Doctorate Program in Molecular Oncology
and Endocrinology
Doctorate School in Molecular Medicine

XX cycle - 2004–2007
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"CaMKII regulates insulin-mediated cell proliferation and glucose uptake in skeletal muscle cells through Erk 1/2 activation"

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“CaMKII regulates insulin-mediated cell proliferation and glucose uptake in skeletal muscle cells through Erk1/2 activation”

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1) Illario M, Cavallo A.L, Monaco S, Di Vito E, Mueller F, Marzano LA, Troncone G, Rossi G, Vitale M.
Fibronectin-induced proliferation in thyroid cells is mediated by alphavbeta3 integrin through Ras/Raf-1/MEK/ERK and calcium/CaMKII signals.
J Clin Endocrinol Metab. 2005;90(5):2865-73.

2) Illario M, Monaco S, Cavallo AL, Esposito I, Formisano P, D'Andrea L, Santella G, Fenzi G, Rossi G, Vitale M.
Calcium-Calmodulin-dependent kinase II (CaMKII) partecipates to insulin-stimulated Erk1/2 activation and modulates proliferation and glucose uptake.
In preparation

ABSTRACT

Insulin has both metabolic and mitogenic effects in several mammalian cell types. The pleiotropic effects of insulin include cellular growth and glucose uptake. These events are regulated by multiple signals generated by the insulin receptor. Insulin stimulates glucose influx and metabolism in muscle and liver cells through the Akt pathway, and induces cell proliferation through Erk activation. Insulin receptor regulates its own signal in target tissues through a negative feedback loop involving the phosphorylation of the Insulin Receptor Substrate 1 (IRS1) at serine 612, thus preventing the binding of the PI3K regulatory subunit p85, and the subsequent activation of the Akt pathway. By this mechanism, insulin-stimulated glucose uptake is down-regulated.

The calcium calmodulin-dependent kinase II (CaMKII) controls Ras/Erk signalling cascade in response to cell adhesion to extracellular matrix. In order to evaluate whether this role of CaMKII is a general phenomenon, I investigated its role in insulin signalling. As a cell model I selected a rat skeletal muscle cell line (L6) and a primary culture of human fibroblasts. Insulin stimulation generated partly independent signals leading to the activation of PI3K/Akt and Ras/ERK1/2 cascades. Furthermore, an increase in $[Ca^{2+}]_i$ and CaMKII activation were observed following insulin stimulation in both cell types. In order to investigate the role of CaMKII in insulin signalling, I evaluated the effect of CaMKII inhibition on individual pathways generated by insulin stimulation. CaMKII inhibition was achieved by KN93 (a specific CaMKs pharmacological inhibitor), antCaNtide (CaMKII specific inhibitory cell-permeant peptide) or by infection with a CaMKII dominant negative recombinant adenovirus.

CaMKII inhibition abrogated insulin induced Erk1/2 activation and thymidine incorporation. Insulin promoted CaMKII association with Raf-1, and this binding was dependent upon CaMKII activity, suggesting that the interaction between CaMKII and Ras/Erk pathway occurred on Raf1.
Following insulin stimulation Erk phosphorylates IRS1 at Ser 612. This mechanism represents a negative feedback loop by which Insulin down regulates its own stimulation of glucose uptake. I investigated the role of CaMKII in this negative feedback by analysing the effects of CaMKII inhibitors on PI3K/Akt pathway. KN93 and antCaNtide prevented insulin induced phosphorylation of IRS1 at Ser 612 by Erk, and prolong the binding of p85 to IRS1. Inhibition of CaMKII abrogated the down-regulation of insulin-stimulated Akt phosphorylation, GLUT-4 translocation to the plasma membrane and glucose uptake.

These results demonstrate that: 1-CaMKII mediates the insulin-induced Erk-1/2 activation and cell proliferation, 2-CaMKII mediates the downregulation of glucose uptake by inhibiting PI3K/Akt pathway. This represents a novel mechanism in the selective control of insulin signals, and a possible novel site for pharmacological intervention.

1. BACKGROUND

1.1 The Ca^{2+}/Calmodulin dependent Kinases (CaMKs)

Calcium (Ca^{2+}) is a universal second messenger in eukaryotic cells. Changes in intracellular Ca^{2+} concentration play a major role in a broad array of cell functions including transcription, cell cycle, apoptosis, exocytosis, protein synthesis and motility, often having multiple roles within a single cell (Anderson and Kane, 1998). Cells typically maintain an intracellular Ca^{2+} level of 10^{-7} M, which is 10^4 times lower than the level outside the cell, by sequestering Ca^{2+} in several intracellular organelles (Hudmon and Schulman, 2002). Cells have an intricate network by which they accurately control cytoplasmic Ca^{2+} level. ATP dependent Ca^{2+} pumps shuttle Ca^{2+} into the two major Ca^{2+} sinks, the endoplasmic reticulum (ER) and the extracellular space (Hook Sara S, 2001). Various signals stimulate either waves or spikes of increased intracellular Ca^{2+} up to a concentration of 10^{-4}M. The source of Ca^{2+} depends on the stimulus. Tyrosine kinases receptors and G-protein coupled receptors classically increase Ca^{2+} concentration levels by producing inositol 1,4,5 triphosphate (IP_3), which induces Ca^{2+} release from the ER via IP_3 receptor (Berridge, 1993). Similarly, Cyclic ADP ribose can release Ca^{2+} from intracellular stores. Ligand gated ion channels and voltage dependent ion channels in the plasma membrane initiate Ca^{2+} entry via extracellular stores.

The predominant intracellular receptor for Ca^{2+} is Calmodulin (CaM), a small, highly conserved Ca^{2+} sensor that is ubiquitously expressed in mammalian cells (Bito, 1998). CaM serves as a receptor to sense changes in Ca^{2+} concentration and, in this way, mediates the second messenger role of these ion. Ca^{2+} binds to CaM by a structural motif called EF-hand, and a pair of these structures are located in both globular ends of the protein. When the four binding sites are filled, CaM undergoes a conformational change exposing a flexible eight-turn α helix, which separates the hydrophobic pockets present at each globular end of the protein. CaM thus become "loaded" with Ca^{2+} and capable to interact with one of its many target proteins in the cell. The interaction with target proteins, while usually of high affinity, is rapidly reversible upon a decline in Ca^{2+} concentration (Means, 2000). One of the major family of Ca^{2+}/CaM effectors is represented by the Ca^{2+}/CaM dependent protein kinases (CaMKs) which can be divided into dedicated CaMKs that phosphorylate a single specific substrate such as myosin light chain kinase (MLCK) and the multifunctional CaMKs including CaMKI, CaMKII and CaMKIV, that phosphorylate a large number of proteins (Braun and Schulman, 1995).

CaMKs exhibit fascinating regulatory properties. Their structure presents a regulatory portion containing an autoinhibitory domain interacting with the catalytic domain and keeping the enzyme inactive at resting calcium levels. According to this "intrasteric inhibition model" (Cruzalegui et al., 1992),

Ca^{2+}/CaM binding to a region adjacent to the autoinhibitory domain causes a change in conformation that allows access of substrate and ATP to the catalytic domain. Thus the CaMK can rapidly sense elevation in intracellular Ca^{2+}, becoming active following Ca^{2+}/CaM binding. Moreover, some of these enzymes are also equipped with mechanism enabling them to prolong their activity after Ca^{2+} has returned to basal levels. This "molecular memory" is documented for CaMKII, a multimeric enzyme composed of 8-12 subunits. Each subunits is a 50/60 kDa polypeptide containing a N-terminal catalytic domain, a central autoinhibitory and CaM binding region and a C-terminal domain responsible for multimerization and intracellular localization. A linearized diagram for the domain structure of CaMKII is shown in Figure 1.1

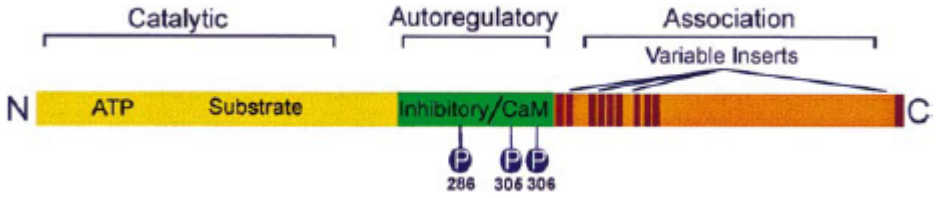


Figure 1.1 Linear diagram of a prototypical CaMKII subunit

The catalytic domain is autoinhibited by a pseudosubstate autoregulatory sequence that is disinhibited following Ca^{2+}/CaM binding. The association domain produces the native form of the enzyme, a multimeric holoenzyme composed of 8-12 subunits. Isoform differences present in α, β, γ, and δ isoforms of CaMKII are contributed primarily by a region of multiple alternatively spliced sequences, termed variable inserts, which reside in the association domain. Conserved sites of autophosphorylation are indicated in the autoregulatory region

The unique multimeric structure of CaMKII allows very rapid trans-phosphorylation of subunits within the complex. As a consequence of trans-phosphorylation on Ser 286, a subunit acquires Ca^{2+}/CaM independent activity (Miller and Kennedy, 1986). In addition, the affinity of the autophosphorylated subunits for CaM increases, making it more sensitive to further Ca^{2+} elevation (Anderson and Kane, 1998) . These properties of CaMKII allow the enzyme to remain active for some time after Ca^{2+} levels have dropped below the activation threshold, which explains why CaMKII activity is stimulated not only by sustained increases in Ca^{2+} concentration but also by Ca^{2+} oscillations (De Koninck and Schulman, 1998). The activity of CaMKI and CaMKIV, which act as monomeric enzymes, is also modulated by phosphorylation. However, in contrast to CaMKII, the regulatory phosphorylation events are catalysed by a distinct group of Kinases termed CaM Kinase kinases (CaMKK) (Soderling, 1999). CaMKKs themselves are Ca^{2+} /CaM dependent enzymes. They phosphorylate Ca^{2+} /CaM-bound CaMKI and CAMKIV on a Thr residue

situated within the activation loop. These phosphorylation events cause the activity of CaMKI and CaMKIV to increase several folds (Chatila et al., 1996).

Apart from the activation features, CaMKs differ in tissue and subcellular localization. CaMKII and CaMKI are ubiquitously expressed, while CaMKIV is tissue specific and expressed mainly in brain, thymus, testis, ovary, bone marrow and adrenal gland (Wang et al., 2001). While CaMKIV is predominantly nuclear (Jensen KF, 1991) and CaMKI appears to be a cytosolic enzyme (Picciotto MR, 1996), the subcellular distribution of CaMKII can vary (Heist and Schulman, 1998). Some of the comparative properties of CaMKI, CaMKII and CaMKIV are listed in Table 1.

Four types of subunits of CaMKII have been identified (α, β, γ, δ) that are encoded by different genes with differing tissue-specific expression. Alternative splicing within the C-terminal sequence of each gene produces further isoforms. Although the biochemical characteristics of CaMKII purified from many tissues are practically identical, the subunit composition, that is dependent on the source, seems to determine the subcellular localization of the complex. The most studied forms of CaMKII in the nervous system are rich in α and β subunits, and are mainly cytoplasmic enzymes. Some splice variants of the α, γ and δ CaMKII genes contain a nuclear localization signal (NLS) (Brocke et al., 1995; Srinivasan et al., 1994), resulting in targeting of the kinase to the nucleus. Expression of the δ isoform together with cytoplasmic isoforms of CaMKII can direct the heteromultimeric enzyme complex to the nucleus, suggesting that the relative abundance of cytoplasmic or nuclear-targeted subunits may determine the subcellular localization (Heist et al., 1998). The nuclear entry of CaMKII can also be regulated by other kinases. In all mammalian nuclear CaMKII isoforms, the NLS is followed by a string of four Ser residues. Phosphorylation of the four Ser by CaMKI or CaMKIV blocks both nuclear translocation of α subunit and binding to importin.(Griffith et al., 2003) . Another peculiarity of CaMKII is its association into a multimeric complex. The enzyme homomultimerizes through the association domain to form a holoenzyme of 8-12 subunits. Within the regulatory domain are overlapping autoinhibitory and CaM-binding domain. In the absence of Ca^{2+} /CaM, CaMKII is inhibited intrasterically by the autoinhibitory region, which is believed to prevent both substrate and ATP from binding to the enzyme. The binding of Ca^{2+}/CaM relieves the autoinhibitory domain thus leading to de-inhibition of the kinase.

Once activated by Ca^{2+}/CaM, CaMKII autophosphorylates on Thr286 located in the regulatory domain. This event has two important consequences: first, the affinity of the enzyme for Ca^{2+}/CaM is increased in a process called "CaM trapping"; Second, the autoinhibitory domain is further disrupted making CaMKII partially independent of Ca^{2+} /CaM or "autonomous" Autophosphorylation occurs between two neighboring subunits in one holoenzyme through an intersubunit reaction (Hudmon and Schulman, 2002) (Figure 1.2).

TABLE. 1.1: Properties of the multifunctional Ca^{2+}/CaM dependent protein kinases

	CaMKI	**CaMKIV**	**CaMKII**
Tissue distribution	Ubiquitous	Limited to: brain, thymus, testis, ovary, bone marrow and adrenal gland	Ubiquitous
Subcellular localization	Cytoplasmic	Nuclear and Cytoplasmic	Cytoplasmic, except α_B , γ_A , δ_B forms can be nuclear
Subunit composition	Monomeric	Monomeric	Homo- or heteromultimeric
Known requirement for complete activation	Ca^{2+}/CaM binding, activation loop phosphorylation	Ca^{2+}/CaM binding, auto-phosphorylation, activation loop phosphorylation	Ca^{2+}/CaM binding
Gains Ca^{2+}/CaM independence	No	Yes (up to 20% of Ca^{2+}/CaM dependent activity)	Yes (with auto-phosphorylation; up to 80% Ca^{2+}/CaM-dependent activity)
Inhibition by KN93 (K_I)	0,8 μM	3 μM	0,8 μM

CaMKII exerts a broad range of biological functions such as regulation of gene expression, cell cycle, proliferation and a number of neuronal functions. CaMKII is one of the most abundant proteins in the brain, comprising 1% of the total proteins in the forebrain and 2% in the hippocampus, a region associated with memory. The first of the CaMKII isoforms to be identified, $CaMKII_{\alpha}$ is a major component of the postsynaptic membrane (PSD) in pyramidal neurons. In the PSD, CaMKII is thought to increase synaptic strength by phosphorylating ion channels and signalling proteins such as glutamate receptors and N-metyl D-aspartate (NMDA) receptors.(Cruzalegui and Bading, 2000).

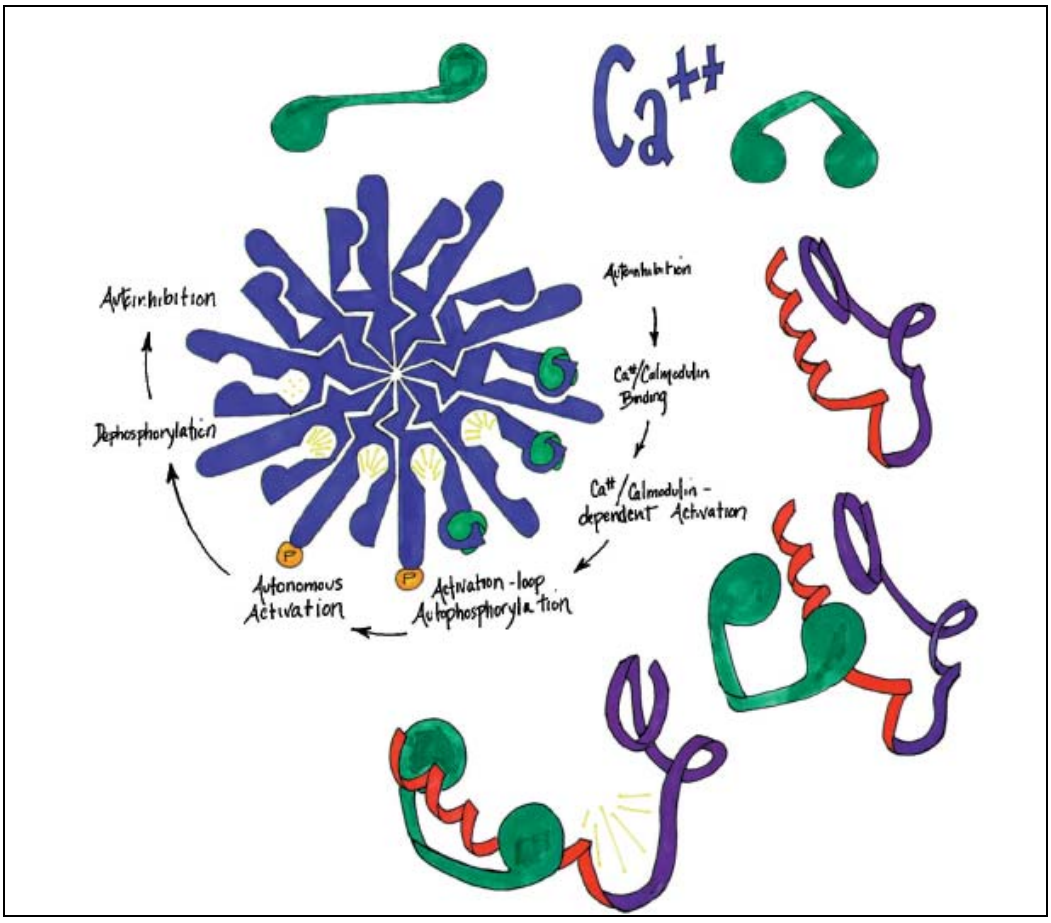


Figure 1.2 Ca^{2+}/CaM-dependent Kinase II activation

Clockwise from the top: CaM undergoes a conformational change upon Ca^{2+} binding. Ca^{2+}/CaM docks at hydrophobic site adjacent to the autoinhibitory domain of a CaMKII subunit. Stepwise activation, potentiation and inactivation of CaMKII are represented schematically in *blue*.(Means A. R. 2000)

In hippocampal neurons, it has been suggested that stimulation of the NMDA receptors regulates association of CaMKII between the F-actin and the PSD in a dynamic manner. Thus, CaMKII is involved both in the mainteinance of dendritic architecture and synaptic plasticity. In addiction CaMKII is required for long lasting changes in synaptic strength such as long-term potentiation (LTP), a process involved in learning and memory (Giese et al., 1998). As a result, modulatory changes in CaMKII have been hypothesized to be implicated in human pathologies of memory including Alzheimer Dementia and Angelman's mental retardation. However, CaMKII has several modulating roles in the neuron in addition to LTP induction, including presynaptic functions such as neurotransmitter synthesis, neurotransmitter secretion, microtubule disassembly and post synaptic functions such as phosphorylation of a number of proteins and kinases in the postsynaptic density of downstream neurons. A list of CaMKII neuronal functions is reported in Figure 1.3

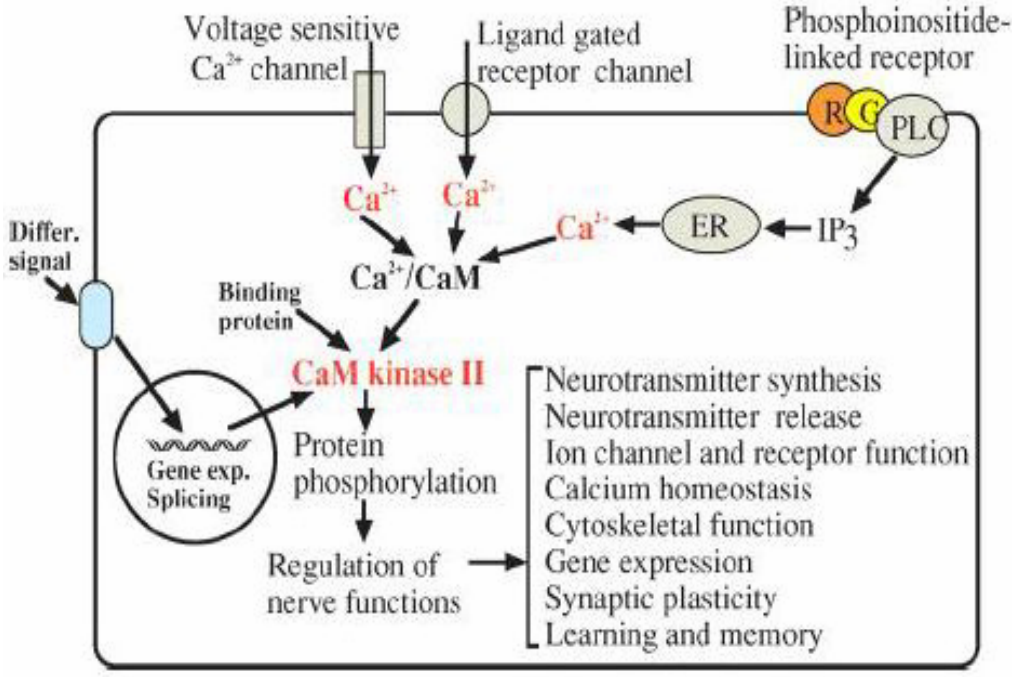


Figure 1.3 Schematic representation of activation and role of CaMKII in neuronal cells.

Intracellular Ca^{2+} is increased by intracellular stimuli, binds to Calmodulin, and activates CaMKII. CaMKII phosphorylates various kinds of proteins and regulates physiological processes. (Yamauchi, 2005)

Whereas the membrane and cytoskeletal roles of CaMKII are beginning to be clarified, the nuclear functions of CaMKII remain obscure. Most of the studies that have addressed nuclear functions of CaMKII have relied on the ability of C-terminal truncated Ca^{2+}/CaM-independent forms of the enzyme to alter transcription when over-expressed in cells along with reporter gene constructs. One of the first such paradigms to be examined was the Ca^{2+} stimulation of immediate early genes that are regulated by cAMP response element (CRE) such as c-fos. Phosphorylation of CRE binding protein (CREB) on Ser133 is essential for transcription because it is required for binding of the ubiquitously expressed CREB binding proteins CBP and p300, which function as transcriptional cointegrator (De Cesare et al., 1999). Ser-133 was originally identified as the target of protein kinase A (PKA), thus explaining the role of cAMP in transcriptional activation. However CaMKII can also phosphorylate this residue leading to the speculation that CAMKII mediates Ca^{2+} requirement for expression of the immediate early genes .Thus CREB was proposed as a target at which cAMP- and Ca^{2+}-mediated signal transduction cascades converge to regulate transcription. However, while the truncated form of CaMKII can stimulate CREB-mediated transcription in some cells, it is inhibitory in others. Sun at al. (Sun P, 1994) discovered that one mode of inhibition was that, in addition to Ser133, CaMKII also phosphorylated a second residue on CREB, Ser142. Indeed phosphorylation of Ser142 was not only inhibitory, but this modification was also dominant and could reverse the activation of CREB resulting from its phosphorylation on Ser133 by PKA. The mechanism by which phosphorylation of Ser142 inhibits CREB-mediated transcription seems to be by destabilizing the association between CREB and CBP (Parker D, 1998). Interestingly, the nature of the effects of CaMKII on transcription seems to be both cell and promoter dependent. Whereas CaMKII inhibits transcription of the interleukin 2 gene in a human T cell line (Nghiem Paul, 2002), it was found to stimulate atrial natriuretic factor (ANF) gene expression in ventricular myocites (Ramirez et al., 1997). Regulatory elements sensitive to CaMKII include C/EBPβ , CRE, SRE, and AP1 (Means et al., 1991). A role for CaMKII in the regulation of cell cycle and cell proliferation have also been investigated. Morris et al. demonstrated that CaMKII mediates Ca^{2+}/CaM-dependent G1 phase progression in mammalian fibroblasts by influencing cyclin D1 levels. Indeed, the inhibition of the endogenous cellular CaMKII by KN93 completely prevents DNA synthesis and leads to decreased levels of cyclin D1, a critical regulatory molecule of the G1 phase protein kinase cdk4 (Morris et al., 1998).

More recent works show that CaMKII regulates cell proliferation in different cell types (An et al., 2007; House et al., 2007; Muthalif et al., 2001)

Illario et al. demonstrated for the first time that CaMKII is a crucial mediator of the Ras/Erk pathway (Illario et al., 2003)

In thyroid cells, integrin stimulation by Fibronectin simoutaneously activates two signalling pathways: Ras/Raf/Mek/Erk and Ca^{2+}/CaMKII. Both signals are necessary to stimulate Erk phosphorylation and cell proliferation as inhibition of Ca^{2+}/CaMKII signal pathway by CaM or CaMKII inhibitors completely abolished the fibronectin induced Erk phosphorylation. The mechanism by which CaMKII modulates Raf1 activation is not fully elucidated. In thyroid cells stimulated by fibronectin, activated CaMKII binds to Raf1 . This event appear to be necessary to subsequent Mek/Erk activation. (Illario et al., 2005)

1.2 The Insulin Signalling Pathway

Insulin is an anabolic hormone secreted by the β cells of the pancreatic islets of Langerhans and it plays a pivotal role in the regulation of glucose homeostasis.

