An Inositol 1,4,5-Triphosphate (IP_3)-IP_3 Receptor Pathway Is Required for Insulin-Stimulated Glucose Transporter 4 Translocation and Glucose Uptake in Cardiomyocytes

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Intracellular calcium levels ($[Ca^{2+}]_i$) and glucose uptake are central to cardiomyocyte physiology, yet connections between them have not been studied. We investigated whether insulin regulates $[Ca^{2+}]_i$ in cultured cardiomyocytes, the participating mechanisms, and their influence on glucose uptake via SLC2 family of facilitative glucose transporter 4 (GLUT4).

Primary neonatal rat cardiomyocytes were preloaded with the Ca^{2+} fluorescent dye fluo3-acetoxymethyl ester compound (AM) and visualized by confocal microscopy. Ca^{2+} transport pathways were selectively targeted by chemical and molecular inhibition. Glucose uptake was assessed using [^{3}H]2-deoxyglucose, and surface GLUT4 levels were quantified in nonpermeabilized cardiomyocytes transfected with GLUT4-*myc*-enhanced green fluorescent protein.

Insulin elicited a fast, two-component, transient increase in $[Ca^{2+}]_i$. Nifedipine and ryanodine prevented only the first component. The second one was reduced by inositol-1,4,5-trisphosphate (IP_3)-receptor-selective inhibitors (xestospongin C, 2 amino-ethoxydiphenylborate), by type 2 IP_3 receptor knockdown via small interfering RNA or by transfected G$\beta\gamma$ peptidic inhibitor βARKct. Insulin-stimulated glucose uptake was prevented by bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid-AM, 2-amino-ethoxydiphenylborate, and βARK-ct but not by nifedipine or ryanodine. Similarly, insulin-dependent exofacial exposure of GLUT4-*myc*-enhanced green fluorescent protein was inhibited by bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid-AM and xestospongin C but not by nifedipine. Phosphatidylinositol 3-kinase and Akt were also required for the second phase of Ca^{2+} release and GLUT4 translocation. Transfected dominant-negative phosphatidylinositol 3-kinase γ inhibited the latter.

In conclusion, in primary neonatal cardiomyocytes, insulin induces an important component of Ca^{2+} release via IP_3 receptor. This component signals to glucose uptake via GLUT4, revealing a so-far unrealized contribution of IP_3-sensitive Ca^{2+} stores to insulin action. This pathway may influence cardiac metabolism in conditions yet to be explored in adult myocardium. **(*Endocrinology* 151: 4665–4677, 2010)**

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Abbreviations: 2-APB, 2-Amino-ethoxydiphenylborate; 2,4-DNP, 2,4-dinitrophenol; Ad, adenovirus; AM, acetoxymethyl ester compound; BAPTA, bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; $[Ca^{2+}]_i$, intracellular calcium; eGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; GLUT, glucose transporter; IP_3, inositol-1,4,5-trisphosphate; IP_3R, inositol-1,4,5-trisphosphate receptor; KD, kinase dead; LTCC, L-type calcium channel; myr, myristoylated; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PTX, pertussis toxin; RyR, ryanodine receptor; SER, sarcoendoplasmic reticulum; si, small interfering; TeTx, tetanus toxin; WT, wild type.

Cardiac muscle is an energy-consuming tissue that requires constant supply of oxygen and metabolic fuels to maintain contractile function. Under physiological conditions, fatty acids are major metabolic substrates for the heart. However, up to 30% of myocardial ATP is generated by glucose and lactate, with lesser contribution from ketones and amino acids. Although glucose is not the major metabolic substrate in the beating heart, it is paramount in several pathological conditions (1).

Insulin is an important regulator of cardiac size and metabolism, and the absence of insulin receptors impedes the switch of cardiac substrate utilization from glucose to fatty acids (2). Like skeletal muscle cells and adipocytes, most insulin actions in cardiomyocytes have been linked to activation of two canonical signaling pathways: the phosphatidylinositol 3-kinase (PI3K)-protein kinase Akt pathway and the Ras-MAPK pathway (3). In addition, a Cbl-CAP-TC10 pathway has also been described in cardiac muscle (4). Interestingly, the insulin receptor has also been reported to activate heterotrimeric G proteins in adipocytes and fibroblasts (5, 6).

Glucose enters cardiomyocytes via the SLC2 family of facilitative glucose transporters (GLUTs) (7). The most abundant GLUT in the heart is GLUT4, essential for insulin-dependent glucose uptake in this tissue (8). Insulin-dependent gains in GLUT4 at the cell surface have been detected in cardiac tissue by immunocytochemistry, subcellular fractionation, and surface photolabeling (9–12). In the heart, there is also significant expression of GLUT1, which under certain circumstances is responsible for a significant component of cardiac glucose uptake (13).

In skeletal muscle, insulin-dependent signals regulating GLUT4 translocation include IRS-1, class IA PI3K, Akt, AS160, and atypical protein kinase C (14). In contrast, beyond PI3K, the insulin signals regulating GLUT4 translocation and glucose uptake in cardiac cells remain to be defined (15). In this context, it is interesting to inquire whether intracellular calcium levels ($[Ca^{2+}]_i$) contribute to regulating glucose uptake. In adipocytes, interfering with $[Ca^{2+}]_i$ decreased insulin-stimulated glucose uptake and Akt phosphorylation (16), and, in skeletal muscle fibers, Ca^{2+} influx was found important for full stimulation of glucose uptake (17, 18). Surprisingly, despite the prominent regulation of Ca^{2+} in cardiomyocyte function (19), its participation in the metabolic actions of insulin in this tissue is unknown. In isolated adult cardiomyocytes, insulin stimulates voltage-activated L-type Ca^{2+} channels (LTCCs) (20) and Na^+-Ca^{2+} exchange (21), but whether this regulates glucose uptake has not been explored.

Cultured neonatal cardiomyocytes are an interesting *in vitro* model to study regulation of glucose uptake by insulin because, at approximately the perinatal period, the predominant metabolism of the heart shifts from non-oxidative glucose utilization to fatty acids oxidation (22). This shift is associated with changes in expression of a number of regulatory proteins of glucose and fatty acids metabolism (23, 24). Importantly, GLUT4 is already expressed in neonatal cardiomyocytes (12, 24) and represents up to 40% of the adult content (25). In these cells, we have observed a contribution of both IGF-1 and testosterone to calcium homeostasis (26, 27).

Here we show that, at physiological doses, insulin induces a transient $[Ca^{2+}]_i$ increase into neonatal rat cardiomyocytes, revealed by a fluorescent Ca^{2+} indicator. The signal has two components: one dependent on extracellular Ca^{2+} influx and a second one dependent on Ca^{2+} release from sarcoendoplasmic reticulum (SER) stores. Notably, the latter contributes to the insulin-dependent increase in glucose uptake through internal GLUT4 vesicle translocation and fusion with the plasma membrane. Finally, we established the participation of a G protein, PI3Kγ and 1,4,5-inositol-triphosphate (IP_3) receptor (IP_3R) axis in the mechanism leading to the Ca^{2+} signal and GLUT4-mediated increase in glucose uptake.

Materials and Methods

Animals

Rats were bred in the Animal Breeding Facility, Faculty of Chemical and Pharmaceutical Sciences, University of Chile (Santiago, Chile). Studies were approved by the Institutional Bioethical Committee, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (28).

Primary culture of neonatal rat cardiomyocytes

Cardiomyocytes were prepared from hearts of 1- to 3-d-old Sprague Dawley rats as described previously (29). Cultured cardiomyocytes were more than 95% pure based on β-myosin heavy chain expression. Serum was removed 24 h before insulin (Novo Nordisk Pharma, Hellerup, Denmark) stimulation. Under these conditions, cardiomyocytes do not show spontaneous Ca^{2+} oscillations.

