic cells.

Although infusion of *db/db* cells with PIP3 or Akt caused a significant increase in $I_{Ca,L}$ density, the maximal current density we measured in the presence of PIP3 plus ISO was 37% lower in *db/db* cells than in wildtype cells. These results are consistent with a decrease in total expression of the $Ca_v1.2$ protein, as demonstrated by Pereira et al.⁵ Loss of $Ca_v1.2$ protein in the diabetic cells may also be due to suppressed PI3K signaling, as Akt-dependent phosphorylation of β_2 has been proposed to stabilize the $Ca_v1.2$ protein in cardiac myocytes.²²

A comparison of the data herein with our earlier results using myocytes from *Ins2^{Akita}* type 1 diabetic mice⁵ indicates that there are some differences in the way $I_{Ca,L}$ is affected in these two models of diabetes. First, the peak $I_{Ca,L}$ density at +10 mV was reduced much more in *db/db* myocytes (53%) than in *Ins2^{Akita}* myocytes (38%) as compared to nondiabetic cells. Second, PIP3 partially restored $I_{Ca,L}$ density in *db/db* myocytes but fully restored $I_{Ca,L}$ density in *Ins2^{Akita}* myocytes. Both of these disparities are probably due to the reduced expression of $Ca_v1.2$ protein in *db/db* myocytes⁴ that is not seen in *Ins2^{Akita}* myocytes.⁵ These differences suggest that hyperglycemia in combination with obesity and insulin resistance in type 2 diabetes is more damaging to $Ca_v1.2$ function than hyperglycemia and lack of insulin in type 1 diabetes. Finally, myocytes from *Ins2^{Akita}* mice exhibited positive shifts in the voltage dependence of steady-state activation and inactivation of $I_{Ca,L}$ and PIP3 infusion corrected the change in inactivation only, whereas PIP3 corrected the change in voltage dependence of steady-state activation in *db/db* cells.

Many ion channels, including $Ca_v1.2$, respond to depletion and resynthesis of phosphoinositides, especially $PI(4,5)P_2$. Alterations in channel activity and voltage dependence have been proposed to be mediated by binding of $PI(4,5)P_2$ to the channel proteins.²³⁻²⁵ Our demonstration that intracellular delivery of Akt or PKC-I mimics the effects of PIP3 in *db/db* myocytes suggests that PIP3 alters $Ca_v1.2$ function indirectly via a phosphorylation mechanism. This idea is supported by our earlier study showing that $I_{Ca,L}$ in myocytes lacking the p110 α isoform of PI3K is increased by infusion of Akt1, and $I_{Ca,L}$ in wildtype myocytes is decreased upon treatment with an Akt inhibitor.¹³ Phosphorylation is already known to be an important mechanism that regulates cardiac $Ca_v1.2$ function.²⁶ β -adrenergic stimulation of $I_{Ca,L}$ in the heart is mediated by PKA-dependent phosphorylation of $Ca_v1.2$.²⁷ Phosphorylation of $Ca_v1.2$ by PKG and a number of PKC isoforms is also associated with changes in channel activity.^{28, 29} In addition, as discussed above, Akt phosphorylation of β_2 subunits promotes increased trafficking of the channel to the plasma membrane and stabilizes the $Ca_v1.2$ protein.^{21, 22} Thus, it is possible that downregulation of $Ca_v1.2$ function in *db/db* myocytes might involve reduced phosphorylation of $Ca_v1.2$ by PKC- λ that alters gating and reduced phosphorylation of β_2 by Akt and/or PKC- λ that decreases the total number of channels and the number of channels on the cell surface.

PI3K signaling pathways can be activated by a variety of hormones and may utilise distinct molecular cassettes to differentially regulate cardiac physiology. For example, cardiac contractility is positively regulated by the PI3K p110 α isoform and negatively regulated by PI3K p110 γ , whereas PI3K p110 β promotes autophagy.^{12, 13, 15, 30} In addition, Akt1 regulates heart size whereas Akt2 regulates cardiomyocyte metabolism and survival.^{9, 31} Coupling of different PI3K signaling pathways to distinct responses may involve compartmentation of receptors, PI3K isoforms and downstream signaling molecules into signalosomes.³² Therefore, strategies that aim to increase overall PI3K signaling in diabetic patients might lead to detrimental effects on the heart and other organs. On the other hand, drugs that improve insulin sensitivity in the heart might provide protection from some of the deleterious effects of diabetes on cardiac function.

