DEPARTAMENT DE BIOQUÍMICA I FISIOLOGIA
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ACCIÓ DE LA INSULINA SOBRE EL SISTEMA A DE TRANSPORT D'AMINOÀCIDS EN EL MÚSCUL ESQUELÈTIC:

ESTUDI DE MECANISMES TRANSDUCTORS I REGULADORS

MEMORIA PER A OPTAR AL GRAU DE
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Insulin-stimulated α-(methyl)aminoisobutyric acid uptake in skeletal muscle

Evidence for a short-term activation of uptake independent of Na^+ electrochemical gradient and protein synthesis

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1. The present study was designed to explore the mechanisms by which insulin stimulates system A of amino acid transport in extensor digitorum longus (EDL) muscles, by using a system A analogue, α-(methyl)aminoisobutyric acid (MeAIB). 2. Insulin stimulation of MeAIB uptake was noted after only 30 min of incubation and was maximal at 60 min. Kinetics of the insulin effect on MeAIB uptake were characterized by an increased $V_{max.}$ without modification of K_m for MeAIB. 3. Incubation of EDL muscles with cycloheximide for 90 min did not modify MeAIB uptake in either the presence or the absence of insulin, indicating the independence of insulin action from protein synthesis *de novo*. Incubations for 180 min with cycloheximide caused a decrease in basal MeAIB uptake; however, the percentage stimulation of amino acid transport by insulin was unaltered. Basal MeAIB uptake was increased by incubation for 180 min, but under these conditions no change in the percentage effect of insulin was found. 4. Ouabain, gramicidin D, or both, markedly decreased basal MeAIB uptake by EDL muscle, but the percentage effect of insulin was unaltered. 5. We conclude that insulin action on amino acid transport through system A in muscle is rapid, is characterized by an increased $V_{max.}$, and is independent of protein synthesis *de novo* and the Na^+ electrochemical gradient. Our data are compatible with insulin acting directly on the system A transporter.

INTRODUCTION

Insulin, on interaction with its receptor, exerts a complex array of cellular actions that profoundly affect the physiology of the plasma membrane. Thus insulin activates enzymes such as $Na^+ + K^+$-dependent ATPase (Moore, 1973; Clausen & Kohn, 1977; Rosic *et al.*, 1985) or phosphodiesterase (Loten & Sneyd, 1970; Kono *et al.*, 1975) in several cell types, it modulates the number of cell-surface receptors for insulin-like growth factor-II or transferrin (Oka *et al.*, 1984; Wardzala *et al.*, 1984; Davis *et al.*, 1986), and it also enhances the transport of several important metabolites such as glucose or neutral amino acids that are taken up by system A (Kipnis & Noall, 1958; Kletzien *et al.*, 1976; Gliemann & Rees, 1983).

Much is known about the mechanisms by which insulin activates glucose transport in adipocytes or in muscle; a major mechanism by which insulin mediates this effect is to increase the number of glucose transporters residing in the plasma membrane of the cell (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Kono *et al.*, 1981; Karnieli *et al.*, 1981; James *et al.*, 1987), as a consequence of a translocation of glucose transporters from an intracellular location to the plasma membrane. However, our knowledge of the mechanism involved in insulin action to activate amino acid transport through system A is scarce, as is structural information on the system A transporter.

In liver, the stimulatory effect of insulin on amino acid transport is totally dependent on protein synthesis and microtubular function, and it is characterized by an increased $V_{max.}$ (Fehlmann *et al.*, 1979; Prentki *et al.*, 1981). In muscle, contradictory findings have been reported about the kinetics of insulin action on amino acid transport (Akedo & Christensen, 1962; Elsas *et al.*, 1968, 1975; Manchester *et al.*, 1971; Le Marchand-Brustel *et al.*, 1982). However, the stimulatory effect of insulin on α-aminoisobutyric acid (AIB) uptake in muscle is observed at an earlier time than in liver, and it is only partially prevented by cycloheximide (Elsas *et al.*, 1968; Le Marchand-Brustel *et al.*, 1982). In addition, insulin action on AIB uptake in perfused muscle seems to be independent of the Na^+–K^+-ATPase activity (Zorzano *et al.*, 1986*b*).

In the present study, we have attempted to delineate further insulin action on system A of transport in skeletal muscle by using the amino acid analogue α-(methyl)-aminoisobutyric acid (MeAIB). This analogue is taken up in skeletal muscle exclusively by system A, unlike AIB, which also enters the cell by the ASC system (Guidotti *et al.*, 1978; Maroni *et al.*, 1986). To that effect, the time course of insulin activation of MeAIB uptake, as well as its kinetics of stimulation by incubated rat skeletal muscle, were investigated. Special attention was paid to the dependence of insulin action on the basal transport activity, and whether insulin action on amino acid transport is mediated via changes in the Na^+ electrochemical gradient.

Abbreviations used: AIB, aminoisobutyric acid; MeAIB, α-(methyl)aminoisobutyric acid; EDL, extensor digitorum longus.

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MATERIALS AND METHODS

Animals and dissection procedures

Male Wistar rats (40–70 g) obtained from our own colony were used. The rats were fed on Purina Laboratory chow *ad libitum*. Animals were housed in animal quarters maintained at 22 °C with a 12 h-light/12 h-dark cycle. The dissection and isolation of the extensor digitorum longus (EDL) muscle were carried out under anaesthesia with pentobarbital (5–7 mg/100 g body wt., intraperitoneally) as described previously (Maizels *et al.*, 1977). The isolated EDL muscle was fixed to a stainless-steel clip in order to maintain the muscle under slight tension (approximating to resting length) during the incubation. Such muscles are able to maintain normal ATP and phosphocreatine concentrations during a 3 h incubation. EDL muscles from several animals were randomly assigned to different experimental groups.

Incubations

EDL muscles were incubated in a shaking incubator at 37 °C for 1.5–3 h in 2 ml of Krebs–Henseleit buffer (as in Zorzano *et al.*, 1985), pH 7.4, containing 5 mM-glucose, 0.1 % bovine serum albumin and 20 mM-Hepes. After addition of the muscles to the vials, they were stoppered and placed in a Dubnoff metabolic shaker set at 37 °C and a shaking rate of 70 cycles/min. Vials were gassed with O_2/CO_2 (19:1) during the whole incubation period. The incubation medium was kept for no longer than 90 min, and during prolonged incubations it was renewed thereafter. At different times, insulin (200 nM) was added to the incubation medium, as well as several inhibitors such as cycloheximide (0.1 mM), ouabain (1 mM) or gramicidin D (25 μg/ml) (see details in Table legends).

Measurement of amino acid uptake into muscle

Amino acid uptake by system A was measured in EDL muscles by using the non-metabolizable amino acid analogue MeAIB. After the incubations with insulin and the above-mentioned inhibitors, muscles were blotted and transferred to a vial with 1.5 ml of Krebs–Henseleit buffer, pH 7.4, containing 5 mM-glucose, 0.1 % bovine serum albumin, 20 mM-Hepes and 0.1 mM-α-[1-^{14}C]-MeAIB (800 μCi/mmol), 10 mM-[^{3}H]mannitol (33 μCi/mmol) and insulin and modulators at the same concentrations as during the preceding incubation period. The vials were stoppered and incubated at 37 °C in a shaking incubator for 30 min. The gas phase in the vials was O_2/CO_2 (19:1). As these studies were conducted over a period of 1 year, and since seasonal variation in amino acid uptake by skeletal muscle has been previously reported (Arvill & Ahrén, 1967; Zorzano *et al.*, 1985, 1986*a*), control and experimental groups were always performed during the same experimental day.

After incubation, muscles were quickly rinsed in cold saline (0.9 % NaCl), blotted briefly on filter paper and frozen in liquid N_2. Samples were weighed and digested in 0.25 ml of NCS tissue solubilizer (The Radiochemical Centre, Amersham, Bucks., U.K.) at 50 °C in Teflon-sealed vials for 2 h. Muscle digests and samples of the incubation media were placed in scintillation vials containing 10 ml of scintillation cocktail and counted for radioactivity in a Packard scintillation counter with channels preset for simultaneous ^{3}H and ^{14}C counting. The amount of each radioisotope present in the samples was determined, and this information was used to calculate the extracellular space. The extracellular space of EDL muscles, estimated by using [^{3}H]mannitol, increased progressively with time, being 0.17 ± 0.01, 0.19 ± 0.01 and 0.25 ± 0.01 ml/g after 10, 20 and 30 min respectively, and it was not modified by the presence of insulin. The intracellular concentration of ^{14}C-labelled amino acid analogue was calculated by subtracting its amount in the extracellular space from the total label found in tissue, as previously reported (Zorzano *et al.*, 1985). Student's *t* test was used for statistical analysis of the data.

RESULTS AND DISCUSSION

Characterization of insulin effect on MeAIB uptake by EDL muscle

Our initial objective was to delineate the stimulatory effect of insulin on MeAIB uptake by EDL muscles. MeAIB is a specific probe for system A of neutral amino acid transport by skeletal muscle (Maroni *et al.*, 1986), unlike AIB, which enters the cell through the A and ASC systems. Preliminary studies showed that insulin-stimulated MeAIB uptake was linear during 30 min of incubation (results not shown). From this, uptake was determined after 30 min of incubation with the radioactive analogue in all subsequent experiments. To investigate the time course of insulin action, muscles were incubated for a total of 3 h, either in the absence of insulin (basal group) or with insulin present during the last 30, 60 or 120 min of incubation. At 30 min after insulin addition, MeAIB uptake was increased by 74 % as compared with the basal group (23.2 ± 5.5 versus 13.3 ± 1.1 nmol/30 min per g respectively). MeAIB uptake after 60 min of insulin addition was already maximal and indistinguishable from that at 120 min (44.4 ± 4.1 and 37.9 ± 1.8 nmol/30 min per g respectively). Kinetic analysis of the stimulatory effect of

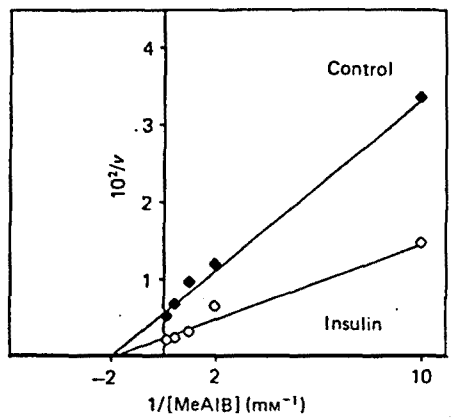

Fig. 1. Effect of insulin on the kinetic analysis of MeAIB uptake by EDL muscle

Muscles were incubated as described in the Materials and methods section for 180 min. Insulin when present was added during the last 60 min of the experiment. Uptake (v, nmol/h per g) was measured at different concentrations of MeAIB (mM) for 30 min. Each point represents the average of four to six muscles. Statistical analysis demonstrated that regression curves were significantly different in control ($r = 0.997$; $y = 0.55 + 0.28\,x$) as compared with insulin-treated group ($r = 0.985$; $y = 0.21 + 0.13\,x$) at $P < 0.05$.

insulin on MeAIB uptake (Fig. 1) indicated that it was characterized by an increased $V_{max.}$ (235 and 486 nmol/h per g in the absence and the presence of insulin respectively), without modifications of K_m for MeAIB (0.70 mM and 0.62 mM in the absence and the presence of insulin respectively).

Thus we have substantiated a short-term effect of insulin stimulating MeAIB uptake by incubated muscle, which is already detected at 30 min after hormone addition, and attains a maximal effect by 1 h of incubation in the presence of hormone. That is in keeping with previous results, which showed acute modulation of AIB transport by insulin, exercise or electrical stimulation in the perfused or incubated muscle (Goldberg *et al.*, 1974; Zorzano *et al.*, 1985, 1986*a*,*b*).

Kinetic analysis of the insulin effect on MeAIB uptake by incubated muscle demonstrated an increased $V_{max.}$, with no modification of K_m. This coincides with other reports of insulin augmenting the $V_{max.}$ of AIB (Manchester *et al.*, 1971; Elsas *et al.*, 1975; Le Marchand-Brustel *et al.*, 1982); however, an effect of insulin on AIB uptake characterized by a decrease in the K_m for AIB has been described in diaphragm (Akedo & Christensen, 1962; Elsas *et al.*, 1968, 1971). The reason for this discrepancy remains to be explained, and heterogeneity between diaphragm and skeletal muscle might be invoked. In any event, our results allow us to discard an effect of insulin increasing the affinity of MeAIB to the system-A transporter in skeletal muscle. Whether insulin modifies K_m for Na^+ in the incubated muscle was not determined in this study; however, it does not invalidate our prior conclusion.

Effect of protein synthesis and adaptive regulation on insulin-stimulated MeAIB uptake by EDL muscle

To assess whether insulin activates amino acid transport system A by a mechanism that involves protein synthesis *de novo*, EDL muscles were incubated in the absence or the presence of 0.1 mM-cycloheximide, a concentration that completely inhibits protein synthesis (Forsayeth & Gould, 1983). When cycloheximide was present during the last 90 min of the experiment (added 30 min before insulin), basal as well as insulin-stimulated MeAIB uptake were unaltered (Table 1). Thus the maximal effect of insulin was not perturbed by cycloheximide added 30 min before the hormone. The data demonstrate that insulin stimulates MeAIB uptake by muscle independently of protein synthesis *de novo*. Thus insulin does not activate amino acid transport by increasing the transcription and/or translation of certain genes which could code for the amino acid transporter or another unknown modulator.

