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Report

Insulin stimulates fibroblast proliferation through calcium-calmodulin-dependent kinase II

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Abbreviations: CaMK, calcium-calmodulin-dependent kinase; IRS, insulin receptor substrates; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinases; PKC, protein kinase C; PLC, phospholipase C

Key words: CaMK, insulin, cell signalling, proliferation, Raf

Insulin effects are mediated by multiple integrated signals generated by the insulin receptor. Fibroblasts, as most of mammalian cells, are a target of insulin action and are important actors in the vascular pathogenesis of hyperinsulinemia. A role for calcium-calmodulin-dependent kinases (CaMK) in insulin signaling has been proposed but has been under investigated. We investigated the role of the CaMK isoform II in insulin signaling in human fibroblasts. A rapid and transient increase of intracellular calcium concentration was induced by insulin stimulation, followed by increase of CaMKII activity, via L type calcium channels. Concomitantly, insulin stimulation induced Raf-1 and ERK activation, followed by thymidine uptake. Inhibition of CaMKII abrogated the insulin-induced Raf-1 and ERK activation, resulting also in the inhibition of thymidine incorporation. These results demonstrate that in fibroblasts, insulin-activated CaMKII is necessary, together with Raf-1, for ERK activation and cell proliferation. This represents a novel mechanism in the control of insulin signals leading to fibroblast proliferation, as well as a putative site for pharmacological intervention.

Introduction

Insulin has both metabolic and mitogenic effects in several cell mammalian cell types. Through its receptor (IR), it modifies the expression or the activity of a variety of enzymes and transport systems, stimulates glucose influx and metabolism in muscle cells and adipocytes, and inhibits gluconeogenesis in the liver. Insulin also stimulates the proliferation in many cell types, and a large body of evidence suggests that it is also an important regulator of cell growth in vivo, as demonstrated by leprechaunism and growth retardation caused by the deletion of the IR.[1] The capability of the IR to regulate such different functions depends upon its ability to generate a number of different intracellular signals initiated by the insulin receptor substrates (IRS-1 and IRS-2), and involving Akt/PKB, mitogen activated protein kinase (MAPK) and protein kinase C (PKC).[2-5]

The role of Ca^{2+} in insulin signaling has been investigated in different studies with various approaches. Whereas some studies suggest that Ca^{2+} is involved in insulin signaling in some cell types, others failed to demonstrate that insulin modifies intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).[6-9] A more recent study in single mouse muscle fibers suggested that insulin induces an increase of near-membrane Ca^{2+} concentration, while global myoplastic Ca^{2+} concentration increase does not significantly change.[10] In another study, insulin induced a very fast and transient $[Ca^{2+}]$ increase in rat myotubes.[11] Phospholipase C γ-1 (PLC γ-1) is an important modulator of the $[Ca^{2+}]_i$. This enzyme is activated by insulin stimulation of the IR and it has recently been shown to participate to insulin stimulated proliferation in 3T3-L1 adipocytes, Rat-1 fibroblasts and hepatocytes.[12-14]

An important mediator of Ca^{2+} signal is the calcium calmodulin-dependent kinase II (CaMKII), a ubiquitous serine-threonine kinase that phosphorylates a large number of substrates.[15,16] There is an extensive cross-talk between CaMKs and other signaling cascades, including those that involve the cAMP-dependent kinase (PKA), ERK and Akt/PKB. In thyroid cells, CaMKII is activated by integrins, and its binding to Raf-1 is necessary for Raf-1 activation.[17,18] Thus CaMKII regulates integrin-stimulated cell proliferation by modulating the Ras→Raf-1→Mek→ERK pathway. Consequently, we extended our studies on the crosstalk between CaMKII and the ERK pathway to insulin signaling. We have recently demonstrated that in a myotube cell model CaMKII is activated by insulin

