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Insulin Induces Calcium Signals in the Nucleus of Rat Hepatocytes

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Abstract

Insulin is an hepatic mitogen that promotes liver regeneration. Actions of insulin are mediated by the insulin receptor, which is a receptor tyrosine kinase. It is currently thought that signaling via the insulin receptor occurs at the plasma membrane, where it binds to insulin. Here we report that insulin induces calcium oscillations in isolated rat hepatocytes, and that these calcium signals depend upon activation of phospholipase C and the inositol 1,4,5-trisphosphate receptor, but not upon extracellular calcium. Furthermore, insulin-induced calcium signals occur in the nucleus, and are temporally associated with selective depletion of nuclear phosphatidylinositol bisphosphate and translocation of the insulin receptor to the nucleus. These findings suggest that the insulin receptor translocates to the nucleus to initiate nuclear, inositol 1,4,5-trisphosphate-mediated calcium signals in rat hepatocytes. This novel signaling mechanism may be responsible for insulin's effects on liver growth and regeneration.

Insulin regulates a wide variety of biological functions in the liver, including glucose uptake, 1 regulation of gene expression,2 and promotion of cell growth.3[–5] The biological actions of insulin are initiated by binding to the insulin receptor, a heterotetrameric receptor tyrosine kinase (RTK) composed of two extracellular α-subunits and two transmembrane β-subunits.[6] The α-subunit possesses insulin-binding activity whereas the β-subunit has intrinsic protein tyrosine kinase activity. Binding of insulin to the α-subunit of its receptor activates the protein tyrosine kinase and results in phosphorylation of tyrosine residues of the β-subunit and of several endogenous substrates. These substrates include proteins containing a *src*-homology 2 domain such as phosphatidylinositol 3-kinase and phospholipase C (PLC).[7] PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), generating two intracellular products: inositol 1,4,5-trisphosphate (InsP3), a universal calcium-mobilizing second messenger, and diacylglycerol, an activator of protein kinase C. Like insulin, Ca^{2+} also regulates glucose metabolism,[8] gene expression,[9,10] and cell growth.[11,12] Although it has not been established how a single second messenger coordinates such diverse effects within a cell, there is increasing evidence that the spatial and temporal patterns of Ca^{2+} signals may determine their specificity. Ca^{2+} signaling patterns can vary in different regions of the cell, and increases in Ca^{2+} in the nucleus have specific biological effects that differ from the

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effects of increases in cytosolic Ca^{2+}.[9,10,13–15] The mechanisms and pathways that promote localized increases in free Ca^{2+} levels in the nucleus have not been entirely defined. It is currently thought that signaling via the insulin receptor occurs only at the plasma membrane, where it binds to insulin.[16] Here we investigate whether and how insulin signaling occurs in the nucleus of hepatocytes, where its downstream messenger Ca^{2+} may act.

Materials and Methods

Cells and Cell Culture

Hepatocytes were isolated from the livers of male Sprague-Dawley rats (190–200 g; Charles River Laboratories, Wilmington, MA) by collagenase perfusion as described.[17] Primary hepatocytes were cultured at 37°C in 5% CO_2/95% O_2 in Williams' medium E containing 10% fetal bovine serum, 50 units/mL penicillin, and 50 g/mL streptomycin (Invitrogen, Carls-bad, CA) and plated on collagen-coated coverslips (50 μg/mL) (BD Biosciences, San Jose, CA). Hepatocytes were used 4–6 hours after isolation. Viability of the hepatocytes was greater than 85% and was measured by trypan blue exclusion.[18,19] SkHep1 cells, a human liver cancer cell line, were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 1 mM sodium pyruvate, 50 units/mL penicillin, and 50 g/mL streptomycin (Invitrogen).

Detection of Ca^{2+} Signals

Nuclear and cytosolic Ca^{2+} were monitored in individual cells by time-lapse confocal microscopy, as described.[14,20] For Ca^{2+} imaging, cells were incubated with fluo-4/AM (6 μM) (Invitrogen) for 30 minutes at 37°C, then coverslips containing the cells were transferred to a custom-built perfusion chamber on the stage of a Zeiss LSM 510 confocal microscope (Thornwood, NY) and the perfusion chamber was maintained at 37°C. The cells were stimulated with insulin (1–500 nM) or vasopressin (10 nM) (Sigma, Saint Louis, MO). In selected experiments cells were perfused for 10 minutes with the PLC inhibitor U-73122 (1 μM) or pretreated for 30 minutes with the InsP3 receptor inhibitor xestospongin C (2.5 μM) (Sigma). Fluo-4 fluorescence was monitored using a 40×, 1.2 NA objective lens, and images were collected at a rate of 1–5 frames/second. Changes in fluorescence F were normalized by the initial fluorescence (F_0) and were expressed as $(F/F_0) \times 100\%$.[11]

InsP3 Buffer Constructs

The InsP3 binding domain (residues 224-605) of the human type I InsP3 receptor was tagged with monomeric red fluorescent protein (mRFP) and then the nuclear localization signal was sub-cloned to generate the nuclear InsP3 buffer expression vector. The nuclear exclusion signal sequence derived from mitogen-activated protein kinase kinase 1 was sub-cloned in the InsP3 binding domain tagged with the mRFP construct to generate the cytoplasmic InsP3 buffer expression vector, as described.[21]

Immunoblotting

Primary hepatocyte immunoblots were performed as described.[22] Briefly, cells were washed twice with ice-cold phosphate-buffered saline, harvested by scraping, and lysed in a lysis buffer (20 mM [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid], pH 7.0, 10 mM KCl, 2 mM $MgCl_2$, 0.5% Nonidet P-40). After incubation on ice for 10 minutes, the cells were homogenized by vortex. The homogenate was centrifuged at 1,500*g* for 5 minutes to sediment the nuclei. The supernatant was then centrifuged at a maximum speed of 16,100*g* for 20 minutes, and the resulting supernatant formed the non-nuclear fraction. The nuclear pellet was washed three times with lysis buffer to remove any contamination from cytoplasmic membranes, and the purity of the nuclei was confirmed by light microscopy. To

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extract nuclear proteins, the isolated nuclei were resuspended in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCL, pH 8.0, 0.5% Nonidet P-40), and the mixture was sonicated briefly to aid nuclear lysis. Nuclear lysates were collected after centrifugation at 16,100*g* for 20 minutes at 4°C. Protease and phosphatase inhibitors (Sigma) were added to all buffers. Blots were visualized by enhanced chemiluminescence, and quantitatively analyzed using a GS-700 imaging densitometer. The purity of nuclear and non-nuclear fractions was confirmed using Lamin B1 (Abcam, Cambridge, MA) as a nuclear marker and α-Tubulin (Sigma) as a non-nuclear (cytosolic) marker.[23] The phosphorylated form of the insulin receptor was detected by immunoprecipitation of the receptor, followed by blotting with a monoclonal antibody directed against phosphotyrosine residues (Millipore, Billerica, MA).