Incubation of EDL muscles with cycloheximide for 180 min caused a decrease in basal MeAIB uptake. Only under these conditions did the presence of cycloheximide result in a decrease in the absolute effect of insulin on MeAIB uptake (Table 1). However, under these circumstances the insulin effect was not modified when expressed as a percentage, in keeping with previous observations (Elsas *et al.*, 1968; Le Marchand-Brustel *et al.*, 1982). These data suggested that cycloheximide acted not by altering the mechanism of insulin action, but through a modification of basal transport activity.

The next series of experiments was designed to test that hypothesis. To that effect, basal MeAIB uptake was increased by prolonged incubation of EDL muscles. It is well known that adaptive regulation is active in skeletal muscle (Guidotti *et al.*, 1975; Le Marchand-Brustel *et al.*, 1982; Logan *et al.*, 1982). A 70% increase in basal MeAIB uptake by EDL muscle was observed by increasing the total incubation time from 90 to 180 min (Table 2). Under those conditions, MeAIB uptake in the presence of insulin was also increased in the 180 min group as compared with the 90 min. That is, the absolute effect of insulin was increased; nevertheless, the percentage effect of insulin was similar in all groups (Table 2). Thus, again the insulin effect was dependent on basal transport activity.

In all, our interpretation of these results is that cycloheximide does not block insulin action on amino acid transport. In addition, when basal MeAIB uptake is either increased or lowered, the effect of insulin persists

Table 1. Effect of cycloheximide on basal and insulin-stimulated MeAIB uptake by EDL muscle

Results are means±S.E.M. for 14–25 observations per group. EDL muscles were incubated for 180 min, with or without 200 nM-insulin (during the last 60 min of incubation). Cycloheximide (0.1 mM) was added either at the beginning of the experiment (180 min group) or during the last 90 min of the experiment (90 min group). MeAIB uptake was determined during the last 30 min. * Value significantly different from that of the basal group ($P < 0.05$), † value significantly different from that of the no-cycloheximide group ($P < 0.05$).

Cycloheximide	MeAIB uptake (nmol/30 min per g)		Increase by insulin (%)
	Basal	Insulin	
None	17.9±0.7	29.9±1.8*	67
90 min	17.1±1.9	27.2±2.5*	59
180 min	14.6±1.4†	20.4±1.6*†	40

Table 2. Effect of adaptive regulation on basal and insulin-stimulated MeAIB uptake by EDL muscle.

Results are means±S.E.M. for 6–17 observations, except for the 150 min group, which represents the mean of two observations. Individual data of MeAIB uptake in the 150 min groups were 11.8 and 15.3 nmol/30 min per g in basal state and 22.4 and 22.8 nmol/30 min per g after insulin addition respectively. EDL muscles were incubated for 90, 150 or 180 min in the absence or in the presence of insulin (200 nM). When indicated insulin was present during the last 60 min of incubation. * Value significantly different from that of the basal group ($P < 0.05$), † value significantly different from that of the 90 min group ($P < 0.05$).

Duration of experiment (min)	MeAIB uptake (nmol/30 min per g)		Increase by insulin (%)
	Basal	Insulin	
90	11.4±1.2	19.0±0.9*	67
150	13.5	22.6	66
180	19.1±0.9†	29.9±1.9*†	59

unaltered. It may be proposed that cycloheximide at long time periods or adaptive regulation alters basal transport activity or another related factor, such as the intracellular pool of transporters, which would be directly modulated by insulin.

Effect of Na^+-electrochemical-gradient disruptors on insulin-stimulated MeAIB uptake by EDL muscle

Insulin induces hyperpolarization (Zierler, 1959) and stimulates the Na^+–K^+ pump (Moore, 1973; Clausen & Kohn, 1977; Flatman & Clausen, 1979; Rosic *et al.*, 1985) in skeletal muscle, and it is still a matter of controversy whether hyperpolarization is related or not to previous activation of Na^+–K^+-ATPase (Zierler & Rogus, 1981). Thus, in a further set of experiments we examined the effect of disruptors of the Na^+ electrochemical gradient, such as ouabain (1 mM), an inhibitor of the Na^+–K^+-ATPase, or gramicidin D (25 μg/ml), an ionophore known to abolish membrane potential (Kristensen & Folke, 1986). It has been previously reported that 1 mM-ouabain is sufficient to occupy the total number of membrane Na^+–K^+ pumps (Clausen & Flatman, 1987), and that 25 μg of gramicidin D/ml abolished membrane potential in liver 5 min after its addition (Kristensen & Folke, 1986). These experiments were carried out at a different time of the year, and, in keeping with previous observations (Arvill & Ahrén, 1967), marked differences were detected in basal MeAIB uptake as compared with the results described above.

Initially we investigated the time course of the ouabain effect on basal MeAIB uptake by incubated EDL muscle. Ouabain rapidly caused a marked decrease in basal MeAIB uptake. After only 30 min of exposure to 1 mM-ouabain, MeAIB uptake decreased by 40% (from 11.1 ± 1.5 to 6.2 ± 1.4 nmol/30 min per g), and the maximal inhibitory effect of ouabain was attained 1 h after its addition (5.0 ± 0.5 nmol/30 min per g). Ouabain action on MeAIB uptake was reversible, and 30 min of ouabain exposure followed by 30 min with no inhibitor caused a partial recovery of transport activity (7.5 ± 1.7 nmol/30 min per g). On the basis of these findings, and in order to investigate whether insulin action on MeAIB uptake required an unaltered Na^+–K^+ pump activity, EDL muscles were incubated in the absence or the presence of insulin and ouabain. Results are presented in Table 3. Ouabain caused a 40% decrease in basal MeAIB uptake. In the presence of insulin, MeAIB uptake was also decreased in the ouabain-treated group compared with the control group; however, the percentage stimulation of MeAIB uptake induced by insulin was similar in ouabain and control groups (Table 3). Again, under those conditions, cycloheximide did not affect insulin-stimulated MeAIB uptake (results not shown).

Next, insulin action in the presence of gramicidin D, or of ouabain plus gramicidin D, in the incubation medium was investigated (Table 3). Incubation with gramicidin D during 30 min caused a 50% decrease in basal MeAIB uptake, whereas ouabain plus gramicidin D caused a 70% decrease in MeAIB uptake by EDL muscle. MeAIB uptake after insulin addition was also decreased in the presence of inhibitors compared with the control group; nevertheless, the percentage stimulation caused by insulin was similar under all these conditions (Table 3). These data demonstrate that insulin action on amino acid transport is not mediated by modification of the Na^+ electrochemical gradient, and is independent of the Na^+–K^+-ATPase and of membrane potential. However, as we discussed above regarding protein synthesis, for insulin action to be maximal a preserved Na^+ electrochemical gradient is required. These data agree with previous work performed in the perfused rat hindquarter, which showed that insulin and exercise stimulate AIB uptake in a fashion independent of Na^+–K^+-ATPase activity (Zorzano *et al.*, 1986*b*).

Finally, a significant correlation was found ($r = 0.909$, $P < 0.001$) when means of basal MeAIB uptake were plotted against insulin-stimulated MeAIB uptake for all

Table 3. Effect of ouabain and gramicidin D on basal and insulin stimulated MeAIB uptake by EDL muscle

Results are means ± S.E.M. for 4 to 12 observations per group. EDL muscles were incubated for 180 min. When indicated, ouabain (1 mM) and insulin (200 mM) were present during the last 60 min of incubation (*a*,*b*). In some studies (*b*), gramicidin D (25 μg/ml) was added during the last 30 min of the experiment. Gramicidin D was dissolved in 60% ethanol, so the final ethanol concentration in the incubation medium was 1%; in those experiments, the control group also contained 1% ethanol in the medium. * Value significantly different from that of the basal (no insulin) group ($P < 0.05$); † value significantly different from that of the control (no ouabain, no gramicidin D additions) group ($P < 0.05$).

	Ouabain	Gramicidin D	MeAIB uptake (nmol/30 min per g) Basal	MeAIB uptake (nmol/30 min per g) Insulin	Increase by insulin (%)
(*a*)	–	–	12.2±1.3	23.6±0.7*	93
	+	–	7.7±0.4†	13.0±0.4*†	69
(*b*)	–	–	15.2±1.2	30.7±1.7*	102
	–	+	7.7±0.8†	18.3±2.1*†	138
	+	+	4.6±0.6†	11.0±1.8*†	139

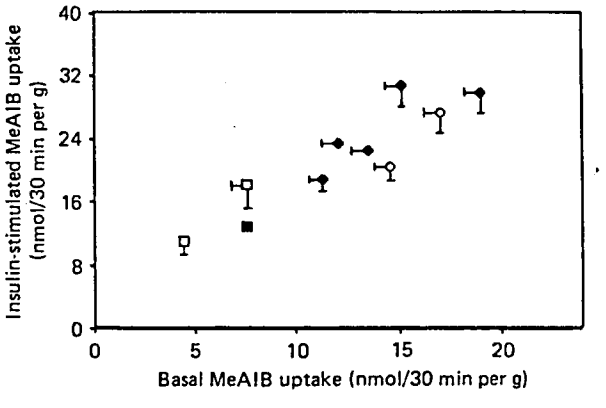

Fig. 2. Relationship between basal and insulin-stimulated MeAIB uptake by EDL muscle

Points are means ± S.E.M. for 2–25 observations (see legends to Tables 1, 2 and 3). ●, Control groups with different durations of incubations with no amino acids in the medium; ○, cycloheximide groups; ■, ouabain group; □, gramicidin D and gramicidin D plus ouabain groups. A significant linear regression was detected, with $r = 0.909$ and $y = 5.32 + 1.32\,x$.

experimental groups (Fig. 2). That is, under conditions characterized by either increasing incubation time or the presence of cycloheximide, ouabain or gramicidin D, stimulation of MeAIB uptake induced by insulin was dependent on basal transport activity. That provides support to the contention that, whatever the mechanisms by which insulin stimulates amino acid transport in skeletal muscle, they are not mediated by protein synthesis *de novo*, adaptive regulation or modification of the Na^+ electrochemical gradient. In addition, the correlation between basal and insulin-stimulated MeAIB uptake by muscle implies that insulin action is somehow dependent on basal transport activity. These findings differ from what occurs in hepatocytes, where it has been described that insulin stimulates AIB uptake by a slower mechanism that involves protein synthesis (Fehlmann *et al.*, 1979, 1981). In fact, it has been proposed that insulin probably stimulates transcription of a gene coding for the A transporter in liver. Therefore it can be concluded that the mechanisms by which insulin stimulates amino acid transport in muscle and in liver might be different.

In conclusion, the present study provides evidence that insulin stimulates system A of amino acid transport in skeletal muscle, by increasing the $V_{max.}$ of transport. The mechanism that mediates this action is independent of protein synthesis and Na^+ electrochemical gradient. However, insulin-stimulated MeAIB uptake depends on basal transport activity in a variety of situations (cycloheximide, adaptive regulation, gramicidin D, ouabain). That fact allows us to postulate a possible direct effect of insulin at the level of the transporters, either by increasing their intrinsic activity (independent of the Na^+ electrochemical gradient) or in consequence of a translocation of them from a hypothetical intracellular pool to the plasma membrane, as described for glucose transporters.

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Protein kinase C activators selectively inhibit insulin-stimulated system A transport activity in skeletal muscle at a post-receptor level

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We have investigated the role of phorbol esters on different biological effects induced by insulin in muscle, such as activation of system A transport activity, glucose utilization and insulin receptor function. System A transport activity was measured by monitoring the uptake of the system A-specific analogue α-(methyl)aminoisobutyric acid (MeAIB), by intact rat extensor digitorum longus muscle. The addition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 0.5 μM) for 60 or 180 min did not modify basal MeAIB uptake by muscle, suggesting that insulin signalling required to stimulate MeAIB transport does not involve protein kinase C activation. However, TPA added 30 min before insulin (100 nM) markedly inhibited insulin-stimulated MeAIB uptake. The addition of polymyxin B (0.1 mM) or H-7 (1 mM), protein kinase C inhibitors, alone or in combination with TPA leads to impairment of insulin-stimulated MeAIB uptake. This paradoxical pattern is incompatible with a unique action of Polymyxin B or H-7 on protein kinase C activity. Therefore these agents are not suitable tools with which to investigate whether a certain insulin effect is mediated by protein kinase C. TPA did not cause a generalized inhibition of insulin action. Thus both TPA and insulin increased 3-*O*-methylglucose uptake by muscle, and their effects were not additive. Furthermore, TPA did not modify insulin-stimulated lactate production by muscle. In keeping with this selective modification of insulin action, treatment of muscles with TPA did not modify insulin receptor binding or kinase activities. In conclusion, phorbol esters do not mimic insulin action on system A transport activity; however, they markedly inhibit insulin-stimulated amino acid transport, with no modification of insulin receptor function in rat skeletal muscle. It is suggested that protein kinase C activation causes a selective post-receptor modification on the biochemical pathway by which insulin activates system A amino acid transport in muscle.