Recombinant adenoviruses

Adenoviruses (Ad) for βARK-ct (AdβARK-ct), green fluorescent protein (GFP) (AdGFP), and an empty construct (AdEmpty) were a gift from Dr. W. J. Koch (Duke University, Durham, NC). AdβARK-ct leads to expression of the C-terminal portion of β-adrenergic receptor kinase that binds G$\beta\gamma$ subunits and behaves as dominant-negative inhibitor of G$\beta\gamma$ signaling (30). Transduction efficiency was more than 95% monitored with AdGFP.

Intracellular Ca^{2+} levels

$[Ca^{2+}]_i$ was determined in cardiomyocytes preloaded with fluo3-acetoxymethyl ester compound (AM) as described previ-

ously (26). Insulin was added directly to the microscope chamber, and fluorescence images were collected every 0.985 s and then analyzed with NIH ImageJ software.

Glucose uptake

Cardiomyocytes were rinsed twice with HEPES-buffered saline containing either 2 mM $CaCl_2$ or 2 mM EGTA as indicated and maintained in this condition for 1 h. Inhibitors were added during the last 30 min and insulin during the last 10 min. Ad transduction was performed 24 h before insulin stimulation. Glucose uptake was measured using 10 μM [^{3}H]2-deoxyglucose (31).

Transfections and immunofluorescence microscopy

Cardiomyocytes were transfected with 2 μg GLUT4-*myc*-enhanced GFP (eGFP) (32), wild-type (WT) PI3Kγ, kinase-dead (KD) PI3Kγ, myristoylated (myr) PI3Kγ, Akt-myr (donated by Dr. T. R. Jackson, University of Newcastle, Newcastle, UK), or tetanus toxin (TeTx) light chain (33) using 2 μl/ml Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfection efficiency was 7%. Type 2 IP_3R mRNA was knocked down using 100 nM of a mix of four different small interfering (si) IP_3R2 (Thermo Fisher Scientific, Waltham, MA) with 1 μl/ml DharmaFECT (Thermo Fisher Scientific). siRNA to the unrelated protein emerin (Sigma-Proligo) served as control. Plasmids or siRNA-treated cells were incubated 24 or 72 h before experiments, respectively. Cell surface GLUT4-*myc*-eGFP was determined as described previously (32), from the ratio of surface *myc* signal to total cellular eGFP fluorescence in each cell.

Membrane potential determinations

Membrane potential was recorded from single, cultured cardiomyocytes using the perforated patch configuration of the whole-cell recording technique. Patch electrodes were formed from thin-walled borosilicate capillary glass using a horizontal puller and filled with the following: 120 mM K-gluconic acid, 20 mM KCl, 8 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, and 240 mg/ml amphotericin B (pH 7.4), tip resistance of approximately 2.5 mW. Experiments were performed with the cells bathed in an extracellular solution containing the following (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, and 5.6 glucose (pH 7.4). The electrode was sealed onto the membrane of a cardiomyocyte with gentle suction, and membrane potential recordings began when the amphotericin B in the pipette had perforated the membrane under the patch to an access resistance less than 40 mW (usually 5–15 min). Membrane potential was recorded in current-clamp mode using an EPC-7 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany) and a Digidata 1200B data acquisition system (Molecular Devices, Sunnyvale, CA), at an acquisition rate of 200 samples/s. Insulin was added to the bath solution from a stock solution in H_2O, after recording basal membrane potential for at least 3 min. In some experiments, the amplifier was switched to voltage-clamp mode to record voltage-dependent membrane currents in response to families of depolarizing voltage pulses, before and after addition of insulin.

Statistical analysis

Results are expressed as mean ± SD of the number of independent experiments indicated (n) and subjected to ANOVA, and comparisons between groups were performed using a protected Tukey's *t* test assigning $P < 0.05$ as limit of statistical significance. Experiments illustrated are representative of assays performed on at least three separate occasions.

Results

Insulin induces a biphasic $[Ca^{2+}]_i$ increase in cultured cardiomyocytes

Changes in $[Ca^{2+}]_i$ were determined in cardiomyocytes preloaded with fluo3-AM. Quiescent cardiomyocytes, maintained in Ca^{2+}-containing media, exhibited basal intracellular Ca^{2+} levels with an average peak fluorescence of 24 ± 15 [ΔF/F] × 100 (n = 10) relative to the first 10 s mean basal reading (Supplemental Fig. 1, A and C, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), whereas in Ca^{2+}-free media, the basal peak fluorescence was 8 ± 1 [ΔF/F] × 100 (n = 10) (Supplemental Fig. 1, B and C). These values correspond to 5 min of recording.

In cardiomyocytes maintained in Ca^{2+}-containing media, insulin (10 and 100 nM) induced a transient $[Ca^{2+}]_i$ increase that exhibited two kinetically distinguishable phases (Fig. 1A). The first phase was a fast, almost instantaneous $[Ca^{2+}]_i$ increase reaching a maximal mean fluorescence intensity of 224 ± 23 [ΔF/F] × 100 (n = 4) within the first second of stimulation. The second phase was recorded as a plateau in $[Ca^{2+}]_i$ levels right after the first phase, with maximal mean fluorescence intensity of 144 ± 10 [ΔF/F] × 100 (n = 4) that lasted 26 ± 5 s before returning to basal fluorescence. In cardiomyocytes maintained in Ca^{2+}-free media, insulin (10 and 100 nM) induced a single-phase $[Ca^{2+}]_i$ increase that lasted 75 ± 6 s, with a maximum fluorescent intensity of 219 ± 38 [ΔF/F] × 100 (n = 4), at 7.8 ± 1.4 s after stimulation (Fig. 1B). To establish the concentration-response relationship, cardiomyocytes were stimulated with different concentrations of insulin in Ca^{2+}-containing and Ca^{2+}-free resting media (Supplemental Fig. 1D). Insulin at 0.01 nM did not increase $[Ca^{2+}]_i$, whereas 0.1–1 nM insulin induced the maximal increase of $[Ca^{2+}]_i$ in approximately 80% of cardiomyocytes analyzed. Insulin at 1 μM induced an increase of $[Ca^{2+}]_i$ in practically all the cells analyzed but showed different Ca^{2+} kinetics profile, possibly related with a nonspecific activation of other receptors (data not shown). We chose 10 nM insulin for all the following experiments because it is near physiological concentration that induced a consistent, large $[Ca^{2+}]_i$ response, ensuring specificity. In either case, the magnitude of these changes in $[Ca^{2+}]_i$ is comparatively smaller than those occurring during normal heart beat and hence would not be expected to modify cardiac function.

To confirm that insulin also caused release of Ca^{2+} from intracellular stores, we first depleted SER Ca^{2+} using