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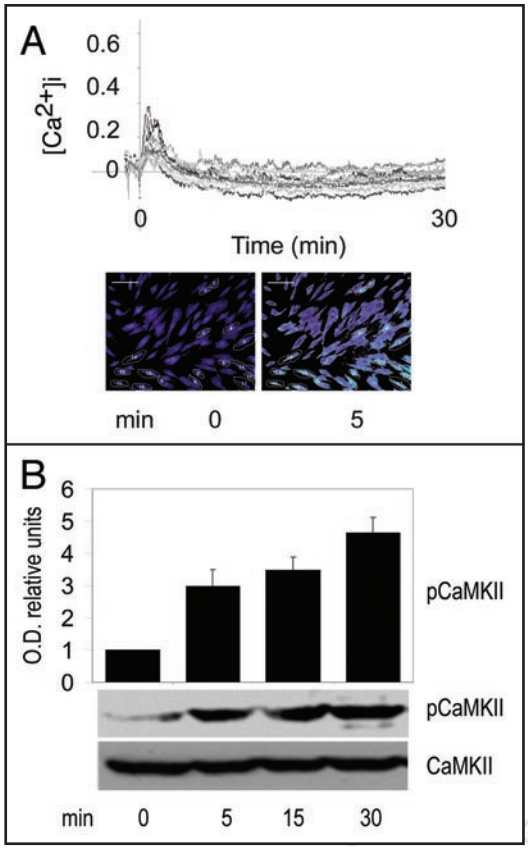

Figure 1. Insulin induces $[Ca^{2+}]_i$ increase and CaMKII activation. (A) fibroblasts cultured on glass coverslip were starved from serum, loaded with Oregon green and stimulated with 100 nM insulin. Selected cells were circled and labelled. $[Ca^{2+}]_i$ of single labelled cells was measured and reported in graph as Relative fluorescence = (fluorescence-fluorescence at 0 point)/fluorescence at 0 point. The white line on top of the images represents 10 μm. (B) starved fibroblasts were treated with 100 nM insulin for the indicated time. The cell lysates were analyzed by western blot with antibodies to total CaMKII or to phosphorylated T286-CaMKII (pCaMKII). (C) fibroblasts were treated with 100 nM insulin or 1 μM Bay K8644 after 30 minutes pre-treatment with 20 μM Nifedipine, and CaMKII phosphorylation was determined by western blot. In (B and C), averages and S.D. of the relative expression of phosphorylated kinases, were determined by scanning densitometry of three independent immunoblots. A value of 1 O.D. arbitrary unit was assigned to the unstimulated samples.

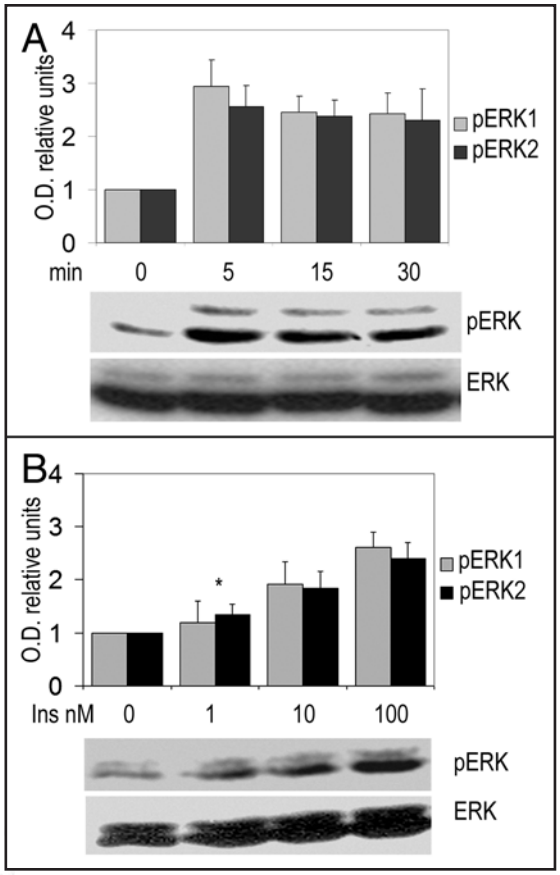

Figure 2. Insulin stimulates ERK activation. Fibroblasts were starved of serum and stimulated with 100 nM insulin for different times (A) or 30 min with different insulin concentrations (B). Cell lysates were analyzed by western blot with antibodies to total (ERK) or to phosphorylated ERK-1 and ERK-2 (pERK). Averages and S.D. of relative expression of phosphorylated kinases were determined by scanning densitometry of three independent immunoblots. A value of 1 O.D. arbitrary unit was assigned to the unstimulated samples. All points vs. 0 were significant, except that marked with *.