Detection of PIP_2

A $PI(4,5)P_2$ Mass Strip Kit (Echelon, Salt Lake City, UT) was used for isolation and PIP_2 detection. Isolated hepatocytes were starved in serum-free William's E medium for 3 hours. Cells were incubated without or with insulin (10 nM) for 10 minutes or vasopressin (10 nM) for 1 minute, and then the medium was aspirated and cellular material precipitated by the immediate addition of 3 mL ice-cold 0.5 M trichloroacetic acid. The lysis buffer was used to prepare the nuclear and non-nuclear cell fractions, as described above.[22] Briefly, cell membranes were disrupted to release cytoplasmic contents. Intact nuclei were recovered from the cytoplasmic extract by centrifugation, and then the nuclei were washed with phosphate-buffered saline and precipitated with 3 mL ice-cold 0.5 M trichloroacetic acid. Isolation of lipids was performed according to manufacturer instructions and as described.[24] The organic phase was collected into a clean tube and dried in a Speed Vac centrifuge. The pellet at this stage was faintly visible. The lipids were then resuspended by sonication in a cold water bath in 10 μL of $CHCl_3$:methanol:H_2O (1:2:0.8), and spotted onto nitrocellulose membrane strips prespotted with $PI(4,5)P_2$ standards, PIP controls, and space for spotting unknown samples for probing with anti-PIP_2 monoclonal antibody (Echelon) to specifically detect PIP_2. Blots were visualized by enhanced chemiluminescence, and quantitatively analyzed using a GS-700 imaging densitometer (Bio-Rad, Hercules, CA).

Immunofluorescence

Confocal immunofluorescence was performed as described.[14,20] Cells were double-labeled with a polyclonal antibody against insulin receptor B (BD Biosciences, CA), which is the predominant form of the receptor in hepatocytes,[25] and a monoclonal antibody against the nuclear membrane marker Lamin B1, and then incubated with secondary antibodies conjugated to Alexa 488 and 555 (Invitrogen), respectively. Images were collected with a Zeiss LSM 510 confocal microscope using a 63×, 1.4 NA objective lens with excitation at 488 nm and observation at 505–550 nm to detect Alexa 488, and excitation at 543 nm and observation at 560–610 nm to detect Alexa 555.

Statistical Analysis

Significance of changes in treatment groups relative to controls was determined by Student *t* test. Data are represented as mean ± standard error.

Results

Insulin Induces Ca^{2+} Oscillations in Rat Hepatocytes

To examine Ca^{2+} signaling induced by insulin, freshly isolated rat hepatocytes were stimulated with a range of insulin concentrations (0.1–100 nM) and observed by time-lapse confocal microscopy. Hepatocytes did not respond to 0.1 nM insulin (n = 30), but responded

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to all higher concentrations tested. The fraction of cells responding to insulin did not vary appreciably with increasing insulin concentrations; 41% of cells responded to stimulation with 1 nM insulin (Fig. 1A), 42% of cells responded to 10 nM insulin (Fig. 1B), and 56% of cells responded to maximal (100 nM) stimulation (Fig. 1C). Ca^{2+} oscillations were elicited in all responding cells stimulated with lower (1–10 nM) insulin concentrations, although higher insulin concentrations elicited Ca^{2+} oscillations in only 10% of responding cells, and instead elicited a sustained increase in Ca^{2+} in the remaining 46% of responding cells (data not shown). Moreover, the response to 100 nM and 500 nM insulin was similar, suggesting that these findings represent the full range of insulin's effect on Ca^{2+} signals in hepatocytes. The frequency of Ca^{2+} oscillations (~5 mHz) was similar regardless of the insulin concentration. These findings show that insulin, like other Ca^{2+} agonists such as vasopressin, phenylephrine, angiotensin, and adenosine triphosphate,[26–28] induces Ca^{2+} signals in hepatocytes that tend to be oscillatory at lower concentrations but can instead be sustained at higher concentrations. However, the frequency of insulin-induced Ca^{2+} oscillations was lower than has typically been reported for other agonists such as phenylephrine (10–50 mHz)[26,27] and vasopressin (10–35 mHz).[26,28] In addition, maximal concentrations of these other agonists generally elicit Ca^{2+} signals in >90% of hepatocytes,[26–28] whereas insulin elicited Ca^{2+} signals in a much lower fraction of cells. Moreover, we stimulated cells with vasopressin (10 nM) and those results confirmed that ~98% of cells responded to that agonist, even though only half of the cells responded to insulin under the same experimental conditions. Vasopressin also induced a greater peak in fluorescence than what was observed in response to insulin stimulation (Fig. 1D). These findings demonstrate that insulin induces Ca^{2+} signals in hepatocytes, including Ca^{2+} oscillations, but that certain characteristics of these signals differ from what is elicited by stimulation of G protein–coupled receptors.

Insulin-Induced Ca^{2+} Signals Are Mediated by InsP3

Several maneuvers were performed to determine the mechanism by which insulin increases Ca^{2+} in hepatocytes. To determine the source of the Ca^{2+}, cells were stimulated in Ca^{2+}-free medium. Insulin induced Ca^{2+} oscillations even in Ca^{2+}-free medium (Fig. 2A), and Ca^{2+} signals were elicited in a similar fraction of cells regardless of the presence of extracellular Ca^{2+} (Fig. 2B). These findings demonstrate that insulin increases cytoplasmic Ca^{2+} by mobilizing intracellular Ca^{2+} stores. Most RTKs increase Ca^{2+} by activation of PLCγ, which forms InsP3 to bind to and release Ca^{2+} from InsP3 receptors in the endoplasmic reticulum.[29] Therefore, we stimulated hepatocytes with insulin in the presence of either the PLC inhibitor U-73122[30] or the InsP3 receptor inhibitor xestospongin C.[31] Both U-73122 (Fig. 3A,B) and xestospongin C (Fig. 3C,D) eliminated insulin-induced Ca^{2+} signals in hepatocytes. Together, these findings suggest that insulin increases Ca^{2+} in hepatocytes through PLC- and InsP3-mediated release of intracellular Ca^{2+} stores.

Insulin-Induced Ca^{2+} Signals Begin in the Nucleus

Ca^{2+} signals in the nucleus and cytoplasm were monitored simultaneously in hepatocytes ($n > 30$). The signals often had a similar temporal profile in both compartments (Fig. 4A), but the Ca^{2+} increase in the nucleus preceded the cytoplasmic increase in some cells, while in other cells an isolated increase in Ca^{2+} in the nucleus was observed (Fig. 4B). The kinetics of vasopressin-induced Ca^{2+}signals differed from this in two ways. First, insulin-induced signals often took up to 50 seconds from the time of onset to reach their peak amplitude (Figs. 1A–C and 4A), whereas the rise time of vasopressin-induced signals always was much shorter (~1 second; Fig. 1D), similar to what has been reported.[18] Second, vasopressin-induced Ca^{2+} signals always began in the cytoplasm rather than the nucleus (Fig. 4C). These findings indicate that the subcellular kinetics of insulin-induced Ca^{2+} signals differ fundamentally from the kinetics of Ca^{2+} signals induced by vasopressin, which

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in turn suggests that insulin may increase Ca^{2+} through a mechanism based in the nucleus rather than in the cytoplasm.