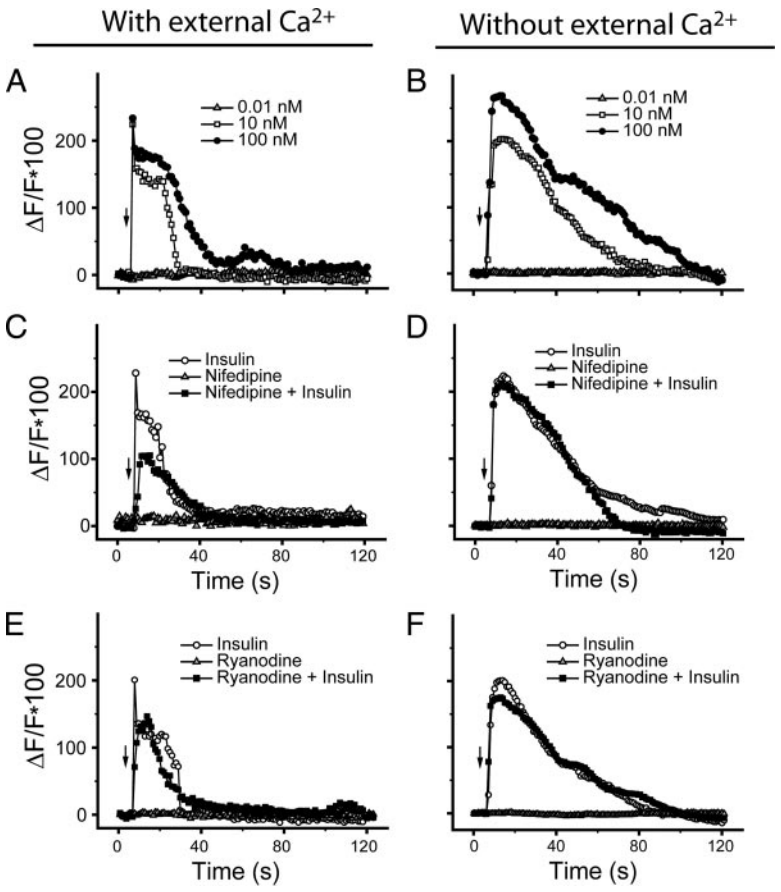

FIG. 1. Insulin increases $[Ca^{2+}]_i$ in neonatal rat cardiomyocytes by a Ca^{2+} influx, followed by a Ca^{2+} release from intracellular stores. Changes in $[Ca^{2+}]_i$ were investigated in individual, fluo3-AM-loaded cells maintained in medium with or without Ca^{2+}. A, B, $[Ca^{2+}]_i$ changes evoked by 0.01 nM (△), 10 nM (□), and 100 nM (■) insulin. C, D, Cardiomyocytes exposed to 1 μM nifedipine 5 min before and during insulin stimulation. E, F, Cardiomyocytes were preincubated with ryanodine (50 μM) for 1 h, and fluo3-AM was added in the final 30 min. A, C, E, Fluorescence of cells in Ca^{2+}-containing medium. B, D, F, Fluorescence of cells in Ca^{2+}-free medium. Results are expressed as relative total fluorescence [ratio of fluorescence difference ($F - F_o$), to basal value (F_o)] × 100 as a function of time and are representative of five independent experiments in which at least 60 cells were analyzed in each case. Response of cardiomyocytes preincubated with fluo3-AM (5.4 μM) for 30 min and throughout insulin (10 nM) stimulation.

thapsigargin (a SER Ca^{2+}-ATPase inhibitor). In the absence of extracellular Ca^{2+}, thapsigargin induced a rapid increase in intracellular fluorescence. Subsequent addition of caffeine or insulin did not evoke any increase in $[Ca^{2+}]_i$ levels (Supplemental Fig. 1E). These results indicate that the insulin-induced $[Ca^{2+}]_i$ gain in cardiomyocytes maintained in Ca^{2+}-free media is mainly attributable to Ca^{2+} release from thapsigargin-sensitive internal stores. To test whether the effect of 10 nM insulin was through its receptors, cardiomyocytes were preincubated with genistein (tyrosine kinase receptor inhibitor). Genistein blocked the insulin-dependent increase in $[Ca^{2+}]_i$ in both the presence or absence of extracellular Ca^{2+} (Supplemental Fig. 1F). These data suggest that the increase in $[Ca^{2+}]_i$ induced by insulin depend on the activation of insulin receptor.

The first phase of $[Ca^{2+}]_i$ rise involves extracellular Ca^{2+} influx through L-type Ca^{2+} channels and Ca^{2+} release through ryanodine receptors

To begin mapping the pathways of Ca^{2+} influx into the cytosol, we used well-defined molecular inhibitors or activators of distinct channels. Nifedipine (1 μM), an exclusive inhibitor of LTCCs at low concentrations, virtually blocked the first phase of insulin-stimulated $[Ca^{2+}]_i$ transient in cardiomyocytes incubated in Ca^{2+}-containing media (Fig. 1C). Conversely, the more delayed insulin-induced $[Ca^{2+}]_i$ increase observed was insensitive to nifedipine (Fig. 1D), confirming distinct participation of intracellular Ca^{2+}stores in this process.

To determine the possible role of plasma membrane depolarization in the insulin-induced intracellular fast Ca^{2+} rise observed in cardiomyocytes, we measured membrane potential from individual cultured cardiomyocytes using the perforated patch recording method. Under basal conditions, the majority of cardiomyocytes fired simple or compound action potentials of variable frequency and duration from a hyperpolarized resting potential (Supplemental Fig. 2A, *left* and *middle panels*). The remaining cells displayed a stable, hyperpolarized membrane potential and fired very few action potentials (Supplemental Fig. 2A, *right panel*). Insulin induced a transient depolarization and volley of action potentials, after which the membrane potential returned to its baseline level (Supplemental Fig. 2B). Another type of response observed was a gradual but sustained depolarization of the membrane, reaching levels between −40 and −20 mV (data not shown). Cardiomyocytes displaying this response ceased firing action potentials, probably as a result of inactivation of voltage-dependent sodium channels at the depolarized membrane potentials. These responses were not artifacts caused by mechanical disruption of the preparation during the addition of solution to the static bath, because such responses were not seen when vehicle alone was added to the bath solution (n = 6). These results support the idea that the nifedipine-sensitive, fast Ca^{2+} component induced by insulin is attributable to calcium influx

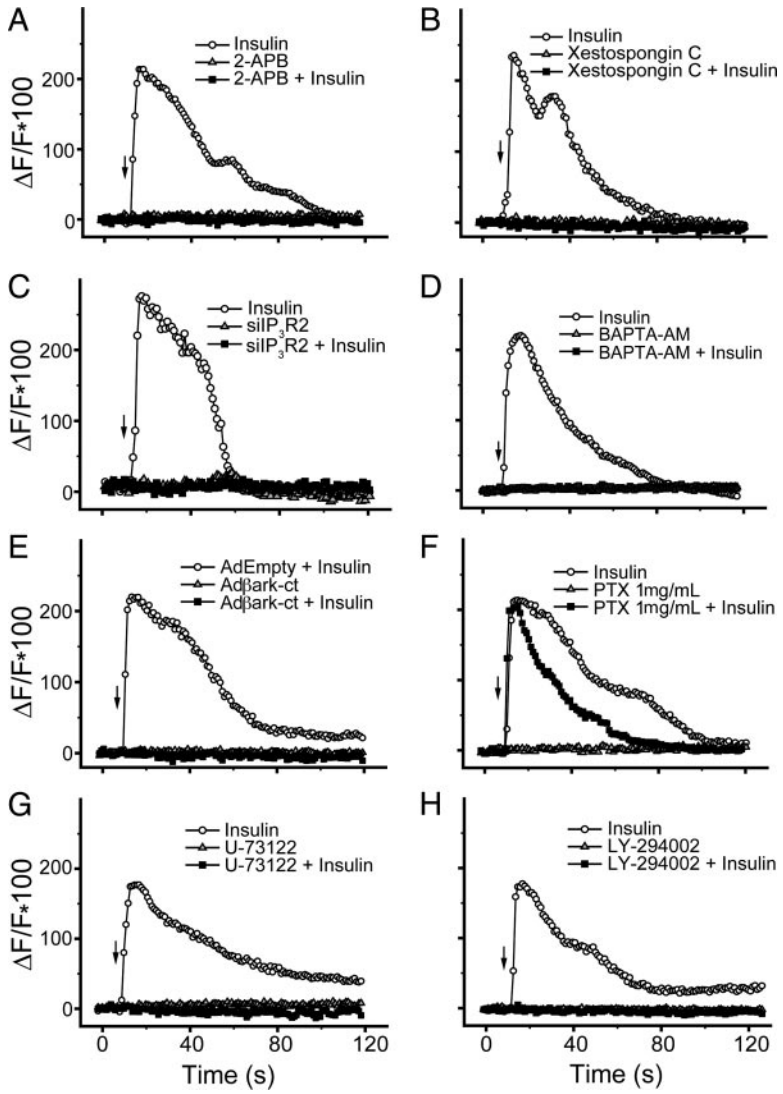

FIG. 2. The second phase of insulin-stimulated Ca^{2+} increase involves Ca^{2+} release through the IP_3R, trimeric G protein, PLC, and PI3K. Cardiomyocytes were analyzed in Ca^{2+}-free media with the respective inhibitors and then stimulated with 10 nM insulin. Fluo3-AM and corresponding inhibitors were added during preincubation and during stimulation. Fluorescence images were acquired, and relative fluorescence was calculated as described in Figure 1. A, 2-APB (20 μM); B, xestospongin C (100 μM); C, siRNA to rat type 2 IP_3R (100 nM); D, BAPTA-AM (50 μM); E, Ad-βARKct (multiplicity of infection of 300) for 24 h; F, PTX (1 μg/ml, preincubated by 1 h); G, U-73122 (50 μM); H, LY-294002 (50 μM). The results are representative of five independent experiments, and at least 60 cells were analyzed.

through LTCCs after plasma membrane depolarization of cardiomyocytes.