and that this in turn modulates cell proliferation and glucose uptake.[19]

In the present study we provide experimental evidences that in fibroblasts, insulin induces the activation of a Ca^{2+}/CaMKII pathway, necessary for the activation of the Ras→Raf-1→Erk pathway leading to cell proliferation. These findings confirm the role of CaMKII in insulin signaling and suggests that this is a general mechanism essential for the control of cell proliferation.

Results

Insulin stimulation induces CaMKII activation. CaMKII activation is achieved by Ca^{2+}/CaM binding and steric displacement of the autoinhibitory domain. In order to evaluate whether insulin was able to initiate the cascade leading to CaMKII activation, we measured $[Ca^{2+}]_i$ in fibroblasts following insulin stimulation (Fig. 1A). Fibroblasts were plated onto 60 mm microscope dishes, cultured for 24 h and serum-starved overnight. $[Ca^{2+}]_i$ was then determined at single cell level by a microscope with a Plan-Neofluar objective in adherent cells loaded with Oregon Green. Insulin induced a modest, sharp and transient $[Ca^{2+}]_i$ increase. A $[Ca^{2+}]_i$ peak occurred by 90 seconds of insulin stimulation in a dose dependent fashion in the range 10–100 nM, while at lower concentration no effect was observed (dose effect not shown). Right after this increase, the $[Ca^{2+}]_i$ decreased below the starting point in the following 30 minutes. A calcium wave is an event necessary to activate CaMKs. To evaluate whether insulin stimulated $[Ca^{2+}]_i$ increase was indeed followed by CaMKII activation, we evaluated CaMKII activity in fibroblasts by western blot with a phospho-specific antibody versus the phosphorylated Thr286 of the regulatory domain of CaMKII. The phosphorylation of this residue is an index of CaMKII autonomous activity (Fig. 2B). Our result displayed a faint band corresponding to phosphorylated

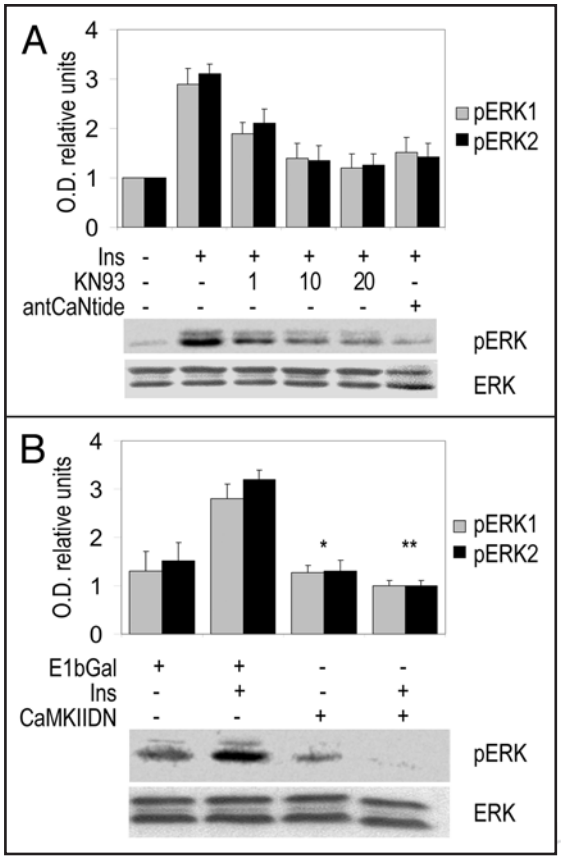

Figure 3. CaMKII is necessary to insulin-dependent ERK phosphorylation. (A) human fibroblasts in primary culture were starved of serum and stimulated for 30 min with 100 nM insulin. The cells were treated with KN93 at the indicated μM concentrations or with 5 μM antCaNtide. (B) the cells were infected with adenoviruses either an empty adenovirus (MOCK) or dominant negative CaMKII (CaMKIIDN) recombinant adenovirus, serum-starved overnight and then stimulated with 100 nM insulin. The level of ERK phosphorylation was evaluated by western blot. Averages and S.D. of relative expressions of phosphorylated ERK, were determined by scanning densitometry of three independent immunoblots. A value of 1 O.D. arbitrary unit was assigned to the unstimulated samples. *, significant vs. insulin alone.