Elevated concentrations of glucose in blood stimulate the release of insulin, and insulin action stimulates uptake, utilization and storage of glucose. The effects of insulin on glucose metabolism vary depending on the target tissue. Two important effects in carbohydrates metabolism are:

1) Insulin facilitates glucose entry in muscle and adipose tissue

The vast majority of cells take up glucose only by facilitated diffusion though a family of hexose transporters. In muscle cells the major transporter used for glucose uptake is GLUT-4. In the absence of insulin GLUT-4 glucose transporters are present into cytoplasmic vesicles, where they are stored in resting conditions. Binding of insulin to its receptors in these cells leads rapidly to the fusion of those vesicles with the plasma membrane and to the insertion of the glucose transporters , thereby making the cells able to efficiently take up glucose. When blood levels of insulin decrease and insulin receptors are no longer occupied, the glucose transporters are recycled into the cytoplasm (Pessin et al., 1999).

2) Insulin induces glucose storing as glycogen in the liver

Insulin activates the enzyme hexokinase, which phosphorylates glucose, trapping it within the cell. In the meantime insulin acts to inhibit the activity of glucose-6-phosphatase. Insulin also activates several enzymes that are directly involved in glycogen synthesis, including phospho-fructokinese and glycogen synthase (Pilkis and Granner, 1992; Sutherland et al., 1996).

Insulin has also a pivotal role in the regulation of lipid metabolism. Important effects of insulin on lipid metabolism include the following:

1) Insulin promotes the synthesis of fatty acids in the liver

As discussed above, insulin stimulates glycogen synthesis in the liver. However, as glycogen accumulates to high levels (roughly 5% of liver mass), further synthesis is strongly suppressed. When the liver is saturated with glycogen, any additional glucose taken up by hepatocytes is shunted into pathways leading to synthesis of fatty acids, which are exported from the liver

as lipoproteins. The lipoproteins are ripped apart in the circulation, providing free fatty acids for other tissues, including adipocytes, which use them to synthesize triglyceride

2) Insulin inhibits breakdown of fat in adipose tissue

Insulin inhibits the intracellular lipase that hydrolyzes triglycerides to release fatty acids. Insulin facilitates entry of glucose into adipocytes and, within those cells, glucose can be used to synthesize glycerol. This glycerol, along with the fatty acids delivered from the liver, is used to synthesize triglyceride. By these mechanisms, insulin is involved in further accumulation of triglyceride in fat cells (Saltiel and Kahn, 2001).

Besides being the most potent anabolic hormone, Insulin is essential for appropriate tissue development, growth and maintenance of whole-body glucose homeostasis. Insulin induces both mitogenic and metabolic effects in most cell type and it can initiate both immediate and long-term cellular responses. The immediate effects of this hormone include an increase in the rate of glucose uptake from the blood into muscle cells and adipocytes and modulation of the activity of various enzymes involved in glucose metabolism. These effects occur within minutes, do not require new protein synthesis, and occur at insulin concentrations of 10^{-9} to 10^{-10} M. Continued exposure to insulin produces long-lasting effects including increased expression of liver enzymes that synthesize glycogen and of adipocyte enzymes that synthesize triacylglycerols. Insulin also functions as a growth factor for many cells (e.g., fibroblasts). These effects are manifested in hours and require continuous exposure to $\approx 10^{-8}$ M insulin.

The capability of insulin receptor to regulate such different functions depends upon its ability to generate a number of different intracellular signals.
The insulin receptor (IR) is a heterotetrameric protein consisting of two extracellular α subunits and two transmembrane β subunits. The binding of insulin to the α subunit of IR stimulates the thyrosine kinase activity intrinsic to the β subunit of the receptor. Extensive studies have indicated that the ability of the receptor to autophosphorylate and phosphorylate intracellular substrates is essential for its mediation of the complex cellular responses to insulin. Structure biology studies revealed that the two α subunits are in a juxtaposition that permits autophosphorylation of thyrosine residues, the first step of Insulin receptor activation. The kinase domain undergoes conformational change upon auto-phosphorylation, providing a basis for activation of the kinase and binding of downstream signalling molecules (Patti and Kahn, 1998).
At the proximal level, phosphorylation of the Insulin receptor Substrates (IRSs) represent one of the earliest events involved in the insulin signalling(White and Kahn, 1994).

The four IRS proteins identified (IRS 1-4) to date are highly homologous with overlapping and differential tissue distribution. Studies with genetic deletion in mouse models and cell lines indicate that these proteins serve complementary functions in different tissues as immediate substrates for Insulin and IGF1 receptors. IRS1 knockout mice exhibit growth retardation due

to resistance to insulin and IGF1, β cell hyperplasia and impaired glucose tolerance(Taylor, 1992). IRS2 knockout mice exhibit more severe insulin resistance in the liver and peripheral tissues, and develop overt type 2 diabetes as a result of profound insulin resistance combined with impaired β cell function(Wojtaszewski et al., 1999). Combined heterozygous deletions of insulin receptor, IRS1 and IRS2 in different tissues results in insulin resistance in skeletal muscle and liver and marked β cell hyperplasia. These data indicate tissue specific differences in the roles of IRSs proteins to mediate insulin action, with IRS1 playing a prominent role in skeletal muscle and IRS2 in liver (Nandi et al., 2004).

IRS1 possesses multiple thyrosine phosphorylaton residues, which allow it to interact with other signalling molecules containing SH2 domains such as Grb2, Syp, Nck, and two isoforms of the regulatory subunit of phosphoinositide 3 kinase (PI3K)(Lee and Pilch, 1994) (Pessin and Saltiel, 2000)

PI3K is a heterodimeric enzyme consisting of the p85 regulatory subunit and the p110 catalytic subunit responsible for its lipid kinase activity. Binding of the regulatory subunit of PI3K, p85, to thyrosine-phosphorilated IRS1 leads to a 3-5 fold stimulation of PI3K activity (Rordorf-Nikolic et al., 1995) and to an increase in the PI 3,4 bisphosphate and 3,4,5 trisphosphate in the cells (Kohn et al., 1996). Acting as second messengers, these phospholipids recruit the PI3K dependent serine-threonine kinases (PDK1) and Akt from the cytoplasm to the plasma membrane by binding to the "plekstrin homology domain" (PH domain) of the kinases. Lipid binding and membrane translocation lead to conformational changes in AKT that is subsequently phosphorylated on the Thr 308 and Ser 473 by PDK1. Activated AKT phosphorylates and regulates the activity of many downstream proteins involved in multiple aspects of cell physiology. Among others, AKT phosphorylates and regulates components of the glucose transporter 4 (GLUT-4) complex, protein kinase C (PKC) and GSK3, all of which are critical in insulin mediated metabolic effects. Insulin-induced GLUT-4 translocation from the intracellular stores to the plasma membrane results in the increased glucose uptake in muscle and adipose tissue (Kohn et al., 1996).

IRS1 interaction with other molecules containing SH2 domain such as Grb2 and SOS also convey mitogenic signal originating from Ras activation. Ras functions as an activator of Raf1, which in turn activates mitogen-activated kinase kinase (MEK) and enables phosphorylation and stimulation of MAPK. These events are necessary for cell proliferation to occur in response to insulin in most cell types (Bandyopadhyay et al., 1997; Nishida and Gotoh, 1993). However, there are also evidences that insulin signal can be conveyed into the MAPK pathway independently of Ras (Carel et al., 1996; Formisano et al., 2000). Neither of the three major MAPK signalling cascades namely Erk1/2, P38 and jun amino terminal kinase (JNK) promote insulin-mediated metabolic responses. Rather, they seem to exert feedback regulatory function. Moreover,

an increased basal MAPK activity seems to contribute to the development of insulin resistance (Pirola et al., 2004).

Several studies show that an increase of IRS1 phosphorylation in Ser/Thr residues leads to a negative modulation of IRS1 activity. Ser phosphorylation of IRS1 can result in subsequent inhibition of its thyrosine phosphorylation by the insulin receptor. If the Ser/Thr phosphorylation sites are located in the IRS1 PTB (phospho thyrosine binding) domain they may hinder the PTB binding to the activated receptor (Aguirre et al., 2000; Aguirre et al., 2002).

IRS1 contains 4 Ser in the motif Pro-X-Ser-Pro, a consensus sequence recognized by members of the MAPK family (Kennelly and Krebs, 1991). All of these 4 Ser are located exactly 4 amino acids downstream of potential thyrosine phosphorylation sites, and one (Ser 612) is close to the major PI3K binding site, thyrosine 608 (Mothe and Van Obberghen, 1996) .

In CHO cells and in the Human kidney fibroblasts cell line 293, PKC activation results in decreased insulin-stimulated IRS1 association with PI3K and subsequent AKT/PKB activation. This inhibition was found to correspond to the phosphorylation of the Ser 612 of IRS1 by ERK1/2 (Bard-Chapeau et al., 2005; De Fea and Roth, 1997) . By this mechanism insulin exerts a negative feedback on its own stimulation of glucose uptake. Thus, after initial stimulation, the glucose entry into the cells is downregulated.

A schematic representation of insulin signalling in muscle cells is reported in Figure 1.4.

A more recent study demonstrated, in a model of liver knockout Grb2 associated binder 1 (Gab1) mice, that Gab1 mediates insulin stimulated ERK1/2 activation (Bard-Chapeau et al., 2005). This scaffolding adaptor protein, while reducing insulin-stimulated glucose metabolism in the liver, promotes hepatocyte proliferation induced by insulin. Thus, ERK1/2 or Gab1 modulators can modulate glucose uptake and participate to glucose homeostasis. Through this mechanism, insulin-induced effects can be modulated by the receptor itself or by endogenous or environmental interfering factors.

In the intricate insulin pathway the role played by Ca^{2+} has long been debated, and it remains still partly unsolved. Indirect evidences suggest that Ca^{2+} is involved in insulin action, as its chelation reduces insulin stimulation of glucose uptake (Pershadsingh et al., 1987a).

Noteworthy, insulin induces an increase in the near-membrane free Ca^{2+} concentration rather than an increase of the cytoplasmic intracellular calcium concentration ($[Ca^{2+}]i$) in skeletal muscle cells.

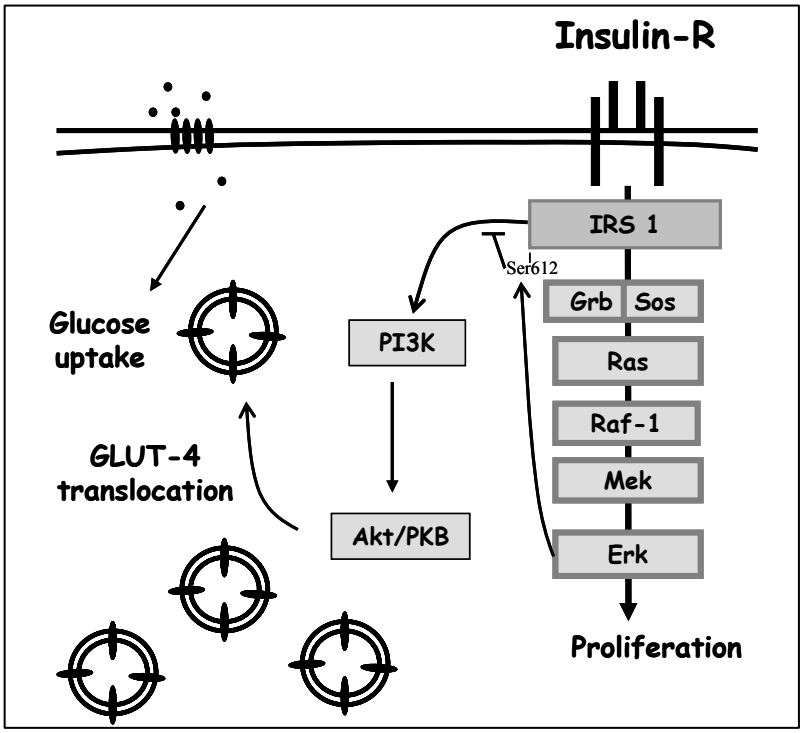


Figure 1.4. Schematic representation of Insulin signalling

Insulin binds to its receptor leading to the auto-phosphorylation of the β-subunits and the tyrosine phosphorylation of insulin receptor substrates (IRS1). IRS1 phosphorylates the SH3 domain of the adaptor molecule Grb2. Activated Grb2 recruits Sos that, in turn, activates the Ras signalling pathway and cell proliferation. IRS1 also activates phosphoinositide 3-kinase (PI3K) through its SH2 domain, thus increasing the intracellular concentration of PIP_2 and PIP. This, in turn, activates PDK-1 (not shown), that subsequently activates Akt/PKB. This results in the translocation of the glucose transporter GLUT-4 from cytoplasmic vesicles to the cell membrane and subsequently to glucose uptake.

The inhibition of the insulin-induced $[Ca^{2+}]_i$ rise in near-membrane by Ca^{2+} nifedipine and nimodipine suggests that entry of Ca^{2+} occurs through L-type channels (Hofmann et al., 1994). The observation that Wortmannin also prevented the Insulin-induced rise in $[Ca^{2+}]_i$ (Bruton et al., 1999, 2001) suggests that the influx of Ca^{2+} in the cells occurs via PI3K dependent modulation of Ca^{2+} channels. This could involve phosphorylation of likely candidates such as protein kinase A, protein kinase C or calmodulin dependent kinases which in turn phosphorylate L-type Ca^{2+} channels as described in neurons and smooth muscle cells (Ahlijanian et al., 1991). The possibility that Ca^{2+} could have biphasic effects on Insulin-stimulated glucose transport was examined by Draznin et al (Draznin et al., 1987b). They found that in adipocytes, large increases in $[Ca^{2+}]$i induced by exposure to ionomicin or depolarization inhibited insulin stimulated glucose transport, whereas small rises in $[Ca^{2+}]$i were stimulatory. It is feasible that Ca^{2+} plays a role in the docking and insertion of the GLUT-4 transporters into the sarcolemma similar to the Ca^{2+} dependent docking of synaptic vesicles in the nerve terminal (Zenisek et al., 2000). Another possibility is that Ca^{2+} activated Calmodulin exerts a modulatory effect at one or more sites in muscle fibre. A role for CaM in glucose transport has long been suspected (Shechter, 1984). Inhibition of CaM significantly reduces the number of GLUT-4 transporters in the sarcolemma after stimulation by insulin or hypoxia. Several studies suggest that CaM might be involved either in the movement of vesicles containing GLUT-4 transporters to the surface membrane or in the insertion of GLUT-4 transporters in the sarcolemma (Bruton et al., 2001). All these findings support the hypothesis that Ca^{2+} is an important second messenger in Insulin signaling even though its role in the insulin complex signaling network has not been clarified in detail.

2. AIMS OF THE STUDY

In thyroid cells, CaMKII is activated by cell adhesion to extracellular matrix and its binding to Raf1 is necessary to Raf1 activation, thus modulating the Ras/Raf/Mek/Erk pathway and cell proliferation (Illario et al. 2005).

In order to determine whether a cross-talk between CaMKII and Erk pathway is restricted to integrins in thyroid cells, or it is a general mechanism in the control of cell proliferation, I extended my studies to the signalling pathways downstream the insulin receptor.

Two important and well characterized cell types responding to insulin are skeletal muscle cells and fibroblasts. In skeletal muscle cells, insulin stimulates proliferation through the Ras/Erk signalling, and glucose uptake through PI3K/Akt/GLUT4 cascade. In fibroblasts, insulin effects are mainly mitogenic as GLUT-4 is not expressed.

The aims of this doctorate thesis are to clarify the role of CaMKII in insulin-induced mitogenic and metabolic effects in skeletal muscle cells and in fibroblasts.

To this purpose I investigated:

1. Whether insulin stimulation modulates the intracellular calcium concentration and CaMKII activity;
2. Whether CaMKII participates to the Ras/Erk signalling and to the stimulation of cell proliferation;
3. Whether CaMKII participates to the PI3K/Akt signalling and the modulation of glucose uptake

A rat skeletal muscle cell line (L6) was chosen as a model as it represents a typical insulin target tissue.

This cells have been extensively characterized and they are one of the most employed models in the study of insulin effects in skeletal muscle. In order to determine if CaMKII involvement in insulin-induced proliferation was a general phenomenon, this study was extended to human fibroblasts.

3. MATERIALS AND METHODS

3.1 Cell Culture

The L6 skeletal muscle cells were seeded (5-9 × 10^3 cells/cm^2) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum. Under these cultural conditions, the myoblasts reach the confluence and then spontaneously differentiate into myotubes. Subsequently L6 skeletal muscle cells were grown in Dulbecco modified eagle medium (DMEM) (Life Technologies, Inc, Grand Island, NY) supplemented with 10% fetal calf serum **(**Sigma, St. Louis, MO) and 2% glutamine, in a humidified CO2 incubator. Human primary fibroblasts were obtained according to the procedure described by Postiglione *et al*. (Postiglione et al., 2005). Briefly, the surgical fragments derived from skin biopsies were mechanically dissociated and subsequently trypsinized 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, USA), 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% CO2. During the following 10 days the percent of serum was progressively reduced to 10%. After 10 days the cells were maintained in culture with DMEM 10% FCS.

3.2 Calcium Measurements

Fluorimetric analysis: A total of 3x10^5 cells were harvested by trypsin and were loaded with cell-permeant Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR), by incubating the cells with Dulbecco's modified Eagle's medium, 10-5 mol/L Fura-2, 0.5% BSA, and 0.01 mol/L HEPES for 30 min at 37 C. The cells were then washed twice for 10 min with 0.001 mol/L CaCl2 in Hanks' balanced salt solution (0.118 mol/L NaCl, 0.0046 mol/L KCl, 0.01 mol/L glucose, and 0.02 mol/L HEPES, pH 7.2). Fluorescence was measured with a fluorimeter (PerkinElmer Life Sciences). Excitation was at 345 and 380 nm, emission was at 510 nm. Rmin and Rmax were obtained by adding 0.01 mol/L EDTA and 2% Triton X-100 or 0.01 mol/L EDTA, 2% Triton X-100, and 0.01 mol/L CaCl2, respectively. The nanomolar concentration of Ca^{2+} was obtained by the Grynkiewiez formula considering a 225 KDa for Fura-2 (Grynkiewicz et al., 1985).

For calcium transients imaging, 2x10^5 cells were plated onto a 60 mm microscope dish, starved over night, and loaded with 12.5 mg Oregon green, 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).

3.3 Western blot and immunoprecipitation procedures

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 ug/mL leupeptin, 5 ug/mL pepstatin).The lysate were quantified by Biorad DC protein assay. An equal amount of proteins from each sample was loaded with laemly buffer. Protein 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 antibodies. Finally, protein bands were detected by an enhanced chemiluminescence system (ECL, Amersham Bioscience). Computer-acquired images were quantified using ImageQuant software (Amersham Bio-sciences).

Immunoprecipitation was performed according to previous reports.(Illario et al., 2005). Briefly 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).

Primary antibodies vs protein of interest and Protein G plus/protein A agarose beads (Oncogene Science, Boston, MA) were used to immunoprecipitate corresponding proteins from 250 μg of total lysate. Non immune rabbit or mouse IgG were used as control. Mouse monoclonal antibodies to p44/p42 MAPK, phospho-p44/p42 MAPK , Raf1 and CaMKII were from Santa Cruz Biotechnology. Polyclonal anti-phospho-CaMKII antibody (pT286-CaMKII) was from Promega (Madison, WI). Anti-IRS-1 and phospho-Ser612-IRS1, Anti- AKT and phospho AKT mouse monoclonal antibodies were from Cell Signaling Technology, Danvers, MA.

3.4 CaMKII activity and inhibitors

To inhibit CaMKII activity two different inhibitors have been used: KN93 and AntCaNtide.

KN93 is a potent, selective and cell permeant pharmacological inhibitor of the CaMKs (IC_{50}=370 nM). This drug is an isoquinolonesulfonamides and

functions as a competitive ATP antagonist (Tokumitsu et al., 1990). It was purchased from BIOMOL international. The CaMKII specific peptidic inhibitor Ant-CaNtide is derived from the endogenous CaMKII inhibitor protein CaMKIIN (Chang et al., 1998) and was made cell permeable by N-terminal addition of an antennapedia-derived sequence (Ant-CaNtide: RQIKIWFQNRRMKWKKR PPKLG QIGRSKRVVIEDDRIDDVLK). The reversed ant-CaNtide (Rev-antcantide) peptide was also used as a control.
The inhibitory activity of AntCantide and Rev-AntCaNtide was tested on active full-length recombinant CaMKII (Signal Chem). In a first reaction step CaMKII was incubated for 30 minutes at 30 °C with 5 mM $CaCl_2$ and 5μM CaM in 50 μl of reaction mixture consisting of 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 0,5 mM dithiothreitol (DTT), 2μM CaM, 100 nM microcystin, 0,5mM cold ATP .A 10 μl aliquot from the first reaction was than incubated with 25mM EGTA, 0,5 mM Autocamtide (Hanson et al., 1989) and 50 μM ATP (1500 cpm/pmol [γ-^{32}P]ATP) in order to determine CaMKII autonomous activity on its peptide substrate Autocamtide. The reaction was carried out for 30 minutes at 30 °C and 20 μl aliquots of the reaction mixture were spotted onto p81 phosphocellulose filters (Upstate Biothechnology, Lake placid, NY) The level of [^{32}P] incorporation into Autocamtide was determined by liquid scintillation counting. Purified CaM and Autocamtide were a kind gift from Dr. AR. Means, (Duke University, Durham, NC).

3.5 Adenoviral Infection of Cells

The catalytically inactive form of CaMKIIα, K42M, was subcloned into pSP72(Promega).

CaMKII Kinase deficient mutant (CaMKII DN) adenoviruses were generated using the AdEasy system first described by He and colleagues and now available from Qbiogene (Draznin et al., 1987a).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% confluency. 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 A.R. Means (Duke University, Durham , NC)

3.6 [^{3}H]thymidine incorporation

To determine DNA synthesis, cells were plated in 24-well plate, and serum-starved for 24 hours in DMEM, 0.5% BSA. Then, 0.5 μCi [^{3}H]thymidine and 100nM insulin were added to the plates. After 24 h, the

plates were gently washed with PBS, 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)

3.7 Measurement of 2-deoxy-D-glucose uptake

Confluent cells were incubated in DMEM supplemented with 0.25% albumin for 18 h at 37°C. The medium was removed and the 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.

3.8 Subcellular fractionation and western blot analysis of GLUT-4

Serum starved L6 cells were exposed to 5μM AntCaNtide for 30 minutes and subsequently stimulated with insulin (100nM) for the indicated time. Cells were washed three times in ice cold PBS, pH 7.4 and homogenized in 500μM fractionation buffer [20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 20 mM β-mercaptoethanol, 1mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin] by passing them 10 times through a 22-gauge needle. Broken cells were centrifuged at 1500 rpm for 15 min at 4°C to pellet nuclei. Supernatants were further centrifuged at 60000 rpm for 1 hour at 4°C to pellet plasma membranes. The last supernatant represented the cytosolic fraction. Plasma membranes (PM) were further purified by resuspending heavy membrane pellets in 500 ml fractionation buffer with 0,1% (v/v) Triton X-100.

After centrifugation at 45000 rpm for 1 hour at 4°C, membranes were recovered from the upper phase. Based on Western blotting, the cell surface marker IR beta (beta subunit of insulin receptor) selectively localized to the plasma membrane fraction.

3.9 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.

4. RESULTS AND DISCUSSION

4.1 Insulin induces Erk1/2 phosphorylation in L6 cells and human fibroblasts

L6 cells and human fibroblasts in primary culture were serum starved for 24 hours and then stimulated with insulin at different concentrations (1-100 nM). The cells were then lysed in RIPA buffer, the proteins were resolved by SDS PAGE, and Erk1/2 phosphorylation was evaluated by Western Blot with a phospho-specific antibody (p-Erk1/2) (Fig 4.1). Although the starvation was maximal, and a longer starvation would result in loss of cell viability, Erk resulted phosphorylated to some extent in L6 cells at resting conditions, thus suggesting that serum starvation was incomplete, or that factors other than serum (i.e integrins engagement, autocrine factors) were responsible for the low-activation state of ERK 1/2 in these cells. Insulin stimulation induced a dose dependent increase of ERK 1/2 phosphorylation ,with a maximal effect at 100nM concentration in both L6 cells and fibroblasts (fig 4.1 A). In a primary culture of human fibroblasts Erk1/2 was barely phosphorylated at basal condition, and Insulin stimulated its phosphorylation in a dose dependent manner with a maximal effect at 100nM concentration. As in both cell types Insulin exerts its maximal effect at the concentration of 100nM, insulin was used at this concentration in the following experiments.