The Insulin Receptor Translocates to the Nucleus

Ca^{2+} signals are initiated in hepatocytes when PIP_2 is hydrolyzed to form InsP3.[18] Both the nucleus and the cytoplasm contain the machinery needed to form InsP3-mediated Ca^{2+} signals, including PLC, PIP_2, and the InsP3 receptor,[32] so we examined the effects of insulin on total cellular and nuclear pools of PIP_2. Insulin reduced the nuclear pool by 38.9 ± 7.1% ($P < 0.05$) without significantly reducing total cellular PIP_2 (Fig. 5). For comparison, vasopressin reduced total cellular PIP_2 by 35.2 ± 7.8% ($P < 0.05$) without significantly reducing nuclear PIP_2. To demonstrate more directly that the insulin receptor forms InsP3 in the nucleus, we targeted the ligand binding domain (residues 224-605) of the type 1 InsP3 receptor[33] to the cytoplasm or nucleus using a nuclear exclusion signal or nuclear localization signal sequence, respectively, plus mRFP to verify localization.21 These targeted InsP3 buffer constructs were expressed in the SkHep1 liver cell line, to circumvent technical difficulties associated with transient transfection of primary hepatocytes. It has previously been shown that the cytoplasmic but not the nuclear InsP3 buffer blocks vasopressin-induced Ca^{2+} signals in SkHep1 cells, reflecting the fact that G protein-coupled receptors such as the vasopressin V_{1a} receptor activate PLC and form InsP3 at the plasma membrane.[21] In contrast, Ca^{2+} signals induced by insulin (100 nM) were nearly abolished in cells expressing the nuclear InsP3 buffer ($P < 0.005$), but were not affected by expression of the cytoplasmic buffer (Fig. 6). Together, these results show that insulin hydrolyzes PIP_2 and increases InsP3 only in the nucleus, and that Ca^{2+} signals throughout the cell result from this. To investigate why insulin preferentially forms InsP3 and increases Ca^{2+} in the nucleus, we examined the location of the insulin receptor during cell stimulation. Immunoblots of non-nuclear and nuclear fractions showed that the insulin receptor was in the non-nuclear fraction of hepatocytes prior to stimulation with insulin. However, the receptor appeared in the nuclear fraction within 2.5 minutes of stimulation, and was detectable within the nucleus until 20 minutes after stimulation (Fig. 7A,B). Similarly, the phosphorylated (active) form of the insulin receptor was absent from the nucleus of hepatocytes prior to stimulation with insulin, but was detected there afterwards (Fig. 7C). To confirm the immunoblot findings, confocal immunofluorescence microscopy was used to monitor the subcellular distribution of the insulin receptor. Confocal imaging demonstrated that the insulin receptor was at the plasma membrane or within the cytoplasm but absent from the nucleus prior to stimulation (Fig. 8A, top panels). Within 5 minutes of exposure to 10 nM insulin, the insulin receptor could also be detected at the nuclear envelope and within the nuclear interior (Fig. 8A, bottom panels). Three-dimensional (3D) reconstruction of serial confocal immunofluorescence images confirmed that the receptor could be identified within the nuclear interior of cells stimulated with insulin (Fig. 8B). Together, these findings demonstrate that stimulation of hepatocytes with insulin induces the insulin receptor to translocate to the nucleus, and this is associated with selective hydrolysis of nuclear PIP_2 and formation of InsP3-dependent Ca^{2+} signals within the nucleus.

Discussion

Insulin is a potent mitogen for hepatocytes *in vitro*[4] and also plays a role in liver regeneration *in vivo*.34 Insulin also plays an essential role in the growth and proliferation of hepatocytes in certain cell culture systems.[35] Insulin acts through the insulin receptor, which is a RTK, and evidence from other RTKs suggests that translocation to the nucleus may be a common feature for this class of receptors. A number of RTKs have been found in the nucleus, including receptors for growth hormone, several cytokines, epidermal growth factor (EGF), hepatocyte growth factor, and fibroblast growth factor (FGF).21,36,37

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Phosphorylated EGF receptor can be found in the nucleus within 1–2 minutes of stimulation with EGF, and reaches peak levels within 15 minutes.36 Phosphorylated hepatocyte growth factor receptor (c-met) appears in the nucleus within a similar time frame after stimulation with hepatocyte growth factor, and its appearance there has been linked to intranuclear formation of InsP3 and initiation of Ca^{2+} signals within the nucleus.[21] Translocation of the FGF receptor to the nucleus occurs over a longer time scale, reaching peak amounts after 3–4 hours.[37] Although these previous studies have demonstrated that RTKs can translocate to the nucleus in cell lines, the current work provides evidence that this also occurs in primary hepatocytes. Intranuclear RTKs can serve functional effects as well. For example, EGF receptors in the nucleus act as a transcription factor that promotes expression of cyclin D1[36] and COX-2,[38] each of which may contribute to the mitogenic effects of EGF. The mechanism by which RTKs reach the nucleus is not known, although transport of the FGF receptor to the nucleus depends on importin β, rather than the presence of a nuclear localization sequence on either the receptor or its ligand,[37] and transport of c-met to the nucleus depends upon both importin β and the adaptor protein GRB2-associated binding protein 1 (Gab1).[21] Early studies based on binding of radiolabeled insulin to nuclear membranes,39 plus autoradiographic studies of hepatocytes and hepatocyte lysates using photolabeled insulin receptors,40 suggested that the insulin receptor can be intranuclear. This conclusion was questioned in later work using immunoblot and immunoelectron microscopic techniques,41 which had led many to conclude instead that the insulin receptor does not translocate to the nucleus.[42] Similarly, previous evidence had suggested that the insulin receptor does not activate PLC, leading to the widely held conclusion that insulin does not stimulate the PLC/InsP3/Ca^{2+} signaling pathway.[43,44] However, recent studies have shown an increase in InsP3 in rat epididymal cells stimulated with insulin,[45] as well as an increase in PLC activity in insulin-stimulated adipocytes.[46] Moreover, PLCγ coprecipitates with the insulin receptor, providing additional evidence that this receptor induces phospholipid hydrolysis.[7] Finally, insulin has been reported to increase cytosolic Ca^{2+} in primary hepatocytes by triggering Ca^{2+} influx,[47] but the current work provides evidence that insulin instead mobilizes intracellular Ca^{2+} stores in hepatocytes, through a PLC-dependent and InsP3-dependent mechanism. The current findings provide both structural and functional evidence that the insulin receptor moves to and acts within the nucleus in hepatocytes. Structural evidence includes immunoblots showing that total as well as phosphorylated insulin receptor accumulates in the nucleus, plus confocal immunofluorescence localization of the receptor within the nucleus. Functional evidence includes studies showing that insulin selectively hydrolyzes the nuclear pool of PIP_2, plus Ca^{2+} imaging studies showing that insulin-induced Ca^{2+} signals can begin in the nucleus, and that these signals depend on intranuclear rather than cytoplasmic InsP3. Thus, previous studies plus the current work together suggest that insulin induces its receptor to move to the nucleus in hepatocytes, and this translocation is associated with PLC-mediated hydrolysis of nuclear PIP_2, leading to formation of InsP3-mediated Ca^{2+} signals. Because Ca^{2+} signals within the nucleus are particularly important for cell growth,[11] the effect of insulin on nuclear Ca^{2+} signaling may explain insulin's action as a mitogen. The metabolic effects of insulin in the liver are mediated by Akt/protein kinase B, and these effects are enhanced in the liver-specific Gab1 knockout mouse.[48] Since Gab1 may mediate nuclear translocation of RTKs,[21] this suggests that the metabolic effects of insulin may be mediated by the non-nuclear insulin receptor, while the effects of insulin on growth and regeneration may be mediated by the insulin receptor that reaches the nucleus.