CaMKII in unstimulated cells. Insulin stimulation induced a progressive, long lasting increase of CaMKII phosphorylation that reached a 5-fold increase after 30 minutes.

In order to determine the source of Ca^{2+} that is responsible for CaMKII activation following insulin stimulation, we tested the effects of L type Ca^{2+} channels antagonist nifedipine on insulin induced CaMKII phosphorylation. Fibroblasts were starved from serum for 24 hours and treated with 100 nM insulin after 30 minutes pre-treatment of 20 μM nifedipine. The L type Ca^{2+} channels antagonist Bay K8644 was used as a control at 1 μM. The phosphorylation of CaMKII was determined by western blot (Fig. 1C). Both insulin and Bay K 8644 induced an increase of CaMKII phosphorylation that was inhibited by nifedipine indicating that Ca^{2+} entry from L type Ca^{2+} channels are involved in CaMKII activation induced by insulin. Conversely, only a slight inhibition of CaMKII phosporylation was visible after treatment with the PLC inhibitor U73122 for 120 min (not shown).

Inhibition of CaMKII abrogates insulin-stimulated ERK phosphorylation. Fibroblasts were stimulated with insulin at different concentrations and for different times, and ERK activation was evaluated by western blot analysis (Fig.2). ERK activation was evident 5 min following insulin stimulation, and 10 nM was the minimum insulin concentration sufficient to generate a significant response.

A cross talk between the Ca^{2+}/CaMKII and ERK signaling pathways was previously demonstrated in integrin signaling.[17,19] To investigate the possible existence of a similar cross talk in insulin signaling, we tested the effects of CaMKII inhibitors on ERK phosphorylation induced by insulin. Fibroblasts were cultured in serum-free medium for 24 h and ERK phosphorylation was determined upon 30 min stimulation with insulin alone or in the presence of CaMKII inhibitors (Fig. 3A). Stimulation of ERK phosphorylation was completely inhibited by both antCantide and, in a dose dependent manner, KN93. In a separate set of experiments, CaMKII inhibition in stimulated fibroblasts was achieved by infection with a defective-recombinant adenovirus expressing a dominant-negative CaMKII mutated form (CaMKIIDN K72M, impaired ATP-binding pocket) (Fig. 3B). ERK phosphorylation was stimulated by insulin in fibroblasts infected with a control adenovirus expressing β-galactosidase. CaMKIIDN expression strongly reduced basal ERK phosphorylation and completely abrogated insulin stimulation.

CaMKII is necessary to Raf-1 activation. Two Raf isoforms have similar and distinct biochemical and functional properties, and vary in their cell-specific expression and subcellular localization. In a previous study, activation of Raf isoforms by insulin was not observed in NIH-3T3, while more recently a pivotal role for Raf-1 has been proposed in the insulin signaling in fibroblasts and in β-cells.[22-24] These results suggest that the Raf isoform/s involvement is cell-specific.

We measured Raf-1 and BRaf activity by insulin stimulation, and found that only Raf-1 was significantly activated. To investigate the possible involvement of CaMKII, serum starved fibroblasts were stimulated with insulin in the presence or in the absence of CaMKII inhibitors and Raf-1 and BRaf activity measured by in vitro kinase assay (Fig. 4). Whereas BRaf activity was not significantly affected by insulin, a two-fold increase of Raf-1 activity was observed. Inhibition of CaMKII abrogated the effects of insulin stimulation indicating that this kinase is required for insulin-induced Raf-1 activation.

Inhibition of CaMKII abrogates insulin-induced thymidine incorporation. [^{3}H]thymidine incorporation was used to measure DNA synthesis in fibroblasts cultured in serum-free medium, and stimulated with 100 nM insulin for 24 h (Fig. 5). Insulin stimulation induced a 2.8-fold increase of [^{3}H]thymidine incorporation. Both CaMKIIDN, KN93 and antCaNtide abrogated such stimulation, demonstrating that insulin-induced DNA synthesis requires CaMKII activity.