In order to study the kinetics of insulin action on Erk phosphorylation, a time course was performed (Fig 4.1 B). L6 cells and fibroblasts were treated with insulin at different time points. In L6 cells strong Erk phosphorylation was visible after 5 minutes of insulin treatment, and it lasted up to 120 minutes, peaking at 30 minutes. In human fibroblasts insulin induction of Erk phosphorylation started after 5 min, the effect was maximal at 15 minutes and it declined after 60 minutes.

Although these data are not original, they were necessary to characterize the cell models employed in this study. L6 cells have been extensively studied, and some difference in the kinetic of the insulin response have been observed between different L6 clones used in different laboratories. These data ensured that the L6 cells available in my laboratory are fully responsive to insulin.

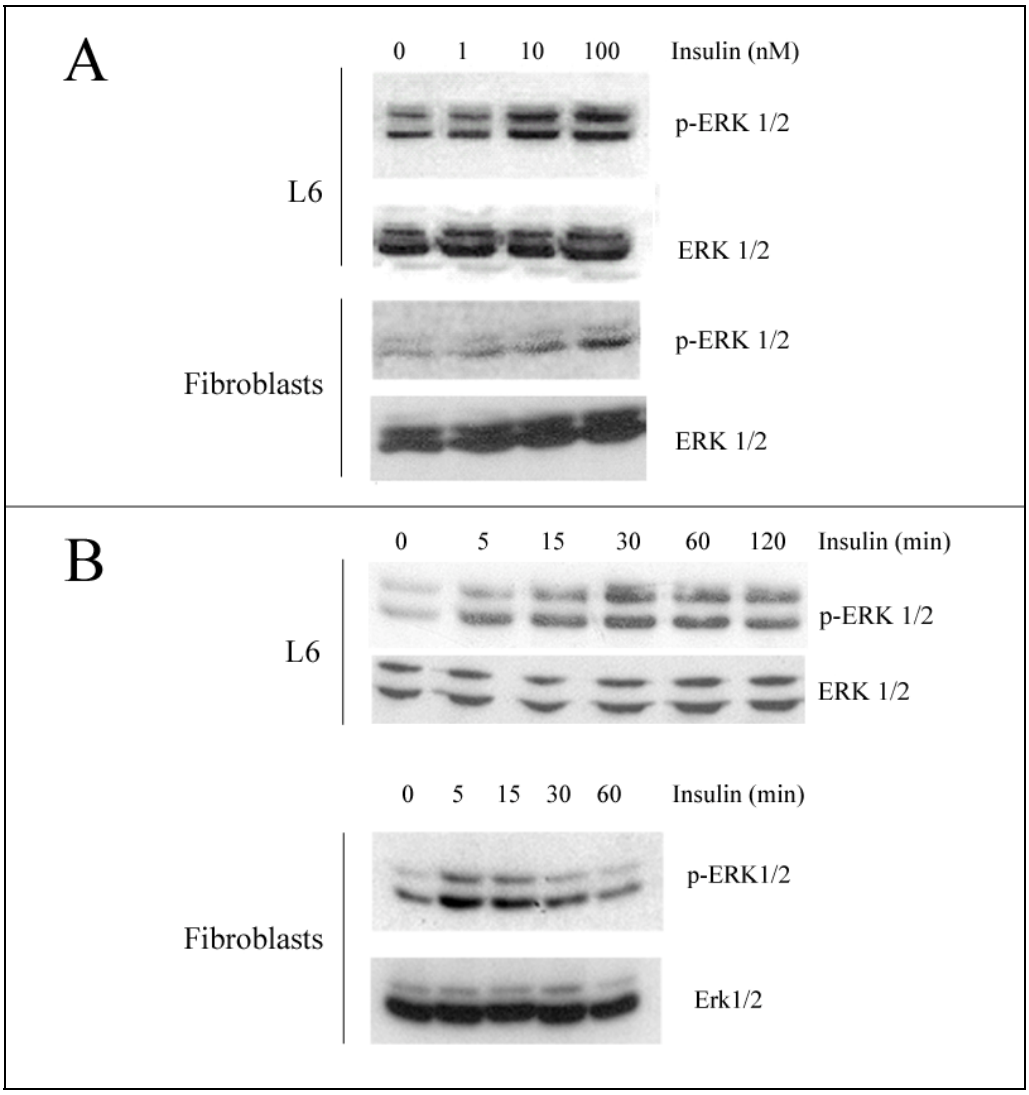


Figure 4.1. Insulin induces Erk1/2 phosphorylation in L6 cells and human fibroblasts

L6 rat muscle cells and human fibroblasts in primary culture were serum starved overnight. The cells were stimulated with different concentration of insulin (1,10,100 nM) **(A)**. In a separate set of experiments L6 cells and human fibroblasts were treated with 100nM insulin for different times **(B)**. The cells were lysed in RIPA and proteins, in equal amount, were resolved by SDS-PAGE.
In either A and B cell lysates were analyzed by Western blot with total or phospho-specific (p-) antibodies to Erk-1/2.

4.2 Insulin stimulation induces intracellular increase of Ca^{2+} concentration

In order to evaluate whether insulin was able to initiate the cascade leading to CaMKII activation, I measured intracellular calcium concentration ($[Ca^{2+}]i$)in the cells following insulin stimulation. To this purpose two different technical approaches were used: measurement of total $[Ca^{2+}]i$ increase by fluorimetric analysis and single cell analysis by confocal microscopy (Fig. 4.2). L6 cells were serum-starved overnight, and were subsequently loaded with Fura-2, detached from the plates by trypsin and examined in suspension by fluorometry. $[Ca^{2+}]i$ remained unchanged for 15 minutes in un-stimulated cells. Subsequently, insulin was added at different concentrations and a $[Ca^{2+}]i$ increase appeared soon after stimulation. The $[Ca^{2+}]i$ increase was rather slow relative to other Ca^{2+} mobilizing agents and was dose-dependent upon the concentration of insulin used, reaching about 5-fold increase by 10 min with 100 nM insulin (Fig. 4.2 A). Although this result clearly demonstrated that insulin modulates $[Ca^{2+}]i$ in L6 cells, the analysis of skeletal cells in suspension is far less physiologic than in adherence condition and the kinetic of the response may be altered. For this reason $[Ca^{2+}]i$ was then determined at single cell level by confocal microscopy of adherent cells. After overnight starvation, L6 cells were loaded with Oregon Green and stimulated with 100 nM insulin. Again, insulin induced a slow increase of $[Ca^{2+}]i$, thus confirming what was observed in the same cells in suspension with FURA-2. (Fig. 4.2 B, C).

The role of Ca^{2+} in insulin signaling has been investigated in different studies with different approaches. Whereas some studies suggest that Ca^{2+} is involved in insulin signaling in several cell types (Draznin et al., 1987a; Pershadsingh et al., 1987b; Youn et al., 1994), others failed to demonstrate that insulin modifies $[Ca^{2+}]i$ (Cheung et al., 1987; Kelly et al., 1989; Klip and Ramlal, 1987). A recent study employing Indo-1 in single mouse muscle fibers suggested that insulin induce an increase of near-membrane Ca^{2+} concentration, while global myoplasmic $[Ca^{2+}]i$ does not significantly change (Bruton et al., 1999). In another study, insulin induced a very fast (2 sec) and transient $[Ca^{2+}]i$ increase in rat myotubes (Espinosa et al., 2004). My results show that insulin induces a slow and global $[Ca^{2+}]i$ in L6 cells. The apparent discrepancy between these results might be explained by differences in calcium homeostasis between the cell models employed and more importantly by the timing of the observation. Indeed, all the mentioned studies investigated only the very early insulin effects and focus their attention to the first 30 min; on the contrary my study examined later effects of insulin, as my interest is focused on subsequent Ca^{2+}-dependent enzyme activation. In my experiments, a very fast global effect in $[Ca^{2+}]i$ was not detectable, however a progressive increase of $[Ca^{2+}]i$ was evident after few minutes of insulin stimulation, and became

maximal by ~25 min. The increase of $[Ca^{2+}]i$ (global or restricted at CaMKII location) induced by insulin was of a magnitude sufficient to activate a signaling pathway involving CaMKII (see later results), whose activation is dependent upon Ca^{2+} . CaMKII activation by insulin was observed in rat soleus muscle but it was not demonstrated in 3T3-L1 adipocytes, suggesting that the insulin dependent CaMKII activation might be tissue-specific (Konstantopoulos et al., 2007; Wright et al., 2004).

I then investigated the effect of insulin on $[Ca^{2+}]i$ in adherent fibroblasts by confocal microscopy (data not shown). According to the literature, insulin induced a sharp and transient $[Ca^{2+}]i$ increase. Again, as in L6 cells, this $[Ca^{2+}]i$, although modest and transient, it was sufficient to induce CaMKII activation (see later results).

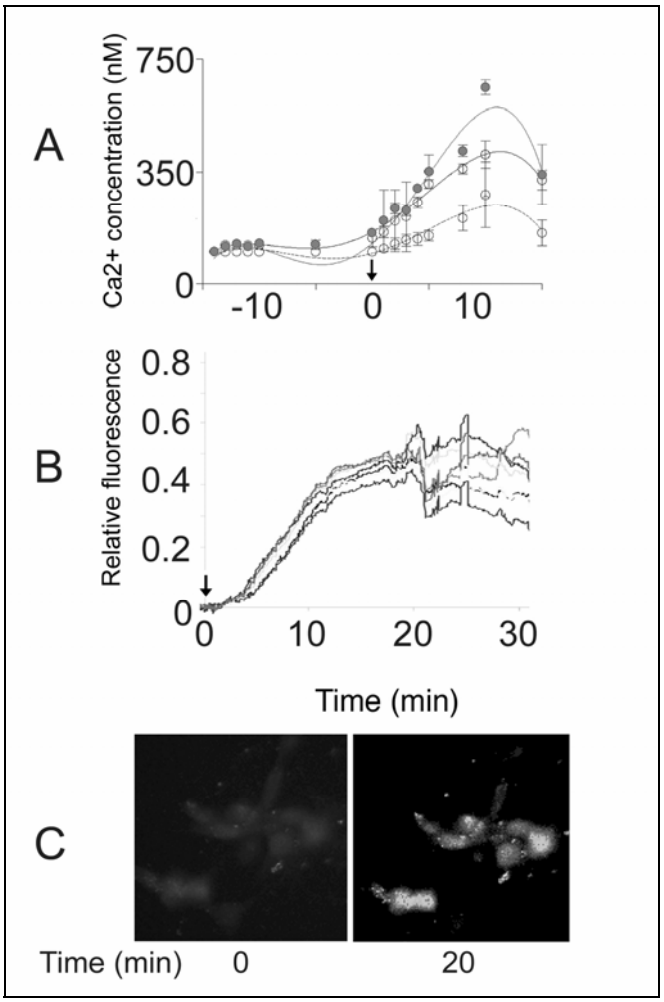


Figure 4.2 Insulin induces $[Ca^{2+}]_i$ increase

A) L6 cells were serum starved and loaded with Fura-2.The cells were then trypsinized and $[Ca^{2+}]$i was measured by a fluorimeter for 15 min before insulin stimulation (arrow) and for the following 15 min. Insulin concentrations were: 1 nM, empty circle, dotted line; 10 nM empty circles continuous line; 100 nM full circles. **B)**and **C)**, L6 cells grown onto glass coverslips were loaded with Oregon green, stimulated with 100nM insulin and observed by confocal microscopy as described in Materials and Methods. $[Ca^{2+}]$i is reported Relative fluorescence = (fluorescence – fluorescence at 0 point)/ fluorescence at 0 point)

4.3 Insulin induces CaMKII phosphorylation in L6 cells and in human fibroblasts

To evaluate whether insulin stimulated $[Ca^{2+}]i$ increase was followed by CaMKII activation, I evaluated CaMKII activity in L6 cells and 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

L6 cells and fibroblasts were exposed to 100nM insulin for the indicated time (Fig. 4.3). In L6 cells insulin treatment induced CaMKII phosphorylation on Ser 286 as early as 5 min and it lasted up to 120 min (Fig. 4.3 A). A similar effect was observed in fibroblasts, where CaMKII phosphorylation was visible from 5 to 60 min. (Fig 4.3 B). Although a basal minimal activation of CaMKII was present, Insulin was able to induce a fast and long lasting increase of Thr286 phosphorylation, demonstrating that also the very modest and transient $[Ca^{2+}]i$ increase observed in fibroblasts was a sufficient activating signal. The long-lasting activation of CaMKII in both cell types is not surprising, because this kinase has unique activation features that enable it to be active after the Ca^{2+} concentration is dropped off. Indeed the binding of Ca^{2+}/CaM to CaMKII is necessary only to initiate the autophosphorylation process of the multimeric kinase, but not for its autonomous activity.

These data show that CaMKII is activated upon insulin stimulation in two different cell types and suggest that it can be considered as a new component of insulin signalling.

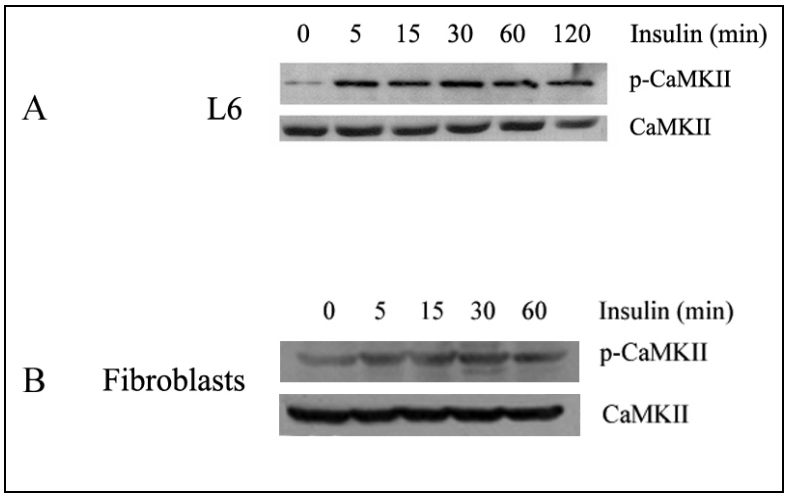


Figure 4.3 Insulin induces CaMKII phosphorylation in L6 cells and in human fibroblasts

L6 cells **(A)** and human fibroblasts **(B)** were serum starved over night and stimulated with 100nM insulin at the indicated times. Cell lysates were analyzed by Western blot with antibodies to total CaMKII or to phosphorylated T286-CaMKII (p-CaMKII).

4.4 Inhibition of CaMKII activity abrogates insulin stimulated Erk-1/2 phosphorylation in L6 cells and in fibroblasts.

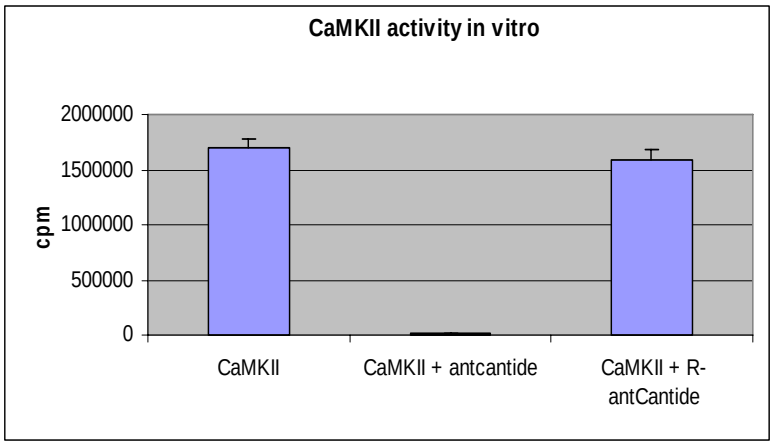
A cross-talk between the Ca^{2+}/CaMKII and Erk-1/2 pathway was previously demonstrated in integrin signalling (Illario et al., 2005). In order to investigate the possible existence of a similar cross-talk in insulin signalling, I tested the effects of two CaMKII inhibitors (KN93 and AntCaNtide) on Erk-1/2 phosphorylation in response to insulin. KN93 is a well known CaMKs pharmacological inhibitor, that function as an ATP antagonist, while AntCaNtide is a CaMKII specific inhibitory peptide (Chang et al., 1998). In the inset AntCaNtide efficiency was tested *in vitro* on recombinant active CaMKII. Reversed AntCaNtide (R-antcantide) was used as a negative control. In a first reaction step, active full-length recombinant CaMKII was incubated with $CaCl_2$, CaM and cold ATP. In a second reaction step an aliquot from the first reaction was incubated with CaMKII peptidic substrate Autocamtide, EGTA and [γ^{32}P]ATP. The reaction mixture was spotted onto p81 phosphocellulose filters and the level of [^{32}P] incorporation into Autocamtide was determined by liquid scintillation counting.
AntCaNtide completely abolished CaMKII activity on its substrate Autocamtide, while R-antcandite had no effect on CaMKII activity, demonstrating the efficiency of AntCaNtide. The specificity of AntCaNtide versus different kinases was previously tested. This peptide demonstrated to have no inhibitory activity on CaMKI, CaMKIV, Raf1, Mek and Erk (Illario et al., 2003).

In order to investigate the crosstalk between Ras/Erk and Ca^{2+}/CaMKII pathways, L6 cells were stimulated with insulin in the presence of KN93 at the indicated μM concentration, or 5 μM AntCaNtide, and the levels of Erk-1/2 phosphorylation were evaluated by western blot (Fig. 4.1 A). KN93 treatment reduced Insulin-induced Erk phosphorylation in a dose dependent manner, with a maximal effect at 10 μM concentration. Treatment with 5 μM AntCaNtide completely abolished Erk-1/2 phosphorylation induced by 30 minutes of insulin stimulation as well as its basal phosphorylation.

These data suggest that in a rat muscle cells model, Erk-1/2 activation requires active CaMKII. To validate the general role of CaMKII in the insulin stimulated Erk-1/2 signalling, similar experiments were replicated in primary culture of human fibroblasts. Fibroblasts from normal subjects were cultured in serum-free medium for 24 h. Erk1/2 phosphorylation was determined by western blot upon stimulation with insulin alone, or in the presence of CaMKII inhibitors KN93 or antCaNtide (Fig. 4.1 B). Likewise in L6 cell line, in these cells Erk-1/2 phosphorylation induced by insulin was abrogated by CaMKII inhibitors. 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 (CaMKII-DN). Erk-1/2 phosphorylation was stimulated by insulin in fibroblasts infected with a mock adenovirus. In the cells infected with the CaMKIIDN-expressing adenovirus,

insulin was not able to induce ERK1/2 phosphorylation. (Fig. 4.4 C). In conclusion, these results strongly suggest that insulin-induced Erk activation is CaMKII dependent both in L6 cell and in primary culture of human fibroblasts, indicating that this is a general mechanism of Insulin action, and is not restricted to a particular tissue.

The role of CaMKII in the modulation of ERK phosphorylation was previously demonstrated also in a thyroid cell model following cell adhesion to the extracellular matrix. (Illario et al. 2005). Altogether, these observations support the hypothesis that CaMKII plays a pivotal role in the activation of ERK pathway in response to different stimuli and in different cell types.



Inset . CaMKII activity on Autocamtide in vitro.

Active recombinant CaMKII was incubated with its peptidic substrate Autocamtide, in the presence of AntCaNtide or R-AntCaNtide and [γ-^{32}P]ATP. The reaction was performed as described in materials and methods. The results are presented as total incorporated cpm. Data are reported as the mean +/- standard deviation from duplicate experimental point.

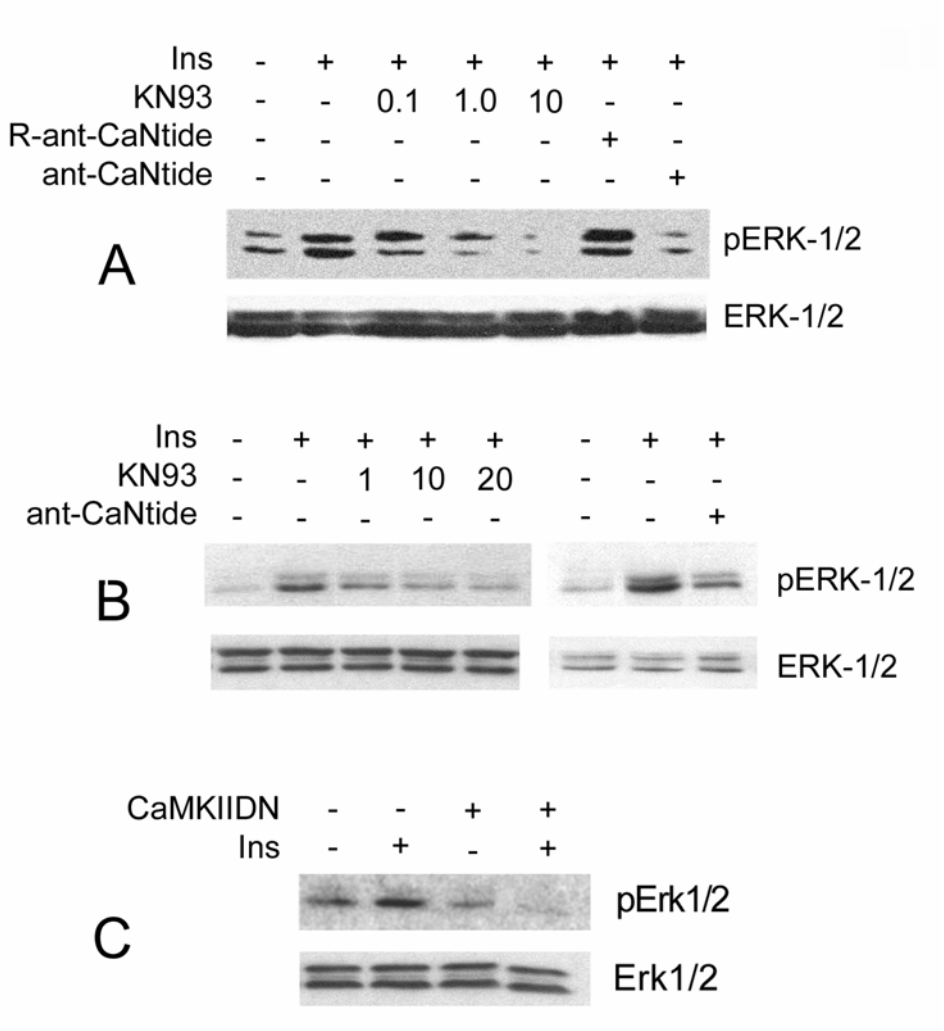


Figure 4.4 Inhibition of CaMKII abrogates insulin stimulated Erk-1/2 phosphorylation in L6 cells and in fibroblasts.

L6 cells **(A)** and human fibroblasts **(B)** were treated with KN93 at indicated μM concentrations, or with 5 μM ant-CaNtide, or with 5 μM R-ant-CaNtide and then stimulated with 100nM insulin (ins). The amount of total ERK1/2 (ERK1/2) and phosphorylated ERK1/2 (p-ERK1/2) was determined by western blot . Human fibroblasts were infected with a dominant negative recombinant adenovirus (CaMKIIDN) or a mock adenovirus as a control. The cells were serum starved overnight and stimulated with insulin. The level of ERK1/2 phosphorylation was evaluated with western blot **(C).**

4.5 Insulin stimulates CaMKII association with Raf1

In a thyroid cell model, FN-induced Raf1 activation was dependent upon CaMKII activity (Illario et al., 2003; Illario et al., 2005b). In order to investigate whether the crosstalk between CaMKII and Ras/Erk pathway in insulin signalling occurred on Raf1, a co-immunoprecitation assay was performed in L6 cells. Serum starved cells were treated, when indicated, with 10 μM KN93 or 5 μM AntCaNtide, and then stimulated with 100 nM Insulin. CaMKII was immunoprecipitated from cell extracts and the Co-immunoprecipitated Raf1 was visualized by western blot. Insulin induced CaMKII binding to Raf1, while KN93 and AntCaNtide treatment disrupted the CaMKII/Raf1 complex, thus indicating that the interaction was dependent upon CaMKII activity (fig 4.5). These data suggest that the interplay between CaMKII and Ras/Erk pathway occurs at Raf1 level also in Insulin signalling. The interaction between CaMKII and Raf1 is a relevant point not yet fully investigated. A molecular approach to investigate this mechanism is on going in the laboratory where I worked for this thesis. Raf1 is a complex kinase requiring phosphorylation at multiple sites to be fully activated. In vitro phosphorylation of Raf1 by CaMKII has been demonstrated, although the site/s have not been identified so far. One hypothesis is that Raf1 is phosphorylated by CaMKII in one or more sites necessary for its activation by other kinases (i.e Src)

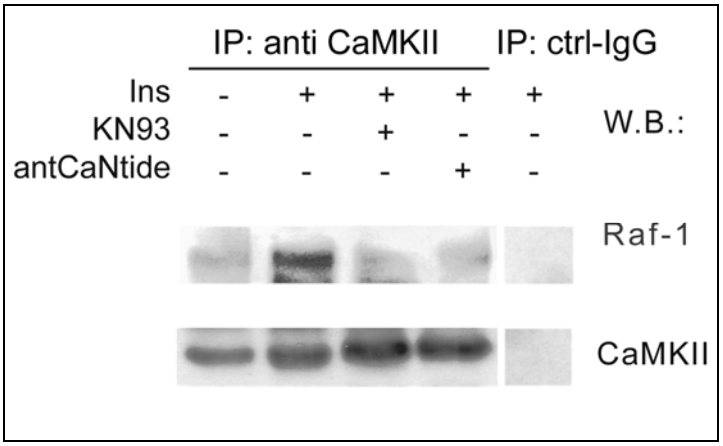


Figure 4.5 Insulin stimulates CaMKII association with Raf1

L6 cells were serum starved and stimulated with 100nM insulin for 30 min in the presence of 10μM KN93 or 5μM antCaNtide. Endogenous CaMKII was immuno-precipitated (IP) from cell extracts by a specific antibody and protein G plus/protein A agarose beads. An unrelated monoclonal antibody was used as a negative control (ctrl-IgG).
After protein separation by SDS-PAGE, immuno-precipitated CaMKII and coprecipitated Raf1 were detected by western blot. (WB)

4.6 Inhibition of CaMKII abrogates insulin-induced thymidine incorporation in L6 cells and human fibroblasts.

In order to study the role of CaMKII in stimulation of cell proliferation following Erk activation, a [^{3}H] thymidine incorporation assay was performed.