INTRODUCTION

The phospholipid, Ca^{2+}- and diacylglycerol-dependent protein kinase (protein kinase C) plays a major role in controlling cell function, through phosphorylation of serine and threonine residues in cellular proteins [1,2]. Extracellular signals, which include neurotransmitters, hormones and growth factors [1], bind to specific cell-surface receptors and generate second messengers that activate protein kinase C. Protein kinase C is also the intracellular receptor of phorbol esters [3], which are potential tumour promoters. Phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), bind to and activate the protein kinase C by interaction at the *sn*-1,2-diacylglycerol site [3,4]. In addition to tumour-promoting effects, phorbol diesters possess insulin-like activity in a variety of tissues. Thus, phorbol esters stimulate glucose transport and oxidation [5–7], lipogenesis [8] and pyruvate dehydrogenase activity [5].

Insulin is the major anabolic and anti-catabolic hormone acting in mammals. The actions of insulin at the cellular level are initiated by insulin binding to its plasma-membrane receptor [9,10]. The insulin receptor is a tyrosine-specific ligand-st.mulated protein kinase [11,12], and there is much evidence indicating that the tyrosine kinase activity of the insulin receptor is essential for insulin action [13,14].

Several types of interaction connecting insulin action and protein kinase C have been postulated. On the one hand, it has been reported that phorbol esters inhibit insulin action and insulin-receptor tyrosine kinase in Fao cells [2,15] and in isolated rat adipocytes [6,16]. This is consistent with the fact that protein kinase C directly phosphorylates the insulin receptor *in vitro* and decreases its tyrosine kinase activity [17]. On the other hand, some investigators have described an insulin-induced activation of protein kinase C activity in several cell types. Insulin activates membrane protein kinase C activity in rat diaphragm [18] and membrane and cytosolic protein kinase C in BC3H-1 myocytes [19]. In adipocytes, insulin causes an increase in [^{3}H]phorbol 12,13-dibutyrate binding to cytosol and a decrease in that to plasma-membrane fractions [20], and stimulates cytosolic protein kinase C activity [21]. Nevertheless, other studies have failed to detect an insulin-induced activation of protein kinase C in adipocytes, L6 or BC3H-1 muscle cell lines [22–24].

In the present work, we have investigated the effects of TPA on basal and insulin-stimulated system A amino acid transport activity, glucose transport and lactate production by intact rat skeletal muscle, as well as the effects of TPA on insulin-stimulated receptor tyrosine kinase activity after lectin-chromatography purification. TPA treatment of incubated muscle caused a blockade on the stimulatory effect of insulin on α-(methyl)-aminoisobutyric acid (MeAIB) uptake. This alteration of insulin action was not detected when 3-*O*-methylglucose uptake or lactate production was assessed. When insulin receptors were purified from TPA-treated muscles, the insulin-stimulated tyrosine kinase activity of the receptors was found to be unaltered. These results suggest that in skeletal muscle protein kinase C does not mediate insulin action on system A transport activity; however, previous activation of protein kinase C modulates insulin action in a negative fashion by a mechanism that lies at a post-receptor level.

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; MA, 4β-phorbol 13α-monoacetate; EDL, extensor digitorum longus; MeAIB, α-(methyl)aminoisobutyric acid; H-7, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine; WGA, wheat-germ agglutinin.

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EXPERIMENTAL

Materials

Pig monocomponent insulin was a gift from Mr. T. L. Jeatran, Eli Lilly and Co. [^{125}I-Tyr^{A14}]Monoiodoinsulin was obtained from New England Nuclear and [^{125}I-Tyr^{B26}]monoiodoinsulin was obtained from Amersham Corp. [γ-^{32}P]ATP was prepared from [^{32}P]P_i (New England Nuclear) by using a Gamma-prep kit from Promega Biotech. All electrophoresis reagents were obtained from Bio-Rad; wheat-germ agglutinin (WGA) bound to agarose was obtained from Vector, and disuccinimidyl suberate was from Pierce. BSA (fraction V, fatty-acid-free), TPA, 4β-phorbol 13α-monoacetate (MA), polymyxin B, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) and most commonly used chemicals were from Sigma.

Animals and dissection procedures

Male Wistar rats (50–60 g) obtained from our own colony were used. The rats were fed on Purina Laboratory chow *ad libitum*. Animals were housed in animal quarters maintained at 22 °C with a 12 h-light/12 h-dark cycle. The dissection and isolation of the extensor digitorum longus (EDL) muscle were carried out under anaesthesia with pentobarbital (5–7 mg/100 g body wt., intraperitoneally) as described previously [25]. The isolated EDL muscle was fixed to a stainless-steel clip in order to maintain the muscle under slight tension (approximating to resting length) during the incubation. Such muscles (20–30 mg wet wt.) are able to maintain normal ATP and phosphocreatine concentrations during a 3 h incubation.

Incubations

EDL muscles were incubated in a shaking incubator at 37 °C for 3 h in 3 ml of Krebs–Henseleit buffer, pH 7.4, containing 5 mM-glucose, 0.2 % BSA and 20 mM-Hepes. After addition of the muscles to the vials, they were stoppered and placed in a Dubnoff metabolic shaker set at 37 °C and a shaking rate of 60 cycles/min. Vials were gassed with O_2/CO_2 (19:1) during the whole incubation period. The incubation medium was kept for no longer than 90 min, and during prolonged incubations it was renewed thereafter. At different times, insulin (100 nM) was added to the incubation medium as well as several drugs such as TPA (0.5 μM), MA (0.5 μM), polymyxin B (0.1 mM) or H-7 (1 mM) (see details in Figure and Table legends). Experimental series were performed by comparing biological activity of one muscle with the contralateral one from the same rat (paired muscles).

Measurements of amino acid and glucose uptake and lactate production by muscle

Amino acid uptake by system A was measured in EDL muscles by using the non-metabolizable amino acid analogue MeAIB. After the incubations with insulin and the above-mentioned agents, muscles were transferred to vials with 1.5 ml of Krebs–Henseleit buffer, pH 7.4, containing 5 mM-glucose, 0.2 % BSA, 20 mM-Hepes and 0.1 mM-[1-^{14}C]MeAIB (800 μCi/mmol), 1 mM-[^{3}H]mannitol (330 μCi/mmol) and insulin and modulators at the same concentrations as for the preceding incubation period. The vials were stoppered and incubated at 37 °C in a shaking incubator for 30 min. The gas phase in the vials was O_2/CO_2 (19:1). In experiments designed to measure 3-*O*-methylglucose uptake, muscles were incubated in Krebs–Henseleit buffer containing 2 mM-pyruvate instead of glucose, and during the last 30 min of incubation the medium contained 0.1 mM-[^{14}C]3-*O*-methylglucose (800 μCi/mmol) and 1 mM-[^{3}H]mannitol (330 μCi/mmol). After incubation, muscles were placed in 0.25 ml of NCS tissue solubilizer (Amersham International), and radioactivity of muscle digests and samples of the incubation media was measured. The amount of each radioisotope present in the samples was determined, and this information was used to calculate the extracellular space. That of EDL muscles, estimated after 30 min of [^{3}H]mannitol addition, was 0.222 ± 0.003 ml/mg and 0.219 ± 0.006 ml/mg in the absence and presence of insulin respectively. The intracellular concentration of [^{14}C]MeAIB or [^{14}C]3-*O*-methylglucose was calculated as previously reported [26,27]. Lactate release to the incubation media was measured during the last 30 min of incubation as in [28]. Student's *t* test for paired data was used for statistical analysis.

Measurement of insulin binding by the incubated muscle

Insulin binding was assessed at 21 °C by the technique of Le Marchand-Brustel *et al.* [29]. EDL muscles were initially incubated for 30 min in the absence or in the presence of 0.5 μM-TPA at 21 °C in 3 ml of Krebs–Henseleit buffer, pH 7.4, containing 5 mM-glucose, 0.2 % BSA and 20 mM-Hepes. The muscles were then incubated with 30 pM-[^{125}I-Tyr^{A14}]monoiodoinsulin for 3 h. After that time, muscles were washed five times in cold 0.9 % NaCl containing 0.25 % BSA. The results presented have been corrected for non-specific binding, which was determined as the amount of ^{125}I-insulin recovered in muscles incubated in the presence of a saturating concentration (1.5 μM) of unlabelled insulin. Radioactivity was quantified in a Packard γ-radiation counter. Non-specific binding was approx. 30 % of the total binding. The degradation of ^{125}I-insulin, assessed by the increase in trichloroacetic acid-soluble radioactivity in the incubation media, was 5–10 % at 3 h in both control and TPA-treated groups.

Preparation of insulin receptors

EDL muscles were incubated for 3 h in 3 ml of Krebs–Henseleit buffer, pH 7.4, containing 5 mM-glucose, 0.20 % BSA and 20 mM-Hepes. For the last 90 min they were incubated in the absence or presence of 0.5 μM-TPA. At the end of the incubation period, muscles were frozen in liquid N_2. Pools from 10–15 muscles (approx. 250–300 mg of tissue) were homogenized and solubilized in 1 % Triton X-100 as described [30]. The solubilized homogenate was centrifuged at 150000 *g* for 90 min at 4 °C. The 150000 *g* supernatant (1.8 ml) was recycled for 30 min (approx. 5–7 times) through a column containing 0.2 ml of WGA bound to agarose, at 4 °C. The resin was washed with buffer (20 ml) containing 25 mM-Hepes and 0.1 % Triton X-100, pH 7.4. Receptors were eluted from the WGA column with buffer containing 25 mM-Hepes, 0.1 % Triton X-100 and 0.3 M-*N*-acetyl-D-glucosamine, pH 7.4.

Ligand binding and receptor cross-linking

Insulin binding was measured as in [30]. WGA eluate (20 μl) was incubated for 1 h at 22 °C in 30 mM-Hepes buffer containing 0.1 % BSA and 100 units of bacitracin/ml (pH 7.6; 200 μl) and 20000 c.p.m. of [^{125}I-Tyr^{A14}]monoiodoinsulin ($\simeq$ 60 pM). Non-specific binding was estimated as ^{125}I-insulin bound in the presence of 1 μM-insulin (5–10 % of total binding). Binding data were expressed per μg of protein, with the latter measured by the method of Bradford [31]. Receptor cross-linking protocols were carried out essentially as described by Pilch & Czech [32], by using 0.5 nM-[^{125}I-Tyr^{B26}]monoiodoinsulin.

Autophosphorylation and phosphorylation of an exogenous substrate

Autophosphorylation assays were performed as described previously [30]. Phosphorylation of an exogenous substrate was carried out with receptor preparations which were preincubated

for 1 h in 30 mM-Hepes buffer, pH 7.6, containing 50 mM-magnesium acetate, and various concentrations of insulin. The receptor kinase activity was initially activated by addition of 50 μM-[γ-^{32}P]ATP (5–10 μCi) for 10 min. The reaction was initiated by the addition of the exogenous substrate (co-polymer of Glu/Tyr, 4:1; 0.25 mg/ml). The reaction was stopped after 30 min by applying samples to filter-paper squares (Whatman 3MM), which were immediately washed in 10 % trichloroacetic acid containing 10 mM-sodium pyrophosphate. Papers were washed, dried and counted as described [30].

RESULTS

Effect of phorbol esters on MeAIB uptake

Incubation of EDL muscles with 0.5 μM-TPA, a concentration that causes maximal effects in other cell types, only caused a minor increase in MeAIB uptake, which did not reach statistical significance (Table 1). This lack of effect on MeAIB uptake was observed after incubation for 60 or 180 min in the presence of TPA. In fact, the same pattern was observed after incubation for 60 min with the inactive phorbol ester MA (11.5±1.4 and 14.1±1.9 nmol/30 min per g in control and MA-treated groups respectively). Dimethyl sulphoxide at a concentration of 0.01 % had no effect on MeAIB uptake (results not shown). This lack of effect of TPA contrasts with the substantial effect of insulin at 60 min after its addition on MeAIB uptake (Table 1). These results indicate that TPA, a protein kinase C activator, does not mimic insulin action, and therefore insulin signalling required to stimulate MeAIB transport does not involve protein kinase C activation in EDL muscle.

Effect of phorbol esters on insulin-stimulated MeAIB uptake

We have previously shown that 100 nM-insulin maximally stimulates MeAIB uptake in EDL muscle after 60 min of incubation [27]. Therefore, in the present study we have selected these conditions to investigate the effect of TPA on insulin-stimulated MeAIB uptake. When 100 nM-insulin and 0.5 μM-TPA were added simultaneously (both during the last 60 min of incubation), insulin-stimulated MeAIB uptake was not compromised (Table 2). However, when 0.5 μM-TPA was added 30 min before 100 nM-insulin (that is, TPA present during the last 90 min and insulin during the last 60 min of incubation), insulin-stimulated MeAIB uptake was largely prevented (Table 2). This effect was specific to TPA, since incubation for 90 min (30 min before insulin addition) with the inactive phorbol MA did not modify insulin action (Table 2). Furthermore, dimethyl sulphoxide (0.01 %) had no effect on insulin-stimulated MeAIB uptake, either (results not shown). These results provide evidence that protein kinase C rapidly compromises insulin action in the incubated skeletal muscle, promoting a situation of insulin resistance.

Table 1. Effect of TPA and insulin on MeAIB uptake by EDL muscle

Results are means±S.E.M. for 9–10 observations per group. EDL muscles were incubated for 180 min by using three different protocols: (i) in the absence or presence of 0.5 μM-TPA during the last 60 min of incubation, (ii) in the absence or presence of 0.5 μM-TPA during the 180 min of incubation, (iii) in the absence or presence of 100 nM-insulin for the last 60 min of incubation. TPA was dissolved in dimethyl sulphoxide, in a final concentration of 0.01 %. Control muscles were also incubated in the presence of 0.01 % dimethyl sulphoxide. Uptake of MeAIB was determined during the last 30 min. * Value significantly different from that of the basal group ($P < 0.05$).