Discussion

The results of this study demonstrate that activated insulin receptor generates two interplaying signals that regulate the proliferation of human fibroblasts.

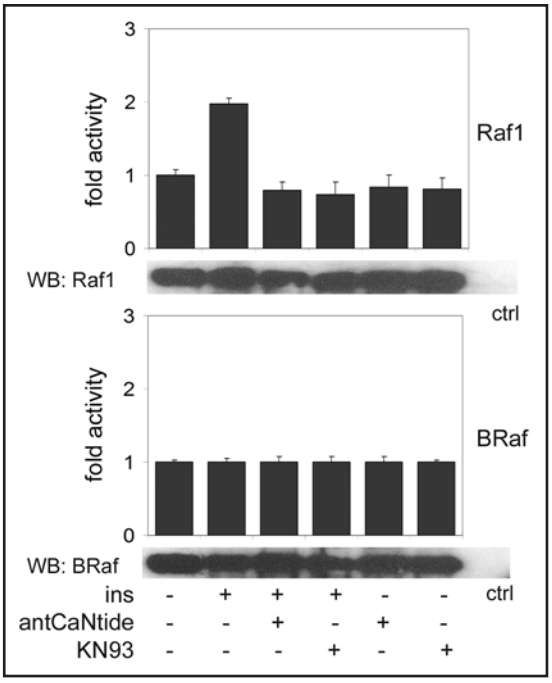

Figure 4. CaMKII is necessary for Raf-1 activation by insulin. Starved fibroblasts were stimulated with 100 nM insulin for 30 min in the presence of CaMKII inhibitors (10 μM KN93 or 5 μM antCaNtide). Raf isoforms were immunoprecipitated from cell lysates and their enzymatic activity was measured in vitro as reported in materials and methods. The total Raf immunoprecipitated was visualized by western blot. In the last lane (ctrl) non immune mouse IgG were used for the immumoprecipitation. Activated kinases was used as positive control in the assay (not shown). The diagrams report the mean and S.D. of fold kinase activity determined in triplicate experiments. In Raf-1, all experimental points were significant vs. insulin alone. Points with the inhibitors were not significant vs. unstimulated as well as each other. In BRaf, no significant differences were observed.

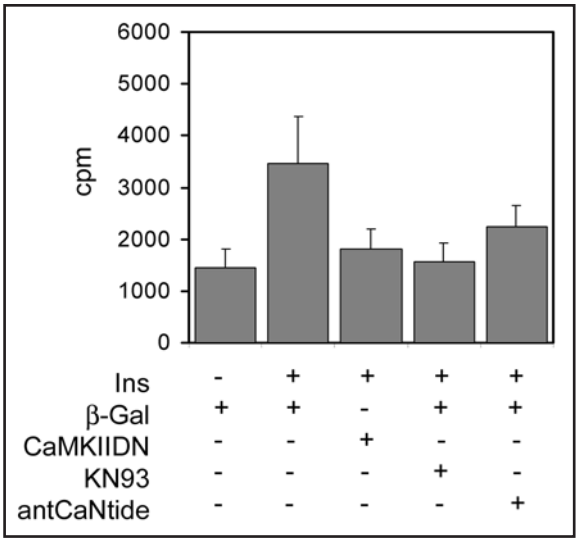

Figure 5. Inhibition of CaMKII abrogates insulin-induced thymidine incorporation. Fibroblasts (2 x 10^5) were infected with β-Gal or dominant negative CaMKII (CaMKIIDN) recombinant adenoviruses plated in 24-well plates, and then serum-starved for 48 hours. [^{3}H]thymidine and 100 nM insulin were then added to the plates. After 24 h, the plates were washed and insoluble TCA radioactivity counted by scintillation fluid in a β-counter. KN93 (10 μM) or antCaNtide (5 μM) were added where indicated. Data are reported as mean ± SD of quadruplicate experiments. *, significant vs. CTRL point. **, significant vs. insulin alone.