There is increasing evidence that the subcellular pattern of Ca^{2+} signals dictates the cellular effects of this second messenger. The InsP3 receptor is the only intracellular Ca^{2+} release channel in hepatocytes,[18] so the subcellular distribution of this receptor determines the form of Ca^{2+} signals in these cells. For example, the type II InsP3 receptor, which is the principle isoform in hepatocytes, is most concentrated in the region of the endoplasmic reticulum

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beneath the canalicular membrane.[18,19] Agonists such as vasopressin, angiotensin, or adenosine triphosphate increase InsP3 in the cytosol, and so the resulting Ca^{2+} signal takes the form of a Ca^{2+} wave that begins in the canalicular region, where the InsP3 receptor is most concentrated.[18] This Ca^{2+} wave directs exocytosis[49] and fluid and electrolyte secretion.[50] Reduced expression of InsP3 receptors in hepatocytes impairs the formation of Ca^{2+} waves,[51] but simple redistribution of the receptors away from the canalicular region impairs Ca^{2+} wave formation as well.[19] This subcellular organization of the Ca^{2+} signaling machinery is relevant for the regulation of secretion, because treatment of cholangiocytes with small interfering RNA to decrease expression of apical InsP3 receptors in these cells results in impaired bicarbonate secretion.[52] Expression of apical InsP3 receptors is also decreased or absent in bile ducts of patients with cholestatic disorders such as primary biliary cirrhosis, sclerosing cholangitis, and biliary atresia,[53] although it has not yet been established that this loss of InsP3 receptors is responsible for the development of cholestasis in these disorders. Localization of InsP3 receptors to other microdomains can affect cell function as well. For example, the type III InsP3 receptor colocalizes more effectively than either the type I or II isoform of the receptor with mitochondria.[54] This is associated with more efficient transmission of Ca^{2+} signals into the mitochondria, which in turn is more effective at inducing apoptosis.[54] A number of Ca^{2+}-mediated events occur in the nucleus; including: activation of cyclic adenosine monophosphate response element-binding transcription factor[10] and the Elk-1 transcription factor[9]; translocation of nuclear protein kinase C to the region of the nuclear envelope[14]; and regulation of progression of the cell cycle through prophase.[11] Although Ca^{2+} can spread passively from the cytosol into the nucleus under certain circumstances,[55,56] intranuclear InsP3 can increase Ca^{2+} directly within the nucleus as well, in both isolated nuclei and in nuclei within intact cells.[14,57] This is because the nuclear envelope[57] and the nucleoplasmic reticulum[14] both express InsP3 receptors, and these receptors can release Ca^{2+} into the nucleoplasm. How much InsP3 receptor is expressed in the nucleus? Although immunofluorescence studies suggest that the InsP3 receptor in hepatocytes is most concentrated in the pericanalicular region,[18] quantitative immunoblots show that the ratio of nuclear:cytosolic InsP3 receptors is nearly 20:1.[14] This ratio reflects InsP3 receptor concentration relative to other proteins in each compartment, but since there is presumably much less total protein per unit volume in the nucleus than in the cytoplasm, this may explain why immunofluorescence studies instead suggest that there is more InsP3 receptor in the cytoplasm. In any case, several mechanisms have been identified to control Ca^{2+} release from nuclear InsP3 receptors. The three InsP3 receptor isoforms have distinct sensitivities to InsP3, so targeting a more sensitive isoform to the nucleus will enable InsP3-mediated Ca^{2+} signals to occur preferentially in the nucleus, relative to the cytosol.[20] Alternatively, selective hydrolysis of the nuclear pool of PIP_2 will lead to local intranuclear formation of InsP3, so that Ca^{2+} will be released preferentially from nuclear InsP3 receptors. In particular, RTKs may selectively activate nuclear isoforms of PLC, particularly PLCβ1, and may also induce PLCγ1 to translocate to the nucleus.[58,59] The current work suggests that the insulin receptor may also act in this fashion. Although insulin-induced Ca^{2+} signals begin before peak accumulation of the insulin receptor occurs within the nucleus, there is likely a threshold relationship rather than a linear relationship between accumulation of insulin receptor within the nucleus and triggering of Ca^{2+} signals, just as there is a threshold, “all-or-none” relationship between accumulation of InsP3 and initiation of Ca^{2+} signals in the cytoplasm.[60] Therefore, although the increase in intranuclear insulin receptor does not become measurable for several minutes, smaller amounts, especially of the phosphorylated receptor, may be sufficient to generate enough InsP3 to initiate Ca^{2+} signals. Further work is needed to determine the mechanism by which the insulin receptor moves to the nucleus in hepatocytes and to demonstrate that this is responsible for insulin’s mitogenic effects.

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Abbreviations

c-met	hepatocyte growth factor receptor
Gab1	CRB2-associated binding protein 1
EGF	epidermal growth factor
FGF	fibroblast growth factor
InsP3	inositol 1,4,5-trisphosphate
KC1	potassium chloride
MKK1	mitogen-activated protein kinase kinase 1
mRFP	monomeric red fluorescent protein
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
RTK	receptor tyrosine kinase