There are two major routes of Ca^{2+} efflux from SER, one mediated by a channel inhibitable by ryanodine [channel hence termed ryanodine receptor (RyR)], the other by a channel activated by IP_3 (IP_3R). The RyR is tightly coupled to activation by surface LTCC for excitation-contraction coupling (34). In cells preincubated with ryanodine (50 μM) and then placed in Ca^{2+}-containing media, the peak of first-phase insulin-induced $[Ca^{2+}]_i$ transient was both reduced and delayed (Fig. 1E). These results suggest that the first phase of the insulin-induced $[Ca^{2+}]_i$ transient requires both nifedipine-sensitive entry of external Ca^{2+} (presumably through LTCCs) and coupled ryanodine-sensitive Ca^{2+}-induced Ca^{2+} release. Importantly, ryanodine did not affect the $[Ca^{2+}]_i$ transient evoked by insulin in cardiomyocytes in Ca^{2+}-free media (Fig. 1F).

The second phase of insulin-dependent $[Ca^{2+}]_i$ rise is mediated by Ca^{2+} efflux through IP_3R

From Fig. 1, it emerges that the second phase of insulin-dependent $[Ca^{2+}]_i$ transient occurs through Ca^{2+} release into the cytosol from intracellular stores but is not mediated by RyR. To evaluate Ca^{2+} release through IP_3R channels, we used two well-described inhibitors, 2-amino-ethoxydiphenylborate (2-APB) and xestospongin C (26), as well as knockdown of the type 2 IP_3R via siRNA (for knockdown efficiency, see Supplemental Fig. 3). Remarkably, all three maneuvers completely inhibited the insulin-induced $[Ca^{2+}]_i$ increase (Fig. 2, A–C). These results suggest that the second phase of insulin-dependent $[Ca^{2+}]_i$ increase is mediated by type 2 IP_3R-gated Ca^{2+} channels. The intracellular Ca^{2+}-chelator bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA)-AM (Fig. 2D) completely suppressed the insulin-induced $[Ca^{2+}]$ increase, confirming that the signal arises from changes in the levels of this cation.

The second phase of insulin-dependent $[Ca^{2+}]_i$ rise is signaled via trimeric G proteins, phospholipase C, and PI3K

The canonical pathways that activate IP_3R are initiated by membrane-bound receptors that engage trimeric G proteins, whose $\beta\gamma$ subunits in turn activate downstream effectors, including phospholipase C (PLC). This latter enzyme generates IP_3, the agonist that opens the IP_3-ligated Ca^{2+}channels (*i.e.* IP_3R) from the endoplasmic reticulum. To explore whether a similar pathway operates in cardiomyocytes stimulated with insulin, we tested the effect of diverse strategies that inhibit $\beta\gamma$ and PLC on the insulin-dependent second phase of the $[Ca^{2+}]_i$ transient.

Overexpression of βARK-ct (inhibitor of $\beta\gamma$ subunits) effectively abolished the insulin-induced $[Ca^{2+}]_i$ transient

in Ca^{2+}-free media (Fig. 2E). However, pertussis toxin (PTX) (an inhibitor of $G_{i/o}$) only partially inhibited the insulin-induced $[Ca^{2+}]_i$ increase (Fig. 2F). These observations suggest that $\beta\gamma$ subunits (dissociated from a heterotrimeric G protein) play a critical role in insulin-induced $[Ca^{2+}]_i$ increase, but such heterotrimeric G protein is unlikely to be exclusively $G_{i/o}$.

To determine whether insulin-stimulated $[Ca^{2+}]_i$ transient involves activation of PLC, we preincubated fluo3-AM preloaded cardiomyocytes with U-73122 [1-[6[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino] hexyl]-1*H*-pyrrole-2,5-dione] (PLC inhibitor, 10 μM) and then stimulated with insulin. Under these conditions, the insulin-dependent second-phase $[Ca^{2+}]_i$ transient was totally eliminated (Fig. 2G), supporting the participation of PLC in this response.

The results in Fig. 3 reveal a novel pathway from the insulin receptor to eventual activation of a putative G protein whose $\beta\gamma$ subunits and PLC contribute to opening IP_3R. Given that insulin classically activates PI3K, we asked whether this enzyme participates in the insulin-dependent Ca^{2+} influx into the cytosol. The second-phase $[Ca^{2+}]_i$ transient was obliterated by LY-294002 [2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one] (Fig. 2H), raising the possibility that such PI3K may be PI3Kγ, a known upstream activator of PLC (35) (see below).

The above experiments were performed in Ca^{2+}-free medium for easier visualization of the second phase, having eliminated the first phase of Ca^{2+} influx. Parallel experiments in Ca^{2+}-containing media rendered comparable results (Supplemental Fig. 4). To investigate whether the parallel, nonrelated signaling pathways are still functional in the presence of inhibitors, we tested the membrane depolarization and excitation-contraction coupling induced by KCl in cardiomyocytes maintained in Ca^{2+}-containing media. Cells preincubated for 30 min with LY-294002, U-73122, or xestospongin C were stimulated with 50 mM KCl. In all conditions, KCl induced a fast increase of $[Ca^{2+}]_i$ and showed Ca^{2+} oscillations related with a Ca^{2+}-induced Ca^{2+}-release response (Supplemental Fig. 5, A–C). These results suggest that unrelated signaling pathways are perfectly operative when the insulin-dependent Ca^{2+} signals are blocked.

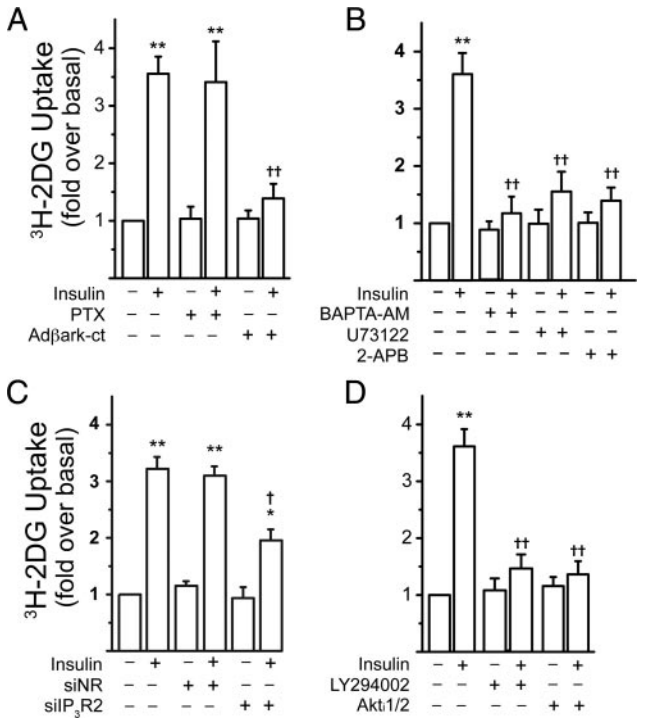

FIG. 3. Participation of G$\beta\gamma$, PI3K, PLC, and IP_3R in insulin-induced glucose uptake. Glucose uptake was measured in cardiomyocytes as described in *Materials and Methods*, in the absence of external Ca^{2+}. A, Cells were transduced with AdβARK-ct (multiplicity of infection of 300) for 24 h or preincubated for 1 h with PTX (1 μg/ml), and increases in glucose uptake induced by insulin (10 nM) were markedly inhibited, as well as the preincubation with BAPTA-AM (50 μM), U-73122 (10 μM), or 2-APB (20 μM) (B). Knockdown of type 2 IP_3R with a specific siRNA (siIP_3R2) decreased significantly the insulin response *vs.* control (nonrelated siRNA, siNR) (C), LY-294002 (50 μM), and $Akt_i1/2$ (10 μM) given 30 min before insulin (10 nM) abolished the response of glucose uptake (D). Values represent the mean ± SD of four different experiments. *, $P > 0.05$ and **, $P < 0.01$ *vs.* control; †, $P < 0.05$; and ††, $P < 0.01$ *vs.* insulin.