Treatment	MeAIB uptake (nmol/30 min per g of muscle)	
	Control group	Experimental group
TPA, 60 min	15.6±1.6	17.9±1.8
TPA, 180 min	17.2±1.2	18.3±1.9
Insulin, 60 min	17.3±1.7	31.8±2.5*

Table 2. Effect of phorbol esters on insulin-stimulated MeAIB uptake by EDL muscle

Results are means±S.E.M. for 8–11 observations per group. EDL muscles were incubated for 180 min in the absence or in the presence of 100 nM-insulin during the last 60 min of incubation. Muscles were incubated in the absence or in the presence of 0.5 μM-TPA, which was added either at the same time as the insulin (i.e. it was present during the last 60 min of incubation) or 30 min before insulin addition (TPA 90 min). In a different experimental series, muscles were incubated in the absence or presence of 0.5 μM-MA during the last 90 min of incubation. Phorbol esters were dissolved in dimethyl sulphoxide (final concn. 0.01 %). Control muscles were also incubated in the presence of 0.01 % dimethyl sulphoxide. MeAIB uptake was determined during the last 30 min of incubation. * indicates a significant difference between control and TPA groups, at $P < 0.05$.

Treatment	MeAIB uptake (nmol/30 min per g of muscle)		
	Control	Phorbol-ester-treated	No insulin
TPA, 60 min	36.5±2.5	37.5±3.1	—
TPA, 90 min	33.2±2.9	23.3±2.0*	19.2±1.7
MA, 90 min	27.8±2.8	29.7±2.9	—

Additive inhibitory effects of TPA and protein kinase C inhibitors (polymyxin B and H-7) on insulin-stimulated MeAIB uptake

Polymyxin B and H-7 are well-known inhibitors of protein kinase C [33–35] in several cell types, and they block different biological effects induced by phorbol esters [36,37]. Thus we attempted to assess whether these agents could also prevent TPA effects on insulin-stimulated MeAIB uptake by muscle. To that end, we investigated, in a separate set of experiments, the effect of both inhibitors on the stimulation of MeAIB uptake induced by incubation for 1 h with 100 nM-insulin. Incubation for 2 h in the presence of 0.1 mM-polymyxin B (100 times its K_i for protein kinase C inhibition; [34]) caused a substantial inhibition of insulin-stimulated MeAIB uptake, with no effects on basal MeAIB uptake (Table 3). In fact, the effect of polymyxin B was very similar to the inhibitory effect caused by preincubation for 90 min with 0.5 μM-TPA on insulin-stimulated MeAIB uptake (Table 3). Thus, whereas insulin induced an increase in MeAIB uptake by control muscle, this stimulation was decreased after incubation with TPA or polymyxin B (Table 3). When both polymyxin B (120 min) and TPA (90 min) were present in the incubation medium, insulin action was maximally inhibited (Table 3). The additive effects of TPA and polymyxin B inhibiting insulin-stimulated amino acid transport indicate that, in muscle, polymyxin B exerts actions other than the inhibitory effect on protein kinase C activity. That is, polymyxin B inhibits insulin action on amino acid transport by a mechanism not mediated by protein kinase C.

We also investigated the effect of H-7 on basal and insulin-

Table 3. Effect of TPA and polymyxin B on insulin-stimulated MeAIB uptake by EDL muscle

Results are means ± S.E.M. for 6–8 observations per group. EDL muscles were incubated for 180 min in the absence or presence of 100 nM-insulin during the last 60 min of incubation. Muscles were incubated in the absence or presence of 0.5 μM-TPA (added during the last 90 min of incubation), 0.1 mM-polymyxin B (added during the last 120 min of incubation) or both TPA (90 min) and polymyxin B (120 min). MeAIB uptake was determined during the last 30 min of incubation. * indicates a significant difference between basal and insulin groups, at $P < 0.05$.

Treatment	MeAIB uptake (nmol/30 min per g of muscle)		Effect of insulin (% of basal)
	Basal	Insulin	
No additions	17.6 ± 1.3	32.3 ± 3.6*	83
TPA	19.3 ± 1.9	28.8 ± 3.4*	49
Polymyxin B	19.3 ± 2.1	25.8 ± 3.3	34
TPA + polymyxin B	13.5 ± 0.9	14.8 ± 0.7	10

Table 4. Effect of TPA and H-7 on insulin-stimulated MeAIB uptake by EDL muscle

Results are means ± S.E.M. for 8–9 observations per group. EDL muscles were incubated during 180 min in the absence or presence of 100 nM-insulin during the last 60 min of incubation. Muscles were incubated in the absence or presence of 0.5 μM-TPA (added during the last 90 min of incubation), 1 mM-H-7 (added during the last 120 min of incubation) or both TPA (90 min) and H-7 (120 min). H-7 was dissolved in ethanol, in a final concentration of 0.2%. Control muscles were also incubated in the presence of 0.2% ethanol. MeAIB uptake was determined during the last 30 min of incubation. * indicates a significant difference between basal and insulin groups, at $P < 0.05$.

Treatment	MeAIB uptake (nmol/30 min per g of muscle)		Effect of insulin (% of basal)
	Basal	Insulin	
No additions	16.6 ± 1.2	34.4 ± 3.5*	107
TPA	18.6 ± 2.0	26.1 ± 3.1*	40
H-7	21.7 ± 2.5	34.7 ± 2.7*	60
TPA + H-7	14.2 ± 1.6	18.0 ± 1.6	27

stimulated MeAIB uptake (Table 4). EDL muscles were incubated for 120 min in the presence of 1 mM-H-7, which is well above the K_i value (6 μM) for inhibition of protein kinase C [35]. Incubation with H-7 caused an increased MeAIB uptake under basal conditions, but not in the presence of insulin (Table 4). Similarly to the effects of polymyxin B, incubation of muscles with 1 mM-H-7 (for 120 min) and 0.5 μM-TPA (for 90 min) caused a further decrease in insulin-stimulated MeAIB uptake (Table 4). In summary, both H-7 and polymyxin B present additive effects with TPA with respect to their inhibitory effect on insulin action, and are not therefore suitable agents to investigate whether a certain insulin effect is mediated by protein kinase C activity.

Effect of TPA on 3-*O*-methylglucose uptake and lactate production

In light of the marked inhibition of insulin-stimulated MeAIB uptake induced by TPA in EDL muscle, we analysed whether

Table 5. Effect of TPA on 3-*O*-methylglucose uptake and lactate production by EDL muscle

Results are means ± S.E.M. for 5–6 observations per group for 3-*O*-methylglucose uptake, and for 14–21 observations per group for lactate production. EDL muscles were incubated for 180 min in the absence or in the presence of 100 nM-insulin during the last 60 min of incubation. Muscles were incubated in the absence or in the presence of 0.5 μM-TPA, which was added 30 min before insulin addition (TPA 90 min). TPA was dissolved in dimethyl sulphoxide (final concn. 0.01%). Control muscles were also incubated in the presence of 0.01% dimethyl sulphoxide. 3-*O*-Methylglucose uptake and lactate production were determined during the last 30 minutes of incubation. * indicates a significant difference between control and TPA groups, at $P < 0.05$. † indicates a significant difference between basal and insulin groups, at $P < 0.05$.

(*a*) 3-*O*-Methylglucose uptake

	Uptake (nmol/30 min per g of muscle)	
	Control	TPA
Basal	6.9 ± 0.3	9.8 ± 0.5*
Insulin	18.8 ± 0.8†	19.6 ± 0.7†

(*b*) Lactate production

	Lactate (μmol/h per g of muscle)	
	Control	TPA
Basal	8.4 ± 0.7	11.5 ± 0.6*
Insulin	11.9 ± 0.6†	14.4 ± 0.8*†

that was a consequence of a generalized TPA-dependent inhibition of insulin action. To that end, we investigated the effect of TPA on insulin-stimulated glucose utilization by muscle. Incubation of muscles in the presence of TPA (0.5 μM, 90 min) caused a 40% increase in the rate of 3-*O*-methylglucose uptake by muscle (Table 5); insulin caused a larger increase (170%) in 3-*O*-methylglucose uptake, and insulin and TPA effects were not additive (Table 5). TPA also caused an enhanced production of lactate, which was quantitatively similar to the activation caused by 100 nM-insulin (Table 5); under these conditions insulin and TPA showed additive effects (Table 5).

Effect of TPA on insulin binding and receptor tyrosine kinase

In order to ascertain the mechanisms by which TPA compromises insulin-stimulated system A transport activity by skeletal muscle, specific insulin binding was determined by using the incubated EDL muscle (Table 6). TPA treatment for 30 min did not cause any significant change in the binding of tracer quantities of insulin by muscle.

Insulin binding was also assessed after lectin-affinity purification of insulin receptors. Insulin receptors from control and 90 min-TPA-treated EDL muscles were partially purified by WGA affinity chromatography. No differences in the yield of glycoproteins was detected in either group (0.44 ± 0.03 and 0.37 ± 0.02 μg/mg of muscle in control and TPA-treated muscles respectively). In addition, no significant differences in insulin binding were detected, either when expressed per μg of protein eluted from the column or when expressed as total insulin binding per mg of muscle (Table 6).

Studies of affinity cross-linking using 0.5 nM-[^{125}I-Tyr^{B26}]-insulin (near high-affinity K_d values) were also performed to determine the M_r of the α subunit of the insulin receptor from

Table 6. Insulin binding by incubated rat EDL muscle and by partially purified insulin receptor: effect of TPA

Values are means ± S.E.M. of 5–6 observations per group. (*a*) EDL muscles were initially incubated for 30 min in the absence or presence of 0.5 μM-TPA. To assess insulin binding, the muscles were then incubated with 30 pM-[^{125}I-Tyr^{A14}]monoiodoinsulin at 21 °C for 3 h in the absence or presence of 1.5 μM-insulin. The ^{125}I-insulin specifically bound was determined by subtracting the binding observed in the presence of 1.5 μM-insulin from that observed in the absence of unlabelled insulin. (*b*) Insulin receptors were partially purified from control and TPA-treated (90 min) EDL-muscle homogenates, after solubilization in Triton X-100 and ultracentrifugation, by lectin affinity chromatography. Each preparation was obtained from 10–15 rats. The WGA eluate (20 μl) was incubated for 1 h at 22 °C in buffer containing 30 mM-Hepes, 0.2% BSA, 100 units of bacitracin/ml and [^{125}I-Tyr^{A14}]monoiodoinsulin, in the absence or presence of 1 μM-insulin. See the Experimental section for further details. Differences between control and TPA groups were statistically insignificant.

	Binding	
	Control	TPA
(*a*) Incubated muscle fmol/mg of tissue ($\times 10^{-2}$)	7.2 ± 1.7	6.5 ± 2.1
(*b*) Purified insulin receptor fmol/μg of protein	0.26 ± 0.06	0.22 ± 0.06
fmol/mg of tissue	0.11 ± 0.03	0.08 ± 0.02

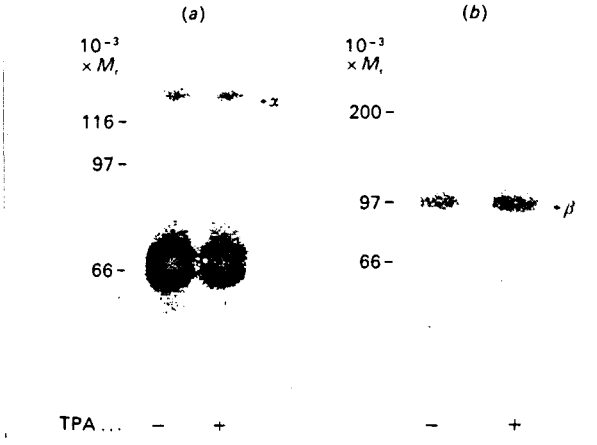

Fig. 1. Labelling of α and β subunits of insulin receptors from control and TPA-treated muscles

Partially purified insulin receptors were obtained from control and TPA-treated (90 min) EDL muscles as described in the Experimental section. Each preparation was obtained by pooling muscles from 10–12 rats. (*a*) Affinity cross-linking of ^{125}I-insulin to the insulin receptor. Partially purified receptor (10 μl) was incubated at 22 °C for 60 min in 30 mM-Hepes containing 0.5 nM-[^{125}I-Tyr^{B26}]insulin in the absence or presence of 1 μM unlabelled insulin. After incubation for 5 min at 0 °C, disuccinimidyl suberate was added (final concn. 1 mM), and samples were incubated for a further 15 min at 0 °C. The reaction was stopped by addition of Laemmli sample buffer with 0.1 M-dithiothreitol. (*b*) Labelling of β subunit of insulin receptors. Partially purified receptors were phosphorylated in the presence of [γ-^{32}P]ATP and insulin. The reaction was stopped by addition of Laemmli sample buffer with 0.1 M-dithiothreitol. Samples were subjected to electrophoresis in 7.5% polyacrylamide gels, followed by autoradiography.