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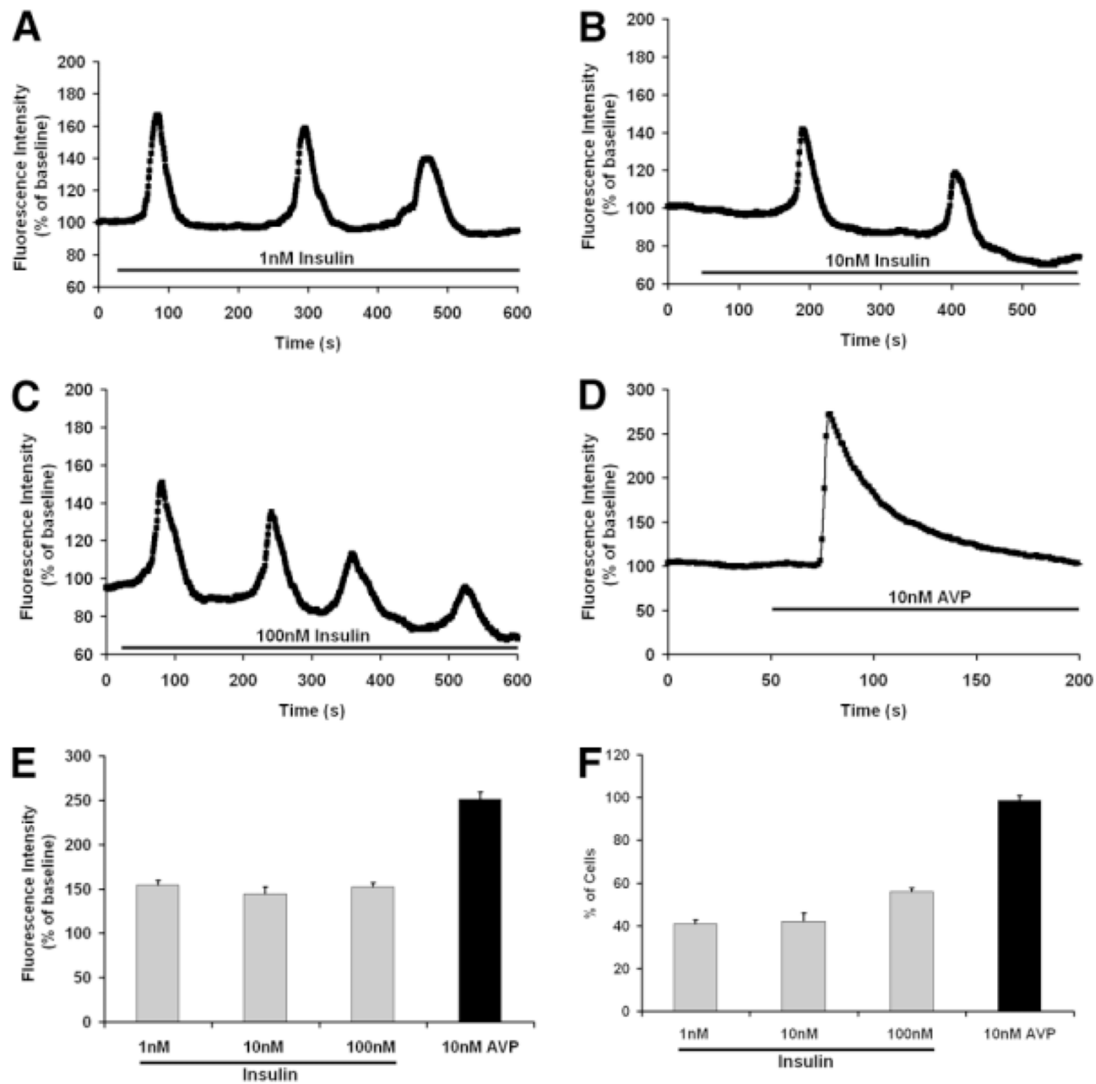

Fig. 1.

Insulin induces calcium signaling in hepatocytes. (A–C) Insulin induces Ca^{2+} oscillations in primary rat hepatocytes. Ca^{2+} was monitored in individual hepatocytes 4–6 hours after isolation using the Ca^{2+} dye fluo-4 and time-lapse confocal microscopy. Tracings are shown from individual cells stimulated with 1, 10, or 100 nM insulin, respectively. No response was observed in cells stimulated with 0.1 nM insulin (n = 30; data not shown), and the response to 500 nM insulin was similar to the response shown here for 100 nM insulin (n = 35; data not shown). Stimulation with 1 and 10 nM insulin induced Ca^{2+} oscillations (n = 58). Stimulation with 100 nM insulin instead induced a sustained Ca^{2+} increase in some cells (n = 25; data not shown). Results are representative of what was observed in >25 responding cells under each condition. (D) Representative tracing of the Ca^{2+} signal induced by maximal (10 nM) stimulation with vasopressin. Note that the response begins and reaches its peak more rapidly in these cells than in cells stimulated with insulin. The result is representative of what was observed in n = 90 separate cells. (E) The amplitude of the Ca^{2+} signal induced by insulin is not concentration-dependent, and is significantly less than the amplitude of the Ca^{2+} signal induced by vasopressin (*$P < 0.001$; n = 30 in each group). (F) The fraction of cells responding to insulin is not concentration-dependent, and is

significantly less than the fraction that responds to vasopressin (* $P < 0.001$; n = 30 in each group).

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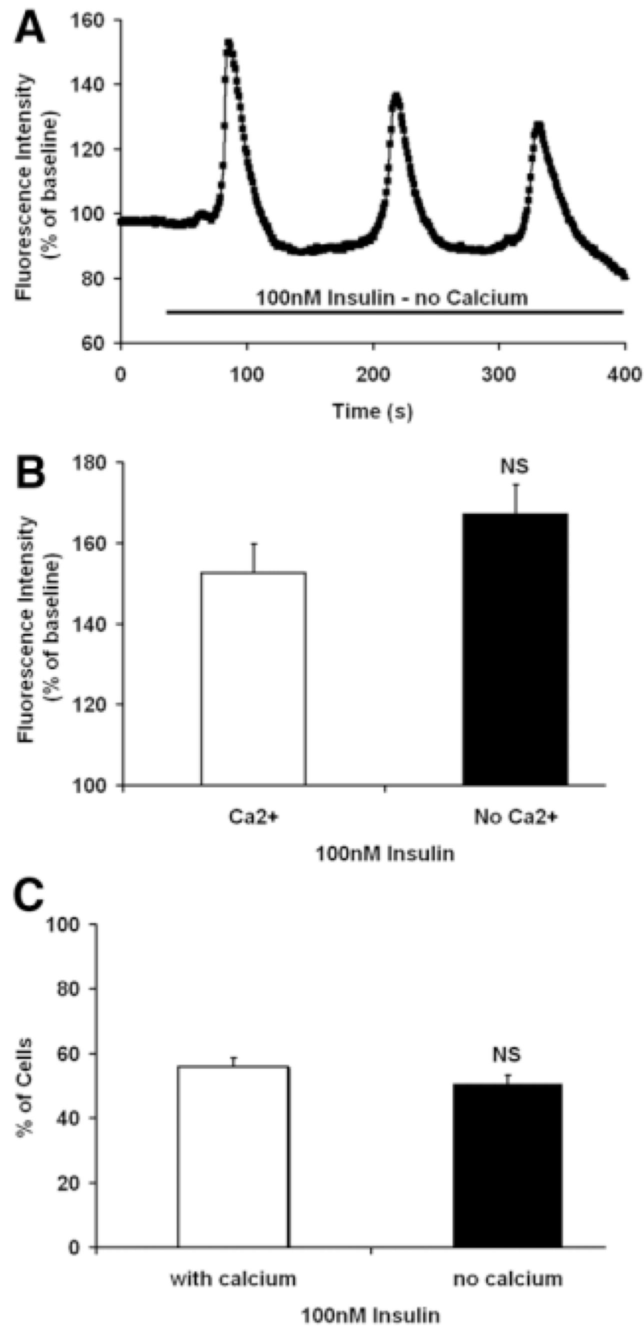

Fig. 2.

