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Calcium-calmodulin-dependent kinase II (CaMKII) mediates insulin-stimulated proliferation and glucose uptake

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ABSTRACT

Cellular growth and glucose uptake are regulated by multiple signals generated by the insulin receptor. The mechanisms of individual modulation of these signals remain somewhat elusive. We investigated the role of CaMKII in insulin signalling in a rat skeletal muscle cell line, demonstrating that CaMKII modulates the insulin action on DNA synthesis and the negative feedback that down regulates glucose uptake. Insulin stimulation generated partly independent signals leading to the rapid activation of Akt, Erk-1/2 and CaMKII. Akt activation was followed by Glut-4 translocation to the plasma membrane and increase of glucose uptake. Then, IRS-1 was phosphorylated at S612, the IRS-1/p85PI3K complex was disrupted, Akt was no more phosphorylated and both Glut-4 translocation and glucose uptake were reduced. Inhibition of CaMKII abrogated the insulin-induced Erk-1/2 activation, DNA synthesis and phosphorylation of IRS-1 at S612. Inhibition of CaMKII also abrogated the down-regulation of insulin-stimulated Akt phosphorylation, Glut-4 membrane translocation and glucose uptake.

These results demonstrate that: 1 — CaMKII modulates the insulin-induced Erk-1/2 activation and cell proliferation; 2 — after the initial stimulation of the IRS-1/Akt pathway, CaMKII mediates the down-regulation of stimulated glucose uptake. This represents a novel mechanism in the selective control of insulin signals, and a possible site for pharmacological intervention.

1. Introduction

Insulin has both metabolic and mitogenic effects in several mammalian cell types. 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 of a variety of cells, and a number of evidence suggest that it is also an important regulator of growth *in vivo*. The capability of the insulin receptor to regulate such different functions depends upon its ability to generate a number of different intracellular signals including those involving Akt, mitogen activated protein kinase, protein kinase C (PKC) and Ca^{2+}. It remains to be determined how all these signals are individually modulated to respond to environmental stimuli and cellular requirements. The insulin receptor is a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity, modulated by insulin binding through auto phosphorylation. Direct substrates of the insulin receptor are the insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) [1]. After insulin binding, the major substrate IRS-1 is phosphorylated in several tyrosine residues [2–5]. Phosphorylation of these tyrosines is required to bind the p85 subunit of PI3-kinase (PI3-K) and to the subsequent Akt activation. Insulin binding to its receptor activates different pathways that transduce signals to the nucleus [6,7]. Notably, the insulin receptor can regulate its own signal through a negative feedback loop involving Erk-1/2. This inhibition was found to correspond to phosphorylation of the serine 612 in IRS-1 by Erk-1/2. Phosphorylation of IRS-1 at serine 612 by Erk-1/2 appears to inhibit phosphorylation at tyrosine 608, the major p85PI3-K binding site [8–10]. More recently, liver knockout experiments demonstrated that Gab1 mediates insulin-stimulated Erk-1/2 activation [11]. This scaffolding adaptor protein, while reducing insulin-stimulated glucose metabolism in the liver, promotes hepatocyte proliferation induced by insulin. Thus, modulation of Erk-1/2 or Gab1 interferes with glucose uptake and metabolism and participates to glucose homeostasis. This may be part of a mechanism by which insulin

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receptor itself or endogenous and environmental interfering factors can modulate insulin action.

The role played by Ca^{2+} in insulin signalling has long been debated, and remains still partly unsolved. Indirect evidence suggest that Ca^{2+} is involved in insulin action, as its chelation reduces insulin stimulation of glucose uptake [12]. Noteworthy, insulin does not induce a significant increase of the cytoplasmic intracellular calcium concentration $[Ca^{2+}]i$ in skeletal muscle cells, however it increases the near-membrane free Ca^{2+} concentration with a mechanism mediated by PI3-K, involving L-type Ca^{2+} channels [13,14]. An important mediator of Ca^{2+} signal is the calcium calmodulin-dependent kinase II (CaMKII). CaMKII is a ubiquitous serine–threonine kinase that phosphorylates a large number of substrates [15,16]. Several isoforms exist of CaMKII derived from alternative splicing, some located in the cytoplasm, others in the nucleus [17]. In the cytoplasm, there is an extensive cross-talk between CaMKs and other signalling cascades, including those that involve the cAMP-dependent kinase (PKA), Erk-1/2 and Akt [18–20].

CaMKII modulates the integrin signal in thyroid cells [18,21]. CaMKII binding to Raf-1 is necessary to Raf-1 activation by Ras, thus modulating the Ras→Raf-1→Mek→Erk-1/2 pathway generated by fibronectin-dependent integrin activation in this cell type [18]. We have now provided experimental evidence for a novel role of CaMKII in the insulin signalling cascade. In L6 skeletal muscle cells, insulin-activated CaMKII has a permissive role in Erk-1/2 phosphorylation and cell proliferation. Moreover, consistent with Erk-1/2 regulatory action, CaMKII is necessary for the attenuation of Akt activation that leads to the down-regulation of insulin-stimulated glucose uptake. This represents a novel mechanism in the selective control of insulin signalling.

2. Materials and methods

2.1. Cell cultures

The L6 skeletal muscle cells were plated (6×10^3 cells/cm^2) and routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 2 mM glutamine. Cultures were maintained at 37 °C, in a humidified atmosphere containing 5% (v/v) CO_2. Before the experiments, cells were cultured with DMEM supplemented with 2% FBS, to allow differentiation. Under these culture conditions, L6 myoblasts spontaneously differentiate into myotubes upon confluence. The myotube stage of differentiation was assayed by evaluating fusion index (the relative proportion of nuclei in myotubes and in mononucleated cells) and quantitation of creatine kinase activity, as described by Caruso et al. [22].