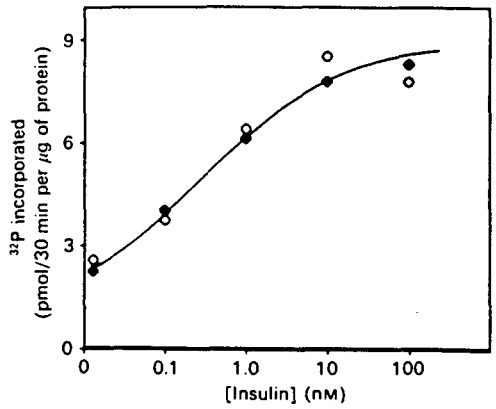

Fig. 2. Effect of TPA on exogenous kinase activity of insulin receptors from muscle

Insulin receptors from EDL muscles treated (○) or not (●) with TPA for 90 min were partially purified as described in the Experimental section. Each preparation was obtained by pooling muscles from 10–12 rats. WGA eluates (10 μl) were incubated at 22 °C for 1 h in 30 mM-Hepes buffer, pH 7.6, containing 50 mM-magnesium acetate and various concentrations of insulin. [γ-^{32}P]ATP (50 μM) was added, and samples were incubated for an additional 10 min. The substrate (copolymer of Glu/Tyr, 4:1; 0.25 mg/ml) was then added and allowed to react for 30 min. The reaction was stopped by applying samples to filter-paper squares and soaking in 10% trichloroacetic acid/10 mM-sodium pyrophosphate. Papers were washed, dried and counted by Čerenkov radiation. All values have been corrected for non-specific association of ^{32}P with the paper, which was estimated by incubating samples in the absence of receptor addition. Each data point is the mean of 6 observations per group performed in triplicate.

control and TPA-treated muscles (Fig. 1). When affinity-labelled insulin receptors from control and TPA-treated muscles were run on SDS/PAGE under reducing conditions, only one band (M_r approx. 130000) was specifically labelled (Fig. 1). The migration characteristics of this specifically labelled band did not differ between control and TPA-treated muscles. The β subunit, detected after autophosphorylation, showed similar apparent M_r values when control and TPA-treated groups are compared (Fig. 1). This supports the observation that the integrity of the α and β subunits of insulin receptors partially purified from control and from TPA-treated EDL muscles were similar.

The kinase activity of the insulin receptor in control and TPA-treated EDL muscle was next characterized by using an exogenous substrate. The dose/response relationship between insulin and ^{32}P incorporation into a copolymer of Glu/Tyr, in the presence of purified insulin receptor, is presented in Fig. 2. Equal amounts of insulin binding (and protein) were used for control and TPA-treated groups. Insulin stimulated the exogenous kinase activity of the insulin receptor from control muscle as previously shown (Fig. 2). Thus, 1 nM-insulin caused more than a half-maximal stimulation of the rate of exogenous substrate phosphorylation, and at 10 nM-insulin stimulation was already maximal. Supra-maximal insulin caused a 3-fold increase in exogenous kinase activity from control insulin receptors. Insulin receptors partially purified from TPA-treated muscles exhibited a similar ability to phosphorylate the exogenous substrate in the absence as well as in the presence of insulin as compared with the control group (Fig. 2).

DISCUSSION

In this report, we have demonstrated that in skeletal muscle

protein kinase C activation inhibits some effects of insulin, as assessed by insulin-stimulated system A transport activity. However, TPA treatment does not modify insulin-stimulated receptor kinase, glucose transport or lactate production by muscle. These are in keeping with previous reports in which no inhibitory effect of phorbol esters on insulin-stimulated receptor kinase or glucose transport by muscle was substantiated [38,39]. Therefore, we propose that activation of protein kinase C must block a step located at a post-receptor level in the biochemical pathway that leads to stimulation of amino acid transport. Whether this is the only effect caused by TPA on insulin action in muscle remains to be established. Our findings differ from other results reported for intact Fao hepatoma cells, in which TPA treatment led to inhibition of insulin action and insulin-stimulated tyrosine kinase activity [2,15], as well as with other reports in adipocytes [7,8]. In our study, we did not find a substantial modification of insulin binding after TPA treatment, which agrees with others [2,15,38,40]. However, alterations of insulin-binding properties have also been reported in isolated rat adipocytes, lymphocytes, macrophages, monocyte-like and promyelocytic leukaemia human cell lines after phorbol ester treatment [6,16,41,42].

The variable response to phorbol esters, as well as the variable interaction between insulin and protein kinase C discussed above, might be understood on the basis of tissue differences in the pattern of expression of protein kinase C isoenzymes. At least seven different subspecies of protein kinase C have been identified in mammalian tissues [43–45], and some kinetic differences among isoenzymes have been reported [44]. Further work is required to define the protein kinase C isoenzymes present in skeletal muscle, their kinetic properties and the similarities between the isoenzyme patterns found in muscles and other hormone-sensitive tissues.

Under our conditions, we have observed that in skeletal muscle the effect of insulin stimulating system A transport activity for neutral amino acids is not mimicked by the addition of TPA, a protein kinase C activator. From a mechanistic viewpoint, our data permit the conclusion that protein kinase C activation does not mediate insulin-stimulated system A transport in skeletal muscle. The lack of effect of TPA on basal system A transport activity contrasts with the marked increase that we found in glucose transport and lactate production. The stimulatory effect of TPA on glucose transport agrees with previous observations obtained in incubated muscle from mice [39] and contradicts another report, in which TPA failed to increase glucose transport in rat skeletal muscle [38]. The reason for these differences is unclear. In any event, the fact that TPA stimulates glucose transport and not MeAIB transport in skeletal muscle substantiates the contention that insulin causes activation of both transport systems by independent mechanisms. This is especially interesting in the light of the parallel regulation of glucose uptake and system A transport activity previously described in skeletal muscle under a variety of conditions [26,46,47].

It is worth noting that, although TPA causes a smaller stimulatory effect on glucose transport as compared with insulin, both agents enhance lactate production to a similar extent. Furthermore, whereas TPA and insulin did not show additive effects on glucose transport, their stimulatory action on lactate production by muscle was clearly additive. In order to explain this metabolic pattern, we should mention that (*a*) insulin-stimulated glucose uptake, in the incubated muscle, is mainly directed into glycogen and lactate production [25], (*b*) TPA inhibits glycogen synthesis from glucose in muscle [38], and (*c*) phorbol esters increase fructose 2,6-bisphosphate levels and activate glycolysis in several cell types [48,49]. Based on that, it might be postulated that in the presence of TPA a smaller proportion of the glucose taken up by the cell is incorporated into muscle glycogen, in favour of a greater activation through the glycolytic pathway.

Many inhibitors of protein kinase C have recently been reported. These include polymyxin B [33,34], calmodulin antagonists [33], H-7 [35], K252a [50], staurosporine [51], sphingosine [52] and sangivamycin [53]. These inhibitors are sometimes used with the intention of gaining information on the role of protein kinase C in different cellular responses. Ideally, protein kinase inhibitors block, under appropriate conditions, different phorbol-ester-induced effects [36,37,50]. However, it has been reported that, for instance, polymyxin B also inhibits insulin-stimulated membrane transport processes in muscle and adipocytes [54,55], and in addition inhibits a Ca^{2+}-activated K^+ channel [56]. In the present study we have substantiated that both H-7 and polymyxin B block, to a different extent, insulin action and, in addition, that these inhibitors and phorbol esters present additive inhibitory effects on insulin-stimulated MeAIB uptake. Our data indicate that protein kinase C does not mediate the mechanism by which H-7 or polymyxin B blocks the insulin effect on system A transport activity. This suggests that H-7 or polymyxin B are not specific inhibitors of protein kinase C activity and are not therefore suitable agents with which to investigate whether a certain insulin effect is mediated by protein kinase C activity.

We have substantiated that phorbol esters do not induce an insulin-resistance-like situation as regards either glucose utilization or receptor tyrosine kinase activity in skeletal muscle. That allows us to hypothesize that situations of muscle insulin resistance characterized by altered peripheral glucose disposal and deficient receptor kinase activity, such as those reported in diabetes or obesity [57,58], cannot be attributable to altered catalytic properties of protein kinase C. However, protein kinase C must be envisaged as a negative modulator of the biochemical pathway by which insulin activates system A amino acid transport activity in skeletal muscle. This offers a molecular explanation for the generation of insulin-resistant states in which the impairment just involves certain biological effects of insulin.

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Gumà, A., Viñals, F., Muñoz, P., Camps, M., Bertran, J., Testar, X., Palacín, M., Zorzano, A.; Benzyl succinate inhibits insulin receptor kinase and insulin action in skeletal muscle.

BENZYL SUCCINATE INHIBITS INSULIN RECEPTOR KINASE AND INSULIN ACTION IN SKELETAL MUSCLE.

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Keywords: Amino acid uptake, lactate production, insulin receptor kinase, benzyl succinate, (skeletal muscle), (rat).

Abreviations used: EDL, extensor digitorum longus; MeAIB, α-(methyl)aminoisobutyric acid; WGA, wheat germ agglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic.

ABSTRACT.

No direct evidence for the involvement of insulin receptor kinase on insulin action has been reported in skeletal muscle. Therefore, in this study we have examined the effect of an inhibitor of insulin receptor kinase, benzyl succinate, on insulin-stimulated system A transport activity - assessed by the uptake of the analogue α-(methyl)aminoisobutyric acid (MeAIB)- and lactate production in the incubated intact muscle. Benzyl succinate inhibited to the same extent tyrosine receptor kinase activity for exogenous substrates and β-subunit phosphorylation in purified insulin receptor preparations from rat skeletal muscle. Benzyl succinate inhibited the effects of insulin on lactate release and MeAIB uptake in the incubated, intact muscle preparation. The inhibition of insulin action was concentration-dependent and 50% reduction was found near 1 mM benzyl succinate.

1. INTRODUCTION.

Insulin stimulates many cellular processes in mammalian cells. Very soon after binding, insulin stimulates a tyrosine kinase activity located in the β subunit of the insulin receptor. This kinase activity seems to play a critical role in the signaling that causes the effects of insulin. This is evidenced by studies of insulin action after inhibition of tyrosine kinase caused by the introduction of cellular monoclonal antibodies against tyrosine kinase activity [1, 2] or by studies of site-directed mutagenesis [3-5]. These studies have been performed either in isolated cells or in cultured cell lines. The evidence to support a role of tyrosine kinase in intact skeletal muscle is only indirect, and consists in the correlation found between in vivo insulin action and insulin receptor kinase activity reported in some conditions [6-8].

A useful strategy to investigate in a more direct way the dependence of tyrosine receptor kinase activation on insulin action in intact skeletal muscle might be the utilization of inhibitors of insulin receptor kinase activity. In this respect, several types of inhibitors of tyrosine receptor kinases, characterized by low molecular weight and hydrophobic nature, have been recently reported [9-11]. Some of these inhibitors, block insulin receptor kinase and some effects of insulin in adipocytes and chinese hamster ovary cells [10, 11]. In the present study, we have investigated the effect of benzyl succinate, a carboxyl compound that contains a hydroxyphenyl moiety [10], on insulin action in skeletal muscle. Our results offer direct evidence for the involvement of tyrosine receptor kinase activity on insulin action in skeletal muscle.

2. MATERIALS AND METHODS

Animals and dissection procedures.

Male Wistar rats (50-60 g), obtained from our own colony were used. The rats were fed on Purina Laboratory chow ad libitum. Animals were housed in animal quarters maintained at 22°C with a 12 h-light/12 h-dark cycle. The dissection and isolation of the extensor digitorum longus (EDL) and soleus muscles were carried out under anaesthesia with pentobarbital (5-7 mg/100 g body wt., intraperitoneally) as described previously [12].

Incubations

Muscles were incubated in a shaking incubator at 37°C for 3 h in 3 ml of Krebs-Henseleit buffer, pH 7.4 containing 5 mM glucose, 0.20% bovine serum albumin and 20 mM Hepes, as previously described [13]. At different

times, insulin (100 nM) or benzyl succinate (from 0 to 2 mM, Sigma) were added to the incubation medium (see details in Figure legends). Amino acid uptake by the system A was measured in muscles using the non-metabolizable amino acid analog α-(methyl)aminoisobutyric acid (MeAIB). Following the incubations with insulin and the above mentioned agents, muscles were transferred to vials with 1.5 ml of Krebs-Henseleit buffer, pH 7.4 containing 5 mM glucose, 0.20% bovine serum albumin, 20 mM Hepes and 0.1 mM α-[1-^{14}C]-methylaminoisobutyric acid (800 μCi/mmol), 1 mM [^{3}H]-mannitol (330 μCi/mmol) and insulin or benzyl succinate at the same concentrations as during the preceeding incubation period. The vials were stoppered and incubated at 37°C in a shaking incubator for 30 min. The gas phase in the vials was 95% O_2 and 5% CO_2. Following incubation, the muscles were processed as previously reported [13]. Intracellular concentration of ^{14}C-amino acid analog was calculated by substracting ^{14}C-amino acid analog in the extracellular space from the total label found in tissue, as previously reported [13]. Lactate release to the incubation media was measured during the last 30 minutes of incubation as in [12].

Preparation of insulin receptors and receptor kinase assays.