The role of Ca^{2+} in insulin signaling has been investigated in several studies. In Swiss 3T3 fibroblasts and in rat myocytes L6 stimulated with insulin, no $[Ca^{2+}]_i$ increase was observed by the calcium florescent indicator 3H-quin 2 acetoxymethyl ester.[25,26] More recently, in single mouse muscle fibers, insulin increased near-membrane Ca^{2+} concentration measured by Indo-1, while global myoplasmic $[Ca^{2+}]_i$ did not significantly change.[10] In the same study, the authors suggested that insulin stimulates Ca^{2+} entry into muscle fibers via L-type Ca^{2+} channels through a PI3K mediated mechanism. These results might be explained by differences in calcium homeostasis and response to insulin between different cell types, as well as by the sensitivity of the methods used to measure the $[Ca^{2+}]_i$. Although modest and transient, the $[Ca^{2+}]_i$ increase following insulin stimulation observed in fibroblasts in our study was sufficient to induce an intense and long lasting CaMKII activation. The amplification of an even modest calcium signal is made possible by the distinctive mechanism of CaMKII activation. CaMKII is a multimeric enzyme composed by 8–12 subunits The activation of a single CaMKII catalytic subunit requires Ca^{2+}/CaM binding. This binding causes a conformational change that relieves the autoinhibitory domain and allow the inter-subunit autophosphorylation in Thr286. After this phosphorylation the kinase gains an autonomous activity that makes it independent from Ca^{2+}. The catalytic domain of one subunit then phosphorylates and activates the adjacent subunits. This mechanism allows CaMKII to potentiate modest and transient increases in $[Ca^{2+}]_i$.[15] While a role for the Ca^{2+}/CaM/CaMKII signaling in the insulin-stimulated glucose uptake has been proposed in 3T3-L1 adipocytes and in rat myotubes, its role in the proliferation of human fibroblasts has not been investigated.[19,27,28]

We recently demonstrated that in L6 rat myotubes, CaMKII mediates insulin stimulated proliferation. In the present study, the observation that CaMKII inhibitors completely abolished ERK phosphorylation as well as [^{3}H]thymidine incorporation induced by insulin stimulation, demonstrates the pivotal role played by CaMKII also in human fibroblasts. To date, data on the interaction between CaMKII and Raf is limited to the Raf-1 isoform, while the interaction with ARaf and BRaf has not yet been investigated. Raf family members are differentially activated by growth factors and display varying abilities to activate MEK isoforms.[29] In thyroid cells, integrin stimulation by fibronectin generates a Ca^{2+}/CaMKII signal that modulates the Ras/ERK pathway.[17,18] In that model, CaMKII binds Raf-1 and participates to its activation together with Ras. Activation of Raf-1 is complex and involves multiple converging signalings, protein-protein interactions and Raf-1 phosphorylation at multiple sites.[30] Seven canonical R/KXXS/T consensus sequences for CaMKII are present in Raf-1 and one of these includes a serine (S338) necessary to full Raf-1 activation. Although to date direct experimental evidences have

not been provided, the presence of consensus sequences and the observation of Raf-1 phosphorylation by CaMKII in vitro, make Raf-1 a possible candidate substrate for CaMKII.[17] Which Raf isoform is activated by insulin and in which function it is involved has been investigated in different cell types. In a previous study, activation of Raf isoforms by insulin was not observed in NIH-3T3, while more recently a pivotal role for Raf-1 has been proposed in the insulin signaling in fibroblasts and in β-cells.[22,23] This supposes that the Raf isoform/s involvement is cell type specific.

We measured Raf-1 and BRaf activity by insulin stimulation, finding that only Raf-1 was significantly activated and that this activation was abrogated by CaMKII inhibition. These data demonstrate for the first time that the Raf-1/CaMKII interplay is necessary for the insulin→ERK signaling in human fibroblasts. The finding that the insulin signaling leading to ERK activation is modulated by Ca^{2+}/CaMKII provides new insights into the mechanism of signal transduction by which insulin stimulates the proliferation of some cell types.