2.2. Western blot and immunoprecipitation procedures

For Western blot analysis, the cells were lysed in Laemmli buffer (125 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 1% β-mercaptoethanol, and 0.006% bromphenol blue), and proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Immobilon P; Millipore Corporation, Bedford, MA). Membranes were blocked by 5% nonfat dry milk, 1% ovalbumin, 5% FBS, and 7.5% glycine, washed and incubated for 1 h at 4 °C with primary antibodies, then washed again and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody. Finally, protein bands were detected by an enhanced chemiluminescence system (ECL, Amersham Biosciences). Computer-acquired images were quantified using ImageQuant software (Amersham Bio-sciences). For immunoprecipitation, the cells were lysed in immunoprecipitation buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 10 mM NaF, 5 mM EGTA, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonylfluoride). Rabbit polyclonal antibody reactive to all CaMKII isoforms and mouse monoclonal antibody to Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and protein G plus/protein A agarose beads (Oncogene Science, Boston, MA) were used to immunoprecipitate corresponding proteins from 1 mg of total lysate. Non-immune rabbit IgG was also used as a control. Mouse monoclonal antibodies to all CaMKII isoforms, Raf-1, p44/p42 MAPK and phospho-p44/p42 MAPK were from Santa Cruz Biotechnology. Polyclonal anti-phospho-CaMKII antibody (pT286-CaMKII) was from Promega (Madison, WI). Anti-IRS-1 and phospho-Ser612-IRS1 mouse monoclonal antibodies were from Cell Signalling Technology, Danvers, MA. Anti phospho-Thr 308 Akt and phospho-Ser 473 Akt were from Santa Cruz Biotechnology and Cell Signalling Technology respectively. Both antibodies gave identical results.

2.3. [^{3}H]thymidine incorporation

To determine DNA synthesis, cells were plated in 24-well plate, and serum-starved for 48 h 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 0.2% 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).

2.4. CaMKII activity and inhibitors

The cells were lysed in 200 μl of RSB buffer [23] with 10 mM CHAPS. CaMKII was immunoprecipitated and 500 μg of kinase was assayed in 50 μl of reaction mixture consisting of 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 2 μM CaM, 100 nM microcystin, 50 μM ATP (1500 cpm/pmol [γ32P]ATP), and 0.1 mM of substrate peptide autocamtide II [24]. Total CaMK activity was determined by including 1 mM $CaCl_2$ in the mixture, while autonomous activity was measured in the presence of 2.5 mM EGTA. Ionomycin (Sigma) at a concentration of 500 ng/ml was used as a positive control for CaMKII activation. The reaction was carried out for 2 min at 30 °C and 20 μl aliquots of the reaction mixture were spotted onto p81 phosphocellulose filters (Upstate Biothechnology, Lake placid, NY) as described previously [25]. Purified CaM and autocamtide II were a kind gift from Dr. AR. Means, Durham, NC. The CaMK inhibitor KN93 and the CaM inhibitors trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-nafthalene-sulfonamide (W7) were purchased from Sigma. The CaMKII specific inhibitor antCaNtide is derived from the endogenous CaMKII inhibitor protein CaMKIIN [26,27] and was made cell permeable by N-terminal addition of an antennapedia-derived sequence (antCaNtide: RQIKIWFQNRRMKWKKRPPKLGQIGRSKRVVIEDDRIDDVLK) [28]. The reversed antCaNtide peptide was also used as a control. Both peptides were synthesized by L. D'A.

2.5. Raf-1 activity assay

Raf-1 activity was evaluated by a Raf-1 immunoprecipitation-kinase cascade assay kit (Upstate Biotechnology). Briefly, Raf-1 was immunoprecipitated from 1 mg of cell extracts. The immunocomplexes were washed and incubated in the presence of magnesium/ATP, inactivated Mek-1, and Erk-2 for 30 min at 30 °C. An aliquot of the mixture was then incubated with 20 μg of myelin basic protein (MBP) in the presence of [γ-32P]ATP. The reaction was quenched with Laemmli buffer, and proteins were separated through a 10% polyacrylamide/tris glycine gel. Radioactive-phosphorylated MBP on dried gels was quantified with the use of a Phosphor-Imager (Amersham Biosciences).

2.6. Subcellular fractionation and Western blot analysis of Glut-4

Serum starved cells were exposed to 5 μM antCaNtide and then stimulated with insulin for the indicated time. Then, the cells were further incubated 10 min in glucose-free buffer, washed in ice-cold phosphate-buffered saline and homogenized in 500 μl of Buffer A

(20 mM Tris–HCl, pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 100 mg/ml leupeptin, 0.15 units/ml aprotinin) by passing them 10 times through a 22-gauge needle. The cell lysates were centrifuged at 800 ×g for 5 min at 4 °C. Supernatants were further centrifuged at 100,000 ×g for 20 min at 4 °C. The membrane pellet was solubilized in Buffer A containing 1% Triton X-100 by bath sonication and centrifuged at 12,000 ×g for 10 min at 4 °C, and the supernatant was used as the membrane fraction and analyzed by Western blot. Purity (>90%) of the fractions was assessed by immunoblot with antibody against Na–K/ATPase, as described in Caruso et al. [22].

2.7. Glucose uptake

Confluent cells were incubated in DMEM supplemented with 0.25% albumin for 18 h at 37 °C. The medium was aspirated and cells were further incubated for 30 min in glucose-free HEPES buffer (5 mmol/l KCl, 120 mmol/l NaCl, 1.2 mmol/l $MgSO_4$, 10 mmol/l $NaHCO_3$, 1.2 mmol/l $KHPO_4$, and 20 mmol/l HEPES, pH 7.8, 2% albumin). The cells were incubated with 100 nmol/l insulin for convenient time, supplemented during the final 10 min with 0.2 mmol/l [^{14}C]2-D-glucose. Cells were then solubilized and the 2-D-glucose uptake was quantified by liquid scintillation counting.