Hindlimb muscle was obtained from pentobarbital-anesthesized rats. Muscles were frozen in liquid nitrogen and then homogenized and solubilized in 1% Triton-X-100 as previously described [6, 14]. The solubilized homogenate was centrifuged at 150,000 x g for 90 min at 4°C and the supernatant was subjected to wheat germ agglutinin affinity-chromatography. Receptors were eluted from the WGA column with buffer containing 25 mM Hepes, 0.1% Triton-X-100 and 0.3 M N-acetyl-D-glucosamine pH 7.4. Insulin binding was measured as in [6] and proteins were measured using the method of Bradford [15]. Assays of β-subunit receptor autophosphorylation and tyrosine kinase for exogenous substrates - copolymer of Glu/Tyr, 4:1- were performed as described previously [6, 14].

3. RESULTS.

To provide evidence that insulin action in muscle is dependent on receptor kinase activity, we investigated the effects of benzyl succinate on several insulin-stimulated parameters. Benzyl succinate is an inhibitor of the insulin receptor tyrosine kinase activity [12]; this inhibition is competitive with respect to the substrate and non-competitive with respect to ATP [12]. In agreement with prior findings [12], benzyl succinate

inhibited ligand-stimulated insulin receptor kinase activity for exogenous substrates in a concentration-dependent manner in partially purified receptor preparations from skeletal muscle (Figure 1). The insulin-induced phosphorylation of the β-subunit of the insulin receptor was also inhibited in the presence of benzyl succinate (Figure 1), and the extent of inhibition was similar to that observed for receptor kinase for exogenous substrates ($K_{o.5}$ about 3 mM).

Based on the capacity of benzyl succinate to inhibit the insulin receptor kinase activity, we next investigated the effect of this inhibitor on insulin action in skeletal muscle. Thus, initially we studied the effect of different concentrations of benzyl succinate on lactate production by extensor digitorum longus (EDL) muscle (Figure 2). Under control conditions, the stimulatory effect of insulin on lactate production by muscle was around 2-fold; the addition of benzyl succinate caused a decrease in the stimulatory effect of insulin, in a dose-dependent manner (Figure 2), so at 1 mM benzyl succinate the effect of insulin was reduced by approximately 50%. Under these conditions, benzyl succinate did not alter intracellular concentrations of ATP and creatine phosphate in muscle (data not shown). The effect of benzyl succinate on MeAIB uptake by muscle was also examined. Insulin activated MeAIB uptake by 84% and 66%, in EDL and soleus muscles, respectively (Figure 3). Under these conditions, benzyl succinate caused a concentration-dependent decrease in the stimulatory effect of insulin on MeAIB uptake (Figure 3). In keeping with previous observations (10) benzyl succinate was more effective in the incubated muscle than in vitro in inhibiting insulin effects. Thus, both in soleus and EDL muscle, the effect of insulin on MeAIB uptake was again reduced approximately a 50% in the presence of 1 mM benzyl succinate.

4. DISCUSSION.

Binding of insulin to its receptor is known to activate tyrosine receptor kinase and, probably as a consequence, a variety of membrane-associated and cytosolic serine/threonine kinases [16-17]. Provided that benzyl succinate is a reasonably specific inhibitor of tyrosine kinases, this compound can be used as a tool to demonstrate the role of insulin receptor tyrosine kinase on insulin action [10]. In this report we have demonstrated that benzyl succinate blocks the stimulatory effect of insulin on amino acid uptake and lactate production in intact rat skeletal muscle. That provides evidence for the involvement of the insulin receptor

tyrosine kinase activity in the regulation of system A transport activity and lactate production by insulin in muscle. In the incubated muscle preparation, lactate production is a good estimate of the rate of glycolysis as well as an indirect measure of glucose transport [18], provided that glucose is mainly converted into lactate, and glycogen synthesis or oxidation only account for less than 10% in each case [12, 18]. Therefore, it is likely that insulin-activated glucose transport also depends on an intact receptor kinase activity in skeletal muscle.

Benzyl succinate inhibited both phosphorylation of the β-subunit of the insulin receptor and tyrosine receptor kinase for exogenous substrates as determined in receptor preparations from skeletal muscle. In fact, the sensitivity of receptor kinase activity for exogenous substrates and β-subunit phosphorylation to benzyl succinate was very similar. In this respect, our data contrast with the initial report by Schechter et al. [10] who described that although benzyl succinate inhibited tyrosine kinase for exogenous substrates, it did not alter β-subunit phosphorylation in partially purified rat liver insulin receptors. These differences might be explained on the basis of the previously reported functional differences in insulin receptors from different tissues [19, 20].

Finally, whether the activation of amino acid transport or lactate production caused by insulin is triggered by protein phosphorylation on tyrosine residues subsequent to the activation of the insulin receptor kinase activity, or as a consequence of a conformational change in the receptor induced by autophosphorylation, remains to be established. The utilization of inhibitors that selectively block one of both receptors activities would be critical to answer this question.

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LEGEND TO FIGURES.

Figure 1. Effect of benzyl succinate on autophosphorylation of β-subunit and kinase activities of insulin receptors from skeletal muscle.

Partially purified insulin receptors from muscle were incubated as described [6, 14] in the absence or presence of varying concentrations of benzyl succinate -dissolved in dimethyl sulfoxide, in a final concentration of 0.5%-, or 100 nM insulin.

Autophosphorylation of β-subunit (□). Relative autophosphorylation levels of β-subunit were determined after scanning bands from autoradiography. Results shown are representative of four different experiments.

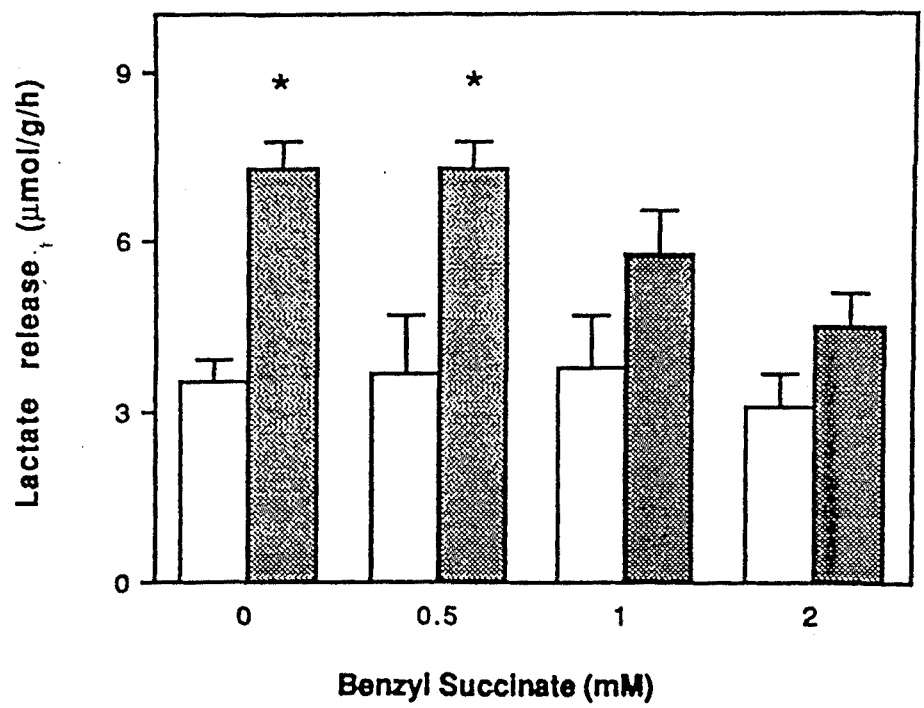
Insulin receptor phosphorylation of exogenous peptide (■). Each data point is the mean of duplicate estimations and the results shown are representative of three different experiments.

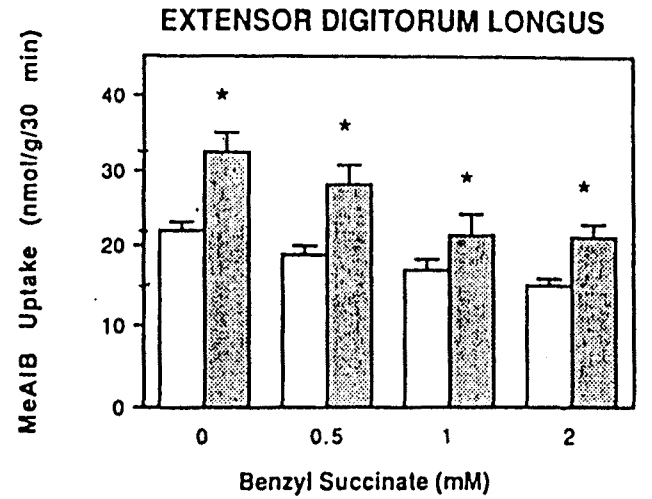
Figure 2. Effect of benzyl succinate on lactate production by extensor digitorum longus muscle

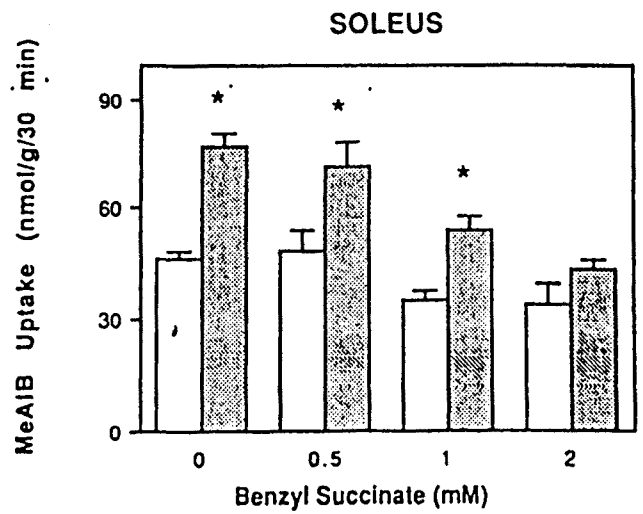
Results are means ± SE for 30 observations in the control group and 6 to 12 observations in groups treated with benzyl succinate. EDL muscles were incubated for 180 minutes in the absence or presence of 100 nM insulin during the last 60 minutes of incubation. Muscles were incubated in the presence of different concentrations of benzyl succinate (added during the last 120 minutes of incubation). Benzyl succinate was dissolved in dimethyl sulfoxide, in a final concentration of 1%. Control muscles were also incubated in the presence of 1% dimethyl sulfoxide. * indicates a significant difference between basal and insulin groups, at $P<0.05$.

Figure 3. Effect of benzyl succinate on MeAIB uptake by extensor digitorum longus and soleus muscles.

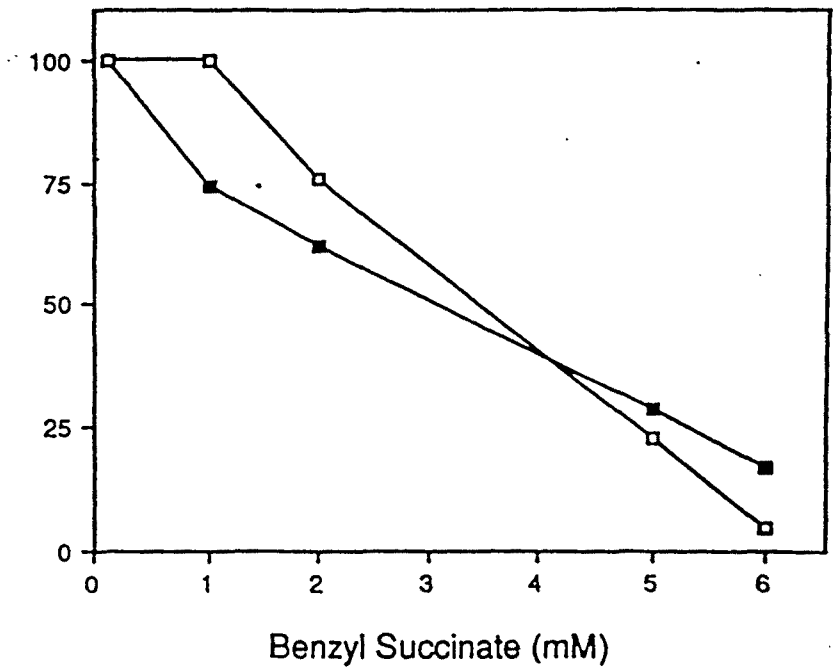
Results are means ± SE for 17 to 22 observations in the control group and 6 to 10 observations in benzyl succinate-treated groups. EDL and soleus muscles were incubated as detailed in legend to Figure 2. * indicates a significant difference between basal and insulin groups, at $P<0.05$.


Lactate release (µmol/g/h)
9
6
3
0
*
*
0
0.5
1
2
Benzyl Succinate (mM)


EXTENSOR DIGITORUM LONGUS
MeAIB Uptake (nmol/g/30 min)
40
30
20
10
0
0
0.5
1
2
Benzyl Succinate (mM)


SOLEUS
MeAIB Uptake (nmol/g/30 min)
90
60
30
0
0
0.5
1
2
Benzyl Succinate (mM)

Tyrosine Kinase Activity and Autophosphorylation (% of maximal insulin effect)



- D -

(Manuscrit en preparació)

Gumà, A., Muñoz, P., Camps, M., Testar, X., Palacín, M., Zorzano, A.; Staurosporine can not be used to prove the role of protein kinase C on insulin action.

STAUROSPORINE CAN NOT BE USED TO PROVE THE ROLE OF PROTEIN KINASE C ON INSULIN ACTION.

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Keywords: staurosporine, protein kinase C, insulin receptor kinase, insulin action, (skeletal muscle), (rat).