Insulin-induced Ca^{2+} signals do not depend on extracellular Ca^{2+}. (A) Insulin (100 nM) induces Ca^{2+} oscillations in an hepatocyte placed in Ca^{2+}-free medium fortified with 1 mM ethylene glycol tetraacetic acid (EGTA). The result is representative of what was observed in 30 cells. (B) The amplitude of the Ca^{2+} signal induced by insulin is not decreased in Ca^{2+}-free medium (n = 10 in each group). (C) The fraction of cells responding to insulin is not decreased in Ca^{2+}-free medium (n = 10 in each group).

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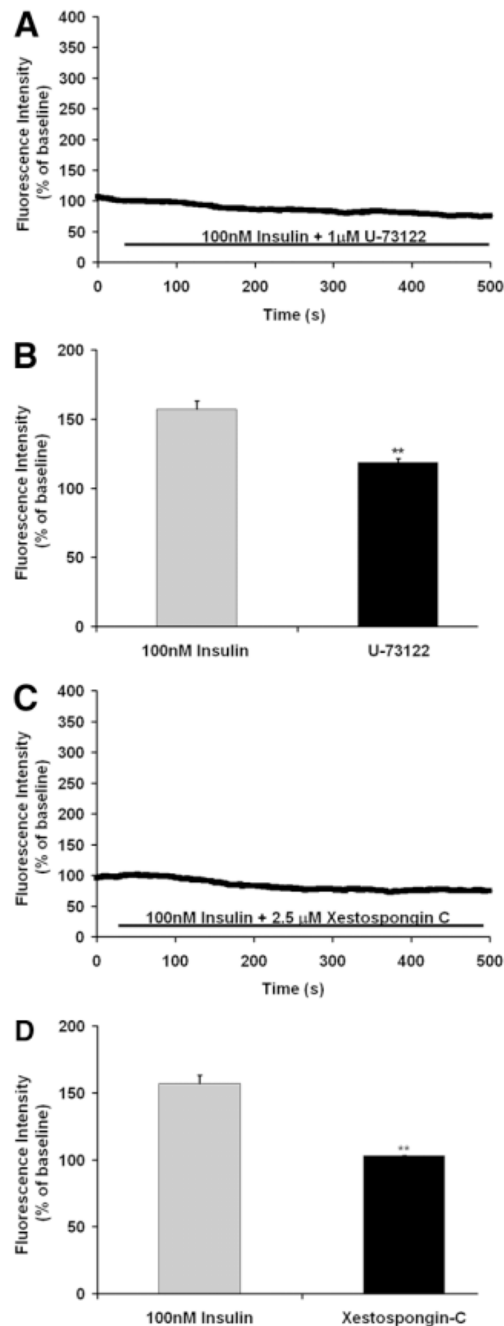

Fig. 3.

Insulin-induced Ca^{2+} signals depend on PLC and InsP3. (A) The Ca^{2+} signal induced by insulin (100 nM) is blocked by the PLC inhibitor U-73122 (1 μM). The result is representative of what was observed in 56 cells. (B) Bar graph summary showing that the amplitude of the Ca^{2+} signal induced by insulin is significantly reduced by U-73122 (**$P <$ 0.001; n = 18 in each group). (C) The Ca^{2+} signal induced by insulin (100 nM) is blocked by the InsP3 receptor inhibitor xestospongin C (2.5 μM). The result is representative of what was observed in 73 cells. (D) Bar graph summary showing that the amplitude of the Ca^{2+} signal induced by insulin is significantly reduced by xestospongin C (**$P < 0.001$; n = 25 in each group).

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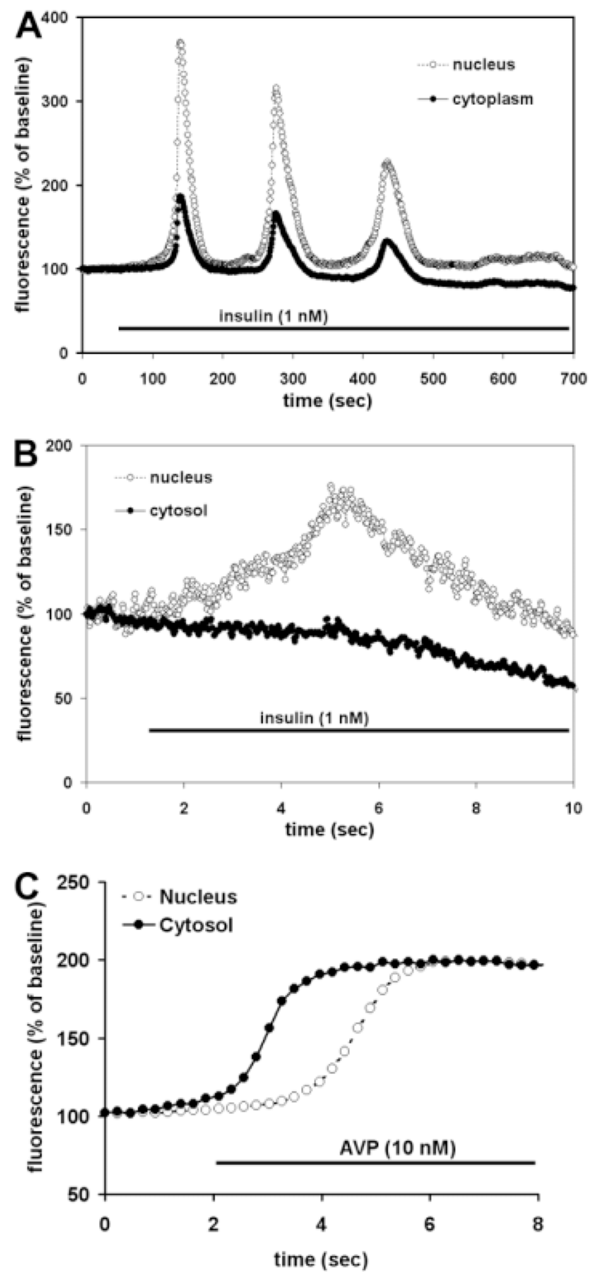

Fig. 4.

Insulin-induced Ca^{2+} signals begin in the nucleus. (A) Examination of the nuclear and cytosolic components of the insulin-induced Ca^{2+} signal reveal that the Ca^{2+} increase occurs in both regions of the hepatocyte. The image is representative of what was observed in >90 cells. (B) Insulin can induce isolated Ca^{2+} increases in the nucleus. All cells responded to insulin in this fashion, with sequential increases in Ca^{2+} in the nucleus and then cytosol, or with a simultaneous increase in Ca^{2+} in the nucleus and cytosol. Note the expanded time scale relative to (A) of this figure and in Figs. 1–3. (C) The vasopressin-induced Ca^{2+} signal begins in the cytosol rather than the nucleus, similar to what has been reported.[18,19] Because the fluorescence intensity of fluo-4 differs in the nucleus and cytosol,[20] fluorescence here was rescaled so that the nuclear and cytosolic signals would have the same baseline and

peak values, to facilitate direct comparison of the time course of each tracing. Results are representative of what was observed in at least 25 cells in each group.