2.8. Statistical analysis

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

3. Results

3.1. Insulin induces Akt, Erk-1/2 and CaMKII phosphorylation in L6 cells

L6 cells were starved from serum and stimulated with insulin for 5 to 120 min. Erk-1/2, Akt and CaMKII phosphorylation were evaluated by Western blot with specific antibodies (Fig. 1A). Although overnight starvation was maximal and longer starvation would result in loss of cell viability, a band obtained by Erk-1/2 phospho-specific antibody was always consistent, suggesting that serum starvation was incomplete or that non-serum factors (i.e. integrins engagement, autocrine factors) are responsible for a constitutive low-activation state of Erk-1/2 in L6 cells. Insulin stimulation induced Erk-1/2 and CaMKII phosphorylation yet at 5 min and both the kinases remained phosphorylated up to 120 min. As observed in other cell types [10] after a rapid increase, Akt phosphorylation decreased to the initial level. Phosphorylation of Akt occurred at 5 min and the kinase was no more phosphorylated upon 120 min of insulin stimulation. Akt phosphorylation occurred in a dose-dependent manner and its down-regulation was independent by the insulin concentration (Fig. 1B).

3.2. Insulin stimulates CaMKII activation and its association with Raf-1

Among CaMKs, the isoform II is ubiquitary and is involved in the control of Ras→Erk-1/2 pathway upon integrin engagement [18]. The expression of α, β and γ CaMKII isoforms in L6 cells was demonstrated by Western blot (Fig. 2A). To evaluate whether insulin induced a dose-dependent CaMKII activation, we determined CaMKII phosphorylation by Western blot analysis and measured the kinase activity by *in vitro* assay. Western blot analysis displayed a faint band corresponding to activated CaMKII in unstimulated cells (Fig. 2B). Insulin stimulation induced a dose-dependent increase of CaMKII phosphorylation. CaMKII kinase activity was measured by the phosphorylation of its specific substrate autocamtide *in vitro* (Fig. 2C). The *in vitro* kinase assay confirmed what we observed by Western blot. Immunoprecipitated CaMKII from unstimulated cells displayed a consistent kinase activity, which was significantly increased by insulin stimulation. As integrins stimulate CaMKII activation and its association with Raf-1 [18], we investigated whether also insulin induces CaMKII/Raf-1 binding and participates to Raf-1 activation. A 3-fold increase of Raf-1 activity was observed by *in vitro* kinase assay in the cells stimulated with insulin, whereas it was inhibited by the CaMKs pharmacological inhibitor KN93 or the cell permeant CaMKII-inhibitory peptide antCaNtide (not shown). In order to determine whether CaMKII and Raf1 associated, CaMKII was immunoprecipitated from extracts of insulin-stimulated cells. Immunoprecipitates were analyzed by Western blot with antibodies to CaMKII and Raf-1 (Fig. 2D). Raf-1 co-precipitated with CaMKII upon insulin stimulation and was completely inhibited by KN93 and antCaNtide. These data suggest that activated CaMKII binds to Raf-1 thus participating to the formation of a multimolecular complex.

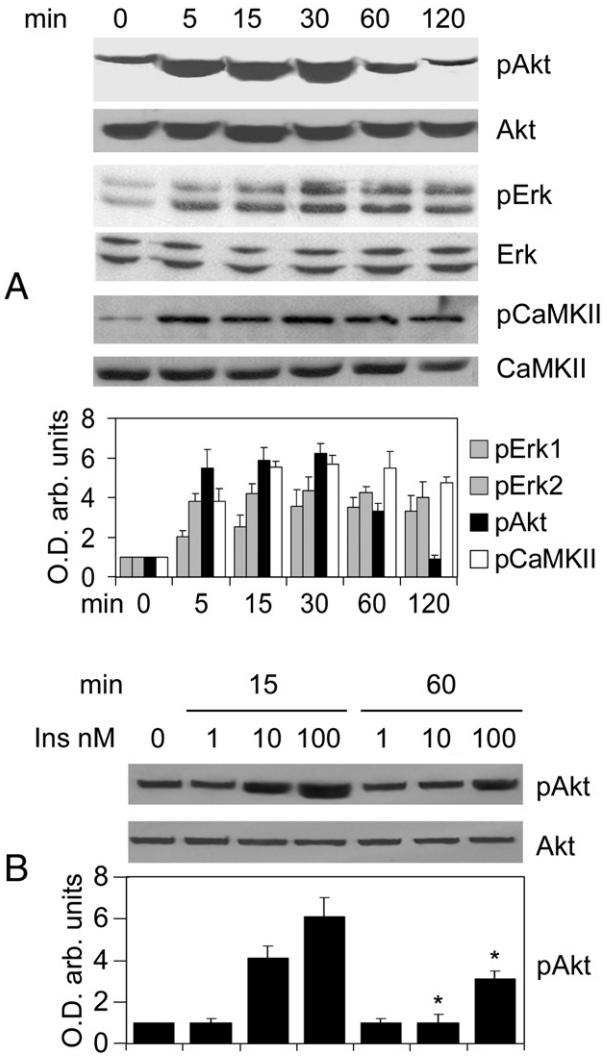

Fig. 1. Time course of insulin-induced signallings. L6 rat muscle cells were starved overnight from serum. A, The cells were stimulated with 100 nM insulin for 5 to 120 min and cell lysates were analyzed by Western blot with total or phospho-specific (p-) antibodies to T308Akt, Erk-1/2 or T286 CaMKII. Averages of relative expressions of phosphorylated kinases were also determined by scanning densitometry of three immunoblots. In each diagram, a value of 1 O.D. arbitrary unit was assigned to the 0 point. Only 120 min pAkt point was not significantly different *vs.* 0 point. pAkt 60 and 120 min were significant *vs.* 30 min. B, The cells were stimulated for 15 or 60 min with the indicated nM insulin concentration, and phosphorylated Akt determined by Western blot. Averages and S.D. of relative expressions of pAkt were determined as before. *Significant *vs.* corresponding nM concentration at 15 min.