[^{3}H] thymidine incorporation was used to measure DNA synthesis in L6 cells and fibroblasts cultured in serum-free medium and then stimulated with 100 nM insulin for 24 h. Insulin stimulation induced a 65% increase of [^{3}H]thymidine incorporation in L6 cells. Both KN93 and AntCaNtide completely abolished such a stimulation demonstrating that, according with the results on inhibition of Erk phosphorylation, insulin-induced DNA synthesis requires CaMKII activity. Human fibroblasts displayed a similar behaviour. In this model CaMKII inhibition was achieved by KN93 or AntCaNtide, and by infecting the cells with the CaMKIIDN recombinant adenovirus. [^{3}H]thymidine incorporation increased 85% following insulin stimulation, and it was reduced by either treatment with CaMKII inhibitors or CaMKIIDN adenovirus infection (fig 4.6).

These data underline the importance of CaMKII in the regulation of Insulin mitogenic effects in different cell types.

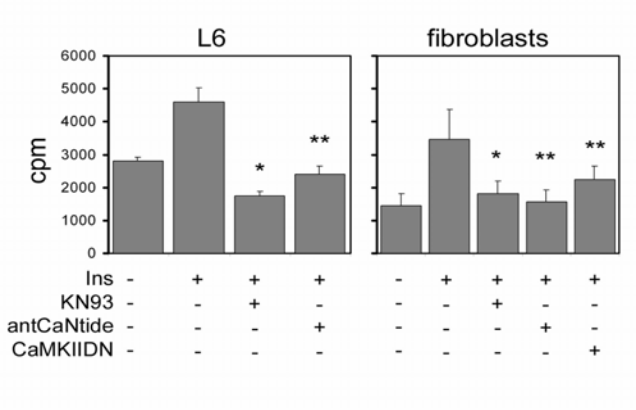


Figure 4.6 Inhibition of CaMKII abrogates insulin-induced thymidine incorporation in L6 cells and human fibroblasts.

L6 cells were plated in 24-well plates, and serum-starved for 24 hours. In a separate experiment fibroblasts were infected with a CaMKII dominant negative recombinant adenovirus (CaMKIIDN) or a mock adenovirus, and serum starved for 24 hours.
Both L6 and fibroblasts were treated, when indicated, with KN93 or antCaNtide for 30 min and then [^{3}H] thymidine and 100 nM insulin were added to the plates. Data are reported as mean ± SD of quadruplicate experiments. *, significant vs. CTRL point. **, significant vs. insulin alone.

4.7 CaMKII inhibition abrogates insulin induced Ser612-IRS1 phosphorylation and enhances the binding of p85 to IRS1

In L6 myotubes both IRS-1 and IRS-2 are responsible for insulin-induced Erk-1/2 activation, but only IRS-1 activates Akt1 and is responsible for GLUT-4 translocation to the plasma membrane, and consequent glucose uptake (Huang et al., 2005). Some studies demonstrate that Erk1/2 is involved in a negative feedback by which, after the initial stimulation of PI3K/Akt/GLUT-4 cascade induced by insulin, this is downregulated (Bard-Chapeau et al., 2005; De Fea and Roth, 1997). Once activated by insulin, Erk phosphorylates IRS1 at Ser612. This residue is located close to Tyr608, the major PI3K binding site (Mothe and Van Obberghen, 1996). The consequence of this phosphorylation is the inhibition of the binding of the regulatory p85 subunit of PI3K to IRS1, and the consequent inhibition of the downstream signal that leads to Akt activation.

In the liver, the major site for glucose production and storage, this mechanism regulates glucose metabolism and finally influences glycemia (Saltiel and Kahn, 2001). Experimental evidences in liver knockout Grb2 associated binder 1 (Gab1) mice, demonstrated that mitogenic and metabolic effects of Insulin can be dissociated by interfering with one component of the Erk-1/2 kinase cascade.

As CaMKII is involved in Erk activation following insulin stimulation, I investigated a possible role for CaMKII in the control of insulin-induced pathways that lead to its major metabolic effect: glucose uptake in its target tissues, like skeletal muscle.

While Fibroblasts do not express GLUT-4 and glucose uptake is not an insulin dependent phenomenon, L6 skeletal muscle cells are responsive to insulin-induced signals leading to GLUT-4 translocation to the plasma membrane and to the increase of glucose uptake. For this reason, L6 cells were chosen as a model to study the role of CaMKII in the metabolic signalling of Insulin.

L6 cells were pre-treated with 5 μM AntCaNtide (ant) or 10 μM KN93 (Kn) for 30 min and then stimulated with 100 nM insulin for the indicated time (Fig. 4.6). Cell extracts were analyzed by Western blot with antibodies versus phosphorylated-Ser612-IRS-1 (pS612) or total IRS-1. Insulin stimulation induced a strong phosphorylation of IRS1 at Ser 612 from 30 to 120 min. CaMKII inhibition by AntCaNtide completely abrogated insulin induced IRS1 phosphorylation at Ser612 for the entire time-course (Fig. 4.6 A).

This data show that CaMKII is important for Erk induced phosphorylation of IRS1-Ser612, and therefore suggest its involvement in the negative feedback loop that leads to the downregulation of the PI3K pathway.

The next step was to evaluate whether CaMKII activation could affect the insulin-induced-binding of p85 to IRS1.

IRS-1 was immunoprecipitated from cell extracts. The co-precipitated p85 subunit of PI3K was detected by a specific antibody. (Fig 4.6 B)

The association between p85 and IRS1 was induced by insulin after 30 min, and it decreased at 120 minutes. The binding of these two proteins was increased after treatment with AntCaNtide at 120 min. These data sustain the hypothesis that CaMKII is involved in the regulation of the negative feedback that leads to the downregulation of PI3K/Akt signal.

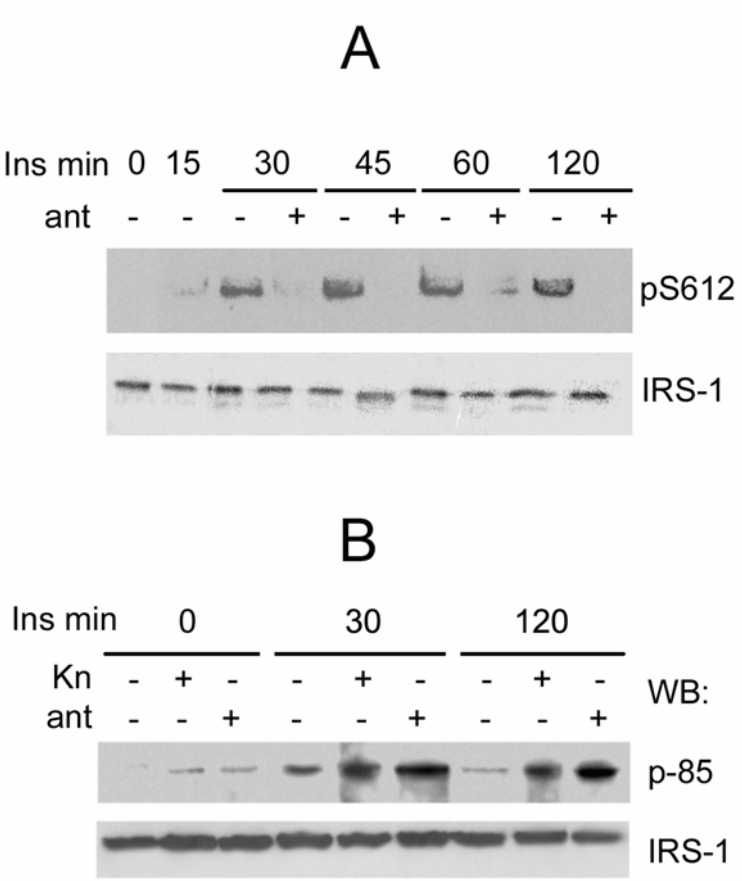


Figure 4.7 CaMKII inhibition abrogates insulin induced Ser612-IRS1 phosphorylation and enhances the binding of p85 to IRS1

L6 cells were pre-treated, when indicated, with 5 μM antCaNtide (ant) or 10 μM KN93 (Kn) for 30 min and stimulated with 100 nM insulin for the indicated time.
A)Cell extracts were analyzed by Western blot with antibodies vs phosphorylated-Ser612-IRS-1 (pS612) or total IRS-1. **B)** IRS-1 was immunoprecipitated from cell extracts. The co-precipitated p85 subunit of PI3K was detected by a specific antibody.

4.8 Insulin-induced Akt phosphorylation is upregulated by CaMKII inhibition.

One of the earliest events that follows Insulin-induced PI3K activation is Akt phosphorylation. As CaMKII is involved in the control of PI3K binding to IRS1 induced by Insulin, I evaluated the effects of CaMKII inhibitor antCaNtide on Akt phosphorylation at Ser 473. The timing of this phenomenon is important to understand the complicated interplay between the different signalling pathways induced by Insulin, therefore a time course was performed. L6 cells were pre-treated with 5μM AntCaNtide and then stimulated with Insulin 100nM for the indicated times. Akt phosphorylation was evaluated by western blot with a phospho-specific-antibody. Insulin induced Akt phosphorylation was maximal at 30 minutes, it decreased at 60 minutes and it was completely abolished after 120 minutes. The treatment with antCaNtide resulted in the increase of Akt phosphorylation at 30 and 60 minutes and it restores Akt phosphorylation at 120 minutes. (fig.4.8)

According to this result, CaMKII participate to the late downregulation of Akt phosphorylation following stimulation by insulin.

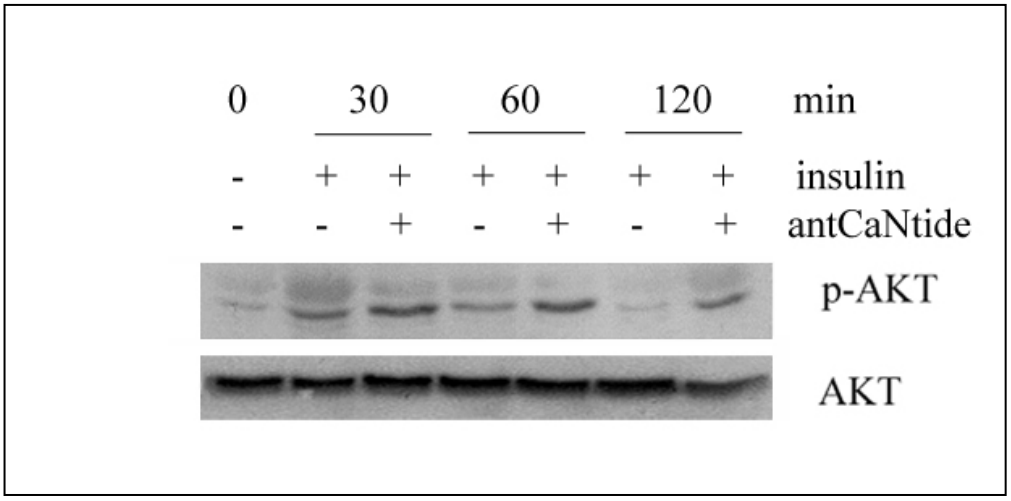


Figure 4.8 Insulin-induced Akt phosphorylation is up regulated by CaMKII inhibition.

Serum starved L6 cells were pre-treated with 5 μM antCaNtide and stimulated with 100nM Insulin for the indicated time. The cell lysates were analyzed by Western blot with total or phospho-specific (p-) antibodies to AKT.

4.9 CaMKII inhibition abrogates the downregulation of insulin-induced GLUT-4 translocation to the plasma membrane.

One of the mechanisms by which Insulin controls glucose homeostasis is glucose uptake in muscle and fat cells, mediated by the insulin-regulated glucose transporter GLUT-4. In resting conditions, GLUT-4 is sequestered inside the cytoplasm of muscle and fat cells. Insulin induces the redistribution of GLUT-4 from intracellular storage sites to the plasma membrane by activating PI3K/Akt pathway.

According to my data, CaMKII inhibition enhances insulin induced Akt signal, therefore I investigated whether CaMKII inhibition could affect insulin induced GLUT-4 translocation. L6 cells were pre-treated with CaMKII inhibitor AntCaNtide (ant) for 30 min and then stimulated with 100nM insulin at the indicated times. Plasma membrane (PM) and intracellular membrane from lysed cells were fractionated by centrifugation. Equal amounts of plasma membrane and intracellular membrane proteins were separated by SDS PAGE and immunoblotted with GLUT-4 antibody. In figure 4.9, GLUT-4 levels in plasma membrane are shown (PM) .GLUT-4 levels in the intracellular membrane didn't change (not shown).

The amount of GLUT-4 translocated to the PM upon Insulin stimulation was maximal at 30 min and decreased after 120 min. The reduction of GLUT-4 translocation after 120 min was prevented by CaMKII inhibition achieved by AntCaNtide treatment. Therefore, CaMKII might represent a novel regulatory element in the control of glucose homeostasis through the regulation of GLUT-4 transporter

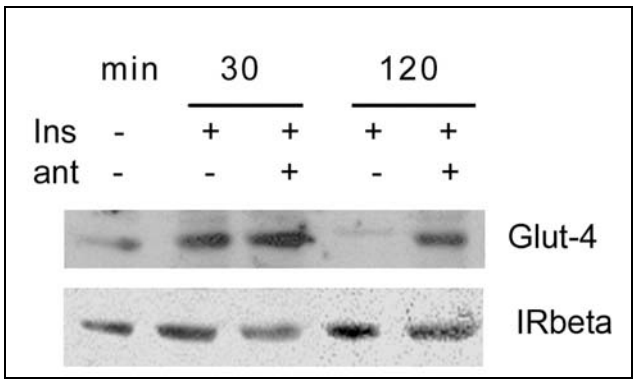


Figure 4.9 CaMKII inhibition abrogates the downregulation of insulin-induced GLUT-4 translocation to the plasma membrane.
L6 cells were incubated in the absence or the presence of AntCaNtide (ant) for 30 min and subsequently treated with 100nM insulin at the indicated times.
The cells were homogenized and subjected to subcellular fractionation to generate fractions enriched in plasma membrane (PM) and intracellular membranes (not shown). Equal amounts of plasma membrane and intracellular membrane proteins were separated by SDS PAGE and the amount of GLUT-4 in the PM was visualized by Western Blot.
The beta subunit of insulin receptor (IR beta) was used as a cell surface marker.

4.10 CaMKII inhibition abrogates the downregulation of insulin-induced Glucose uptake in L6 cells

According to the previous experiments, CaMKII is involved in the regulation of the pathways leading to GLUT-4 translocation to the cell surface, therefore I evaluated the effects of CaMKII inhibitor KN93 and AntCaNtide on one of the most important insulin metabolic effect: glucose uptake by muscle cells.

L6 cells were pre-treated with KN93 or AntCaNtide for 30 min, and then stimulated with 100nM insulin at the indicated times. [^{14}C] 2-D glucose was added to the medium in the last 10 min of Insulin treatment, and 2-D-glucose uptake was determined (Fig. 4.10). Glucose uptake was stimulated by Insulin by 30 min, and it returned to the basal level within 120 min. KN93 treatment increased basal glucose uptake and, consistent with the previous results, it inhibited the downregulation of glucose uptake at 120 min. A similar result was obtained with AntCaNtide (data not shown). These data demonstrate that CaMKII has a pivotal role in insulin metabolic effects, participating to the downregulation of glucose uptake mediated by Erk activation.

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 (Cartee et al., 1992; Youn et al., 1994; Young and Balon, 1997). 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 (Konstantopoulos et al., 2007). However, other studies raised the possibility that Ca^{2+} might have biphasic effects on insulin-stimulated glucose transport, depending on the magnitude of [Ca^{2+}]i variations: large increases in [Ca^{2+}]i induced by exposure to ionomicin or depolarization inhibits insulin stimulated glucose transport, whereas small rises in [Ca^{2+}]i are stimulatory.(Draznin et al., 1987b)

The disaccording results obtained to date are probably the consequences of Ca^{2+}/CaMKII effects exerted at multiple sites and at different times. My results are not in conflict with the positive role of CaMKII proposed in some studies. All these studies investigated the role of Ca^{2+} signaling within 30 min of insulin stimulation, whereas I focused my attention on the downstream negative feedback loop involving the Erk/IRS-1 interplay. Thus, in the model I propose, Ca^{2+}/CaMKII signaling pathway mediates both the early insulin-stimulated glucose uptake as well as its following downregulation.

In this model, CaMKII is an important regulator of insulin pathway. The role of CaMKII might spam in both metabolic and mitogenic effects of insulin on human diseases, such as hypertension and diabetes, both characterized by insulin resistance.

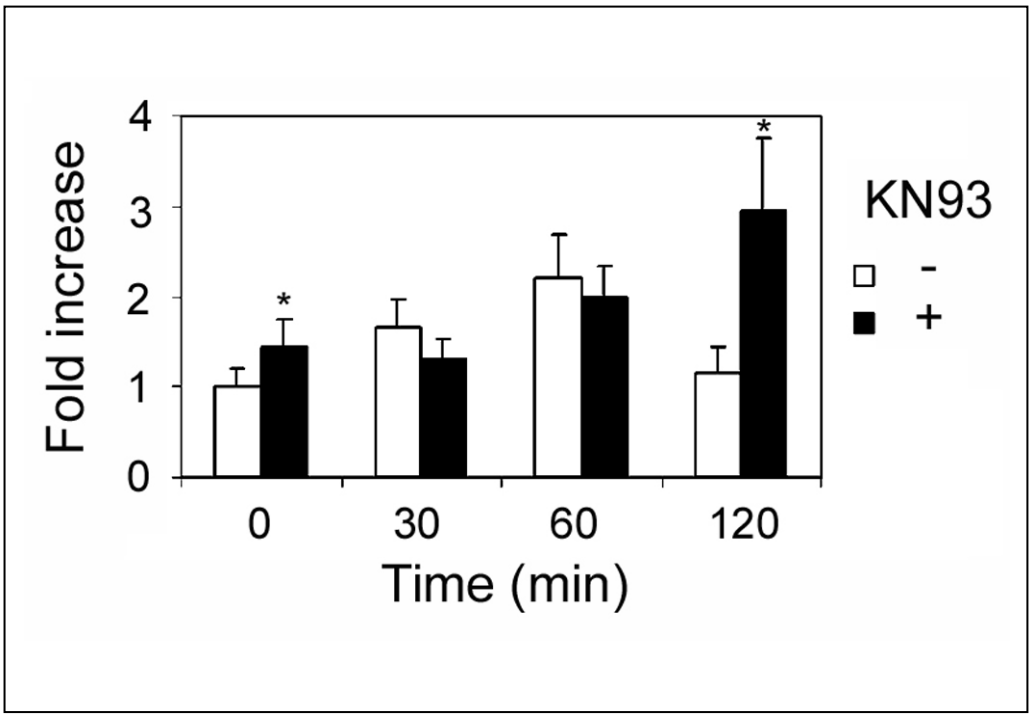


Figure 4.10 CaMKII inhibition abrogates the downregulation of insulin-induced Glucose uptake in L6 cells

L6 cells were treated with 10 μM KN93 and then stimulated with 100nM insulin at the indicated times. [^{14}C]2-D glucose was added to the medium in the last 10 min of Insulin treatment and 2-D-glucose uptake was determined by liquid scintillation counting. Data are reported as mean ± SD of 3 independent experiments in triplicates. *, significant *vs.* insulin alone.

5. CONCLUSIONS

I investigated the role of CaMKII in Insulin signaling cascade in a skeletal muscle cell line (L6) and in human fibroblasts. The results of the present thesis demonstrate that insulin receptor activates a Ca^{2+}/CaMKII dependent pathway that integrates with insulin signaling network, and controls cell growth and glucose uptake. (Fig 5.1)

CaMKII plays a pivotal role in the modulation of Erk activation in a number of models. A crosstalk between CaMKII and Erk pathway was first demonstrated in response to cell adhesion to extracellular matrix in a model of thyroid cells. Illario et al. demonstrated that CaMKII participates to Raf1 activation and controls Erk phosphorylation following integrin stimulation by fibronectin. (Illario et al., 2003; Illario et al., 2005).

To determine whether CaMKII/Erk pathway interplay was a general mechanism in the control of cell proliferation, I investigated the role of CaMKII in insulin signaling.

In skeletal muscle cells, insulin initiates different signaling pathways. The PI3K/Akt cascade is responsible for GLUT-4 translocation to the plasma membrane and glucose transport, whereas Ras/Erk pathway regulates cell growth and proliferation.

In the first part of my study I investigated the role of CaMKII in insulin mitogenic effects. I provide evidences that insulin promotes an increase of intracellular calcium concentration and CaMKII activation, in both L6 cells and fibroblasts. CaMKII inhibitors completely abolished Erk-1/2 phosphorylation as well as [^{3}H] thymidine incorporation induced by insulin stimulation. These observations extend the role of CaMKII and indicate that this mechanism is not restricted to integrins, but it is a general phenomenon that may be relevant for the biological effects of many growth factors and hormones.

In some cell types, like skeletal muscle cells and adipocytes, after the initial stimulation of glucose uptake, insulin promotes its downregulation by activating a negative feedback. The molecular mechanism of this feedback consist in the phosphorylation of IRS1 at Ser-612 by Erk, that impairs PI3K binding to IRS1 and the consequent Akt activation, thus arresting the translocation of GLUT-4 to the plasma membrane and finally reducing glucose uptake. My study demonstrate that CaMKII, through Erk1/2, modulates this negative feedback. CaMKII inhibition, indeed, abrogates the downregulation of insulin induced glucose uptake. As several factors are able to modify [Ca^{2+}]i, this might represent a site where different effectors converge to modulate insulin metabolic effects at single tissue level.

In conclusion, my results can be summarized as follows:

1. **A Ca^{2+}/CaMKII signal is activated following insulin stimulation**
2. **CaMKII participates to insulin induced Erk activation and cell proliferation**

3. **CaMKII is involved in the control of Akt signalling by mediating Erk- induced phosphorylation of IRS1 at Ser 612**
4. **CaMKII inhibition leads to a sustained GLUT-4 translocation to the plasma membrane and glucose uptake in response to insulin**

Therefore, CaMKII can be considered as a new target of insulin signaling, involved in the regulation of two important effects of insulin action such as cell proliferation and glucose homeostasis. This kinase, by modulating the insulin signal that leads to Erk1/2 activation, might have a role in the pathogenesis of insulin resistance. The contribution of CaMKII to these changes in insulin receptor function is worthy to be further investigated.

The identification of a novel molecular mechanism involved in the modulation of insulin signaling and glucose metabolism provides new targets for pharmacological interference, and CaMKII might represent a novel therapeutic target, offering new insights to the treatment of hyperglycemia.