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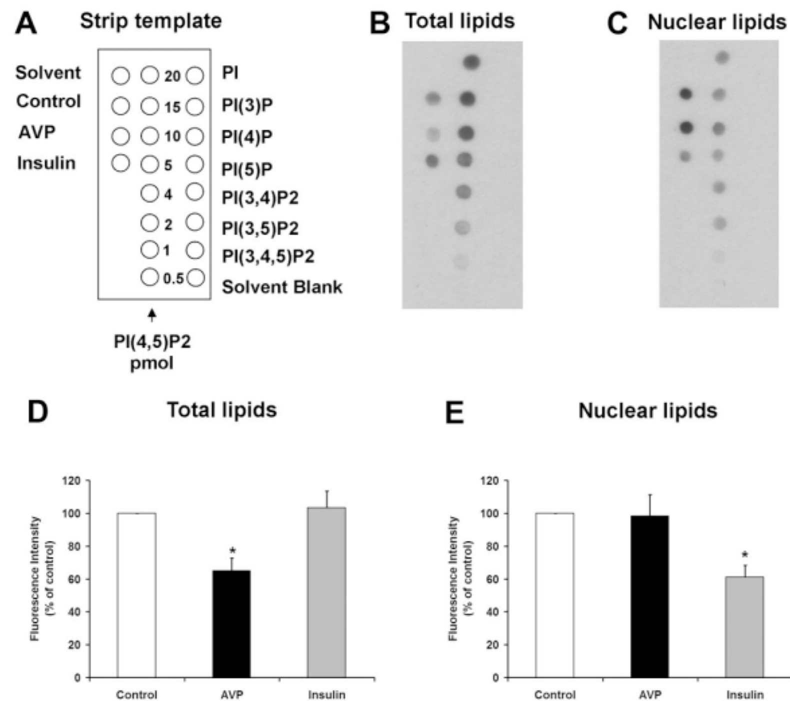

Fig. 5.

Insulin selectively hydrolyzes nuclear PIP_2. Immunoassay of total (B) and nuclear (C) lipids, plus a key to the strip template (A). The lipid samples were spotted onto nitrocellulose membranes, as illustrated by the strip template, and the PIP_2 levels were detected using an anti-PIP_2 monoclonal antibody. This antibody reacts with a higher degree of specificity to $PI(4,5)P_2$ than to other inositol polyphosphates (right column). A total of 20 pmol of each of the other PIP controls was used. (D) Densitometric measurement shows that arginine vasopressin (AVP) hydrolyzes 35.2 ± 7.8% of PIP_2 in whole cell preparations (n = 3, $*P < 0.05$), whereas insulin does not hydrolyze significant amounts of total cellular PIP_2. (E) Insulin hydrolyzes 38.9 ± 7.1% of PIP_2 in the nucleus (n = 3, $*P < 0.05$), whereas vasopressin stimulation does not hydrolyze significant amounts of nuclear PIP_2. Total and nuclear lipids were isolated 10 minutes after stimulation of hepatocytes with insulin (10 nM) or 1 minute after stimulation with vasopressin (10 nM). Data are mean ± standard error of the mean (SEM).

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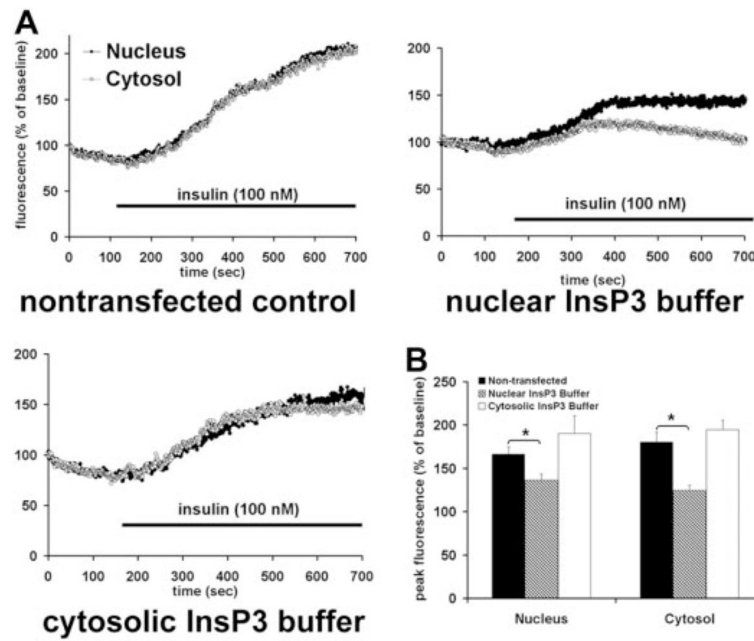

Fig. 6.

Insulin generates InsP3 in the nucleus rather than the cytoplasm. (A) Insulin-induced Ca^{2+} signals are attenuated by the nuclear but not the cytosolic InsP3 buffer. SkHep1 cells loaded with fluo-4 were stimulated with insulin (100 ng/mL) while examined by time-lapse confocal microscopy. Graphical representation of the nuclear and cytosolic Ca^{2+} signal detected in a representative cell from each experimental group stimulated with insulin is shown. Ca^{2+} increases in the nucleus and cytosol are similar in nontransfected cells and in cells expressing the InsP3 buffer targeted to the cytosol, but Ca^{2+} signals in both compartments are attenuated when the InsP3 buffer is targeted to the nucleus. (B) Summary of InsP3 buffer studies confirms that insulin-induced Ca^{2+} signaling are significantly attenuated by buffering nuclear but not cytosolic InsP3. Values are mean ± standard error of the mean (SEM) of the peak fluo-4 fluorescence attained during the observation period (expressed as % of baseline) and include the response from 23 nontransfected cells, seven cells expressing the InsP3 buffer targeted to the nucleus, and five cells expressing the InsP3 buffer targeted to the cytosol (*$P < 0.005$).