3.3. Inhibition of CaMKII abrogates insulin-stimulated Erk-1/2 phosphorylation and thymidine incorporation

A cross talk between the Ca^{2+}/CaMKII and Erk-1/2 signalling pathways was previously demonstrated in integrin signalling. To investigate the possible existence of a similar cross talk in insulin signalling, we first tested the effects of CaMKII inhibitors on Erk-1/2 phosphorylation induced by insulin. A knockdown approach by small interfering RNA was considered, although to date its successful use has not been reported. However, because multiple isoforms of CaMKII are expressed in L6 cells, pharmacological inhibition and highly specific inhibition of enzymatic binding site were preferred [18,26,27]. The cells were stimulated with insulin in the presence or in the absence of KN93 or antCaNtide, and the levels of Erk-1/2 and Akt phosphorylation were evaluated by Western blot (Fig. 3A). Both inhibitors completely abolished Erk-1/2 phosphorylation induced by 30 min insulin stimulation. In addition, also basal phosphorylation was strongly reduced. These data suggest that in L6 cells, Erk-1/2 activation requires active CaMKII. [^{3}H]thymidine incorporation was used to measure DNA synthesis in cells stimulated with 100 nM insulin for 24 h (Fig. 3B). Insulin stimulation induced 65% increase of [^{3}H] thymidine incorporation. Both KN93 and antCaNtide completely abolished such stimulation, demonstrating that insulin-induced DNA synthesis requires CaMKII activity. Together with the reduction of ERK phosphorylation in non-stimulated cells, these data suggest

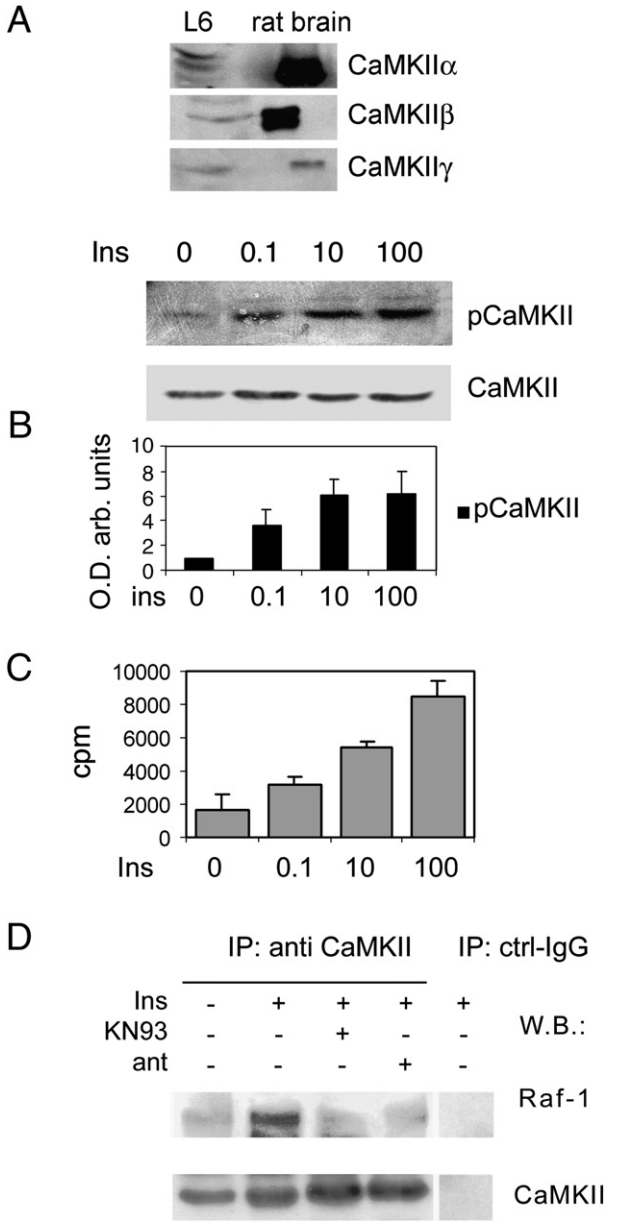

Fig. 2. Insulin induces CaMKII activation and association with Raf-1. Panel A, Extracts from L6 cells and rat brain were analyzed by Western blot with antibodies specific for the different CaMKII isoforms. In the other panels, the cells were starved from serum and stimulated with insulin at the indicated nM concentrations. Panel B, Cell lysates were analyzed by Western blot with antibodies to total CaMKII or to phosphorylated T286-CaMKII (p-CaMKII). Averages and S.D. of relative expressions of pCaMKII were also determined by scanning densitometry of three immunoblots. A value of 1 O.D. arbitrary unit was assigned to the point 0. All experimental points were significant *vs.* 0 point. Panel B, The cells were stimulated for 30 min with 0.1 to 100 nM insulin and total CaMKII was immunoprecipitated with a specific antibody. CaMKII activity was measured by a phosphorylation assay of the CaMKII peptide substrate autocamtide. The results are presented as total incorporated cpm. Data are reported as the mean+/−standard deviation from quadruplicate experimental points. All insulin concentrations but 0.1 nM *vs.* BSA, $p<0.001$. D, The cells were serum starved and stimulated with 100 nM insulin for 30 min in the presence of 10 μM KN93, or 5 μM antCaNtide (ant). The cell extracts were immunoprecipitated with a specific rabbit polyclonal anti-CaMKII antibody or an unrelated monoclonal antibody (ctrl-IgG). After protein separation by SDS-PAGE and transfer to a nitrocellulose membrane, immunoprecipitated CaMKII and coprecipitated Raf-1 were detected by specific mouse monoclonal antibodies. IP, immunoprecipitation; WB, Western blot.