Insulin and other growth factors and nutrients, also activate the mammalian target of rapamycin (mTOR) pathway, which in turn increases protein synthesis, stimulates cell mass growth and inhibits autophagy. It is believed that mTOR plays a pivotal role also in aging.[31] In normal human fibroblasts, stimulation of mTOR-mediated protein synthesis (i.e., by serum) coupled with cell cycle arrest leads to senescence, introducing the notion that block of the cell cycle without block of cell growth would cause cell senescence.[32]

Thus, inhibition of the Ca^{2+}/CaMKII/ERK pathway in the presence of insulin-stimulated mTOR pathway could lead to cell senescence.

The number of factors that modify the $[Ca^{2+}]_i$ is large. Thus, through the regulation of CaMKII activity they can modulate insulin signaling. Most factors increase the $[Ca^{2+}]_i$, while few others have an opposite effect, therefore fibroblasts proliferation induced by insulin can be activated or inhibited in different physiological and pathological situations.[33] CaMKII might have a role also in the pathogenesis of insulin resistance by modulating the insulin signal that leads to ERK and in turn to Akt/PKB activation. Insulin resistance represents the relevant metabolic abnormality that is at the fundament of hypertension, obesity and diabetes. Several evidences support the increase of ERK activation and the reduction of IRS-1/2 tyrosine phosphorylation and association with PI3-K in patients with type 2 diabetes.[34] Furthermore, calcium antagonists treatment is the suggested therapy for hypertensive, pre-diabetic patients, since their favorable effects on metabolism. The contribution of CaMKII to the changes in insulin receptor function should be further investigated.

Materials and Methods

Cell cultures. Human primary fibroblasts were obtained according to the procedure described by Postiglione et al.[20] Briefly, the surgical fragments derived from skin biopsies were mechanically dissociated and subsequently tripsinized for 30 min at 37°C. After repeated washes with PBS, the microfragments were plated and cultured in Dulbecco's minimal essential medium (BioWhittaker, Virviers, Belgium), containing 50% fetal calf serum (GIBCO, Grand Island, NY), 200 mM L-glutamine, penicillin (100 mg/ml) and streptomycin (100 mg/ml). The plates were incubated at 37°C, in the presence of 5% CO_2. The medium was removed after 10 days during which the percent of serum was progressively reduced. After 30 days the cells were maintained in culture with DMEM 10% FCS.

Calcium measurement in adherent cells. For calcium transients imaging, 2 x 10^5 cells were plated onto a 60 mm microscope dish, starved over night, and loaded with 12.5 mg Oregon green 488 BAPTA1, 100 μl of standard buffer (in mM: 137 NaCl; 2.7 KCl; 1 Na_2HPO_4; 20 Hepes, 7.4 pH; 1 $MgCl_2$; 2 $CaCl_2$; 2.5 glucose) for 30 min at 37°C. The cells were then washed once with standard buffer, and acquisition started in 500 μl standard buffer. For stimulation, insulin was used at 100 nM final concentration. Cytosolic Ca^{2+} changes were detected using a cooled CCD camera (Coolsnap HQ, Princeton Instruments, Inc., Trenton, NJ) mounted on a Zeiss Axiovert 200 microscope with a Plan-Neofluar 63 x/1.25 oil objective. The quantified Ca^{2+} signal was normalized to the baseline fluorescence (F0) following the formula Relative fluorescence = [F - F0]/F0, where F represents the average fluorescence level of the region of interest at a given time point. Fluorescent Ca^{2+} images were analyzed with the MetaMorph Imaging System software (Universal Imaging Corporation, West Chester, PA).

Western blot. For western blot analysis, the cells were washed in PBS buffer and lysed on ice for 30 min in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 2 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL pepstatin). An equal amount of protein from each sample was loaded with Laemmli buffer. Proteins were resolved by SDS-PAGE and transferred to an Immobilion P membrane (Millipore Corporation, Bedford, MA). Membranes were blocked by incubation with PBS 0.2% tween, 5% nonfat dry milk for one hour at room temperature. The membranes were then incubated overnight with primary antibodies at 4°C, washed for 40 minutes with PBS 0.2% tween and incubated for 1 hour with a horseradish peroxidase-conjugated secondary antibody. Finally, protein bands were detected by an enhanced chemiluminescence system (ECL, Amersham Bioscience). Computer-acquired images were quantified using ImageQuant software (Amersham Bio-sciences).