CONCLUSIONS

We conclude that reduced activation of the PI3K/Akt/PKC- λ signaling pathway in the heart of type 2 diabetic *db/db* mice causes a decrease in Cav1.2 function in these animals. We speculate that a similar defect in signaling also contributes to diabetic cardiomyopathy in type 2 diabetic patients.

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Figure 1. PIP3 increases $I_{Ca,L}$ density in myocytes from db/db mice

(A) $I_{Ca,L}$ activation in myocytes from WT or db/db mice infused with internal solution.

Upper panels: Representative recordings of $I_{Ca,L}$ activation. *Lower panel:* The corresponding peak current density-voltage (I-V) relationships of $I_{Ca,L}$ activation. (B) $I_{Ca,L}$ activation in myocytes from WT or db/db mice infused with internal solution containing 1 μ M PIP3. *Upper panels:* Representative recordings of $I_{Ca,L}$ activation. *Lower panel:* The corresponding I-V relationships. $n = 10$ cells for each group.

Figure 2. Effect of PI3K effectors and isoproterenol (ISO) on $I_{Ca,L}$ density

Summarized $I_{Ca,L}$ density at +10 mV in myocytes from WT and *db/db* mice.

Phosphoinositides (1 μ M) and kinases (20 nM) in internal solution were infused through the patch pipette and ISO (5 μ M) was applied to the external solution. ** indicates a significant difference vs. *db/db* control ($p < 0.01$). The number of cells studied in each group is above each bar.

Figure 3. Time-dependent stimulation of $I_{Ca,L}$ by PIP3 and ISO

Time dependence of $I_{Ca,L}$ activation in myocytes from WT (*left panels*) and *db/db* (*right panels*) mice infused with or without 1 μ M PIP3 (A), treated with extracellular application of 5 μ M ISO (B), or infused with PIP3 and then treated with ISO (C). $n = 10$ cells for each group.

Figure 4. Akt and PKC-I increase $I_{Ca,L}$ density in *db/db* myocytes

Upper panels: Representative recordings of $I_{Ca,L}$ activation in *db/db* myocytes infused with internal solution without (control) or with inactive Akt1 or active Akt1, Akt2 or PKC-I (20 nM each). *Lower panel:* The corresponding I-V relationships for $I_{Ca,L}$ activation. The voltage protocol was the same as for Fig. 1.

Figure 5. Effect of PI3K effectors on steady-state activation of $I_{Ca,L}$

(A) Steady-state activation curves of $I_{Ca,L}$ in WT and *db/db* myocytes infused with or without 1 μ M PIP3. (B) Steady-state activation curves of $I_{Ca,L}$ in *db/db* myocytes infused with inactive Akt1 or active Akt1, Akt2 or PKC-I (20 nM each). Biophysical parameters derived from each curve and the number of cells in each group are shown in Table 1.

Table 1Biophysical properties of $I_{Ca,L}$ in WT and *db/db* myocytes

		Steady-State Activation		Steady-State Inactivation		Recovery from Inactivation τ (ms)
		V_h (mV)	K (mV)	$V_{1/2}$ (mV)	K (mV)	
WT	Control	-17.8 ± 0.7 (10)	3.3 ± 0.6 (10)	-30.4 ± 1.1 (10)	4.6 ± 1.1 (10)	330 (7)
	PIP3	-17.9 ± 0.8 (7)	3.4 ± 0.4 (7)	-31.0 ± 1.2 (5)	4.5 ± 1.0 (5)	328 (5)
<i>db/db</i>	Control	-13.8 ± 0.4 (10) **	5.0 ± 0.3 (10)	-28.9 ± 1.0 (10)	4.8 ± 0.9 (10)	365 (5)
	PIP3	-18.5 ± 0.6 (10) ##	4.0 ± 0.6 (10)	-28.9 ± 1.6 (10)	4.0 ± 1.4 (10)	348 (5)
	Unact Akt1	-14.0 ± 0.8 (7)	4.6 ± 0.6 (7)			
	Act Akt1	-15.6 ± 0.3 (10)	4.5 ± 0.2 (10)			
	Act Akt2	-15.2 ± 0.6 (10)	4.0 ± 0.4 (10)			
	PKC-I	-18.7 ± 0.6 (7) ##	4.3 ± 0.4 (7)			

Numbers in parentheses indicate the number of myocytes tested.

** significantly different from WT control ($P < 0.01$).## significantly different from *db/db* control ($P < 0.01$).