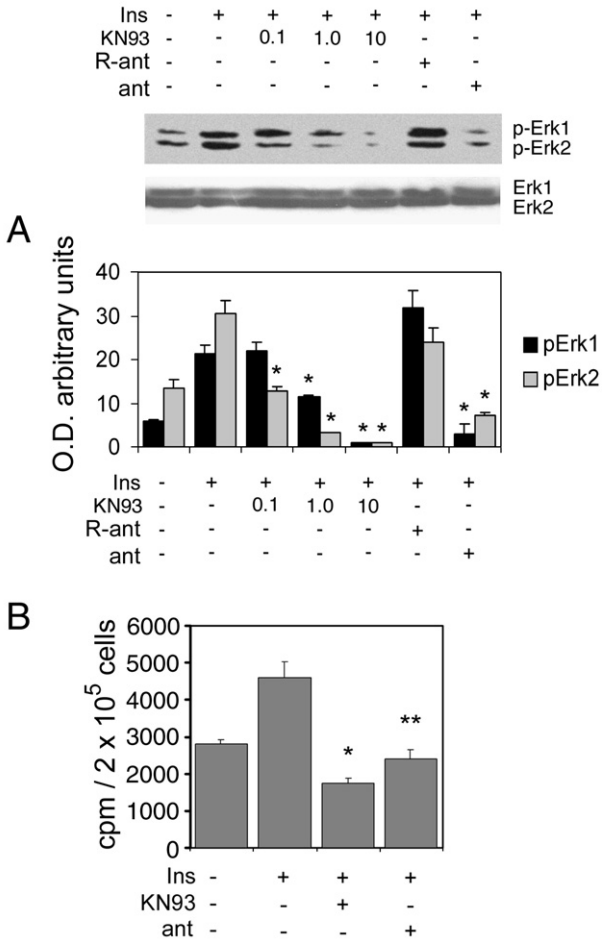

Fig. 3. CaMKII is necessary to insulin-dependent Erk-1/2 phosphorylation and DNA synthesis. Panel A, Serum-starved cells were stimulated for 30 min with 100 nM insulin. The cells were treated with KN93 at indicated μM concentrations or with 5 μM antCaNtide or R-antCaNtide (R-ant). Averages and S.D. of relative expressions of phosphorylated Erk were also determined by scanning densitometry of three independent immunoblots. A value of 1 O.D. arbitrary unit was assigned to the minor point. *, significant *vs.* insulin alone. Panel B, 2×10^5 cells were plated in 24-well plates, and serum-starved for 48 h. [^{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) was added where indicated. Data are reported as mean±S.D. of quadruplicate experiments. *, significant *vs.* CTRL point. **, significant *vs.* insulin alone.

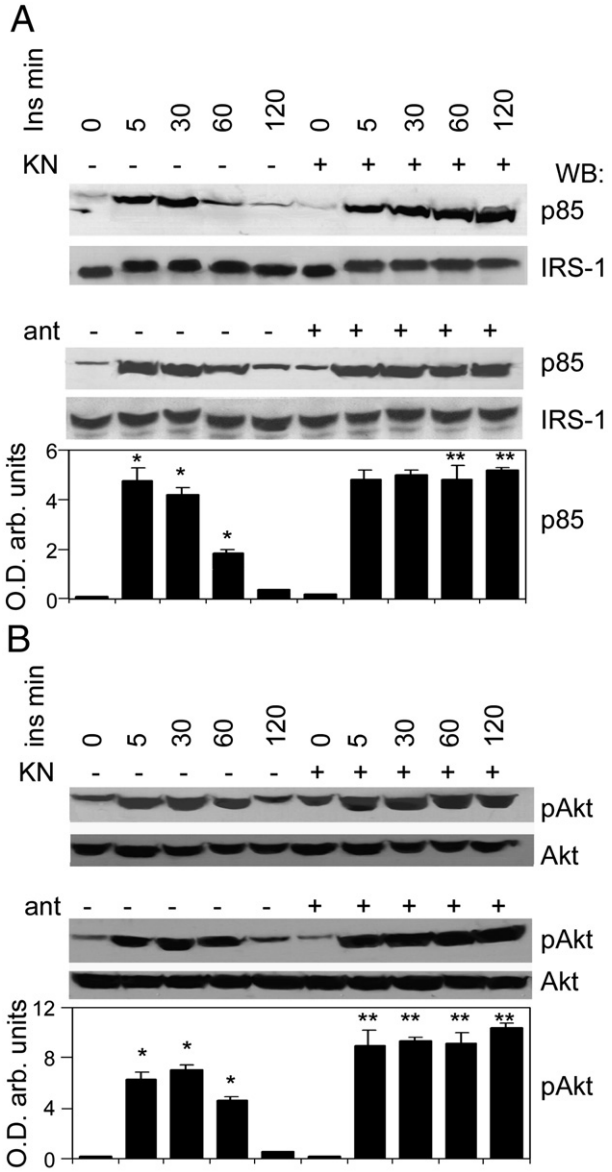

Fig. 4. CaMKII inhibition preserves insulin-stimulated IRS-1/p85PI3K complex and Akt phosphorylation. Panel A, The cells were pre-treated with 10 μM KN93 or 5 μM antCaNtide for 30 min and stimulated with 100 nM insulin for the indicated time. IRS-1 was immunoprecipitated from cell extracts and after protein separation by SDS-PAGE and transfer to a nitrocellulose membrane, immunoprecipitated IRS-1 and co-precipitated p85 subunit of PI3K were detected by specific antibodies. Panel B, The same cell extracts of panel A were analyzed by Western blot with total or phospho-specific antibodies to T308 Akt. In both panels, averages of co-precipitated p85PI3K or phosphorylated Akt with and without antCaNtide, were determined by scanning densitometry of three immunoblots. In each diagram, a value of 1 O.D. arbitrary unit was assigned to the 0 point. *, significant *vs.* CTRL point. **, significant *vs.* insulin alone.

that CaMKs participate also to the basal non-insulin mediated proliferation in L6.