IP_3-dependent Ca^{2+} release is required for glucose uptake and GLUT4 translocation in cardiomyocytes

Although there is evidence in both adipocytes and skeletal muscle that Ca^{2+} may play a role in insulin stimulation of glucose uptake (16, 17), the underlying mechanisms are unresolved, and moreover it is not known whether this occurs in cardiomyocytes. Uptake of [^{3}H]2-deoxyglucose was significantly elevated by insulin in cells incubated in Ca^{2+}-containing or Ca^{2+}-free media. Glucose uptake increased during the first minutes of insulin stimulation, reaching values 3.2 ± 0.2 and 3.5 ± 0.3 times higher than the control, in the presence and absence of extracellular Ca^{2+}, respectively (Supplemental Fig. 6A). Cytochalasin B markedly reduced insulin-stimulated glucose uptake in both extracellular Ca^{2+} conditions (Supplemental Fig. 6A), confirming that glucose uptake occurred through GLUT-mediated transporters. Indinavir (100 μM), a blocker of glucose influx through GLUT4 (36), inhibited insulin-stimulated glucose uptake to reach basal values, suggesting that GLUT4 is the preeminent pathway for glucose uptake in insulin-stimulated cardiomyocytes (Supplemental Fig. 6A).

Neither nifedipine nor ryanodine affected the insulin-dependent stimulation glucose uptake, whether in Ca^{2+}-containing or Ca^{2+}-free media, suggesting that stimulation does not require extracellular Ca^{2+} influx or Ca^{2+} release through RyR (Supplemental Fig. 6B). Notably, however, insulin-stimulated glucose uptake was com-

pletely inhibited by BAPTA-AM, AdβARK-ct, U-73122, and 2-APB and was also partially inhibited by siRNA to type 2 IP_3R (Fig. 3, A–C). These results strongly suggest that insulin-dependent stimulation of glucose uptake requires the second-phase Ca^{2+} transient, which was similarly sensitive to these selective inhibitors of the G$\beta\gamma$-PLC-IP_3R axis. Interestingly, the insulin-dependent stimulation of glucose uptake was not inhibited by PTX (Fig. 3A). The results illustrated refer to cells in Ca^{2+}-free media, and similar results were obtained in the presence of external Ca^{2+} (data not shown). These findings suggest that glucose uptake depends on a signaling pathway involving G$\beta\gamma$, PLC, type 2 IP_3R, and Ca^{2+}.

Hence, a Ca^{2+}-dependent signal is fundamental for the insulin-dependent stimulation of glucose uptake into cardiomyocytes. This response requires PI3K and its downstream target Akt in adipose and skeletal muscle cells, and we confirmed its role in cardiomyocytes (Fig. 3D). The observation that both G$\beta\gamma$-PLC-IP_3R-Ca^{2+} and PI3K-Akt inputs are required raised the question whether Ca^{2+} might influence Akt activation. Indeed, although insulin increased Akt phosphorylation in cardiomyocytes maintained in the presence or absence of extracellular Ca^{2+}, U-73122 and BAPTA-AM decreased the insulin-induced Akt phosphorylation in Ca^{2+}-free media (Fig. 4A). Moreover, the IP_3R inhibitor 2-APB precluded insulin-dependent Akt phosphorylation (Fig. 4B). These data reveal that the second phase of the $[Ca^{2+}]_i$ transient induced by insulin is required to allow Akt activation, which also requires classical input from PI_3K (given its inhibition by LY-294002).

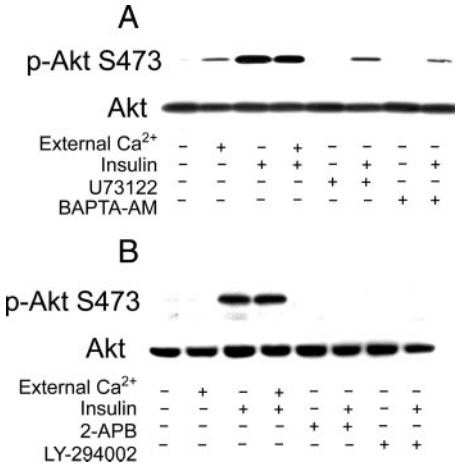

FIG. 4. Activation of Akt induced by insulin is dependent on the intracellular Ca^{2+} rise. Cell lysates were separated by 10% SDS-PAGE and electrotransferred to nitrocellulose, blocked with 5% BSA, Tris-buffered saline (pH 7.6), and 0.1% Tween 20, then incubated with phospho-Akt, total-Akt, type 2 IP_3R, or β-actin antibodies (1:1000 in blocking buffer), and revealed with horseradish peroxidase-linked secondary antibody (1:5000). Bands were detected using ECL and quantified by densitometry. Akt phosphorylation at Ser473 induced by 10 nM insulin for 10 min was independent of extracellular Ca^{2+} and was reduced by U-73122 (10 μM) or BAPTA-AM (50 μM) (A) and by 2-APB (20 μM) or LY-294002 (50 μM) (B). Results shown are representative of three independent experiments.

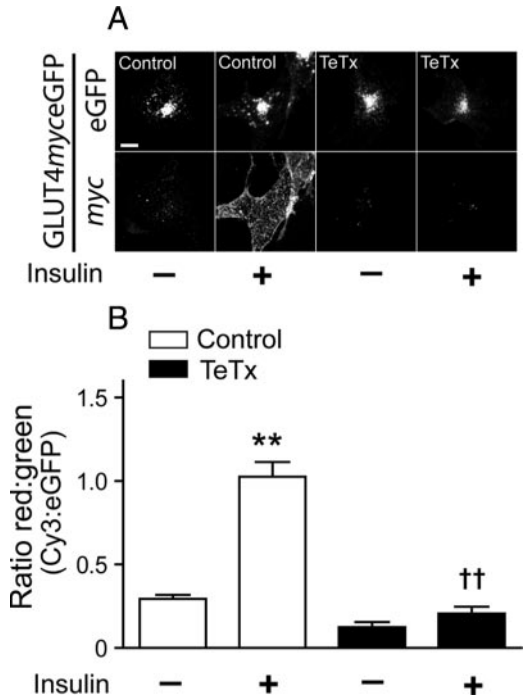

FIG. 5. VAMP-2 is required for insulin-dependent exofacial exposure of *myc* epitope in cultured cardiomyocytes. Cells were transiently cotransfected with GLUT4-*myc*-eGFP and TeTx construct for 24 h (A, B) and stimulated with 10 nM insulin for 10 min in Ca^{2+}-free medium. The effect of light chain of TeTx in insulin-dependent exofacial exposure of *myc* epitope is showed in single cells (A) and quantified (B). Values represent the average ± SD of four different experiments. **, $P < 0.01$ *vs.* basal condition. ††, $P < 0.01$ *vs.* maximal response.