Mouse monoclonal antibodies to p44/p42 MAPK, phospho-p44/p42 MAPK, B-Raf, Raf-1 and CaMKII were purchased from Santa Cruz Biotechnology. Polyclonal anti-phospho-CaMKII antibody (pT286-CaMKII) was bought from Promega (Madison, WI).

[^{3}H]thymidine incorporation. To determine DNA synthesis, cells were plated in 24-well plates, and serum-starved for 48 hours in DMEM, 0.5% BSA. 0.5 μCi [^{3}H]thymidine and 100 nM insulin were then added to the plates. After 24 h, the plates were gently washed with PBS and then with 10% trichloroacetic acid (TCA), and incubated 10 min with 20% TCA at 4°C. TCA was

removed and cells were lysed with 0.5 N NaOH, 0.5% SDS for 15 min at 4°C. The lysates were then resuspended in 5 ml scintillation fluid and counted in a β-counter (Beckton Dickinson).

Raf-1 and B-Raf activity assay. Raf-1 and B-Raf activity was evaluated by a kinase cascade assay kit (Upstate Biotechnology). Briefly, the cells were lysed in RIPA buffer. Primary antibodies vs. Raf-1 and B-Raf and Protein G plus/Protein A agarose beads (Oncogene Science, Boston, MA) were used to immunoprecipitate corresponding proteins from 500 μg of total lysate. Non immune rabbit or mouse IgG were used as controls. The immunocomplexes were washed in PBS, resuspended in Assay Dilution Buffer I (ADBI, Upstate Biotechnology) and incubated in the presence of magnesium/ATP cocktail (Upstate Biotechnology), 0.4 mg inactive Mek-1 (Upstate Biotechnology), and 1 mg Erk-2 inactive (Upstate Biotechnology) for 30 min at 30°C. An aliquot of the mixture was then incubated with 20 μg of myelin basic protein (MBP, Upstate) in the presence of [γ-^{32}P]ATP. The mixture was incubated for 10 minutes at 30°C. Twenty ml aliquots were spotted onto p81 phosphocellulose filters (Upstate Biotechnology, St. Louis, MO). The level of [^{32}P] incorporation into MBP was determined by liquid scintillation counting.

Reagents and inhibitors. The PLC inhibitor U73122 and the L type calcium channels inhibitor Nifedipine were purchased from SIGMA (St. Louis, MO). BayK 8644 was purchased from Research Biochemicals Inc., (Natick, MA). The CaMK inhibitor KN93 was purchased from Biomol (Plymouth Meeting, PA).

The CaMKII specific inhibitor antCaNtide is derived from the endogenous CaMKII inhibitor protein CaMKIIN and was made cell permeable by N-terminal addition of an antennapedia-derived sequence (antCaNtide: RQI KIW FQN RRM KWK KRP PKL GQI GRS KRV VIE DDR IDD VLK).[21]

Adenoviral infection of cells. The kinase deficient mutant of CaMKIIα, K42M, was subcloned into pSP72 (Promega, Madison, WI). CaMKII kinase deficient mutant (CaMKII DN) adenoviruses were generated using the AdEasy system first described by He and colleagues and now available from Qbiogene (Montreal, Canada).[6] The methods used to generate these viruses were based on the initial description of the system, and the protocols published by Qbiogene (formerly, Quantum Biotechnologies).

For fibroblasts infections: Approximately 1.5 x 10^6 cells were plated in 100 mm dish 24 hours before infection, by which time they reached 70–80% confluence. The cells were incubated at 37°C with 1 ml of cell medium containing an amount of adenovirus of 100 viral particle/cell (vp/cell). After 30 min of incubation, 6 ml of DMEM supplemented with 10% FBS were added to the plates. The experiments were performed 24 after the infection.

CaMKII DN adenovirus and the mock control virus were a generous gift provided by Dr. A.R. Means (Duke University, Durham, NC).

Statistical analysis. Results are presented as the mean ± SD. Statistical analysis was performed by using the t test. The level of significance was set at p less than 0.05.

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