3.4. CaMKII is necessary for the negative-feedback of insulin-stimulated IRS-1/p85PI3K complex and IRS-1 phosphorylation on S612

Erk-1/2 phosphorylates IRS-1 at Ser612 and inhibits the insulin-stimulated binding of IRS-1 to PI3-K, thus down-regulating Akt activation [8]. Based on our results, CaMKII inhibition was expected to suppress IRS-1 phosphorylation at Ser612, down-regulation of IRS-1/p85PI3K binding and Akt dephosphorylation. The cells were stimulated with insulin for 5 up to 120 min and then IRS-1 was immunoprecipitated. After protein separation, the phosphorylation at Ser 612 or the p85 subunit of PI3K bound to IRS-1 was detected by Western blot with specific antibodies. Co-precipitated p85PI3K appeared by 5 min of insulin stimulation, decreased at 60 min and disappeared by 120 min (Fig. 4A). CaMKII inhibition by both KN93 and antCaNtide preserved the IRS-1/p85PI3K complex up to 120 min of insulin stimulation. Also Akt phosphorylation was determined (Fig. 4B). After a rapid increase following insulin stimulation, Akt was no more phosphorylated at 120 min, whereas it was preserved by the inhibition of CaMKII.

Phosphorylation of IRS-1 at Ser612 initiated after 30 min of insulin stimulation and it remained up to 120 min (Fig. 5). AntCaNtide completely abrogated the phosphorylation of both Erk-1/2 and IRS-1 at Ser612. These results indicate that CaMKII is necessary for Erk-1/2 activation that in turn phosphorylates S612 IRS-1 that inhibits the formation of the IRS-1/p85PI3K complex.

3.5. CaMKII is necessary for the negative feedback of insulin-stimulated Akt phosphorylation, Glut-4 translocation and glucose uptake

The effect of CaMKII inhibition on the down-modulation of Akt phosphorylation was further evaluated (Fig. 6A). After 120 min of insulin stimulation, Akt was no more phosphorylated, whereas the phosphorylation was maintained by antCaNtide in a dose-dependent manner. We then determined the effect of CaMKII inhibition on Glut-4 translocation induced by insulin (Fig. 6B). Glut-4 translocation to the cell membrane was observed by 30 min of insulin treatment. By 120 min, plasma membrane Glut-4 was barely detectable. Pre-treatment of the cells with antCaNtide maintained Glut-4 in the membrane up to 120 min. The effect of CaMKII inhibition on insulin-induced glucose uptake was also determined. The cells were stimulated with insulin for the indicated time with or without 30 min antCaNtide pre-treatment. [^{14}C]2-D-glucose was added to the cells for the last 10 min of stimulation and the radioactivity determined (Fig. 6C). Glucose uptake was stimulated by insulin reaching its maximum at 60 min, and it returned to the basal level by 120 min. In the absence of insulin, no significant changes were

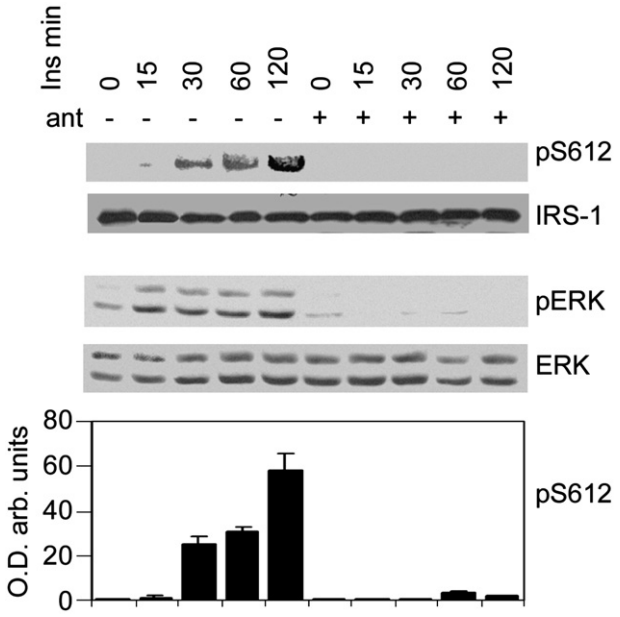

Fig. 5. CaMKII inhibition abrogates the insulin-stimulated phosphorylation of S612-IRS-1. The cells were treated as in Fig. 6, IRS-1 was immunoprecipitated from cell extracts and analyzed by Western blot with antibodies to phosphorylated-Ser612-IRS-1 (pS612) or total IRS-1. Averages and S.D. of relative expressions of coimmunoprecipitated p85PI3K or phosphorylated-S612-IRS-1 were also determined by scanning densitometry of three independent immunoblots. A value of 1 O.D. arbitrary unit was assigned to the control point.