Ultimately, mechanistic information on the stimulation of glucose uptake requires demonstration of regulation of GLUT4 translocation. To evaluate the participation of cytosolic Ca^{2+} in the process of GLUT4 exposure at the plasma membrane, cardiomyocytes were transiently transfected with a cDNA encoding a GLUT4-*myc*-eGFP chimera. Insulin caused a notable and consistent 2-fold to 3-fold gain in surface GLUT4-*myc* at the cell surface. To demonstrate that insulin-dependent exofacial exposure of *myc* epitope relies on fusion of GLUT4-containing vesicles, the involvement of the vesicle soluble N-ethylmaleimide sensitive factor attachment protein receptor vesicle-associated membrane protein 2 (VAMP-2) was tested. Transfection of TeTx light chain (which proteolyses VAMP-2) completely abrogated the GLUT4 exposition (Fig. 5).

In nonpermeabilized cells maintained in Ca^{2+}-free medium, insulin induced a significant increase in exofacial exposure of the *myc* epitope. Nifedipine did not alter this response, and ryanodine only slightly decreased the insulin effect from 0.95 ± 0.04 to 0.70 ± 0.07 (normalized cyanine 3/eGFP ratio) (Fig. 6, A and B). In contrast, major reductions in insulin-induced surface gain in GLUT4 were provoked by BAPTA-AM, $Akt_i1/2$, or the IP_3R blocker xestospongin C.

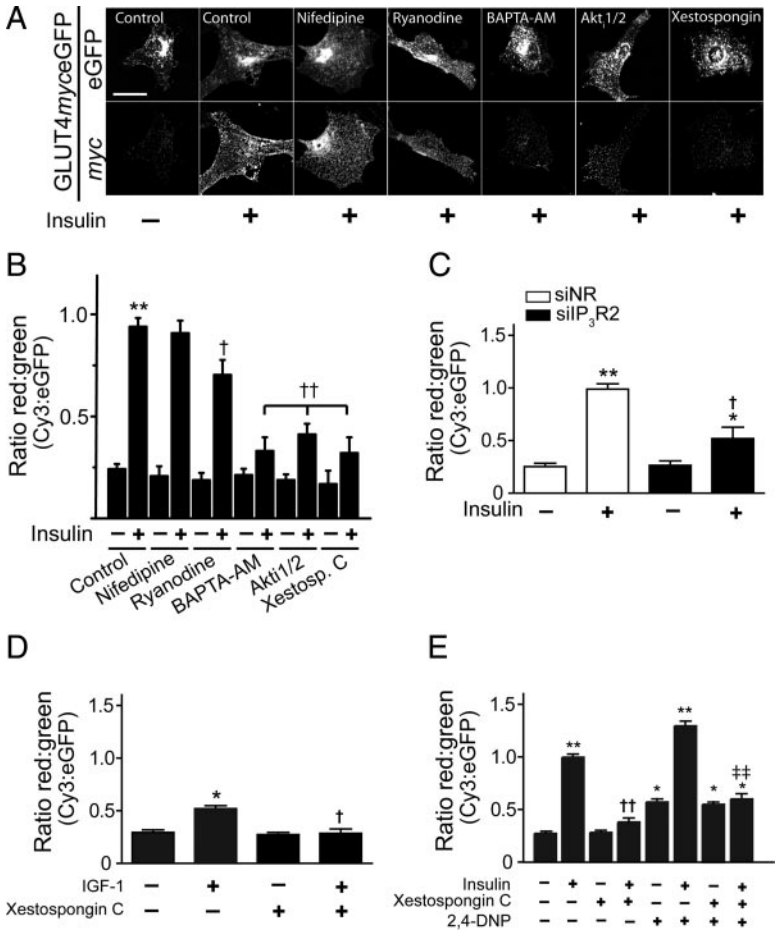

FIG. 6. Insulin-induced exofacial exposure of GLUT4-*myc*-eGFP depends on $[Ca^{2+}]_i$, Akt, and IP_3R type 2 activation. Cells were transiently transfected with GLUT4-*myc*-eGFP cDNA for 24 h (A, B) or cotransfected with GLUT4-*myc*-eGFP and siRNA type 2 IP_3R (siIP_3R2) or nonrelated siRNA (siNR) for 72 h (C, D) and stimulated with 10 nM insulin for 10 min in Ca^{2+}-free medium. Extracellular exposure of the *myc* epitope was detected by immunofluorescence in nonpermeabilized cells as described in *Materials and Methods*. Insulin induced an increase in cyanine 3/eGFP ratio independently of extracellular Ca^{2+} presence. Cardiomyocytes were preincubated with nifedipine (1 μM) for 5 min, with ryanodine (50 μM) for 1 h, or with BAPTA-AM (50 μM), $Akt_i1/2$ (10 μM), or xestospongin C (100 μM) for 30 min, before insulin stimulation (A). The quantification was calculated as described in *Materials and Methods* (B). The effect of knockdown of type 2 IP_3R in insulin-dependent exofacial exposure of *myc* epitope is showed in single cells (C) and quantified (D). Values represent the average ± SD of four different experiments. **, $P < 0.01$ *vs.* control basal conditions. †, $P < 0.05$ and ††, $P < 0.01$ *vs.* insulin-stimulated control condition (siNR). D, IGF-1 (10 nM) for 10 min induced a modest increase in exofacial exposure of *myc* epitope that was inhibited by xestospongin C (100 μM). E, 2,4-DNP (0.5 mM) for 10 min induced an increase in exofacial exposure of *myc* epitope that was insensitive to xestospongin C (100 μM) pretreatment. Values represent the average ± SD of four different experiments. *, $P < 0.05$ and **, $P < 0.01$ *vs.* basal conditions. †, $P < 0.05$ and ††, $P < 0.01$ *vs.* insulin or IGF-1-stimulated condition. ‡‡, $P < 0.01$ *vs.* insulin plus 2,4-DNP stimulated cardiomyocytes.

Moreover, specific knockdown of type 2 IP_3R by siRNA also markedly decreased the exofacial exposure of *myc* epitope induced by insulin (Fig. 6C). These observations strongly support a role of Ca^{2+} release through type 2 IP_3R in GLUT4 translocation and insertion into the cardiomyocyte plasma membrane. To determine whether this mechanism is specific to insulin pathways, we studied the effect of IGF-1 and 2,4-dinitrophenol (2,4-DNP) on the exofacial exposure of myc epitope. IGF-1 (10 nM) induced a small increase in exofacial exposure of GLUT4-*myc*, and exposure was completely inhibited by xestospongin C (Fig. 6D). Conversely, 2,4-DNP increased exofacial exposure of GLUT4-*myc* that was not inhibited by xestospongin C in cardiomyocytes maintained in Ca^{2+}-free resting media (Fig. 6E). These results suggest that, although free cytosolic Ca^{2+} by itself triggers translocation/fusion of GLUT4 with the plasma membrane, the IP_3R-dependent release participate in specific pathways aimed to that purpose.

Finally, the involvement of $\beta\gamma$ subunits of a heterotrimeric G protein in GLUT4 translocation raised the possible participation of the γ isoform of PI_3K in this process. To test this hypothesis, cardiomyocytes were cotransfected with GLUT4-*myc*-eGFP and WT-PI3Kγ, KD-PI3Kγ, or myr-PI3Kγ. Remarkably, the non-active form of PI3Kγ decreased insulin-dependent exofacial exposure of *myc* epitope, whereas WT-PI3Kγ had no effect (Fig. 7, A and B). Moreover, myr-PI3Kγ caused a small, if not statistically significant, elevation of the basal level of surface GLUT4-*myc* and also failed to inhibit insulin-mediated GLUT4-*myc* translocation (Fig. 7C). As positive control, myr-Akt was shown to produce the expected gain in surface GLUT4-*myc* in the absence of insulin (Fig. 7D). Together, these results support the participation of PI3Kγ in the mechanism of GLUT4 membrane translocation and insertion induced by insulin.