Abreviations used: EDL, extensor digitorum longus; MeAIB, α-(methyl)aminoisobutyric acid; WGA, wheat germ agglutinin;

ABSTRACT.

Staurosporine, a previously reported protein kinase C inhibitor, also inhibits insulin receptor kinase activity partially purified from rat skeletal muscle. Insulin, but not phorbol esters, stimulates α(methyl)-aminoisobutyric acid (MeAIB) uptake in extensor digitorum longus (EDL) muscle; furthermore, phorbol esters as well as insulin activates lactate in an additive manner by the incubated EDL. Thus, our data indicate that insulin-stimulation of amino acid and glucose uptake is not mediated by protein kinase C activation. Under these conditions, staurosporine caused a substantial inhibition of insulin effects on lactate production and MeAIB uptake in the incubated muscle. It is concluded that staurosporine being a nonspecific kinase inhibitor, can not be used as a tool to investigate the dependence of insulin action on protein kinase C activity in insulin-sensitive tissues.

1. INTRODUCTION.

Protein kinase C plays a complex role in insulin action. Thus, insulin has been reported to activate protein kinase C in several cell types [1, 2], and in this regard, phorbol esters, known activators of protein kinase C, mimick some of insulin effects [3-5]. In addition, phorbol esters can also inhibit insulin action, concomitant to inhibition of insulin receptor tyrosine kinase [6-8] or acting at a post-receptor level [9].

Inhibitors of protein kinase C, such as polymyxin B, H-7, staurosporine or sphingosine, have been used with the intention of gaining information on the role of protein kinase C in insulin action [10, 11, 12]. However, inhibitors such as polymyxin B and H-7 block insulin action in the incubated muscle, in an additive manner to the inhibitory effect displayed by phorbol esters [9]. Thus, polymyxin B and H-7 behave as nonspecific inhibitors of protein kinase C in skeletal muscle. In the presence study we have investigated the effects of staurosporine, also known to inhibit insulin receptor kinase activity [13], on insulin action in skeletal muscle. Our data demonstrate that staurosporine inhibits insulin effects that are not mediated by protein kinase C. That allow us to conclude that staurosporine can not be used as a tool to ascertain whether a certain insulin effect is mediated by protein kinase C.

2. MATERIALS AND METHODS

Animals and dissection procedures.

Male Wistar rats (50-60 g), obtained from our own colony were used. The rats were fed on Purina Laboratory chow ad libitum. Animals were housed in animal quarters maintained at 22°C with a 12 h-light/12 h-dark cycle. The dissection and isolation of the extensor digitorum longus (EDL) and soleus muscles were carried out under anaesthesia with pentobarbital (5-7 mg/100 g body wt., intraperitoneally) as described previously [14]. The isolated EDL and soleus muscles were fixed to a stainless-steel clip in order to maintain the muscle under slight tension (approximating to resting length) during the incubation. Such muscles (20-30 mg weight) are able to maintain normal ATP and creatine phosphate concentrations during a 3 h incubation.

Incubations

Muscles were incubated in a shaking incubator at 37°C for 3 h in 3 ml of Krebs-Henseleit buffer, pH 7.4 containing 5 mM glucose, 0.20% bovine serum albumin and 20 mM Hepes, as previously described [9, 15]. At different times, insulin (100 nM) or staurosporine (1μM, Boehringen Manheim) were added to the incubation medium (see details in Figure legends). Amino acid uptake by the system A was measured in muscles using the non-metabolizable amino acid analog α-(methyl)aminoisobutyric acid (MeAIB). Following the incubations with insulin and the above mentioned agents, muscles were transferred to vials with 1.5 ml of Krebs-Henseleit buffer, pH 7.4 containing 5 mM glucose, 0.20% bovine serum albumin, 20 mM Hepes and 0.1 mM α-[1-^{14}C]-methylaminoisobutyric acid (800 μCi/mmol), 1 mM [^{3}H]-mannitol (330 μCi/mmol) and insulin and modulators at the same concentrations as during the preceeding incubation period. The vials were stoppered and incubated at 37°C in a shaking incubator for 30 min. The gas phase in the vials was 95% O_2 and 5% CO_2. Following incubation, the muscles were processed as previously reported [9, 15]. The extracellular space of EDL muscles was not modified by insulin or staurosporine treatment (data not shown). Intracellular concentration of ^{14}C-amino acid analog was calculated by substracting ^{14}C-amino acid analog in the extracellular space from the total label found in tissue, as previously reported [9, 15]. Lactate release to the incubation media was measured during the last 30 minutes of incubation as in [16].

Preparation of insulin receptors and receptor kinase assays.

Hindlimb muscle was obtained from pentobarbital-anesthesized rats. Muscles were frozen in liquid nitrogen and then homogenized and solubilized in 1% Triton-X-100 as previously described [17]. The solubilized homogenate was centrifuged at 150,000 x g for 90 min at 4°C and the supernatant was subjected to wheat agglutinin affinity chromatography (WGA). Receptors were eluted from the WGA column with buffer containing 25 mM Hepes, 0.1% Triton-X-100 and 0.3 M N-acetyl-D-glucosamine pH 7.4. Insulin binding was measured as in [17] and proteins were measured using the method of Bradford [18]. Assays of β-subunit receptor autophosphorylation and tyrosine kinase for exogenous substrates -copolymer of Glu/Tyr, 4:1- were performed as described previously [9, 17].

3. RESULTS.

Staurosporine, a previously reported inhibitor of protein kinase C [19], has also been described as an inhibitor of insulin receptor kinase from human placenta [13]. In agreement with this, staurosporine caused a similar dose-dependent inhibition of insulin-stimulated receptor kinase partially purified from rat skeletal muscle (Figure 1). In fact, the $K_{0.5}$ value of inhibition was approximately 100 nM, in close agreement with the previous observation [13]. Staurosporine at a final concentration of 1 μM totally blocked insulin receptor kinase (Figure 1). Staurosporine also inhibited the autophosphorylation of insulin receptor, although with less potency than its action on receptor kinase activity, and 1 μM staurosporine only caused a 64% inhibition of β-subunit phosphorylation (data not shown). This is also in keeping with the original report by Fujita-Yamaguchi and Kathuria [13].

In a further step we investigated the effect of staurosporine or phorbol esters on insulin-stimulated MeAIB uptake and lactate production by muscle. To that end, we initially used 0.5 μM 12-O-tetradecanoyl-13-acetate (TPA), a concentration high enough to activate protein kinase C as well as several biological processes in the incubated muscle [9, 20]. Under control conditions, insulin increased MeAIB uptake in EDL muscle by 127% (Table 1). Incubation of EDL muscles with TPA for 90 min did not modify MeAIB uptake (Table 1). However, TPA treatment caused a 50% reduction in the extent of MeAIB uptake stimulation obtained in response to insulin (Table 1). Furthermore, insulin stimulated lactate production in EDL muscle by 42% (Table 1); TPA also caused a stimulatory effect on lactate production and the effects of insulin and TPA were additive (Table 1). In all, these data show that insulin effects on MeAIB uptake and lactate production are not mediated by protein kinase C. We next examined the effect of 1 μM staurosporine, a concentration that causes maximal inhibition of tyrosine kinase activity in the partially purified receptor preparation. Staurosporine caused a marked blockade of insulin-stimulated MeAIB uptake and lactate production in EDL muscle (Table 2).

4. DISCUSSION.

Staurosporine, a microbial alkaloid, has been widely used as a protein kinase C inhibitor [19, 21, 22] to investigate the regulatory role of protein kinase C. However, it also inhibits the activity of a

variety of protein kinases in vitro such as cAMP-dependent protein kinase, insulin receptor kinase or p60^{v-src} tyrosine protein kinase [13, 23, 24]. This rather nonselective inhibitory action of staurosporine seems to be consequence of its interaction with the ATP binding site on the catalytic domains of the protein kinases, which share a certain level of homology [25].

In spite of the nonspecific action of staurosporine, it has been utilized to examine the role of protein kinase C in different cellular responses [21, 22]. In fact, it has also been used to determine whether the stimulatory effect of insulin on glucose transport in skeletal muscle is dependent on protein kinase C activity [12]. In the present study, we have demonstrated that staurosporine can not be used for such a purpose since a) it inhibits tyrosine kinase activity of the insulin receptor, that is, a more initial step in insulin action, and b) effects of insulin that are independent of protein kinase C in skeletal muscle such as the stimulation of system A transport activity or lactate production [9] are also markedly blocked in the presence of staurosporine.

Based on previous studies [9] and in the present investigation, we conclude that the utilization of protein kinase C inhibitors, such as staurosporine, polymyxin B and H-7, should be avoided in order to investigate the relationship between protein kinase C and insulin action in different target cells.

Acknowledgments.

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Legend to Figure 1. Effect of staurosporine on tyrosine kinase activity of insulin receptors from skeletal muscle.

Insulin receptors were partially purified from rat skeletal muscle as described in Methods. Partially purified receptor (20 µl) was incubated for 1 h at 22°C in 30 mM Hepes buffer, pH 7.6, containing 50 mM Mg Acetate, 4 mM $MnCl_2$, 100 nM insulin and varying concentrations of staurosporine. Samples were further incubated with 50 µM [γ-^{32}P]-ATP (5-10 µCi) for 10 min and then the reaction was initiated by the addition of the substrate (copolymer of Glu/Tyr). See Methods for further details. Each data point is the mean of duplicate estimations and the results shown are representative of three different experiments.

Table 1

EFFECT OF PHORBOL ESTERS AND INSULIN ON MeAIB UPTAKE AND LACTATE PRODUCTION BY EXTENSOR DIGITORUM LONGUS MUSCLE.

A) MeAIB Uptake

	Control	TPA-treated	n
	(nmol MeAIB/g muscle/30 min)		
Basal	23.4±3.2	24.5±2.4	6
Insulin	53.2±2.1 +	42.6±3.2 *+	10

B) Lactate Production

	Control	TPA-treated	
	(µmmol lactate/g muscle/h)		
Basal	8.4±0.7	11.5±0.6 *	15
Insulin	11.9±0.6 +	14.4±0.8 *+	20

Results are means±SE for n observations per group. EDL muscles were incubated for 180 min in the absence or presence of 100 nM insulin during the last 60 min of incubation. Muscles were incubated in the absence or presence of 0.5 µM 12-O-tetradecanoyl-13-acetate (TPA), which was added 30 min before insulin addtion (TPA 90 min). TPA was dissolved in dimethyl sulfoxide, in a final concentration of 0.01%. Control muscles were also incubated in the presence of 0.01% dimethyl sulfoxide. MeAIB uptake and lactate production were determined during the last 30 min of incubation. * indicates a significant difference between control and TPA groups, at $P<0.05$. +, indicates a significant difference between basal and insulin groups, at $P< 0.05$.

Table 2

EFFECT OF STAUROSPORINE ON MeAIB UPTAKE AND LACTATE PRODUCTION BY EXTENSOR DIGITORUM LONGUS MUSCLE.

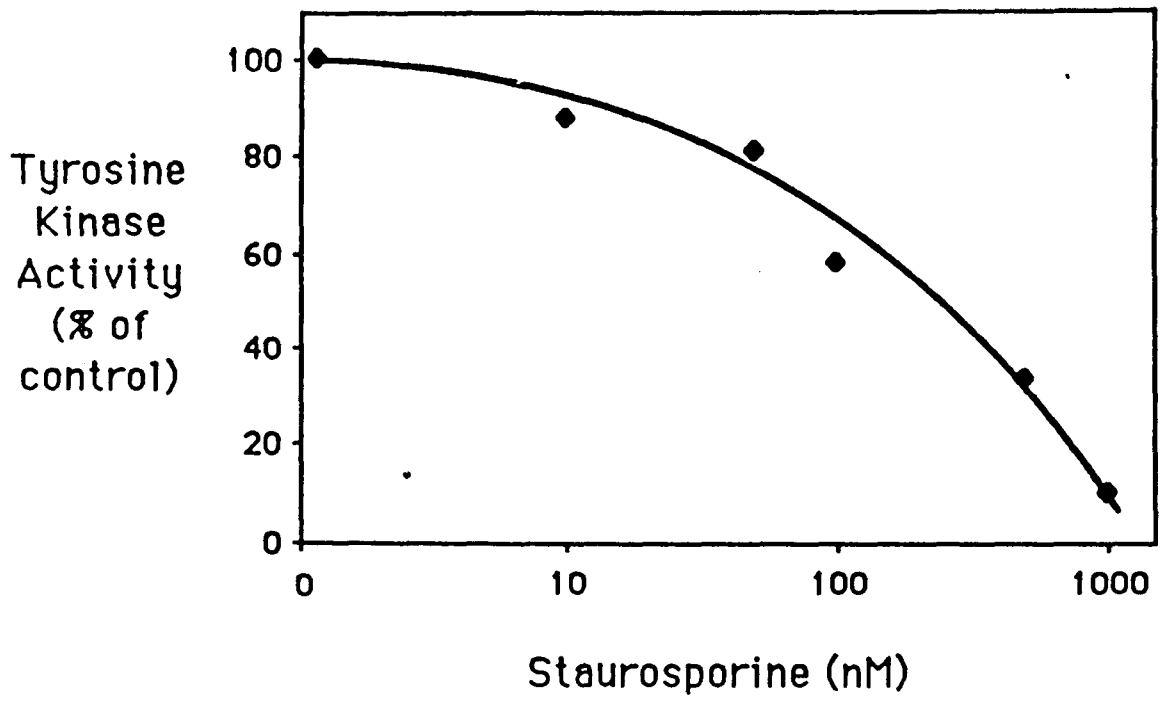
A) MeAIB Uptake

	Basal	Insulin	Effect of Insulin
	(nmol MeAIB/g muscle/30 min)		(%)
Control	25.2±1.3	41.0±2.1 *	63
Staurosporine	26.0±1.6	32.5±0.9 *+	25

B) Lactate Production

	Basal	Insulin	Effect of Insulin
	(µmol lactate/g muscle/h)		(%)
Control	4.8±0.4	10.7±0.8 *	123
Staurosporine	5.0±0.7	6.6±0.9 +	32

Results are means ± SE for 5-6 observations per group. EDL muscles were incubated for 180 minutes in the absence or presence of 100 nM insulin during the last 60 minutes of incubation. Muscles were incubated in the absence or presence of 1 µM staurosporine (added during the last 120 minutes of incubation). Staurosporine was dissolved in dimethyl sulfoxide, in a final concentration of 1%. Control muscles were also incubated in the presence of 1% dimethyl sulfoxide. MeAIB uptake and lactate production were assessed during the last 30 minutes of incubation as detailed in METHODS. * indicates a significant difference between basal and insulin groups, at $P<0.05$. + indicates a significant difference between control and staurosporine groups, at $P<0.05$.