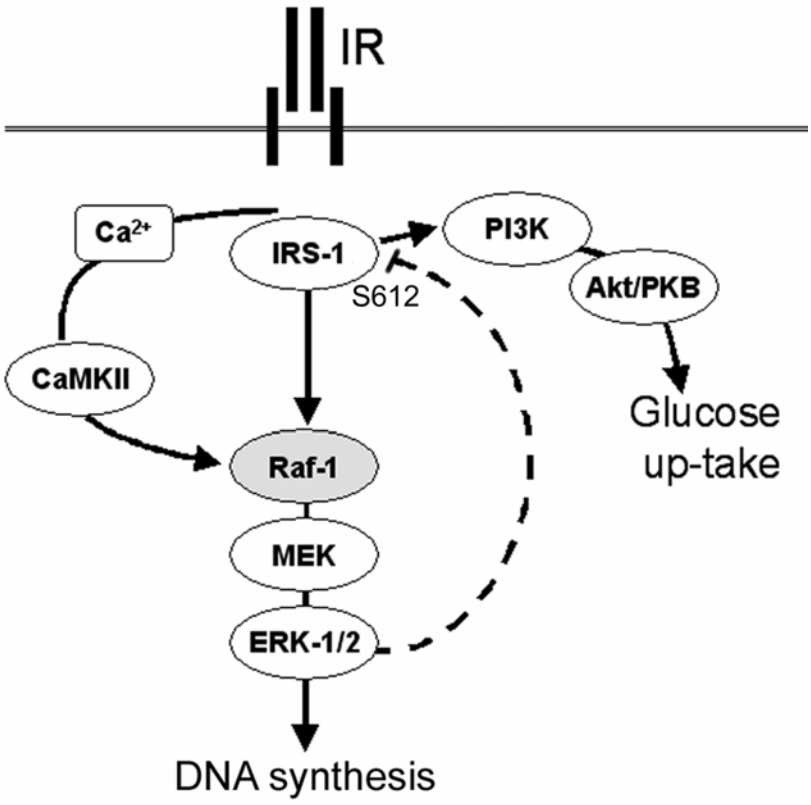


Figure 5.1 Schematic diagram of the insulin receptor signaling in L6 cells.

Activation of the insulin receptor generates the IRS-1/2→PI3K→Akt signaling 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 PI3K 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, phosphatydidlinositol 3-kinase; Akt, protein kinase B; CaMKII, calcium-calmodulin dependent kinase II.

6. ACKNOWLEDGEMENTS

I thank Prof. Guido Rossi for giving me the opportunity to join his group, and for supporting me for the entire period of my Doctorate.

Thanks to Prof. Giancarlo Vecchio, who allowed me to attend the doctorate program in Molecular Oncology and Endocrinology under his supervision.

Thanks to Prof. Mario Vitale, who supervised my work and encouraged my interest in biomedical research.

A special thank to Doctor Maddalena Illario, who followed my work since my first steps in science, and helped me during the development of the experiments and the elaboration of this thesis. I'm grateful to her enthusiasm, and to her confidence in my ability.

Thanks to all my colleagues of the Rossi's Lab, for their friendship and cooperation. In particular, I would like to thank Doctor Maria Rosaria Rusciano, whose collaboration was fundamental for this thesis. Thanks to Serena Maione, Raffaella Bonavita, Michela Di Salvio, Marcella Salzano, Francesca Gatta, for their continuous help and extraordinary dedication to the work in the lab.

Thanks to Prof. Lucio Pastore and Doctor Dalila Astone for providing the essential viral tools and expertise.

Thanks to Prof. Pietro Formisano for critical discussion of the data, and for his precious advises.

I'm really grateful to Prof. Anthony R. Means for the opportunity he gave me to spend three months in his laboratory at Pharmacology and Cancer Biology department of Duke University Medical Center. I thank Prof. Means for his teachings, his criticism and for allowing me to work in the most stimulating scientific environment.

Many thanks to all my colleagues and friends from Durham, who made my stay in the US really unforgettable.

For financial support, I thank the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) .

I cannot end without thanking my family and friends, on whose constant encouragement and love I have relied throughout my life.

Thanks to my mother, for supporting my dreams and aspirations;

Thanks to my sister Tiziana, for sharing with me joys and sorrows;

Thanks to my friend Silvana Libertini for never abandon me in tough moments, and for making these three years much happier;

Thanks to Massimo for the revolution he brought into my life.

This thesis is dedicated to the memory of my father, who transmitted to me the love for science.

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0021-972X/05/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 90(5):2865–2873

doi: 10.1210/jc.2004-1520

Fibronectin-Induced Proliferation in Thyroid Cells Is Mediated by αvβ3 Integrin through Ras/Raf-1/MEK/ERK and Calcium/CaMKII Signals

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We recently demonstrated in an immortalized thyroid cell line that integrin stimulation by fibronectin (FN) simultaneously activates two signaling pathways: Ras/Raf/MAPK kinase (Mek)/Erk and calcium (Ca^{2+})/calcium calmodulin-dependent kinase II (CaMKII). Both signals are necessary to stimulate Erk phosphorylation because CaMKII modulates Ras-induced Raf-1 activity. In this study we present evidence that extends these findings to normal human thyroid cells in primary culture, demonstrating its biological significance in a more physiological cell model. In normal thyroid cells, immobilized FN-induced activation of p21Ras and Erk phosphorylation. This pathway was responsible for FN-induced cell proliferation. Concurrent increase of intracellular Ca^{2+} concentration and CaMKII activation was observed. Both induction of p21Ras activity and increase of intracellular Ca^{2+} concentration were mediated by FN binding to αvβ3 integrin. Inhibition of the Ca^{2+}/CaMKII signal pathway by calmodulin or CaMKII inhibitors completely abolished the FN-induced Erk phosphorylation. Binding to FN induced Raf-1 and CaMKII to form a protein complex, indicating that intersection between Ras/Raf/Mek/Erk and Ca^{2+}/CaMKII signaling pathways occurred at Raf-1 level. Interruption of the Ca^{2+}/CaMKII signal pathway arrested cell proliferation induced by FN. We also analyzed thyroid tumor cell lines that displayed concomitant aberrant integrin expression and signal transduction. These data confirm that integrin activation by FN in normal thyroid cells generates Ras/Raf/Mek/Erk and Ca^{2+}/CaMKII signaling pathways and that both are necessary to stimulate cell proliferation, whereas in thyroid tumors integrin signaling is altered. (*J Clin Endocrinol Metab* 90: 2865–2873, 2005)

THYROID FOLLICULAR CELLS are arranged in the thyroid gland to form follicles, round-shaped structures that represent the functional unit of the gland. A basal lamina surrounds the follicles, and thyroid follicular cells, like any other epithelial cell, are attached to the extracellular matrix (ECM) proteins that constitute the basal lamina. Fibronectin (FN) together with collagen and laminin represents one of these ECM proteins (1, 2). We previously demonstrated that FN together with other soluble factors (TSH, epidermal growth factor, insulin, and IGF) controls essential biological functions in the thyroid cell, including proliferation and survival (3–5). Control of cell proliferation and survival exerted by ECM is of fundamental relevance for tissue homeostasis and is important to neoplastic cells that must proliferate and survive in ectopic environments or denied adhesion, while metastasizing through the bloodstream (6). Cell adhesion to ECM is mainly mediated by integrins, a large family of cell surface receptors widely expressed in all tissues. Integrins are heterodimeric receptors located at basal cell membrane whose expression pattern is tissue type dependent (7–9). Membrane distribution of some integrins is restricted to subcellular structures known as focal adhesions, which contain structural and signaling molecules including actin, focal adhesion kinase (Fak), Srk, protein kinase C, and paxillin. Integrin activation can generate multiple signals that regulate cell behavior through the modulation of ion concentration (Ca^{2+}, Na^{+}, H^{+}) or lipid metabolism. Although integrins do not display direct kinase activity, their activation by binding to ECM proteins generates signals that include kinase cascades. In a thyroid cell line obtained by immortalizing human fetal thyroid cells (TAD-2), integrin clustering by binding to FN generates two signaling pathways: Fak/Ras/Raf-1/MAPK kinase (Mek)/Erk that mediates FN-induced proliferation, and the phosphatidylinositol-3 kinase (PI-3K) pathway involved in cell survival (10).

We recently demonstrated in the same cell type that integrin binding to FN also generates a third pathway that is calcium/calcium calmodulin-dependent kinase II (Ca^{2+}/CaMKII). The latter modulates the Ras/Raf-1/Mek/Erk pathway by binding to Raf-1 and regulating its activity (11). Although TAD-2 cells and normal thyroid cells in primary cultures display common integrin profile and FN-induced

First Published Online February 1, 2005

Abbreviations: BSA/PBS, 0.5% BSA and PBS; Ca^{2+}, calcium; $[Ca^{2+}]i$, intracellular free calcium concentration; CaMKII, calcium calmodulin-dependent kinase II; CDK, cyclin-dependent kinase; ECM, extracellular matrix; F-12, Ham's F-12 medium; Fak, focal adhesion kinase; FCS, fetal calf serum; FN, fibronectin; Mek, MAPK kinase; PI-3K, phosphatidylinositol-3 kinase; PTC, papillary thyroid carcinoma; RFI, relative fluorescence index; RGD, arginine-glycine-aspartic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

behavior (4, 12), a direct demonstration of the existence of such integrin-generated signal in normal thyroid cells in primary cultures is lacking. Moreover, because the pattern expression of adhesion molecules changes after cell transformation, it is important to determine which integrin receptor generates the signals that mediate FN-induced proliferation.

Thus, we investigated the signal pathways generated by FN-dependent integrin activation in normal human thyroid cells in primary culture. We provide evidence that FN binding to the integrin $\alpha v\beta 3$ activates the Ras/Raf-1/Mek/Erk and the Ca^{2+}/CaMKII pathways and that both are necessary to promote FN-induced proliferation.

Materials and Methods

Cell cultures

Tissue specimens were obtained at surgery from contralateral lobes of thyroid papillary carcinomas or internodular tissue of nodular goiters of subjects undergoing thyroidectomy. Informed consent for cell culture preparation was obtained from patients undergoing thyroidectomy.

Cell cultures were prepared as previously described (4). Briefly, tissues were chopped by scalpels in small pieces and digested by type IV collagenase (Sigma Chemical Co., St. Louis, MO) 1.25 mg/ml in Ham's F-12 medium (F-12) and 0.5% BSA overnight at 4 C under rotation. Cells were pelleted by centrifugation at 150 × *g* for 5 min, washed twice in BSA-F-12, seeded in petri dishes, and cultured in 5% CO_2 atmosphere at 37 C in F-12 supplemented with 10% fetal calf serum (FCS). Medium was changed every 3–4 d, and the cells were harvested by treatment with 0.5 mM EDTA in calcium- and magnesium-free PBS containing 0.05% trypsin. The follicular origin of the cultures was confirmed by flow cytofluorometry searching for cytokeratin and thyroglobulin as previously described (12). More then 98% of the cells in culture were positive for cytokeratin and thyroglobulin. For each experiment, single individual cultures were used and results pooled for statistical analysis.

The TAD-2 cell line, obtained by Simian virus 40 infection of human fetal thyroid cells was generously donated by Dr. T. F. Davies (Mount Sinai, New York, NY) (13). Thyroid papillary carcinoma cell lines NPA and TPC-1 were kindly donated by M. Nagao (Tokyo, Japan) (14). All cell lines were cultured in F-12 supplemented with 10% FCS.

Coated plates were prepared as follows: the plates were filled with PBS, 1% heat-denatured BSA (Sigma, St. Louis, MO), or 100 μg/ml human FN (Collaborative Research, Bedford, MA). After overnight incubation at 4 C, the plates were washed with PBS three times and used.

Antibodies and flow cytometric analysis

For intracellular immunofluorescence (cytokeratin and thyroglobulin), cells were fixed in 3.5% paraformaldehyde, 0.2% Tween 20 in PBS, washed twice in PBS, and resuspended in 0.5% BSA and PBS (BSA/PBS); immunostaining was then performed using fluorescein-conjugated anticytokeratin antibodies (Ortho, Raritan, NJ) or rabbit antihuman thyroglobulin serum followed by sheep antirabbit IgG as a fluorescein-conjugated secondary antibody. Serum from nonimmunized rabbits or nonspecific fluoresceinated immunoglobulins of the same isotype was used as controls. Cells were then analyzed by flow cytometry using a FACScan apparatus (Becton Dickinson, Mountain View, CA). Monoclonal antibody of mouse origin against β1-subunit (clone A1A5) was kindly donated by Dr. M. E. Hemler (Dana Farber Cancer Institute, Boston, MA) and anti-α3 (J143) by Dr. L. J. Old (Ludwig Institute for Cancer Research, New York, NY). Monoclonal antibody to α5 was purchased from Telios (San Diego, CA); anti-$\alpha v\beta 3$ and anti-$\alpha v\beta 5$ were purchased from Chemicon (Temecula, CA); fluorescein-conjugated antimouse and antirabbit IgG and horseradish peroxidase-conjugated antirabbit IgG were purchased from Ortho. Flow cytometric analysis was performed as follows: cells harvested from subconfluent cell cultures by PBS containing 0.05% trypsin were incubated with the primary monoclonal antibody for 1 h at 4 C in BSA/PBS, washed in the same buffer, and incubated again with the secondary fluorescein-conjugated antibody for 30 min at 4 C. Cells were resuspended in BSA/PBS and analyzed by flow cytometry. Nonspecific immunoglobulins of the same isotype were used as controls. The expression of each integrin was represented as: relative fluorescence index (RFI) = experimental mean fluorescence/control mean fluorescence.

Western blot and immunoprecipitation

For Western blot analysis, the cells were lysed in Laemmli buffer [0.125 mol/liter Tris (pH 6.8), 5% glycerol, 2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, and 0.006% bromphenol blue], and proteins were resolved by 7–10% SDS-PAGE and transferred to a nitrocellulose membrane (Immobilon P; Millipore Corp., Bedford, MA). Membranes were blocked by 5% nonfat dry milk, 1% ovalbumin, 5% FCS, and 7.5% glycine in PBS, washed, and incubated for 1 h at 4 C with primary antibodies and 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 (Amersham Biosciences, Piscataway, NJ). Computer-acquired images were quantified using ImageQuant software (Amersham Biosciences). For immunoprecipitation, the cells were lysed in immunoprecipitation buffer [0.05 mol/liter Tris-HCl (pH 8.0), 0.005 mol/liter EDTA, 0.15 mol/liter NaCl, 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 0.01 mol/liter NaF, 0.005 mol/liter EGTA, 0.01 mol/liter sodium pyrophosphate, and 0.001 mol/liter phenylmethylsulfonylfluoride]. Rabbit polyclonal antibody reactive to all CaMKII isoforms (Santa Cruz Biotechnology, Santa Cruz, CA) and protein G plus/protein A agarose beads (Oncogene Science, Boston, MA) were used to immunoprecipitate CaMKII from 1 mg of total lysate. Raf-1 and BRaf antibodies were from Santa Cruz Biotechnology. Mouse monoclonal antibodies to Erk-2 and phospho-Erk-1/2 were from Santa Cruz Biotechnology. Polyclonal anti-phospho-CaMKII antibody (pT286-CaMKII) was from Promega (Madison, WI).

p21Ras activity assay

Ras activity was assayed by affinity precipitation using a Ras activation assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, 4×10^6 cells were lysed with Mg^{2+} lysis buffer [0.125 mol/liter HEPES (pH 7.5), 0.75 mol/liter NaCl, 5% Igepal CA630, 0.05 mol/liter $MgCl_2$, 0.005 mol/liter EDTA, and 10% glycerol] and incubated with 5 μl of a 50% slurry of Raf-1 Ras binding domain peptide for 30 min at 4 C. The beads were then boiled in reducing sample buffer, and adsorbed proteins were resolved by electrophoresis, transferred to nitrocellulose, and probed with a monoclonal anti-Ras (1 μg/ml) (Mg^{2+} lysis buffer, Raf-1 Ras binding domain peptide, and anti-Ras were included in a Ras activation assay kit; Upstate Biotechnology, Lake Placid, NY). Proteins were visualized using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

Calcium measurement

A total of 3×10^5 cells harvested by trypsin were loaded with cell-permeant fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) by incubating the cells with DMEM, 10^{-5} mol/liter fura-2, 0.5% BSA, and 0.01 mol/liter HEPES for 30 min at 37 C. The cells were then washed twice for 10 min with 0.001 mol/liter $CaCl_2$ in Hanks' balanced salt solution [0.118 mol/liter NaCl, 0.0046 mol/liter KCl, 0.01 mol/liter glucose, and 0.02 mol/liter HEPES (pH 7.2)]. When indicated, cells were incubated with integrin-FN-binding arginine-glycine-aspartic acid (RGD)-containing peptides (Gly-Arg-Gly-Asp-Ser-Pro) or control peptides RGE (Gly-Arg-Gly-Glu-Ser-Pro) (Calbiochem, EMD Biosciences, Darmstadt, Germany). Fluorescence was measured with a fluorometer (PerkinElmer Life Sciences, Norwalk, CT). Excitation was at 345 and 380 nm and emission was at 510 nm. Minimal and maximal relative fluorescences were obtained by adding 0.01 mol/liter EDTA and 2% Triton X-100 or 0.01 mol/liter EDTA, 2% Triton X-100, and 0.01 mol/liter $CaCl_2$, respectively. The nanomolar concentration of Ca^{2+} was obtained by the Grynkiewiez formula considering 225 Kd for fura-2 (15).

[^{3}H]Thymidine incorporation

To determine DNA synthesis, cells were plated in F-12 , 0.5% BSA, and 0.5 μCi [^{3}H]thymidine in 24-well plates coated with FN. After 24 h,

the plates were gently washed with PBS avoiding cell loss 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. Lysates were then resuspended in 5 ml scintillation fluid and counted in a β-counter (Beckton Dickinson).

Results

Expression of FN receptors of the integrin family in thyroid cells in culture

Follicular cells from normal glands obtained by collagenase digestion were cultured for a maximum of 7 d *in vitro*. The expression of the FN receptors of the integrin family was evaluated by flow cytometry with monoclonal antibodies specific for β1-, α3, and α5-chains and for αvβ3 and αvβ5 heterodimers. Figure 1 reports the average expression of integrin subunits and heterodimers measured in four different cultures at 75% confluence. Analysis was performed on 4- to 5-d-old cultures after two passages. As previously demonstrated for α3β1 (16), the level of αvβ3 expression also changed during the culture under the regulatory effect of cell-to-cell contact. The expression of these integrins was higher at 75% confluence than at full confluence. For this reason all experiments were performed on cells after two passages from 4- to 5-d-old cultures at 75% confluence. The β1- and α3-chains that associate to constitute the α3β1 etherodimer were strongly expressed as previously demonstrated in normal thyroid cells in culture as well as in tissue sections (4). The αvβ3 receptor was expressed as previously reported in the thyroid cell line TAD-2 (12), whereas α5 and αvβ5 were not expressed. Thus, normal thyroid cells in primary culture express only two integrin receptors for FN: α3β1 and αvβ3.

FN activates the Ras/Erk pathway and stimulates cell proliferation

Thyroid cells were starved from serum for 24 h, harvested, and plated onto FN- or BSA-coated plates. Active p21Ras and phosphorylated Erk were determined in cell extracts (Fig. 2, A and B). Strong p21Ras activation was achieved 15 min after plating the cells onto immobilized FN. Whereas comparable p21Ras activation was observed at 15 and 30 min, induction of Erk phosphorylation was slower and 30 min stimulation was required to induce a powerful phosphorylation. Also cell proliferation was influenced by FN. Cell number was calculated after 24 and 48 h culture onto FN-coated plates in the absence of serum (Fig. 2C). Whereas in the absence of FN and serum cell adhesion was denied, FN alone was sufficient to allow cell adhesion and spreading. FN induced a 60% increase of cell number after 48 h, whereas the in the presence of the Mek inhibitor PD98052, cell proliferation was completely abrogated. The number of cells plated onto BSA-coated plates was not calculated because cell adhesion was very weak in the absence of serum or FN and most of the cells were detached by 48 h culture.

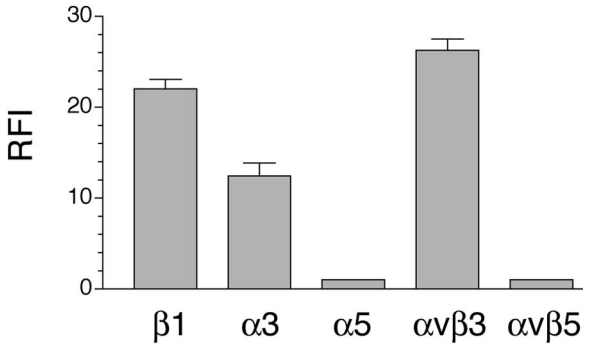

FIG. 1. Expression of integrin FN receptors in normal thyroid cells from primary cultures. Cells were harvested by mild trypsinization from subconfluent cultures and incubated with monoclonal antibodies specific for single integrin subunits (β1 and α5) or whole receptors (αvβ3 and αvβ5) followed by the secondary fluorescein-conjugated antibody. The relative fluorescences were measured by flow cytometry as described in *Materials and Methods* in different cultures. The expression of each integrin etherodimer or single subunit is reported as: RFI = experimental mean fluorescence/control mean fluorescence.

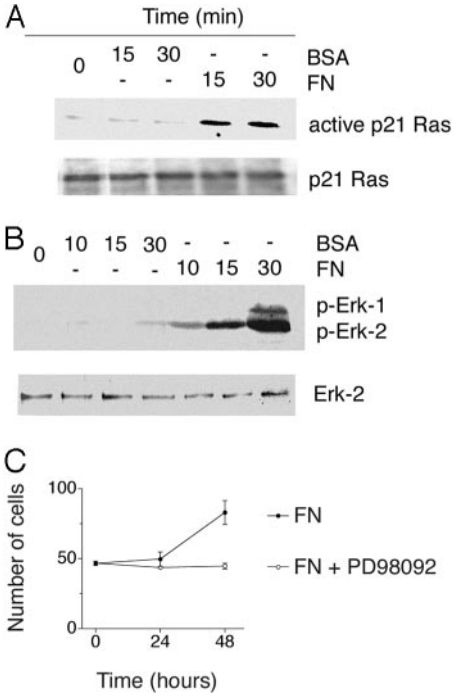

FIG. 2. Activation of the Ras/Erk pathway and stimulation of cell proliferation by FN. Cells were harvested by trypsinization from subconfluent cultures and plated onto BSA- or FN-coated plates. After the indicated time, the cells were lysed. A, Activated p21Ras was immunoprecipitated with Raf-1 Ras binding domain peptide-conjugated agarose beads and visualized by Western blot (active-p21Ras), and total Ras in cell extracts was visualized by Western blot (p21Ras). B, The extracts were analyzed by Western blot with antiphosphotyrosine-Erk (p-Erk-1, p-Erk-2) or anti-total-Erk-2 (Erk-2) antibodies. C, Fifty thousand cells were plated in serum-free medium onto FN-coated plates with or without 20 μM PD98052. After 24 and 48 h, the cells were harvested and counted by a hemocytometer.

Inhibition of FN-integrin binding impairs p21 Ras activation

To determine which integrin/s generate/s the Ras/Erk pathway, p21Ras activity was determined in cells plated onto FN in the presence of binding-inhibiting antibodies (Fig. 3). An anti-αvβ3 binding-inhibiting antibody strongly inhibited p21Ras activation induced by FN, whereas anti-α3 was ineffective. Inhibition of p21Ras activation by anti-αvβ3 anti-

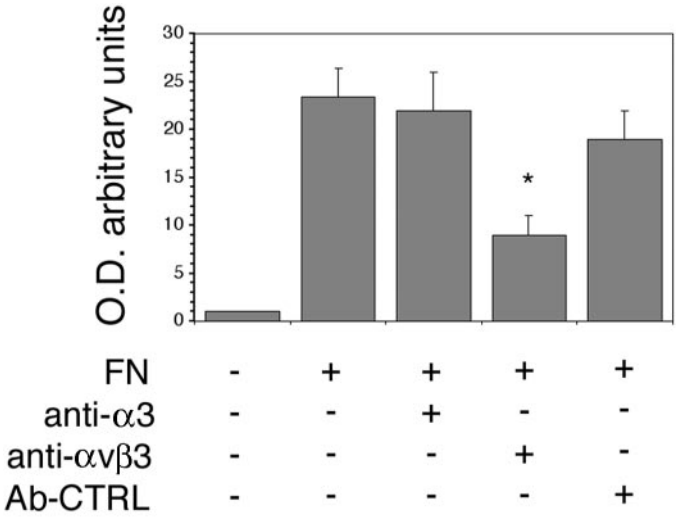

FIG. 3. Inhibition of FN-induced p21Ras activation by antiintegrin antibodies. Cells were harvested by trypsinization from serum-starved cultures and plated onto BSA- or FN-coated plates. Cells were preincubated for 30 min with 12 μg/ml of antiintegrin antibodies inhibiting binding to FN (Ab-$\alpha v\beta 3$, Ab-$\alpha 3$) or nonrelevant antibody (Ab-CTRL). After 30 min, p21Ras activity was determined as described. Relative expression of activated p21Ras was determined by scanning densitometry in three independent experiments. A value of 1 OD arbitrary unit was assigned to BSA point. Results are presented as mean ± SD. *, Significant *vs.* CTRL point. CTRL, Control.

bodies was not complete. Although this could be explained by incomplete inhibition of FN-$\alpha v\beta 3$ binding, participation of other factors (other adhesion molecules or FN contaminants) in the p21Ras activation is possible. These data suggest that the integrin $\alpha v\beta 3$ participates in the activation of the Ras/Erk signal pathway in normal human thyroid cells.