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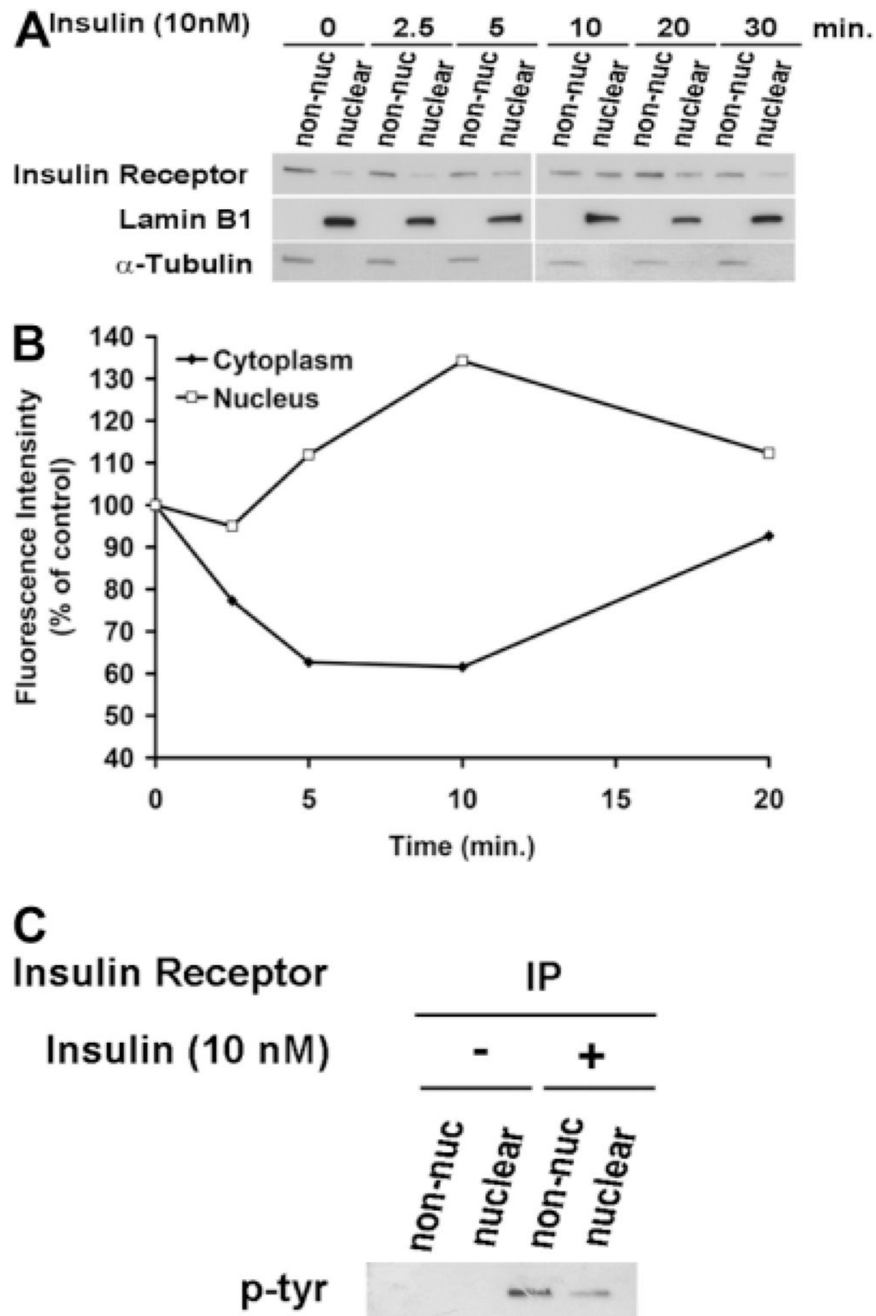

Fig. 7.

The insulin receptor translocates to the nucleus. (A) Immunoblots show the insulin receptor in nuclear and non-nuclear fractions of primary hepatocytes before and at serial time points after stimulation with insulin (10 nM). Trace amounts of the receptor are found in the nucleus at baseline, and this increases within 5 minutes and reach peak intensity within 10 minutes of stimulation. Blots are representative of what was observed in n = 3 separate experiments. Alpha-tubulin and Lamin B were used as purity controls for the non-nuclear and nuclear fraction, respectively.[23] (B) Densitometric measurement of subcellular fractions of the insulin receptor. The amount of the insulin receptor in the nucleus is maximal within 10 minutes of stimulation (n = 3). Note that the increase in insulin receptor in the nucleus is

transient and is temporally associated with a transient decrease in the receptor elsewhere in the cell. Measurements were normalized by alpha-tubulin and Lamin B levels in non-nuclear and nuclear fractions, respectively. Values are representative of what was observed in three separate experiments. (C) The phosphorylated insulin receptor reaches the nucleus. Insulin receptor was immunoprecipitated from the non-nuclear and nuclear fractions of hepatocytes before and 5 minutes after stimulation with insulin (10 nM), then probed with a phosphotyrosine-specific antibody. The phosphorylated receptor is found after but not before stimulation with insulin in both cell fractions.

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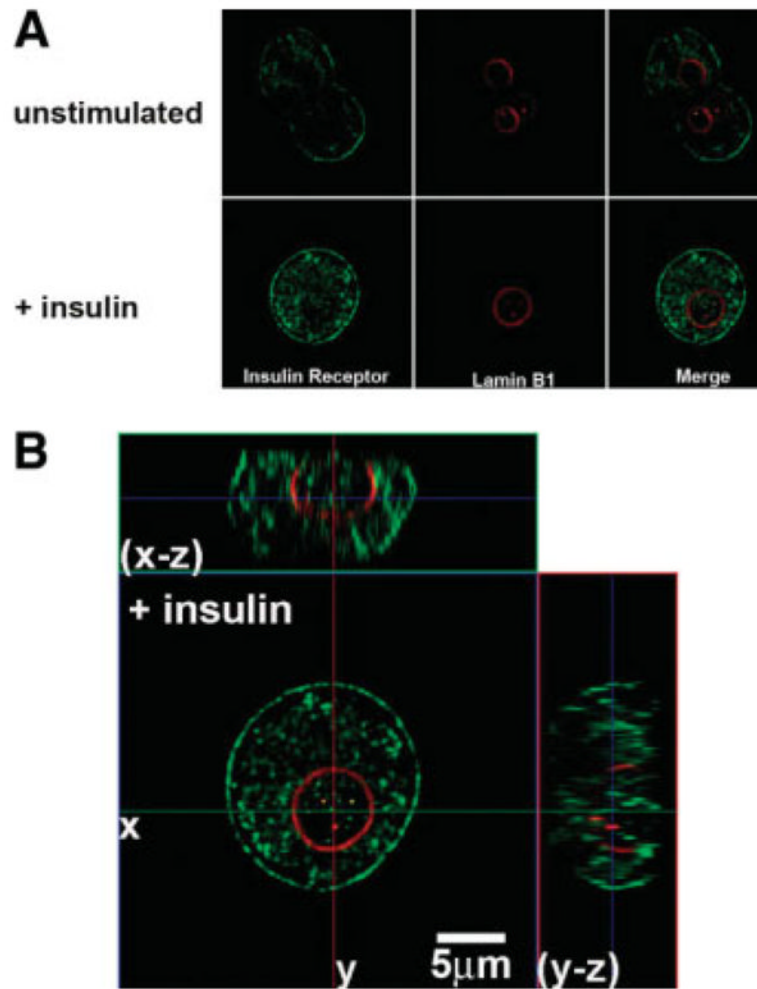

Fig. 8.

The insulin receptor translocates to the nucleus. (A) Confocal immunofluorescence images of the insulin receptor before and 5 minutes after stimulation with insulin (10 nM), respectively. Insulin receptor labeling is in green and the nuclear envelope is stained with Lamin B1 in red. Note that the receptor initially labels the plasma membrane and is heterogeneously distributed in the cytosol as well, but is excluded from the nuclear interior until after stimulation with insulin. (B) Serial confocal sections were collected for three-dimensional reconstruction. These double-labeled images confirm that the receptor is heterogeneously distributed within the nuclear interior after stimulation with insulin.