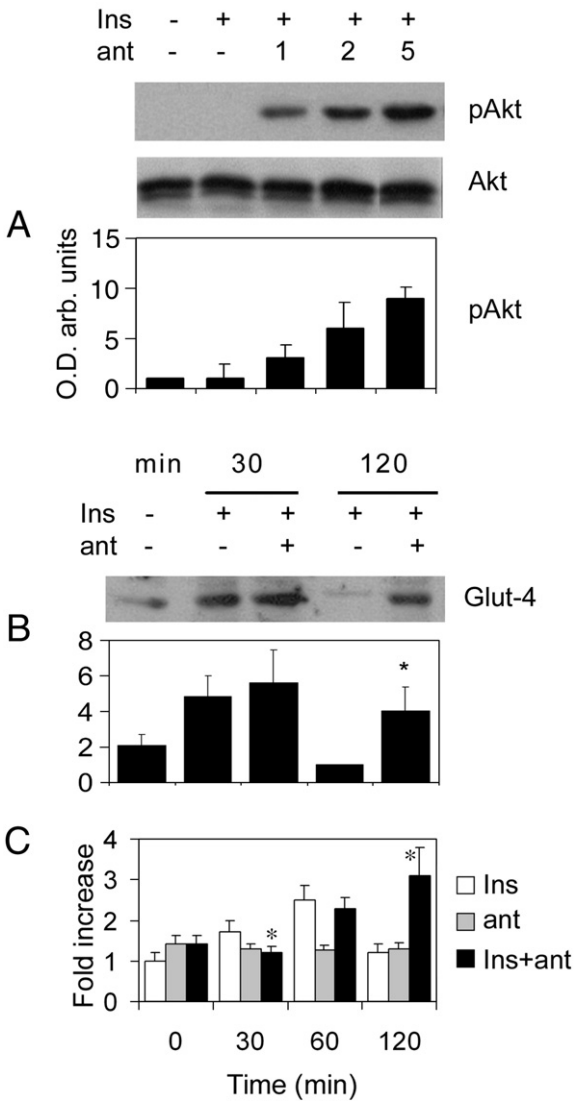

Fig. 6. CaMKII inhibition abrogates the down-regulation of insulin-stimulated Akt phosphorylation, Glu-4 membrane translocation and glucose uptake. Panel A, Phosphorylated S473 Akt and total Akt were visualized by Western blot in cells treated with insulin for 120 min and pretreated for 30 min with the indicated μM concentration of antCaNtide. Data are also presented as relative expressions of phosphorylated kinases determined by scanning densitometry of three immunoblots. A value of 1 O.D. arbitrary unit was assigned to the 0 point. Points with antCaNtide were significant *vs.* insulin. Panel B, Cell membrane extracts were prepared as described in Materials and methods from cells treated for 30 or 120 min with 100 nM insulin with or without 5 μM antCaNtide. Glut-4 and Na–K/ATPase (used as a membrane marker, not shown) were determined by Western Blot. Blots were quantitated by scanning densitometry and Glut-4 was normalized to Na–K/ATPase. A value of 1 O.D. arbitrary unit was assigned to the minor point. Averages and S.D. were calculated from three independent immunoblots. *, significant only *vs.* unstimulated cells. Thirty min insulin significant *vs.* unstimulated cells. Panel C, The cells were stimulated with insulin with or without 5 μm antCaNtide for the indicated time and [^{14}C]2-D-glucose was added to the medium in the last 10 min. Then, 2-D-glucose uptake was determined. Data are reported as mean ± S.D. of 3 independent experiments in triplicates. *, significant *vs.* insulin alone.

induced by antCaNtide pre-treatment, while a significant decrease of glucose uptake was induced upon insulin stimulation at 30 min. As predicted by the observation of the sustained Akt phosphorylation and Glut-4 in the membrane, CaMKII inhibition increased glucose uptake at 120 min.

4. Discussion

We have investigated the function of CaMKII in insulin action in L6 myotubes. The results of this study demonstrate that activation of insulin receptor generates time-dependent interplaying signalling systems that regulate cell growth and glucose uptake (Fig. 7).

The role of Ca^{2+} in insulin signalling has been investigated in different studies with different approaches. While some studies suggest that Ca^{2+} is involved in insulin signalling in several cell types, others failed to demonstrate that insulin modifies $[Ca^{2+}]i$ [29–31]. Klip et al. found that insulin did not induce $[Ca^{2+}]i$ increase in L6 cells [32]. Indeed, a subsequent study employing Indo-1 in single mouse muscle fibers suggested that insulin increases near-membrane Ca^{2+} concentration, while global myoplasmic $[Ca^{2+}]i$ does not significantly change [14]. In another study, insulin induced a very fast (2 s) and transient $[Ca^{2+}]i$ increase in rat myotubes [33]. The increase of $[Ca^{2+}]i$ (global or restricted at CaMKII location) induced by insulin was sufficient to activate a signalling pathway involving CaMKII, a kinase whose activation is dependent by Ca^{2+} and calmodulin. CaMKII activation by insulin was observed in rat soleus muscle but it was not demonstrated in 3T3-L1 adipocytes suggesting that the link between insulin and CaMKII may be tissue-dependent [34,35]. More recently, others suggested in rat adipocytes the involvement of CaMKII in glucose transport in a permissive role [35].

We previously demonstrated that in thyroid cells integrin stimulation by fibronectin, besides activating the Ras→Raf-1→Mek→Erk-1/2 pathway, also generates a Ca^{2+}→CaMKII signal that modulates the Ras→Erk pathway [18,21]. In thyroid cells, CaMKII is activated by integrin engagement and binds to Raf-1, participating with Ras to its activation. In the present study, the observation that CaMKII inhibitors completely abolished Erk-1/2 phosphorylation as well as [^{3}H]thymidine incorporation induced by insulin stimulation, demonstrate that in our model of rat myotube, Erk-1/2 activation requires active CaMKII. These results were reproduced also in human fibroblasts, where CaMKII inhibition achieved by a dominant-negative CaMKII expression demonstrated the pivotal role of this kinase in the insulin signal that leads to Erk-1/2 activation (manuscript in preparation). These results extend what we previously observed,