Activation of the integrin αvβ3 induces increase of intracellular calcium concentration

To test whether integrin activation by FN also generates a calcium signal, intracellular free calcium concentration ($[Ca^{2+}]i$) was measured by fluorometric analysis in cells in suspension in response to soluble FN binding (Fig. 4). Cells were incubated with soluble FN in the presence of peptides inhibiting the integrin-FN binding (RGD) or control peptides (RGE), anti-$\alpha v\beta 3$, or nonrelevant antibodies. After 30 min of suspension, FN plus peptides or antibodies were added to the cells. In the cells treated with FN alone or FN plus control peptide (not shown), $[Ca^{2+}]i$ increased, reaching a 4-fold increase by 60 min. The FN receptor antagonist peptide RGD completely inhibited the $[Ca^{2+}]i$ increase, demonstrating that FN effect was mediated by integrins. The anti-$\alpha v\beta 3$ antibody inhibited the $[Ca^{2+}]i$ increase induced by FN, whereas a control antibody was ineffective. These data suggest that FN activates a Ca^{2+} signal through the integrin $\alpha v\beta 3$.

Integrin binding to FN induces CaMKII activation

To determine whether the FN-induced $[Ca^{2+}]i$ increase could activate CaMK enzymatic activity, we evaluated the phosphorylation level of CaMKII (Fig. 5). CaMKII autophosphorylation at T286 residue indicates CaMKII activation. Serum-starved cells were plated onto FN or BSA, and

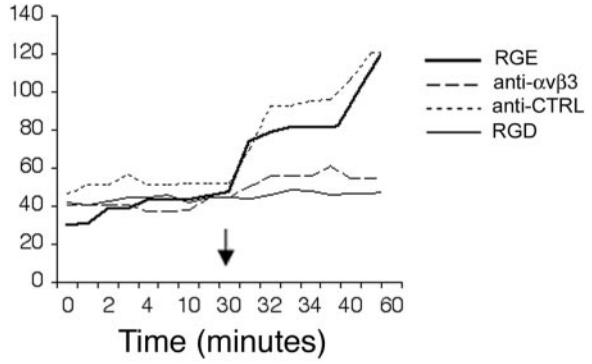

FIG. 4. Activation of integrin $\alpha v\beta 3$ by FN induces a Ca^{2+} signal. The cells were serum starved for 24 h, harvested by trypsin, washed with culture medium, and loaded with fura-2. At the time indicated by the *arrow*, soluble FN alone or with integrin binding inhibitor (RGD) or control peptide (RGE), anti-$\alpha v\beta 3$ or nonrelevant (anti-CTRL) antibodies were added to the cells, and $[Ca^{2+}]i$ was measured by fluorometric analysis. Results are presented as mean ± SD nanomolar concentration of Ca^{2+} from quadruplicates. CTRL, Control.

CaMKII T286-phosphorylation was evaluated by Western blot. In the absence of FN no signal was visible by antiphosphothreonine CaMKII antibody at any time. After 15 min of FN stimulation, a sharp band that increased by 30 min stimulation was visible, thus demonstrating that cell binding to FN induces CaMKII activation in thyroid cells.

Inhibition of the Ca^{2+}/CaMKII pathway inhibits FN-induced Erk phosphorylation

We previously demonstrated that in an immortalized thyroid cell line, Erk phosphorylation induced by FN requires both Ras/Raf-1/Mek and Ca^{2+}/CaMKII signals. To investigate whether this is a general mechanism or it is restricted to that specific cell type, we tested the effects of Ca^{2+}/CaMKII pathway inhibitors on the phosphorylation of Erk after FN binding. To perform this analysis, we used the CaM inhibitors (W7 and TFP), the nonisoform specific CaMK in-

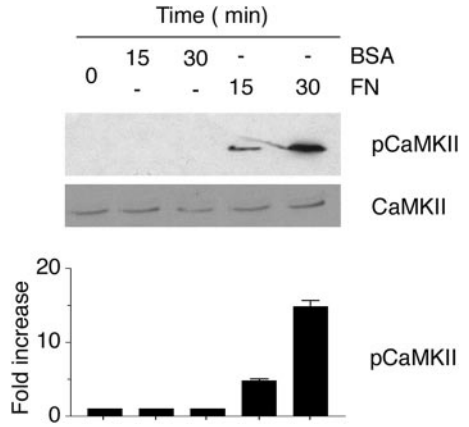

FIG. 5. CaMKII phosphorylation in response to FN stimulation. Serum-starved cells were seeded onto FN- or BSA-coated plates in the absence of serum and analyzed by Western blot. Total CaMKII and phosphorylated T286-CaMKII (p-CaMKII) were visualized by specific monoclonal antibodies. Averages and SD of relative expressions of phosphorylated CaMKII were also determined by scanning densitometry of three immunoblots. In each diagram, a value of 1 OD arbitrary unit was assigned to 0 point.

hibitor (KN93), and a CaMKII-specific inhibitor (ant-CaNtide). This short peptide is derived from the endogenous CaMKII inhibitor-protein CaMKIIN and was made cell permeable by the Antennapedia N-terminal sequence. The cells were plated onto immobilized FN with different inhibitors and the level of Erk-1/2 phosphorylation was evaluated by Western blot (Fig. 6). Erk-1/2 phosphorylation induced by FN was higher than that induced by 10% FCS. KN93, W7, and TFP displayed a dose-dependent inhibitory effect on Erk-1/2 phosphorylation. Ant-CaNtide abolished FN-induced Erk-1/2 phosphorylation at a 5 μM concentration. These data demonstrate that Erk phosphorylation is dependent on CaMKII activation. Thus, the requirement of Ca^{2+}/CaMKII signals in the FN-induced Erk activation is not a feature restricted to a cell line but is a general mechanism in thyroid cells.

FN induces Raf-1/CaMKII association

To determine whether the interaction of Ras/Raf-1/Mek and Ca^{2+}/CaMKII signals occurs at the Raf-1 level, we tested whether CaMKII formed with Raf-1 a multiprotein complex. CaMKII was immunoprecipitated in extracts from cells plated onto BSA or FN. Immunoprecipitated CaMKII and coprecipitated Raf-1 were detected by Western blot by specific antibodies (Fig. 7). Coimmunoprecipitated Raf-1 was visible only in cells stimulated with FN. The CaMK inhibitor KN93 and the CaMKII inhibitor ant-CaNtide completely inhibited the FN-induced Raf-1/CaMKII association. This experiment suggests that FN induces Raf-1 and CaMKII to form a protein complex that depends on CaMKII activation.

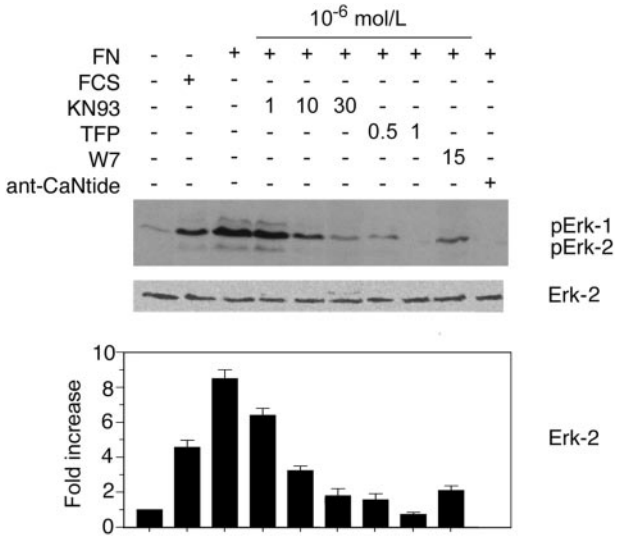

FIG. 6. Inhibition of the Ca^{2+}/CaMKII pathway blocks FN-dependent Erk phosphorylation. Serum-starved cells were plated for 30 min in uncoated plates in the presence of 10% FCS or in serum-free medium onto BSA-coated plates or in FN-coated plates with or without KN93, W7, TFP, or ant-CaNtide. Cell extracts were analyzed by Western blot with antiphosphotyrosine-Erk-1/2 (p-Erk) or anti-total-Erk-1 (Erk-1) antibodies. Averages and SD of relative expressions of phosphorylated Erk1/2 were also determined in three immunoblots. A value of 1 OD arbitrary unit was assigned to untreated point.

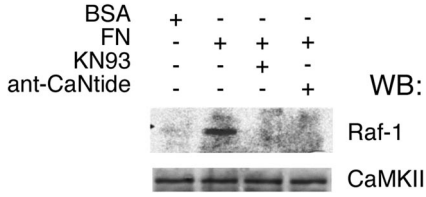

FIG. 7. FN induces CaMKII binding to Raf-1. The cells were plated for 30 min onto BSA- or FN-coated plates in serum-free medium without or with KN93 or ant-CaNtide. Cell extracts were immunoprecipitated with a specific anti-CaMKII antibody. After protein separation by SDS-PAGE and transfer to a nitrocellulose membrane, immunoprecipitated CaMKII and coprecipitated Raf-1 were detected by anti-CaMKII and anti-Raf-1 antibodies, respectively. WB, Western blot.

BRaf/Raf-1 heterodimerization induced by FN

BRaf overexpression obtained by transfection in human embryonic kidney cells suggested that Ras induces the Raf-1/Braf heterodimerization through the exposure of 14–3-3 binding sites in the COOH terminus of Raf-1 (17). Thus, BRaf heterodimerized with Raf-1 may cooperate with Raf-1 in cellular response to FN. BRaf appears largely expressed in thyroid cells analyzed by Western blot (Fig. 8A). To determine whether BRaf heterodimerized with Raf-1 in the CaMKII/Raf-1 complex induced by FN, the cells were plated onto FN, and CaMKII was immunoprecipitated. Coprecipitated Raf-1 and BRaf were detected by Western blot by specific antibodies (Fig. 8B). Whereas Raf-1 was evident in FN-induced CaMKII coprecipitate, a band for BRaf was barely visible. These results do not support a significant role for BRaf in the signal generated by FN-integrin binding in thyroid cells.

Inhibition of CaMKII blocks FN-induced thymidine incorporation

[^{3}H]thymidine incorporation was used to measure the DNA synthesis in thyroid cells cultured in serum-free me-

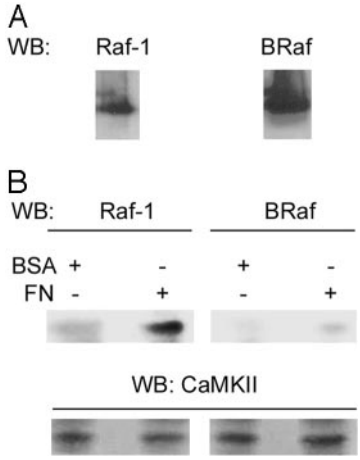

FIG. 8. BRaf coprecipitation with the CaMKII/Raf-1 complex. A, Raf-1 and BRaf in total cellular extracts were visualized by Western blot with specific antibodies. B, The cells were plated for 30 min onto BSA- or FN-coated plates in serum-free medium. Cell extracts were immunoprecipitated with a specific anti-CaMKII antibody. After protein separation by SDS-PAGE and transfer to a nitrocellulose membrane, immunoprecipitated CaMKII and coprecipitated Raf-1 and BRaf were detected by specific antibodies. WB, Western blot.

dium on immobilized FN or untreated wells for 24 h (Fig. 9). In the absence of FN, cells were weakly attached to the plates, round shaped, and spreading was limited by 24 h. In FN-coated wells, the cells were well adherent and flat shaped. The presence of inhibitors did not change the attachment, shape, or spreading. The FN stimulation induced 66% increase of [^{3}H]thymidine incorporation. Both KN93 and ant-CaNtide completely abolished such stimulation, demonstrating that FN-induced DNA synthesis requires CaMKII activity.

Thyroid cancer cells display aberrant integrin expression and signaling

The surface expression of integrin receptors for FN was determined in a cell line originated from normal thyroid (TAD-2), and cell lines originated from papillary carcinomas (NPA and TPC-1). Although thyroid cancer cells have a broad pattern of integrin expression (18), within FN receptors only quantitative changes of $\alpha3\beta1$ and $\alpha v\beta3$ were found, and *de novo* expression of $\alpha v\beta5$ was not observed (Fig. 10A). Because integrin expression is regulated by several factors, data in cultured cells must be confirmed by tissue analysis. Immunohistochemistry with anti-$\alpha v\beta3$ antibodies confirmed altered expression of this integrin in papillary carcinomas (Fig. 10B). As for cell lines, the level of $\alpha v\beta3$ expression was demonstrated to be variable. To evaluate the signal response to FN, the cells were plated onto immobilized FN with or without CaMK inhibitor, and the level of Erk-1/2 phosphorylation was evaluated by Western blot (Fig. 9B). Erk-1/2 phosphorylation was induced in TAD-2 cells and inhibited by KN93 as previously demonstrated (11). In unstimulated papillary carcinoma cell lines, Erk-1/2 was phosphorylated and FN stimulation did not produce any change. CaMKII inhibition by KN93 decreased Erk-1/2 phosphorylation in TPC-1 cells, whereas it was ineffective in NPA cells. The explanation for the insensitivity to CaMK inhibition in NPA cells lies in the presence of BRaf^{Val599} oncogene (19) that is independent from CaMKII activation (our unpublished data).

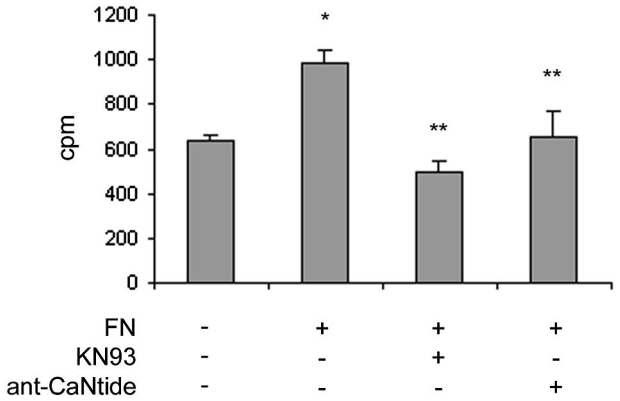

FIG. 9. Stimulation of thymidine incorporation by FN requires CaMKII activity. Thyroid cells were seeded in microtiter plates previously coated by overnight incubation with 10 μg/ml FN in PBS. Cells were cultured for 24 h in serum-free medium, with 0.5 μCi [^{3}H]thymidine in the presence of 0.01 mol/liter KN93 or 0.005 mol/liter ant-CaNtide where indicated. Data are reported as mean ± SD of quadruplicate experiments. *, Significant *vs.* CTRL point. **, Significant *vs.* FN point, not significant *vs.* CTRL.

Discussion

We recently demonstrated that integrin activation in the TAD-2 cell line generates three distinct signals: Fak/Ras/Mek/Erk, Ca^{2+}/CaMKII, and PI-3K signal (10, 11). In the same studies, we also provided evidence that the Ca^{2+}/CaMKII signal is necessary to Raf-1 activation by Ras in the Fak/Ras/Mek/Erk pathway. Although TAD-2 cells represent a reliable human thyroid cell model to study integrins and thyroid ECM interaction, it was generated from fetal thyroid cells by Simian virus 40 infection. Thus, a direct demonstration of the signals generated by integrin activation by FN and their role in the control of proliferation in primary thyroid cells in cultures was not yet provided.

Within integrins, the two FN receptors, $\alpha5\beta1$ and $\alpha v\beta3$, are recognized to be important for generating signals that drive proliferation, survival, and spreading (20–23). Previous studies demonstrated that thyroid cells do not express $\alpha5\beta1$ or $\alpha v\beta5$, whereas they express $\alpha v\beta3$ and minimal $\alpha v\beta1$ in certain conditioned medium (24, 25). Within the FN receptors expressed in thyroid cells, $\alpha3\beta1$ is likely to play only a structural role because its signaling capacity is not yet established, whereas $\alpha v\beta3$ has a recognized signaling function (8, 26–28). The complexity and tissue specificity of signaling generated in response to integrin engagement is made possible by the complexity of interactions between multiple factors that take place in the focal adhesion. In response to $\alpha5\beta1$ and αvb3 engagement, the Ras/MAPK signal pathway is activated through the adapter molecules Shc and Grb2/Sos involving FAK, paxillin, or c-Srk in fibroblasts and epithelial and endothelial cells (29–31). Thyroid cells cultured *in vitro* in medium also containing hypothalamus and pituitary extracts express $\alpha v\beta3$ integrin in a latent state characterized by its inability to cluster at focal adhesions and promote cell adhesion. However, $\alpha v\beta3$ recruitment at focal adhesions and ligand-binding activity occurred on treatment with hepatocyte growth factor/scatter factor (25). In the normal thyroid cell line TAD-2, $\alpha v\beta3$ membrane expression is restricted to focal adhesions, whereas in the present study, in normal thyroid cells cultured in the absence of growth factors, $\alpha v\beta3$ stained by immunofluorescence displayed a fine grainy pattern of distribution (not shown). This membrane localization not restricted to focal adhesions suggests the possibility that the effect measured in our system might be of a higher magnitude in a more physiological environment in the presence of those endocrine and paracrine factors abundant *in vivo*. All together these data indicate that culture conditions and growth factors are relevant modulators of expression and function of integrins in thyroid cells and that normal thyroid cells potentially express active $\alpha v\beta3$ integrin.

Cell proliferation is regulated by converging signals on the cell cycle machinery that determine whether the cell stays in the G_1 phase or proceeds to S phase. The progression through G_1 into the DNA synthesizing S phase is driven by cyclin-dependent kinase (CDK)4 and CDK6, which interact with the cyclin D family of proteins, and CDK2, which interacts with cyclins A/E (32). The Ras/Raf/Mek/Erk cascade plays a

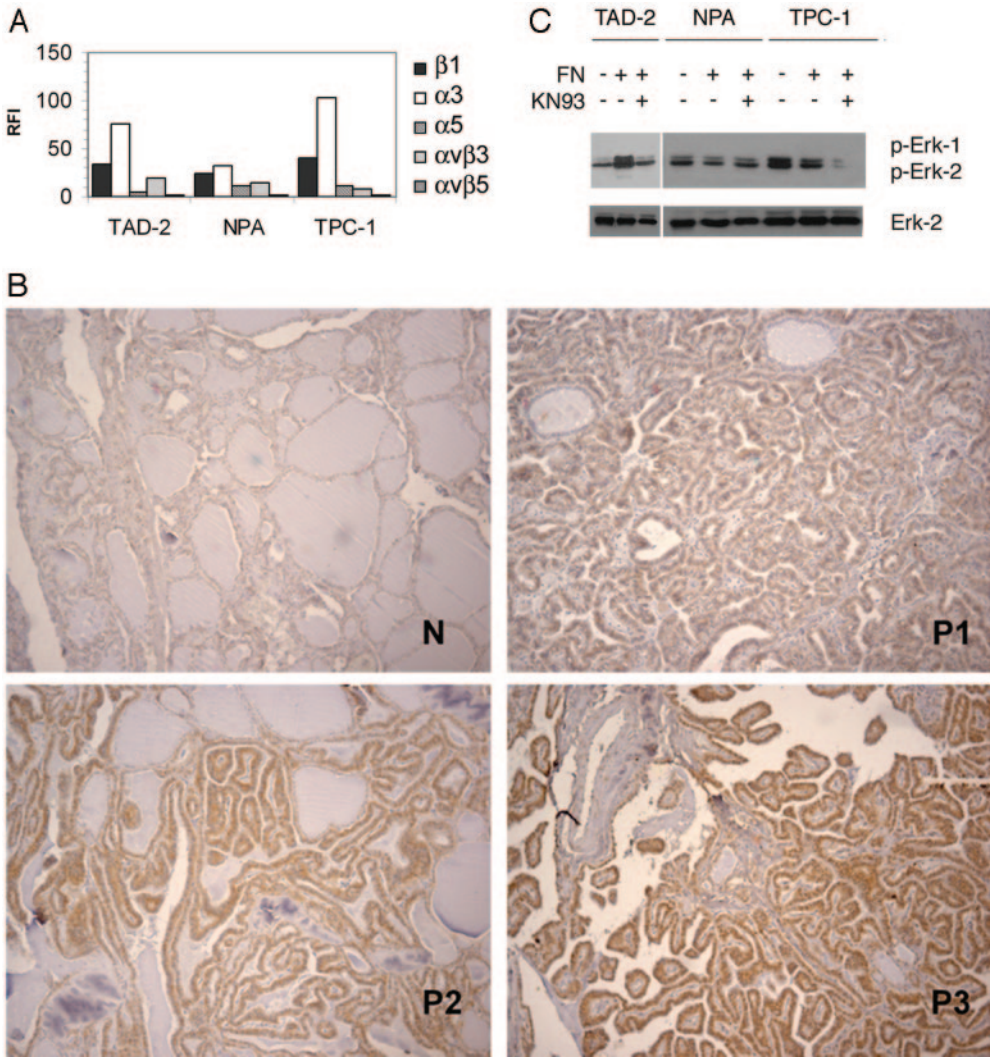

FIG. 10. Aberrant integrin expression and signaling in thyroid cancer cells. A, Cells were harvested by mild trypsinization, stained by indirect immunofluorescence with monoclonal antibodies, and relative fluorescences were measured by flow cytometry. The expression of each integrin etherodimer or single subunit is reported as: RFI = experimental mean fluorescence/control mean fluorescence. B, Immunohistochemical analysis of integrin $\alpha v\beta 3$ expression in normal thyroid and in papillary cancer. The staining was equally developed in all follicles in normal thyroid samples (N). The $\alpha v\beta 3$ expression displayed a variable degree of intensity in papillary carcinomas, being from faint to intense (P1-P3). C, Serum-starved cells were plated in serum-free medium for 30 min onto BSA-coated plates or in FN-coated with or without KN93. Cell extracts were analyzed by Western blot with antiphosphotyrosine-Erk-1/2 (p-Erk) or anti-total-Erk-1 (Erk-1) antibodies.

pivotal role in the control of this process. Sustained Erk activation is required to pass the G_1 restriction point and regulate cyclin D1 expression during mid-G1 phase (33). Inhibition of FN-stimulated proliferation by Mek inhibitor demonstrates the role of Erk in this process in thyroid cells. In normal cells, it is unlikely that integrin might sustain prolonged proliferation in the absence of other growth factors. This topic may not be investigated in primary cultures of thyroid cells because they have a very limited growth potential. Our results demonstrate the involvement of $\alpha v\beta 3$ and the Ras/Erk signal in FN-stimulated proliferation in culture, whereas *in vivo* the entire pathway or part of it must be abrogated or counteracted. Integrins are constantly engaged by the ECM of the basal lamina surrounding the thyroid follicles. Thus, whereas the PI-3K signal might be continuous and relevant in survival, the Ras/Erk signal must be downmodulated to avoid aberrant stimulation of proliferation. Thyroid-stimulating hormone that has been proposed to inhibit Ras signaling to Raf/MAPK might represent one of these factors (34, 35).

Although this and other factors might be responsible of such a regulation, the complexity of signals generated by integrins makes possible their self-modulation. Within signals generated by integrins, PI-3K/Cdc42/Pak represents a costimulatory signal that modulates the Ras/Erk pathway. In COS-7 cells, PI-3K inhibition induces severe attenuation of integrin-dependent Erk activation (36). Integrin-FN binding activates the serine/threonine kinase Pak through PI-3K and Cdc42. Pak then phosphorylates Raf-1 on Ser338, ensuing Erk activation by Ras (36, 37). Whereas Ser338-Raf-1 phosphorylation is necessary for Raf-1 to be fully activated by Ras (38, 39), integrins are not the sole activators of Pak-1, and in any

system Pak-1 is not always necessary for Raf-1 activation, possibly because it is substituted by other factors. Indeed, Pak-1 inhibition in Rat-1 fibroblasts abrogates Ras-induced transformation, whereas it is ineffective in NIH-3T3 fibroblasts in which other factors must coactivate Raf-1 (40, 41). Also in TAD-2 cells, FN binding induces a modest level of Pak-1 phosphorylation whose role was not investigated (11). In TAD-2 cells and primary thyroid cultures, besides the Ras/Erk signal, integrin engagement activates the Ca^{2+}/CaMKII signal. CaMKII and Ras are both necessary to activate Raf-1, and inhibition of CaMKII abrogates Erk phosphorylation and proliferation induced by FN. Thus, integrins can self-modulate their own proliferation signal through the PI-3K/Pak-1 and Ca^{2+}/CaMKII signals. Coimmunoprecipitation experiments demonstrate that CaMKII and Raf-1 participate in a common multiprotein complex. The question whether Raf-1 is a direct CaMKII substrate was not addressed in this study. However, this is a possibility worthy of consideration because Raf-1 is a substrate for many kinases and presents a number of consensus sequences for CaMKII.