Discussion

Here we show a novel, insulin-induced transient increase in $[Ca^{2+}]_i$ levels in neonatal rat cardiomyocytes. This Ca^{2+} transient consists of a first phase of extracellular Ca^{2+} influx and a second phase of SER Ca^{2+} release into the cytosol. The latter appears to be a physiologically relevant signal and can be studied in isolation in the absence of external Ca^{2+}. This second phase of insulin-induced $[Ca^{2+}]_i$ increase involves $\beta\gamma$ subunit of a heterotrimeric G protein, PI3Kγ, PLC, and type 2 IP_3R channels. We speculate that these signals act as a linear sequence of events,

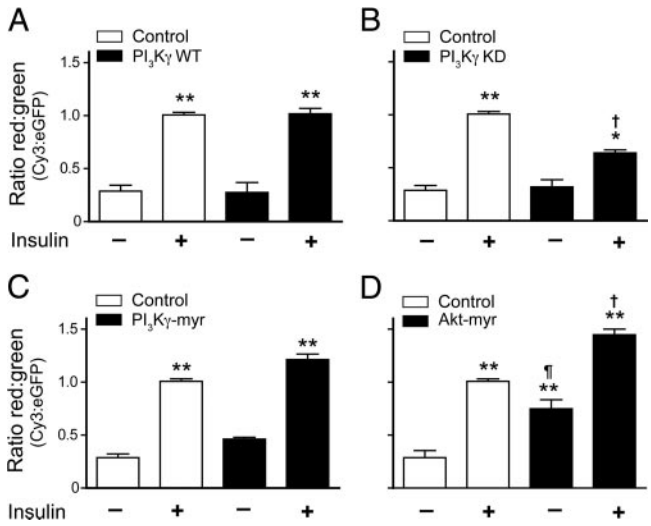

FIG. 7. Insulin-dependent exofacial exposure of GLUT4-*myc* requires the PI3Kγ isoform. Cells were transiently cotransfected with GLUT4-*myc*-eGFP and WT-PI3Kγ (A) or KD-PI3Kγ (B), or myr-PI3Kγ (C) or myr-Akt (D) constructs for 24 h and stimulated with 10 nM insulin for 10 min in Ca^{2+}-free medium. KD-PI3Kγ decreased significantly the insulin-dependent exofacial exposure of *myc* epitope as showed in single cells. Values represent the mean ± SD of four different experiments. *, $P <$ 0.05 and **, $P < 0.01$ *vs.* control; †, $P < 0.05$ *vs.* basal conditions; ¶, $P < 0.05$ *vs.* maximal response.

as summarized in Fig. 8. We further show that each of the above elements is required for insulin-dependent GLUT4 translocation, revealing an unrealized connection between IP_3R-mediated Ca^{2+} release and insulin action. The intersection between Ca^{2+} and the canonical PI3K-Akt insulin pathway may occur at several levels, including Ca^{2+}-dependent facilitation of Akt activation, and potentially vesicle traffic and/or fusion with the membrane (Fig. 8). These conclusions are expanded below.

Ca^{2+} entry pathways into the cytosol

The $[Ca^{2+}]_i$ debate in insulin action has centered around the difficulty in detecting changes in the levels of the cation in response to the hormone. Whereas ratiometric dyes sampling the entire cytosol have failed to detect any rise in $[Ca^{2+}]_i$ in response to insulin in 3T3-L1 adipocytes (37), L6 myotubes (38), and isolated skeletal muscle (39), the hormone activated the signal of a membrane-associated Ca^{2+} sensor (FIP18) in the latter system (39). Here we show that fluo3, a dye with dynamic range higher than the ratiometric dye indo-1, clearly detected $[Ca^{2+}]_i$ transients in the cytosol of neonatal cardiomyocytes. This suggests that, in these cells, the change in $[Ca^{2+}]_i$ may be more pronounced than in the other cell types and/or that fluo3 can reveal $[Ca^{2+}]_i$ transients more effectively than ratiometric dyes.

The first phase of insulin-dependent $[Ca^{2+}]_i$ increase in cultured rat cardiomyocytes involves Ca^{2+} influx through LTCCs, because this rapid effect was totally prevented by both Ca^{2+}-free/EGTA-containing resting solution and nifedipine. The inhibition by ryanodine further suggests involvement of a Ca^{2+}-induced Ca^{2+}-release mechanism, *i.e.* Ca^{2+} influx through the LTCC activates the RyR which in turn release Ca^{2+} from the SER. This mechanism participates in normal cardiac muscle contraction, albeit producing comparatively larger elevations in $[Ca^{2+}]_i$. The changes in $[Ca^{2+}]_i$ produced by insulin are clearly insufficient to drive contraction.

In the heart, IP_3 is generated through the action of distinct PLCs on phosphatidylinositol 4,5-bisphosphate (40, 41). Some neurohumoral agonists (*e.g.* acetylcholine, endothelin, catecholamines, prostaglandins) activate a G_q-dependent PLCβ (42, 43). In contrast, purines or angiotensin II stimulate a tyrosine kinase-dependent PLCγ (40, 44). IP_3 induces slow release of Ca^{2+} from vesicular preparations and activates contraction in skinned ventricular rat muscle and chick heart preparations (45). In the adult rat, ventricular (46) and atrial (47) myocytes express mainly type 2 IP_3Rs, and all isoforms of IP_3R have been identified in neonatal rat cardiomyocytes, with individual intracellular localization (26). The $[Ca^{2+}]_i$ increase induced by insulin reported here was inhibited by the expression of βARK-ct, LY-294002, and type 2 IP_3R knockdown, suggesting participation of Gβγ subunits from heterotrimeric G protein as well as the PI_3K and type 2 IP_3R.

Participation of Gβγ and PI3Kγ in insulin action in cardiomyocytes

According to the canonical insulin signaling pathway, insulin binding to its receptor activates class I PI3K (catalytic p110 α and β subunits) through binding of the regulatory p85 subunit to insulin receptor substrate proteins, a mechanism that is independent of G protein action. However, βγ subunits of heterotrimeric G proteins can activate PI_3Kγ by direct interaction with two domains of the catalytic p110γ subunit (48). During pressure load hypertrophy, PI3K is activated by a Gβγ-dependent process in mice (49). PI3Kγ is crucial for the purinergic regulation of spontaneous Ca^{2+} spiking in rat cardiomyocytes, in which LY-294002 prevented membrane translocation of PLCγ and IP_3 generation (50). By analogy, we hypothesize that insulin might activate PI_3K (p110γ) through βγ subunits of a heterotrimeric G protein. In support of this hypothesis, p110γ physically associates with and phosphorylates $G\alpha_{q/11}$ in fibroblasts overexpressing insulin receptors (5). This may explain the inhibition of insulin-induced $[Ca^{2+}]_i$ increase and GLUT4 translocation caused by the PI3K inhibitor LY-294002, as well as the inhibition of GLUT4 translocation by KD-PI3Kγ.