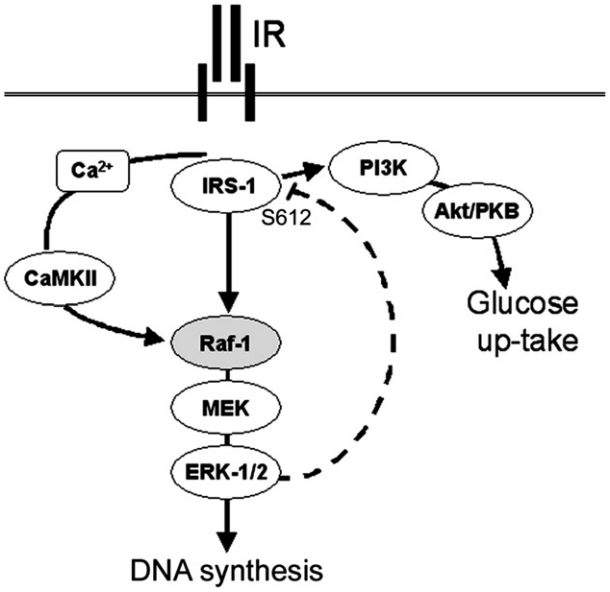

Fig. 7. Schematic diagram of the insulin receptor signalling in L6 cells. Activation of the insulin receptor generates the IRS-1/2→PI3-K→Akt signalling pathway and promotes glucose uptake. Insulin receptor activation generates two other signals: $[Ca^{2+}]i$→CaMKII and IRS-1/2→Erk-1/2. They both participate to Raf-1 activation, leading to stimulation of cell proliferation. Activated Erk-1/2 phosphorylates S612-IRS-1 and inhibits its association with PI3-K and in turn Akt activation, thus generating a negative feedback loop that down-regulates insulin stimulated glucose uptake. Abbreviations: IR, insulin receptor; IRS, insulin receptor substrate; Erk, extracellular regulated kinase; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; CaMKII, calcium-calmodulin dependent kinase II.

suggesting that the CaMKII/Raf-1 interplay is not a mechanism restricted to integrins in thyroid cells.

The finding that the insulin signalling leading to Erk-1/2 activation is modulated by CaMKII provides new insights into the mechanism of signal transduction that allows insulin to exert its pleiotropic effects. Glucose production by the liver and glucose uptake in certain tissues, such as fat, fibroblasts and muscle, depend upon insulin. At the same time, insulin also stimulates cell proliferation. Metabolic and mitogenic responses to insulin are paired in cell cultures, however *in vivo* the prevalence of one effect on the other may be more appropriated depending upon cell type, variations of environmental conditions or to maintain systemic homeostasis. Prior studies have indicated that phosphorylation of IRS-1 at S612 by Erk-1/2 inhibits IRS-1/PI3K binding and in turn Akt activation, thus generating a negative feedback loop regulating some of insulin effects. In L6 myotubes, both IRS-1 and IRS-2 are responsible of Erk-1/2 activation stimulated by insulin, whereas only IRS-1 phosphorylates Akt1 and is responsible of Glut-4 translocation and glucose uptake [36]. In the liver, the major site for glucose production and storage, this mechanism regulates glucose metabolism and finally influences the glycemia [37]. Liver knockout Gab1 mice demonstrated that mitogenic and metabolic effects can be dissociated by interfering with one component of the Erk-1/2 kinase cascade. By inhibiting Erk-1/2 activity, the cell might selectively increase the strength of the metabolic effects induced by insulin, and increased glucose uptake can be obtained only where required without increasing insulin secretion and generalized systemic effects. Because several are the factors that modify $[Ca^{2+}]$I and in turn CaMKII activation, this might represent a site where different effectors converge to modulate insulin signalling.

The involvement of Ca^{2+} in insulin-stimulated glucose transport has long been debated, and a large body of evidence associates $[Ca^{2+}]$i variation with modulation of glucose transport. Ca^{2+} channel blockers such as nifedipine, or the calmodulin inhibitor W7, reduced insulin-stimulated glucose transport in skeletal muscle cells [29–31]. Recently, another study proposed in 3T3-L1 adipocytes a permissive role for CaMKII in the insulin-stimulated glucose transport through a mechanism not involving Glut-4 translocation [35]. However, other disaccording results raised the possibility that Ca^{2+} might have biphasic effects on insulin-stimulated glucose transport, depending on the magnitude of $[Ca^{2+}]$i variations, i.e. ionomycin increased $[Ca^{2+}]$i and inhibited insulin-stimulated glucose transport in skeletal muscle cells [38].

The disaccording results obtained to date are probably the consequence of Ca^{2+}/CaMKII effects exerted at multiple sites and at different time. Our results are not in conflict with the positive role of CaMKII proposed in some studies. All these studies investigated the role of Ca^{2+} signalling at 30 min of insulin stimulation, and indeed CaMKII inhibition reduced the insulin-stimulated glucose uptake at 30 min (Fig. 6C). Nevertheless, no inhibition of Glut-4 plasma membrane expression was detected following CaMKII inhibition. This apparent discrepancy could be due to inhibition of CaMKII effects on other factors that participate in the glucose transport (i.e. Glut-1) or inhibition of phosphorylation events linked to CaMKII. However, we focused our attention on the following negative feedback loop involving the Erk/IRS-1 interplay, demonstrating a role for CaMKII also at this site. Thus, in the model we propose, the Ca^{2+}/CaMKII signalling modulates both Erk-1/2 activation as well as the following down-regulation of Akt activation (Fig. 7).

In this model, CaMKII is a pivotal kinase in both metabolic and mitogenic signals of insulin and its role in some insulin involving pathologies should be considered. Several evidence support the increase of Erk-1/2 activation and the reduction of IRS-1/2 tyrosine phosphorylation and association with PI3K in patients with type 2 diabetes [39]. CaMKII might have a role in the pathogenesis of insulin resistance by modulating the insulin signal that leads to Erk-1/2 and in turn to Akt activation. The contribution of CaMKII to these changes in insulin receptor function is worthy to be further investigated.

5. Conclusions

We identified novel molecular mechanisms by which CaMKII modulates the insulin signallings and final cellular effects. By modulation of CaMKII, insulin controls its own signals and effects on cellular growth and glucose metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2009.01.022.

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