The malignant behavior of cancer cells is determined by not simply uncontrolled growth but also the ability to migrate, proliferate, and survive in denied adhesion or ectopic body districts. Tumor cells metastasized into ectopic sites or moving in the vascular or lymphatic system loose adhesion to the basal lamina mediated by integrins. The adhesion receptors expressed on the surface of invading cells play a fundamental role in this process. Changes in the level of surface expression of integrins or *de novo* expression or activation state occur in a number of neoplastic lesions including thyroid cancer (18, 42–46). The two thyroid cancer cell lines we examined in this study displayed aberrant integrin expression, and Erk resulted in a phosphorylated state also in denied adhesion. Independently whether Erk activation resulted from activated integrins, oncogenes, or other activating pathways, aberrant integrin expression and activation might still play a relevant role as a costimulatory factor. In TPC-1 cells the Ca^{2+}/CaMKII signal is still necessary to Erk phosphorylation. In this cell line harboring RET/PTC-1, this oncogene might be responsible of sustained Erk phosphorylation, and CaMKII can be activated by integrins or other effectors including RET/PTC-1 oncogene. Conversely, tumor cells harboring oncogenes that do not lie on the tyrosine kinase/Raf-1 pathway elude the Raf-1/CaMKII checkpoint. The mutation at site T1796 is sufficient to generate a BRaf mutant constitutively activated (47). One relevant difference between Raf-1 and BRaf is the presence of the S445 in BRaf. This serine is equivalent to S338 of Raf-1 and is constitutively phosphorylated (39). Also for this reason, Ras alone is sufficient to activate BRaf and Val599E mutation activates BRaf with no need for other factors. A number of biological consequences with clinical relevance arise from the Raf-1/CaMKII interaction. Future studies must determine the factors responsible for CaMKII activation in tumors. Besides altered integrins and known physiological factors, mutated Ras, Trk, and RET/PTC have the potentiality to autonomously activate the Ca^{2+}/CaMKII signal. A different ability to activate CaMKII might be responsible for the different aggressive behavior observed within RET/PTC mutants and different oncogenes.

Acknowledgments

We thank K. Ulrich Bayer for providing us with purified ant-CaNtide.

Received July 31, 2004. Accepted January 20, 2005.

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This work was supported in part by the Ministero dell'Istruzione, dell'Università e della Ricerca (to M.V., G.F., and G.R.) and Ministero della Salute (to M.V.).

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JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

CALCIUM-CALMODULIN-DEPENDENT KINASE II (CaMKII) PARTICIPATES TO INSULIN-STIMULATED ERK-1/2 ACTIVATION AND MODULATES PROLIFERATION AND GLUCOSE UPTAKE

Running title: Role of CaMKII in insulin signaling

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ABSTRACT 1

The pleiotropic effects of insulin include cellular growth and glucose synthesis and uptake. These events 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 signaling in a rat skeletal muscle cell line, demonstrating that CaMKII modulates the stimulation of the DNA synthesis and the negative feedback that down regulates glucose uptake.

Insulin stimulation generated partly independent signals leading to the activation of Akt, Erk-1/2 and CaMKII. Phosphorylation of these three kinases and IRS-1/p85PI3K association began by 5 min stimulation. By 30 min IRS-1 was phosphorylated at S612, the IRS-1/p85PI3K complex was disrupted and Akt was no more phosphorylated. Akt activation was followed by Glut-4 translocation to the plasma membrane and increase of glucose uptake. Then, both Glut-4 translocation and glucose uptake were reduced. Insulin, promoted CaMKII association with Raf-1 and inhibition of CaMKII abrogated the insulin-induced Erk-1/2 activation, thymidine incorporation and the 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 CaMKII promotes insulin-induced Erk-1/2 activation and cell proliferation, while attenuating the IRS-1/Akt pathway and promoting 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.

INTRODUCTION

Insulin has both metabolic and mitogenic effects in several cell 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 evidences 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 phosphorylation of the receptor itself. 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 the subsequent Akt activation. Insulin binding to its receptor activates different pathways that can signal to the nucleus, including extracellular-regulated kinase (Erk-1/2) (6, 7). Notably, the insulin receptor can regulate its own signal through a negative feedback loop involving Erk-1/2 and Grb-2 associated binder 1 (Gab1). In CHO cells and in the human kidney fibroblast cell line 293, PKC activation results in decreased insulin-stimulated IRS-1 association with PI3-K and subsequent Akt activation (8). 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 PI3-K binding site (8-10). More recently, liver knockout Gab1 mice 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, Erk-1/2 or Gab1 modulators can enhance or reduce glucose uptake and metabolism in the liver and participate to glucose homeostasis. By this mechanism, insulin induced effects can be modulated by the receptor itself or by endogenous or environmental interfering factors.

The role played by Ca^{2+} in insulin signaling has long been debated, and remains still partly unsolved. Indirect evidences 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 signaling 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). To determine the relevance of the CaMKII/Erk-1/2 interplay in other models, we extended our study to the insulin signaling in a rat skeletal muscle cell line (L6) stably expressing the human insulin receptor, and in human fibroblasts. We provided experimental evidences for a novel role of CaMKII in the insulin signaling cascade. According to our results, insulin-activated CaMKII promotes Erk-1/2 phosphorylation and cell proliferation, while attenuating the IRS-1/Akt pathway promotes the down-regulation of stimulated glucose uptake. This represents a novel mechanism in the selective control of insulin signaling.

MATERIALS AND METHODS

Cell cultures

The L6 skeletal muscle cells were grown in Dulbecco modified eagle medium (DMEM) (Life Technologies, Inc, Grand Island, NY) supplemented with 10% fetal calf serum **(**Sigma, St. Louis, MO) and 2% glutamine, in a humidified CO_2 incubator by the method of Caruso *et al*. (22).

Calcium Measurement

Fluorimetric analysis: A total of 3x105 cells harvested by trypsin were loaded with cell-permeant Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR), by incubating the cells with Dulbecco's modified Eagle's medium, 10-5 mol/L Fura-2, 0.5% BSA, and 0.01 mol/L HEPES for 30 min at 37 C. The cells were then washed twice for 10 min with 0.001 mol/L CaCl2 in Hanks' balanced salt solution (0.118 mol/L NaCl, 0.0046 mol/L KCl, 0.01 mol/L glucose, and 0.02 mol/L HEPES, pH 7.2). Fluorescence was measured with a fluorimeter (PerkinElmer Life Sciences). Excitation was at 345 and 380 nm, emission was at 510 nm. Rmin and Rmax were obtained by adding 0.01 mol/L EDTA and 2% Triton X-100 or 0.01 mol/L EDTA, 2% Triton X-100, and 0.01 mol/L CaCl2, respectively. The nanomolar concentration of Ca2+ was obtained by the Grynkiewiez formula considering a 225 KDa for Fura-2 (23).

For calcium transients imaging, 200.000 cells were plated onto a 60 mm microscope dish, starved o.n., and loaded with 12.5 mg Oregon green /100 µl of Standard Buffer (in mM: 137 NaCl; 2.7 KCl; 1 Na2HPO4; 20 Hepes, 7.4 pH; 1 MgCl2; 2 CaCl2; 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 Ca2+ 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 Ca2+ 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 Ca2+ images were analyzed with the MetaMorph Imaging System software (Universal Imaging Corporation, West Chester, PA).

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% FCS, 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 Biosciences). 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 (Santa Cruz Biotechnology, Santa Cruz, CA), Raf-1 (Santa Cruz Biotechnology), 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 were also used as a control. Mouse monoclonal antibodies to 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 Signaling Technology, Danvers, MA.

[^{3}H]thymidine incorporation

To determine DNA synthesis, cells were plated in 24-well plate, and serum-starved for 48 hours in DMEM, 0.5% BSA. 0.5 μCi [^{3}H]thymidine and 100nM 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).

CaMKII activity and inhibitors

The cells were lysed in 200 μl of RSB buffer (24) with 10 mM CHAPS and 20 μl of the extracts were assayed in 50 μl of reaction mixture consisting of 50 mM HEPES pH 7.5, 10 mM MgCl2, 0,5 mM dithiothreitol (DTT), 2μM 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 CaCl2 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 minutes 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 ant-CaNtide 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 (ant-CaNtide:
RQIKIWFQNRRMKWKKRPPKLGQIGRSKRVVIEDDRIDDVLK) (28). The reversed ant-CaNtide peptide was also used as a control. Both peptides were synthesized by L. D'A.

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).

Cell membrane expression of Glut-4

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 MgSO4, 10 mmol/l NaHCO3, 1.2 mmol/l KHPO4, 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.

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.

RESULTS

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

L6 cells starved from serum were stimulated with Insulin for 5 to 120 min, lysed and Erk-1/2, Akt and CaMKII phosphorylation were evaluated by Western blot with a phospho-specific antibody (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 CaMKII phosphorylation yet at 5 min and both the kinases remained phosphorylated up to 120 min. As observed in other cell types (9) after an initial increase, Akt phosphorylation resulted down-regulated. Phosphorylation of Akt occurred at 5 min and the kinase was no more phosphorylated by 30 min of insulin stimulation. Akt phosphorylation occurred in a dose-dependent manner and its down-regulation was independent by the insulin concentration (Fig. 1B).

Insulin stimulation induces intracellular [Ca^{2+}]i increase and CaMKII activation.

CaMKII is activated by its binding to Ca^{2+}-activated calmodulin. This is the only mechanism for CaMKII activation. In order to evaluate whether insulin was able to initiate the cascade leading to CaMKII activation, we measured [Ca^{2+}]i in the cells following insulin stimulation. (Fig. 2). The cells were serum-starved overnight, and were subsequently loaded with Fura-2, detached from the plates and examined in suspension by fluorometry (Fig. 2A). [Ca^{2+}]i remained unchanged for 15 minutes in unstimulated cells. Subsequently, insulin was added at different concentrations and a [Ca^{2+}]i increase appeared soon after stimulation. The [Ca^{2+}]i increase was rather slow relative to other Ca^{2+} mobilizing agents and was dose-dependent upon the concentration of insulin used reaching about 5-fold increase by 10 min with 100 nM insulin. [Ca^{2+}]i was then determined at single cell level by confocal microscopy in adherent cells loaded with Oregon Green (Fig. 2B, 2C). 100 nM insulin induced a slow increase of [Ca^{2+}]i, confirming what observed in the same cells in suspension. Among CaMKs, the isoform II is ubiquitary and is involved in the control of Ras→Erk-1/2 pathway upon integrin engagement (18). To evaluate whether insulin-stimulated [Ca^{2+}]i increase 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. 3A). 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. 3B). 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.

Insulin stimulates CaMKII association with Raf-1.

As integrin stimulates CaMKII activation and its association with Raf-1, we investigated whether also insulin induces CaMKII/Raf-1 binding (18). 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 KN93 and 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. 4). 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.

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 signaling pathways was previously demonstrated in integrin signaling. To investigate the possible existence of a similar cross talk in insulin signaling, 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. However, because multiple isoforms of CaMKII are expressed in L6 cells (not shown), pharmacological inhibition and highly specific inhibition of enzymatic binding site were preferred (26)(18)(27).The cells were stimulated with insulin in the presence or in the absence of the CaMKs pharmacological inhibitor KN93 or the cell permeant CaMKII-inhibitory peptide ant-CaNtide, and the levels of Erk-1/2 and Akt phosphorylation were evaluated by Western blot (Fig. 5A). Both inhibitors not only completely abolished Erk-1/2 phosphorylation induced by 30 min insulin stimulation but also basal phosphorylation was strongly reduced. These data suggest that in our model muscle cells, Erk-1/2 activation requires active CaMKII. [^{3}H]thymidine incorporation was used to measure DNA synthesis in the cells cultured in serum-free medium stimulated with 100 nM insulin for 24 h (Fig. 5B). Insulin stimulation induced 65% increase of [^{3}H]thymidine incorporation. Both KN93 and ant-CaNtide completely abolished such stimulation, demonstrating that insulin-induced DNA synthesis requires CaMKII activity.

CaMKII is necessary for the negative-feedback of insulin-stimulated IRS-1/p85 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/PI3-K 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 PI3-K bound to IRS-1 were detected by Western blot with specific antibodies. Co-precipitated p85 appeared by 15 ATTENZIONE VERIFICARE SE AKT SI ATTIVA A 5 MIN. DEVE ESERE UGUALE ALL'ATTIVAZIONE DI AKT min of insulin stimulation and disappeared by 30 min (Fig. 6A). IRS1 was phosphorylated at Ser612 by 30 min and it remained phosphorylated up to 120 min (Fig 6B). Both KN93 and antCaNtide completely abrogated phosphorylation of S612 IRS-1. By 15 min of insulin stimulation, the association of IRS-1 and p85 was consistently increased by CaMKII inhibitors.

CaMKII inhibition also preserved the IRS-1/p85 complex after 30 min and up to 120 min of insulin stimulation. These results indicate that CaMKII is necessary for Erk-1/2 activation that in turn phosphorylates S612 IRS-1 and inhibits the formation of the IRS-1/p85 complex.

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

We investigated the effect of CaMKII inhibition on Akt phosphorylation stimulated by insulin (Fig. 7A). CaMKII inhibition had no effect on AKT phosphorylation induced by 15 min of insulin stimulation. After 30 min of insulin stimulation, Akt was no more phosphorylated, whereas the phosphorylation was maintained by KN93 in a dose-dependent manner and was stimulated at higher KN93 concentrations. We then determined the effect of CaMKII inhibition on Glut-4 translocation induced by insulin (Fig. 7B). Glut-4 translocation to the cell membrane was observed by 30 min of insulin treatment, and it remained constant up to 60 min. By 120 min, membrane Glut-4 was minimal. CaMKII inhibition 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 KN93 pre-treatment. [^{14}C]2-D-glucose was added to the cells for the last 10 min of stimulation and the radioactivity determined (Fig. 7C). Glucose uptake was stimulated by insulin, and it returned to the basal level by 120 min. KN93 increased basal glucose uptake and, as predicted by the observation of the sustained Akt phosphorylation and Glut-4 in the membrane, CaMKII inhibition increased glucose uptake that lasted up to 120 min.

DISCUSSION

The results of this study demonstrate that activation of insulin receptor generates three interplaying signaling systems that regulate cell growth and glucose uptake (Fig. 9).

The role of Ca^{2+} in insulin signaling has been investigated in different studies with different approaches. While some studies suggest that Ca^{2+} is involved in insulin signaling in several cell types (29-31), others failed to demonstrate that insulin modifies $[Ca^{2+}]i$ (32-34). Klip et al. found that insulin did not induce $[Ca^{2+}]i$ increase in L6 cells (35). However, the low sensitivity of the Ca^{2+}-indicator Quin-2 used in that study bias their results and raises doubts on their conclusions. Partly according to Klip observations, a more recent 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 an other study, insulin induced a very fast (2 sec) and transient $[Ca^{2+}]i$ increase in rat myotubes (36). Our results demonstrate that insulin induces a slow and global $[Ca^{2+}]i$ in L6 cells. The discordance between these results might be explained by differences in calcium homeostasis between the cell models employed and more importantly by the timing of the observation. Our results are not in conflict with previously published results as all the studies mentioned investigated only the very early insulin effects , whereas in our study we examined later effects as our interest was focused on subsequent enzyme activation mediated by Ca^{2+}. In our experiments a very fast global effect on $[Ca^{2+}]i$ was not present or was hidden, however a progressive increase of $[Ca^{2+}]i$ was evident after few minutes of insulin stimulation and became maximal by ~25 min. The increase of $[Ca^{2+}]i$ (global or restricted at CaMKII location) induced by insulin was of a magnitude sufficient to activate a signaling pathway involving CaMKII, a kinase whose activation is dependent by Ca^{2+}. 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 (41, 42).

We 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 $[^3H]$thymidine incorporation induced by insulin stimulation, demonstrate that in our model of rat muscle cells Erk-1/2 activation requires active CaMKII. These results were reproduced also in human fibroblasts, where CaMKII inhibition achieved by y 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, suggesting that the CaMKII/Raf-1 interplay is not a mechanism restricted to integrins in thyroid cells.

The finding that the insulin signaling leading to Erk-1/2 activation is modulated by Ca^{2+}→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/PI3-K binding and in turn Akt activation, thus generating a negative feedback loop regulating some of insulin effects. In L6 mytubes, 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 (43). In the liver, the major site for glucose production and storage, this mechanism regulates glucose metabolism and finally influences the glycemia (44). 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 $[Ca^{2+}]i$, this might represent a site where different effectors converge to modulate insulin signaling.

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, 45, 46). Recently, an other 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 (42). 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 {Lee, 1995 #417}.

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+} signaling at 30 min of insulin stimulation, while we focused our attention on the following negative feedback loop involving the Erk/IRS-1 interplay. Thus, in the model we propose, the Ca^{2+}/CaMKII signaling mediates both the early insulin-stimulated glucose up-take as well as its following down-regulation.

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 evidences support the increase of Erk-1/2 activation and the reduction of IRS-1/2 tyrosine phosphorylation and association with PI3-K in patients with type 2 diabetes (48). 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.

The identification of a novel molecular mechanisms involved in the modulation of insulin signaling and glucose metabolism provides new targets for pharmacological interference: CaMKII might represent another novel therapeutic target offering new insights to the treatment of hyperglycemia.

ACKNOWLEDGMENTS

This work has been supported in part by the Italian Ministero dell'Istruzione, dell'Universita` e della Ricerca and Associazione Italiana per la Ricerca sul Cancro.

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FIGURE LEGENDS

Figure 1. Time course of insulin induced signalings. 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 Akt, Erk-1/2 or CaMKII. For the latter, we used the specific antibody recognizing the phosphorylated T286 of the activated kinase. 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. B, the cells were stimulated for 15 or 30 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. Variations of pAkt at 30 min were not significant.

Figure 2**.** Insulin induces $[Ca^{2+}]i$ increase. A, the cells were starved from serum and loaded with Fura-2. Then, the cells were resuspended by tripsinization and $[Ca^{2+}]i$ was measured by a fluorimeter for 15 min before insulin stimulation (arrow) and for the following 15 min. Insulin concentrations were: 1 mM, empty circle, dotted line; 10 nM empty circles continuous line; 100 mM full circles. B and C, the cells grown onto glass coverslips were loaded with Oregon green, stimulated with 100mM insulin and observed by confocal microscopy as described in Materials and Methods. $[Ca^{2+}]i$ is reported Relative fluorescence = (fluorescence – fluorescence at 0 point)/ fluorescence at 0 point)

Figure 3. Insulin induces CaMKII activation. The cells were starved from serum and stimulated with insulin at the indicated nM concentrations. Panel A, 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. Panel B, The cells were stimulated for 30 min with 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. BSA *versus* Insulin, $p < 0.001$.

Figure 4. Insulin stimulates CaMKII association with Raf-1. The cells were serum starved and stimulated with 100 nM insulin for 30 min in the presence of 10 μM KN93, or 5 nM antCaNtide. The cell extracts were immunoprecipitated with a specific 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 was detected by specific antibodies. IP, immunoprecipitation; WB, Western blot.

Figure 5. 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 nM ant-CaNtide or R-ant-CaNtide. Averages and S.D. of relative expressions of phosphorylated Erk were also determined by scanning densitometry of independent immunoblots. A value of 1 O.D. arbitrary unit was assigned to the minor point. Panel B, $2x10^5$ cells were plated in 24-well plates, and 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.

Figure 6. CaMKII inhibition preserves insulin-stimulated IRS-1/p85 complex and abrogates IRS-1 phosphorylation on S612. Panel A, the cells were pre-treated with 5 μM KN93 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 coprecipitated p85 subunit of PI3K were detected by specific antibodies. Panel B, the cell were treated as before, IRS-1 was immunoprecipitated from cell extracts and analyzed by Western blot with antibodies to phosphorylated-Ser612-IRS-1 (pS612) or total IRS-1. In both panels, averages and S.D. of relative expressions of

coimmunoprecipitated p85 or phosphorylated-S612-IRS-1 were also determined by scanning densitometry of independent immunoblots. A value of 1 O.D. arbitrary unit was assigned to the minor point.

Figure 7. CaMKII inhibition abrogates the down-regulation of insulin-stimulated Akt phosphorylation, Glu-4 membrane translocation and glucose uptake. Panel A, Phosphorylated and total Akt were visualized by Western blot in cells treated with insulin for 15 or 30 min and pretreated for 30 min with increasing μM concentration of KN93. Panel B, Cell membrane extracts were prepared as described in Material and Methods from cells treated for 30 or 120 min with 100 nM insulin with or without 5 nM antCaNtide (ant). Glut-4 and insulin receptor beta chain (IRbeta, used as a membrane marker) were determined by Western Blot. Panel C, the cells were stimulated with insulin with or without 10 μm KN93 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 ± SD of 3 independent experiments in triplicates. *****, significant *vs.* insulin alone.

Figure 8. A schematic time course of insulin signaling. Diagrams are a schematic representation of time-dependent insulin-stimulated Erk, Akt and CaMKII activation, increase of [Ca^{2+}]i, S612-IRS1 phosphorylation, IRS1/p85 interaction, Glut-4 translocation to the membrane and glucose uptake. The diagrams are only indicative of time variations and are not quantitative.

Figure 9. Schematic diagram of the insulin receptor signaling in L6 cells. Activation of the insulin receptor generates the IRS-1/2→PI3-K→Akt signaling 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; PI3-K, phosphatydidlinositol 3-kinase; Akt, protein kinase B; CaMKII, calcium-calmodulin dependent kinase II.