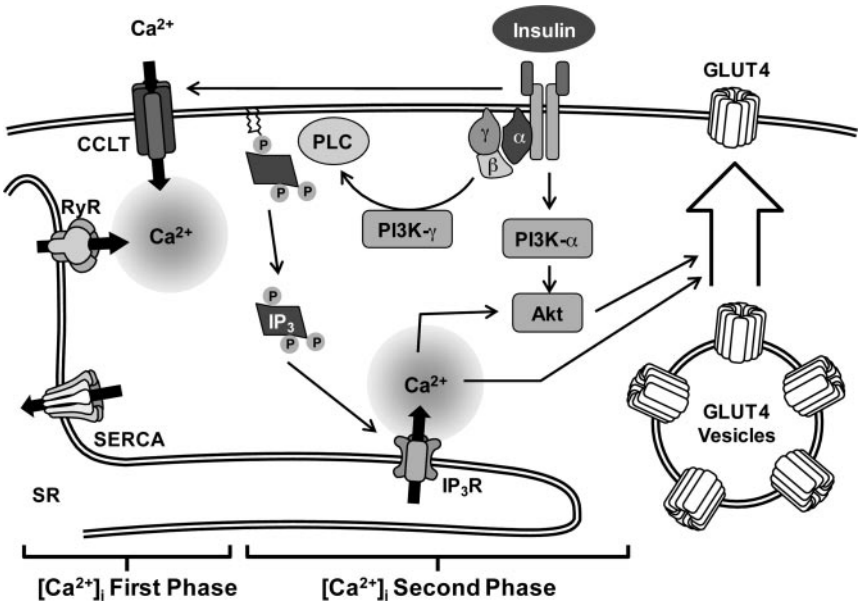

FIG. 8. Proposed model. Insulin binding to its receptor activates a biphasic $[Ca^{2+}]_i$ response. The first phase involves the LTCC activation, extracellular Ca^{2+} influx, activation of RyR Ca^{2+} channel, and Ca^{2+} release from SER. The second phase involves a non-inhibitory G protein-coupled receptor, the $G\beta\gamma$ subunits that activates the lipid-kinase PI3Kγ to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2). This is a strong signal for recruitment of the pleckstrin homology domain of PLC, leading to its binding to the inner side of the cell surface. PLC then converts PIP_3 to IP_3 and diacylglycerol. IP_3 activates the IP_3R, leading to calcium release from SER. This Ca^{2+} is involved in GLUT4 vesicle traffic and/or fusion with the cell surface, finally producing an increase in glucose uptake. CCLT, L-type Ca^{2+} channels; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoendoplasmic reticulum.

Participation of Ca^{2+} released via IP_3R in insulin-dependent glucose uptake and GLUT4 translocation

Here we show that insulin induces an increase in glucose uptake in cardiomyocytes maintained in both Ca^{2+}-containing and Ca^{2+}-free resting media, suggesting that the effect of insulin is independent of extracellular Ca^{2+}. Extracellular Ca^{2+} also does not regulate glucose uptake in isolated working heart (51). Although that study speculated that cytoplasmic Ca^{2+} may not contribute to the regulation of glucose uptake in working conditions, our results clearly show that $[Ca^{2+}]_i$ is required for insulin-induced glucose uptake in cardiomyocytes, and this effect seems to be independent of excitation-contraction $[Ca^{2+}]_i$ signals.

The participation of $[Ca^{2+}]_i$ in insulin-dependent stimulation of glucose uptake has been debated in studies using primary and cultured adipocytes, L6 myotubes, and isolated skeletal muscle. Early studies in rat adipocytes showed that, by changing extracellular Ca^{2+}, a window of $[Ca^{2+}]_i$ ensued that was permissive for insulin-stimulated glucose uptake (52), and that beyond a narrow value of 140–270 nM, $[Ca^{2+}]_i$ instead correlated with insulin resistance (53). In 3T3-L1 adipocytes, as shown here for cardiomyocytes, BAPTA-AM reduced the stimulation of Akt and the gain in surface GLUT4 (16, 54). Although neither study determine whether the hormone increases the levels of $[Ca^{2+}]_i$ or merely requires the resting $[Ca^{2+}]_i$ levels, they both showed inhibition of Akt phosphorylation, a finding we have corroborated in neonatal cardiomyocytes. Worrall and Olefsky (54) proposed that $[Ca^{2+}]_i$ has negative input in insulin signals proximal to the receptor and a positive input on more distal ones, including Akt. Whitehead *et al.* (16) further proposed two possible positive inputs for the cation based on the use of BAPTA-AM and Ca^{2+} ionophores: one allowing the transporter to reach the plasma membrane and another one allowing its fusion with the membrane. This was ascertained from comparisons of the levels of GLUT4 associated with membrane lawns and the extent of hemagglutinin-GLUT4 epitope exposure to the outer medium. The authors presented a lucid discussion of potential molecules affected, and to date these remain attractive Ca^{2+} targets. Finally, that study found that BAPTA-AM directly inhibited glucose uptake in adipocytes, consistent with the high susceptibility of GLUT4 to inhibition by very diverse chemical agents, which however does not invalidate the effects of BAPTA-AM on GLUT4 traffic. In contrast to these findings, a less effective chelator of intracellular $[Ca^{2+}]_i$, Quin2-AM (2-[(2-bis[carboxymethyl] amino-5-methylphenoxy]-6-methoxy-8-bis[carboxymethyl]-aminoquinolinetetrakis acetoxymethyl ester), did not reduce glucose uptake in L6 myotubes or 3T3-L1 adipocytes (37, 38), and dantrolene, an inhibitor of Ca^{2+} release from the SER through RyRs, did not prevent GLUT4 translocation in L6 myotubes expressing GLUT4-*myc* (55). Finally, in rat skeletal muscle, GLUT4 coprecipitates with the TRPC3 channel, and TRPC3 knockdown decreased in insulin-mediated glucose uptake (18), suggesting that, in this tissue the channel, and possibly ions flowing through it, are required for insulin-stimulated glucose uptake. All these findings beg for analysis of cell-specific responses of Ca^{2+} to insulin and for a more in depth exploration of the source of the regulatory Ca^{2+}. In the present study, we find that this cation is likely released from endomembranes through IP_3R and propose a signaling pathway leading to such release. This mechanism may be unique to cardiomyocytes or may be more amply operative,

and future work in each of the insulin-sensitive tissues should provide answers to these possibilities.

We also show that either IGF-1 or 2,4-DNP can induce an increase in plasma membrane GLUT4 levels independent of the presence of extracellular Ca^{2+}. In these conditions, the pretreatment of cardiomyocytes with xestospongin C inhibited only the effect of IGF-1. We have shown previously that IGF-1 also increases Ca^{2+} in cardiomyocytes but with clear difference in the amplitude, frequency, duration, or subcellular localization of the Ca^{2+} signals elicited by insulin (26). These last findings show that a link exists between IP_3R-dependent Ca^{2+} release and GLUT4 translocation, but this mechanism is particular for certain stimuli.

In particular, the link between IP_3R-dependent Ca^{2+} release and mobilization of GLUT4 is a novel contribution of this study. Moreover, our study provides an explanation for the documented contribution of endogenous $G\alpha_{q/11}$ in insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (5) and the emulation of this process by a constitutively active form of $G\alpha_q$ (Q209L-$G\alpha_q$) acting via a PI3K-dependent mechanism (6).

The observations made in adipocytes, skeletal muscle, and ours in cardiomyocytes beg the question of what is the precise role of $[Ca^{2+}]_i$ that impacts on GLUT4 translocation. The regulation of Akt shown both here and by James *et al.* (16) may be an important point of action. Additionally, Ca^{2+} may regulate GLUT4 vesicle fusion, potentially through interaction with molecules functionally akin to synaptotagmin. Furthermore, Ca^{2+} ions may participate in the recently described calmodulin kinase II-dependent regulation of GLUT4 traffic (16, 33). Future studies should reveal the molecular action of $[Ca^{2+}]_i$ and whether the IP_3R implicated in this study is directly present on GLUT4 vesicles, thereby providing local levels of elevated Ca^{2+} in the vicinity of the membrane.

In summary, insulin induces $[Ca^{2+}]_i$ transients in neonatal cardiomyocytes, and Ca^{2+} release from IP_3-sensitive stores is an important physiological regulator of the insulin-dependent stimulation of glucose uptake via GLUT4. These novel observations suggest a link between intracellular Ca^{2+} homeostasis and metabolism and raise the concept that alterations in Ca^{2+} regulation, so common in cardiomyopathies, may impinge on the ability of cardiomyocytes to regulate nutrient availability. Because cardiac glucose uptake is paramount for energy procurement in conditions of ischemia and hypoxia, the concomitant impairment in Ca^{2+} fluxes may be fundamental for energy availability in the failing heart. Such a scenario would be important in cardiomyopathy accompanying insulin resistance and diabetes.

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S.L. is on sabbatical leave at the University of Texas Southwestern Medical Center (Dallas, TX).

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