FIGURES

FIGURE 1

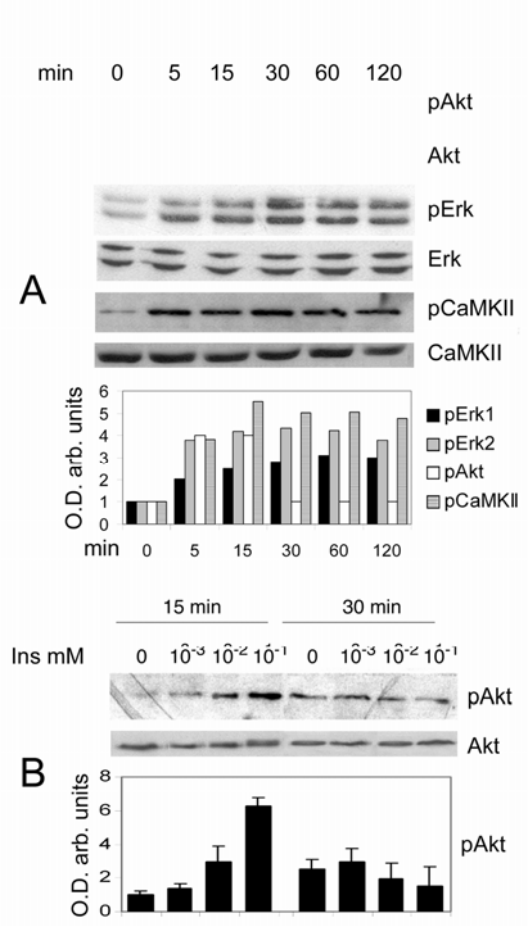
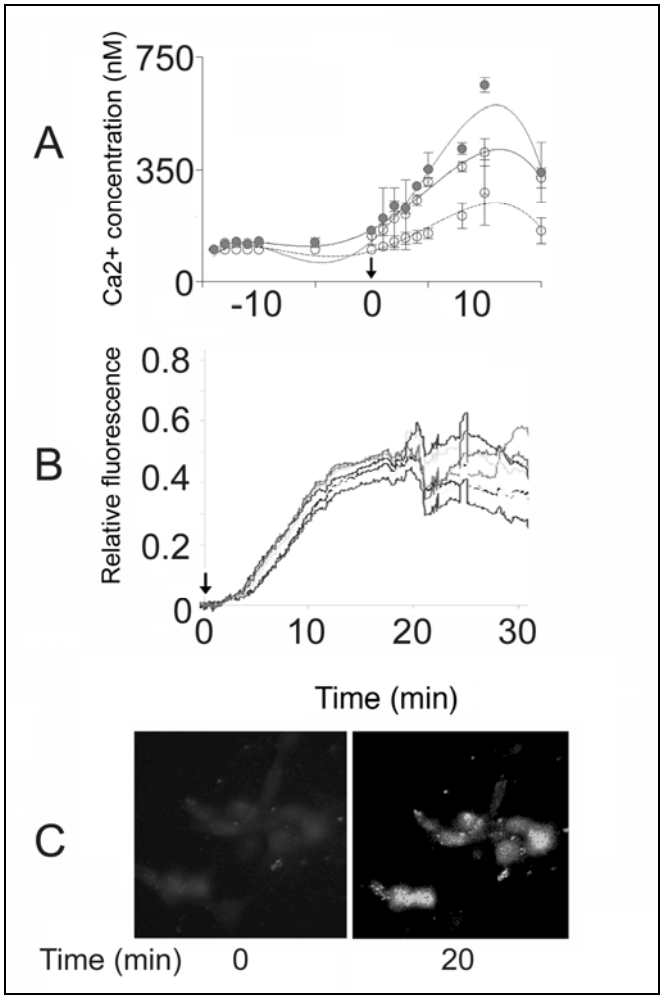


FIGURE 2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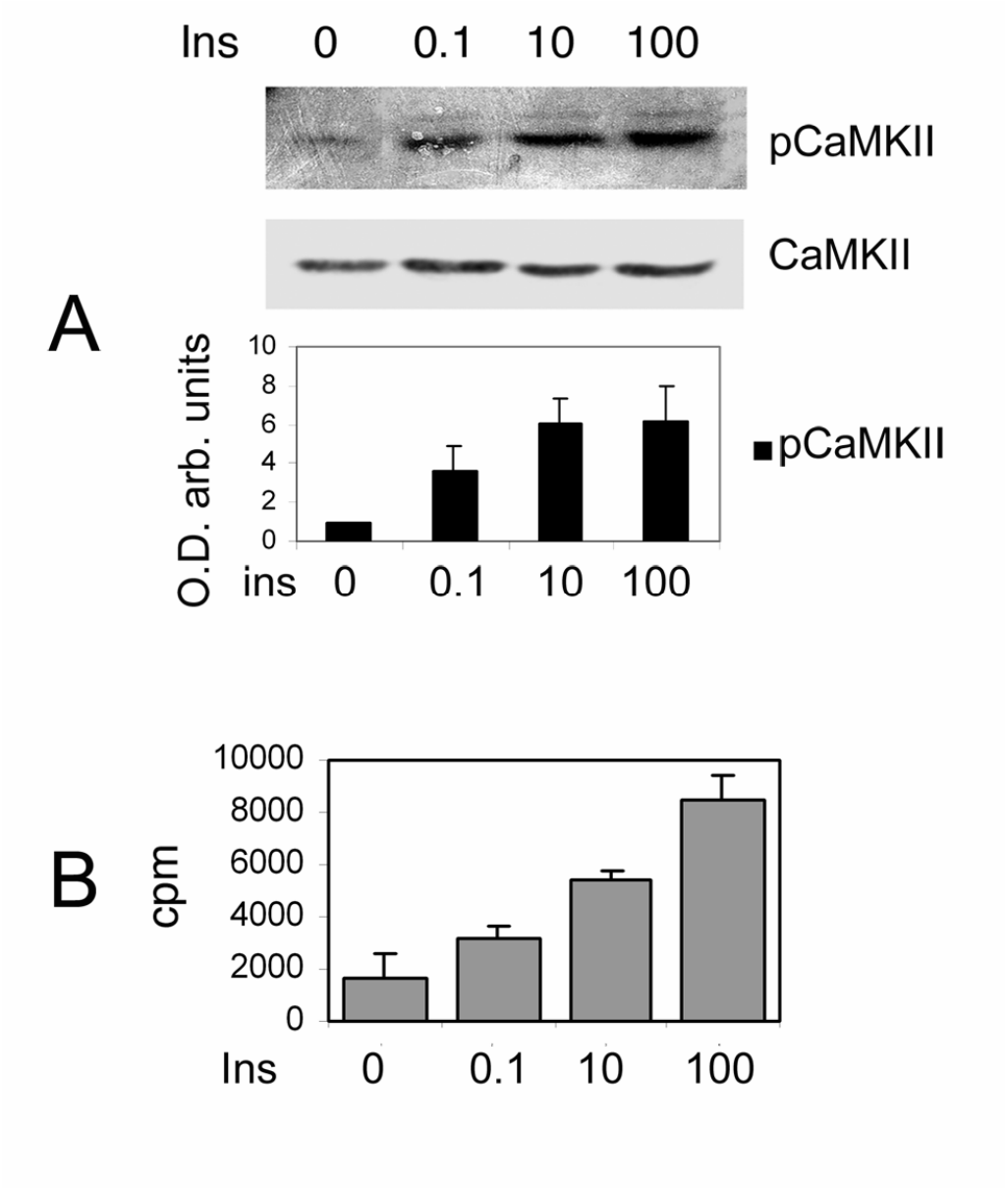
Ca2+ concentration (nM)
750
350
0
-10
0
10
B
Relative fluorescence
0.8
0.6
0.4
0.2
0
0
10
20
30
Time (min)
C
Time (min)
0
20

FIGURE 3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pCaMKII
CaMKII
A
O.D. arb. units
10
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2
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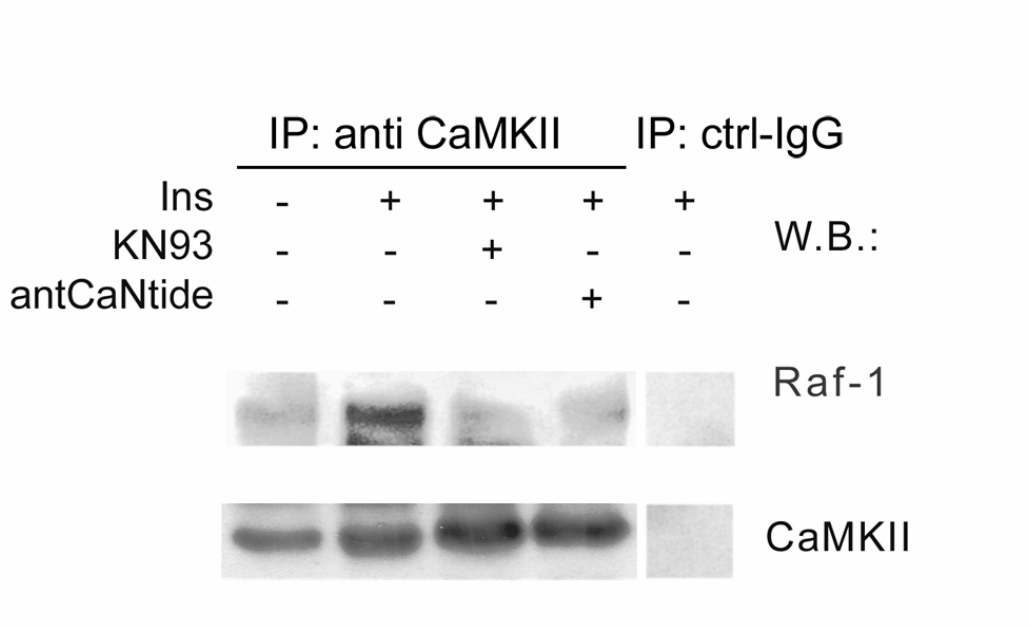
10000
8000
6000
4000
2000
0
Ins 0 0.1 10 100

FIGURE 4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IP: ctrl-IgG
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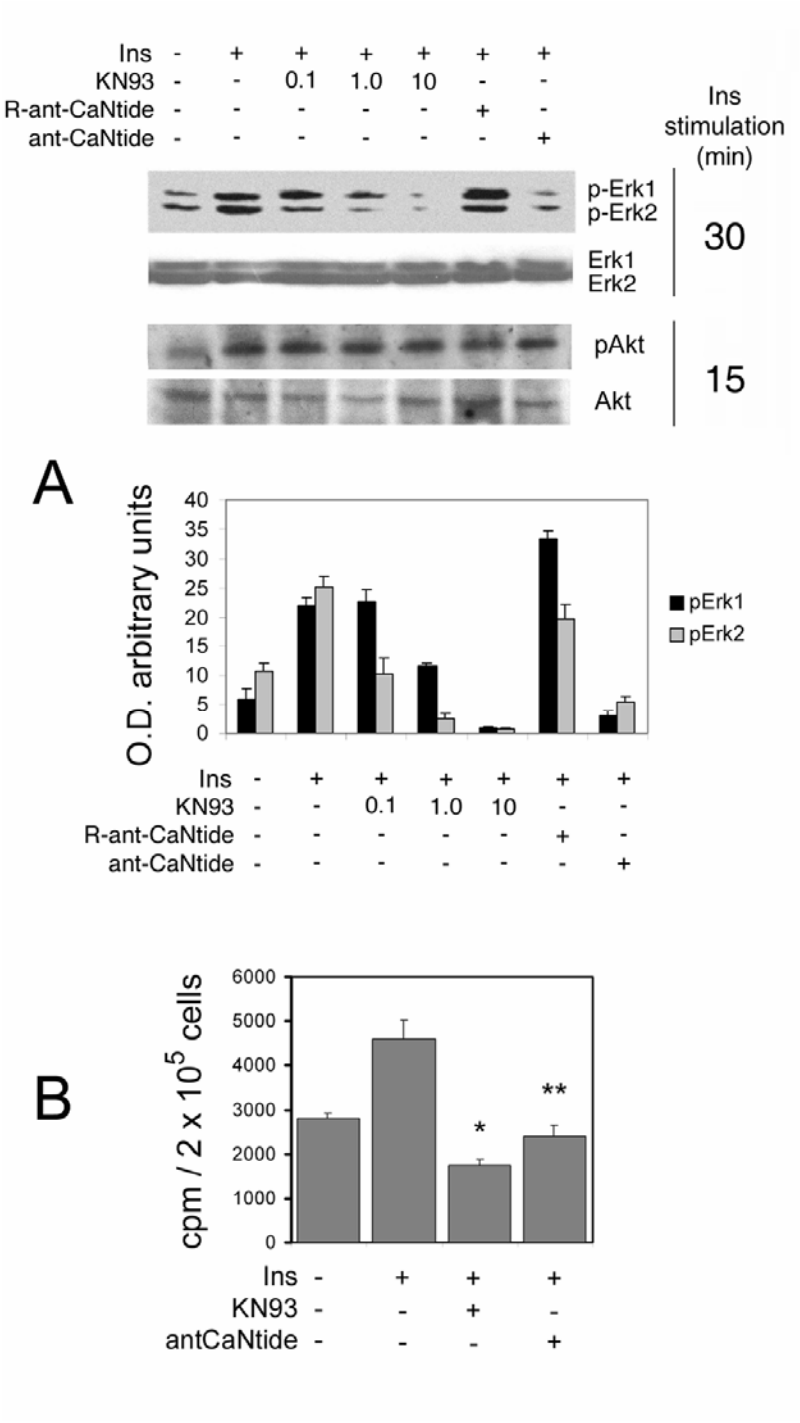
W.B.:
Raf-1
CaMKII

FIGURE 5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KN93 - - 0.1 1.0 10 - -
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ant-CaNtide - - - - - - +
Ins stimulation (min)
p-Erk1
p-Erk2
30
Erk1
Erk2
pAkt
15
Akt
A
O.D. arbitrary units
40
35
30
25
20
15
10
5
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pErk1
pErk2
Ins - + + + + + +
KN93 - - 0.1 1.0 10 - -
R-ant-CaNtide - - - - - + -
ant-CaNtide - - - - - - +
B
cpm / 2 x 10^5 cells
6000
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2000
1000
0
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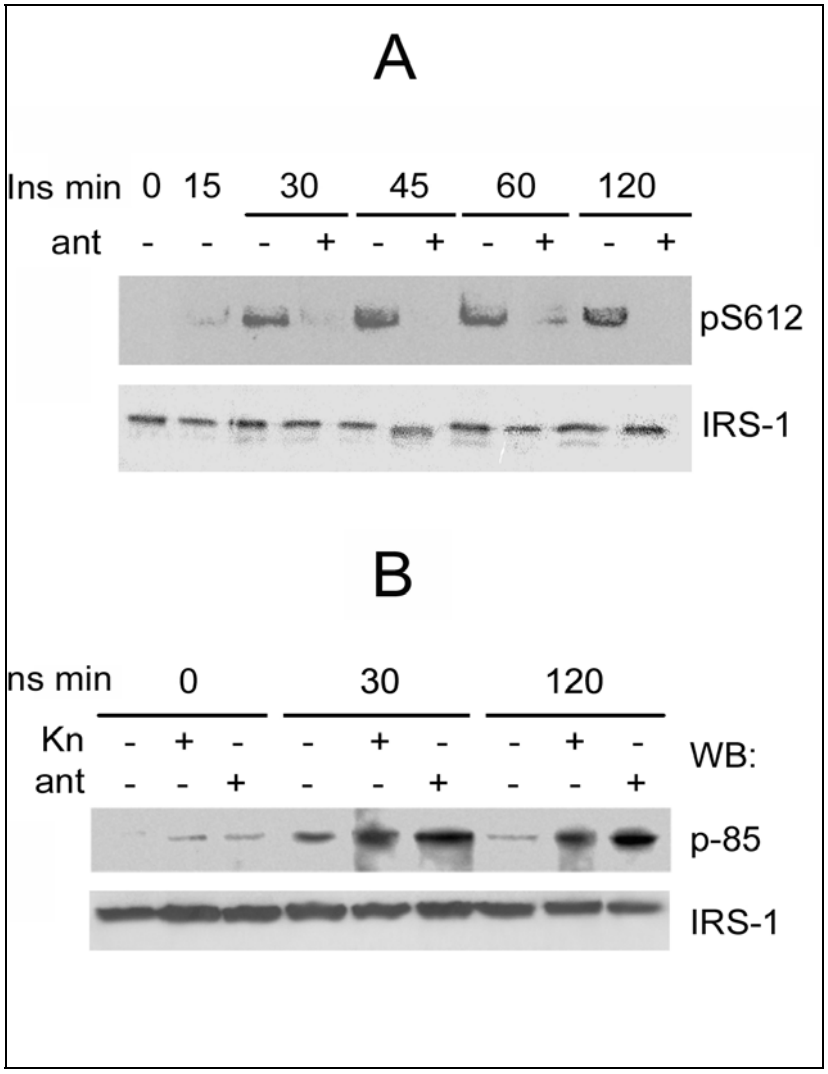
Ins - + + +
KN93 - - + -
antCaNtide - - - +

FIGURE 6


A
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ant - - - + - + - + - +
pS612
IRS-1
B
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Kn - + - - + - - + -
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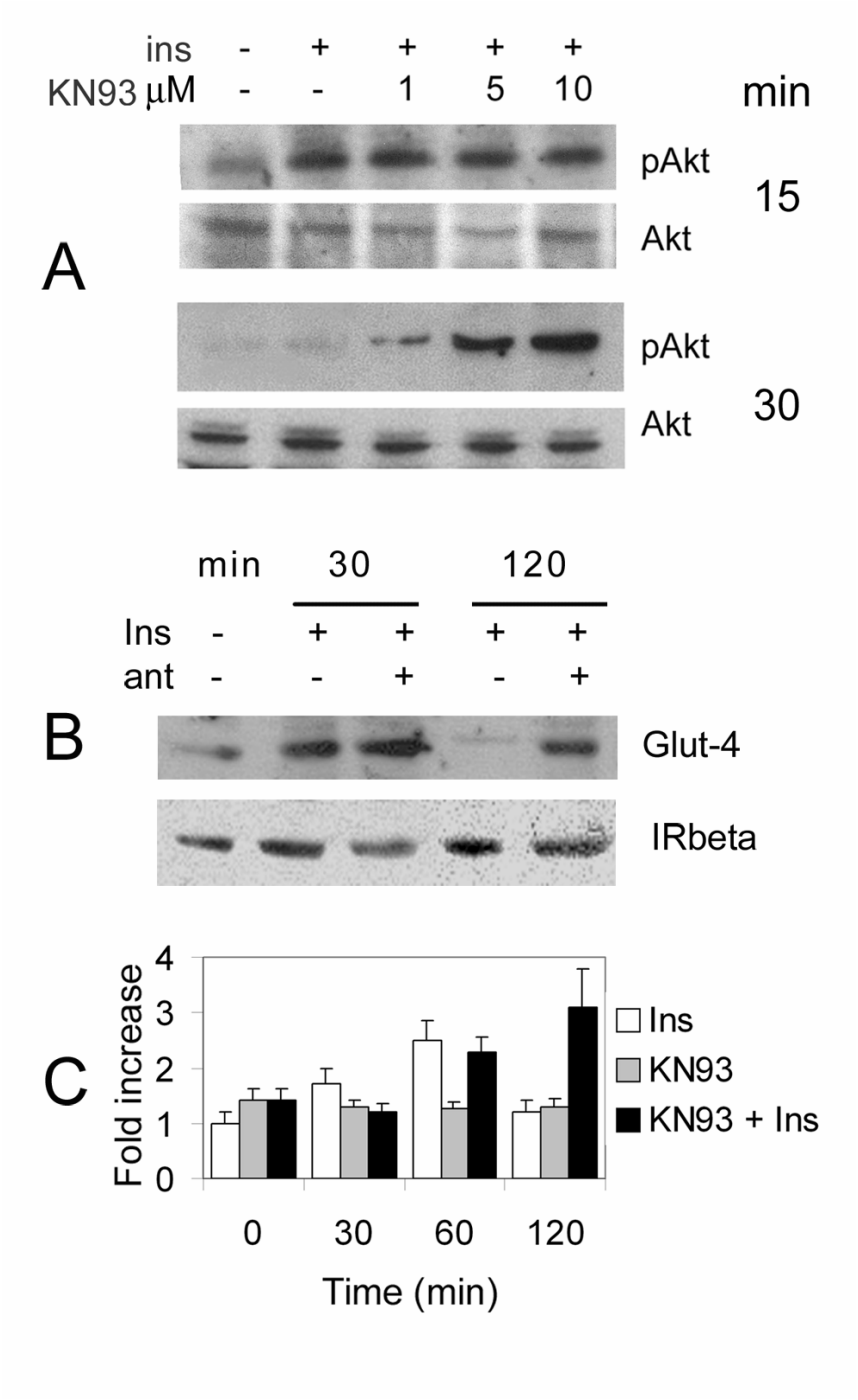
WB:
p-85
IRS-1

FIGURE 7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pAkt
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B
Glut-4
IRbeta
C
Fold increase
0
1
2
3
4
Ins
KN93
KN93 + Ins
0 30 60 120
Time (min)

FIGURE 8

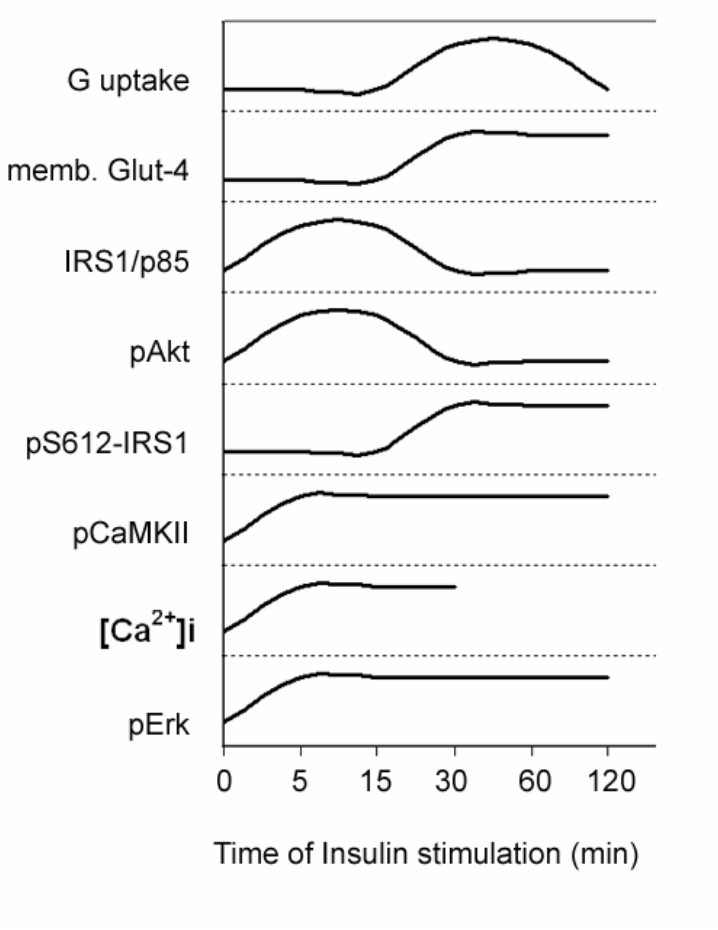
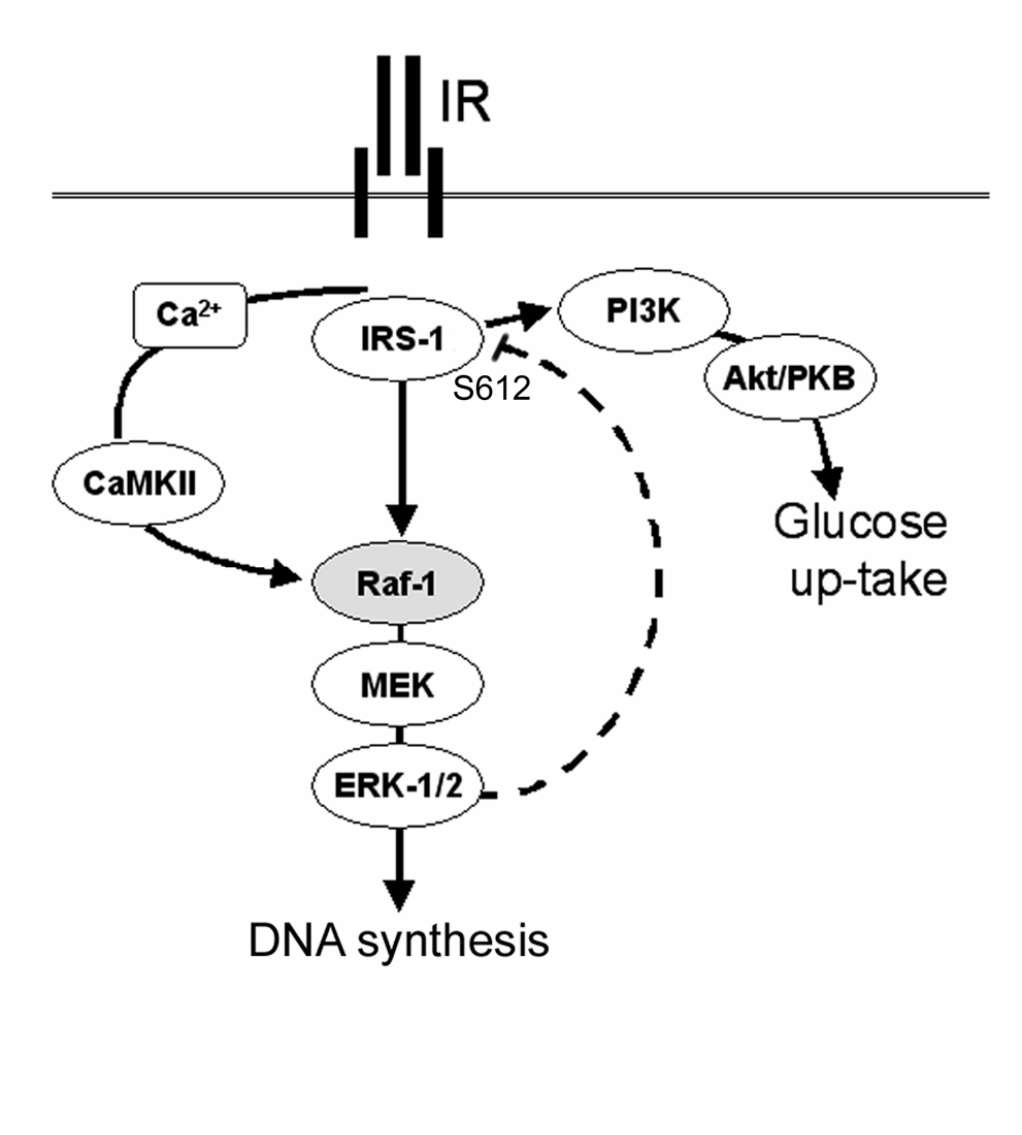

G uptake
memb. Glut-4
IRS1/p85
pAkt
pS612-IRS1
pCaMKII
[Ca2+]i
pErk
0
5
15
30
60
120
Time of Insulin stimulation (min)

FIGURE 9


IR
Ca2+
IRS-1
PI3K
S612
Akt/PKB
CaMKII
Glucose
up-take
Raf-1
MEK
ERK-1/2
DNA synthesis