Tyrosine Kinase Activity (% of control)
100
80
60
40
20
0
0
10
100
1000
Staurosporine (nM)

- E -

(Manuscrit sotmés a revisió al "Biochemical Journal")

Gumà, A., Castelló, A., Testar, X., Palacín, M., Zorzano, A.; Differential sensitivity of insulin- and adaptative regulation-induced system A activation to microtubular function in skeletal muscle.

DIFFERENTIAL SENSITIVITY OF INSULIN- AND ADAPTIVE REGULATION-INDUCED SYSTEM A ACTIVATION TO MICROTUBULAR FUNCTION IN SKELETAL MUSCLE.

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Running Title: Microtubules, microfilaments and System A in muscle

ABSTRACT.

1. Insulin and adaptive regulation are known to stimulate system A amino acid transport activity in skeletal muscle. The present study was designed to investigate whether activation of system A in muscle is a consequence of processes which rely upon microtubule or microfilament function. To that end, extensor digitorum longus (EDL) muscles were incubated in the presence of colchicin and cytochalasin D, well-known inhibitors of microtubule and microfilament activity, respectively. 2. Basal α-(methyl)aminoisobutyric acid (MeAIB) uptake decreased after incubation with 5 μM colchicin in a time-dependent manner. In keeping with this, adaptive regulation of MeAIB uptake caused by prolonged incubation in the absence of amino acids was substantially reduced in the presence of colchicin. 3. Under these conditions, stimulation of MeAIB uptake by insulin was unaltered in muscle in the presence of colchicin. This was opposed to the insulin-induced stimulation of MeAIB uptake by isolated rat hepatocytes, which was markedly reduced by colchicin. 4. Cytochalasin D, an agent that disrupts microfilaments did not inhibit basal or insulin-stimulated MeAIB uptake by the incubated muscle. 5. Neither colchicine nor cytochalasin D modified the stimulatory effect of insulin on 3-O-methylglucose uptake by EDL muscle. 6. We conclude that up-regulation of system A by synthesis of new carriers depends on the integrity of microtubular function both in skeletal muscle and in hepatocytes. Microtubules might play a role in the movement of system A-containing vesicles from the Golgi network to the plasma membrane.

INTRODUCTION.

Insulin is known to produce profound effects on membrane transport processes in a variety of tissues. In this regard, the most classical effect of insulin is the rapid activation of glucose transport that occurs in adipocytes and muscle. This activation is the result of a very complex process which, at least in isolated rat adipocytes, is partially due to the translocation of two different types of glucose transporters, GLUT-4 and GLUT-1 (Wheeler et al., 1982; Shanahan et al., 1982; James et al., 1988; Zorzano et al., 1989). Inhibition of microtubular function induced by colchicin in isolated rat adipocytes has been reported to delay somewhat the effect of insulin on glucose transport but it does not reduce the maximal stimulation induced by the hormone (Haring et al., 1979). In skeletal muscle, insulin has also been reported to promote the translocation of glucose carriers to surface membranes (Wardzala & Jeanrenaud, 1981, 1983; Klip et al., 1987; Hirshman et al., 1990).

Amino acid uptake is also modulated in response to insulin in hepatocytes and in skeletal muscle due to stimulation of system A transport activity (Kipnis & Noall, 1958; Kletzien et al., 1976). In liver, the stimulatory effect of insulin on system A amino acid transport activity is dependent on protein and RNA synthesis and microtubular function and this is characterized by an increased Vmax (Fehlmann et al., 1979; Prentki et al., 1981). On the other hand, insulin-induced stimulation of system A transport activity in skeletal muscle is rapid, characterized by an increased Vmax, and independent of protein synthesis and the Na^+ electrochemical gradient (Gumà et al., 1988). Data obtained in muscle are compatible with insulin acting directly on the system A transporter; however, it is not possible to prove whether insulin induces the translocation of system A carriers or activates intrinsic activity of transporters in the plasma membrane, since no tools for quantitation of these carriers are available. This requires the advance in our knowledge on the structure/function relationship of the A carrier. In this regard, recently published data, which might be useful to develop methods for the quantification of

system A, indicate that hepatic system A possesses essential hystidine and cysteine residues (Pola et al., 1990; Bertran et al., 1991) and that it is encoded by mRNA of 1.9-2.5 kb in length as judged by expression in Xenopus laevis oocytes (Palacín et al., 1990; Tarnuzzer et al., 1990).

Previous studies have described that glucagon- and insulin-induced stimulation of system A in hepatocytes is blocked by the addition of colchicin to the incubation medium (Prentki et al., 1981). In addition, insulin-stimulated α-aminoisobutyric acid uptake in KB cells -derived from human epidermoid carcinoma- was inhibited by pretreatment with agents disrupting microfilaments (Goshima et al., 1984). This allows us to postulate that microtubules or microfilaments might be essential to the arrival of newly synthesized system A carriers to the cell membrane. Thus, we have examined the sensitivity of system A to colchicin and cytochalasin D in response to insulin and to adaptive regulation in skeletal muscle. In addition, we have also examined whether insulin-induced stimulation of glucose transport is sensitive to these blockers.

EXPERIMENTAL PROCEDURES

Materials.

Porcine monocomponent insulin was a gift from Mr T.L. Jeatran, Eli Lilly & Co. Bovine serum albumin (fraction V albumin, fatty acid free), colchicin, cytochalasin D and most commonly used chemicals were from Sigma.

Muscle incubations.

Muscle studies were performed in extensor digitorum longus (EDL) muscles from male Wistar rats (50-70 g), obtained from our own colony. The rats were fed on Purina Laboratory chow ad libitum. Animals were housed in animal quarters maintained at 22°C with a 12 h-light/12 h-dark cycle. The dissection and isolation of the EDL muscle was carried out under anaesthesia with pentobarbital (5-7 mg/100 g body wt., intraperitoneally) as described previously (Maizels et al., 1977). The extensor digitorum longus muscle was fixed to a stainless-steel clip in order to maintain the muscle under slight tension (approximating to resting length) during the incubation. Such muscles (20-30 mg weight) are able to maintain normal ATP and creatine phosphate concentrations during a 3 h incubation.

EDL muscles were incubated in a shaking incubator (45 cycles/min) at 37°C for 3 h in 3 ml of Krebs-Henseleit buffer, pH 7.4 containing 5 mM glucose, 0.20% bovine serum albumin and 20 mM Hepes. Vials with intact muscles were gassed with 95% O_2 and 5% CO_2 during the whole incubation period. The incubation medium was kept for no longer than 90 min, and during prolonged incubations it was renewed thereafter. At different times, insulin (100 nM) was added to the incubation medium as well as several drugs such as colchicin or cytochalasin D (see details in Figure legends). Experimental series were performed by comparing biological activity of one muscle to the contralateral one from the same rat (paired muscles).

Measurements of amino acid and glucose uptake by muscle.

Amino acid uptake by system A was measured in EDL muscles using the non-metabolizable amino acid analog α-(methyl)aminoisobutyric acid (MeAIB). Following the incubations with insulin and the above mentioned agents, muscles were transferred to vials with 1.5 ml of Krebs-Henseleit buffer, pH 7.4 containing 5 mM glucose, 0.2% bovine serum albumin, 20 mM Hepes and 0.1 mM α-[1-^{14}C]-methylaminoisobutyric acid (800 uCi/mmol), 1 mM [^{3}H]-mannitol (330 uCi/mmol) and insulin and modulators at the same concentrations as for the preceeding incubation period. The vials were stoppered and incubated at 37°C in a shaking incubator. The uptake of MeAIB was linear with time for at least 30 min (data not shown). Therefore, in all subsequent studies, uptake was measured over a 30 min period. In experiments designed to measure 3-O-methylglucose uptake, muscles were incubated in Krebs-Henseleit buffer containing 2 mM pyruvate instead of glucose, and for the last 30 min of incubation the medium contained 0.1 mM [^{14}C]3-O-methylglucose (800 μCi/mmol) and 1 mM [^{3}H]-mannitol (330 uCi/mmol). Following incubation, muscles were digested in 0.25 ml of Protosol tissue solubilizer (New England Nuclear) and radioactivity of muscle digests and aliquots of the incubation media was measured. The amount of each isotope present in the samples was determined and this information was used to calculate the extracellular space. The extracellular space of EDL muscles, estimated after 30 min of ^{3}H-mannitol addition, was 0.222±0.003 ml/mg and 0.219±0.006 ml/mg in the absence and presence of insulin, respectively. Incubation in the presence of cytochalasin D or colchicin did not alter mannitol distribution space, under any of the conditions investigated (data not shown). Intracellular concentration of ^{14}C-amino acid analog or [^{14}C]3-O-methylglucose was calculated by subtracting its amount in the extracellular space from the total label found in tissue, as previously reported (Zorzano et al., 1985; Gumà et al., 1988). Muscle ATP and creatine phosphate concentrations were determined spectrophotometrically (Lamprecht &Stein, 1963; Lamprecht & Trautschold, 1963). Student's t test was used for statistical analysis.

Isolation of hepatocytes and incubation procedure.

Hepatocytes were isolated from fed male Wistar rats (200-250 g) by collagenase digestion of the liver as previously described (Katz et al., 1975). Hepatocytes were incubated at 37°C in Krebs-Henseleit bicarbonate buffer, pH 7.4 containing 1% BSA (dialyzed and fatty acid free) and bacitracin (0.8 mg/ml) in the presence of O_2/CO_2 (95:5). Colchicin was added to the cell suspension 30 min after the initiation of the incubation which was continued for 2.5 h. Insulin, when used, was added during the last 2 h of incubation.

Measurement of amino acid transport in hepatocytes.

After incubation for 3 h, hepatocytes were collected by centrifugation (600 x g for 10 s) and resuspended in Krebs-Henseleit bicarbonate buffer. Viability of hepatocytes was 88±1%, as assessed by trypan blue exclusion, after 3 h of incubation in the absence or presence of different hormones or blockers. MeAIB transport assays were initiated by the addition of 1.35 ml of the cell suspension (about 0.5-1 x 10^6 cells/ml) to 90 µl of Krebs-Henseleit bicarbonate buffer containing [^{14}C]MeAIB (0.125 µCi) to give a final concentration of 0.1 mM MeAIB. The tubes were incubated at 37°C and the reaction was terminated at different times by a 30 s centrifugation of 750 µl samples through 400 µl of an oil mixture of dinonylphthalate:dibutylphthalate (1:3, v/v) at 8,000 x g in a microcentrifuge. Following centrifugation, supernatants were obtained without removing oil and the radioactivity of one aliquot was counted. Tubes were then refilled with cold saline poured on top of the oil and saline plus oil were then discarded. The insides of the tubes above the cell pellets were thereafter wiped with cotton swabs. Cell pellets were then solubilized in 100 µl of 10% SDS for 2 h at room temperature and after addition of scintillation fluid their radioactivity was counted. Blank tubes were identical to the experimental ones, except that no cells were present. Blanks never exceeded 6% of total radioactivity taken up at 0.1 mM [^{14}C]MeAIB. In keeping with this, mannitol distribution space was only 0.8±0.1 µl/10^6 cells. Thus, the contamination of extracellular water in the cell pellet accounts for only 6% of basal MeAIB uptake, and therefore, it exerts a negligible effect on the measurement of MeAIB uptake. The

uptake of MeAIB was linear with time for at least 20 min (data not shown). Therefore in all subsequent studies transport was measured in triplicate over a 10 min period.

RESULTS.

Effect of colchicin on system A transport activity in muscle and hepatocytes.

Incubation of extensor digitorum longus (EDL) muscles in the presence of 5 μM colchicin for the last 120 or 180 min, caused a progressive decrease in basal MeAIB uptake (Table 1). This occurred in the absence of changes in the intracellular muscle concentration of ATP (3.4 and 3.0 μmol/g in control and colchicin-treated groups, respectively) or creatine phosphate (19.3 and 18.4 μmol/g in control and colchicin-treated groups, respectively). In spite of such an inhibition of basal system A transport activity, the stimulatory effect of insulin on MeAIB uptake was not altered to any extent in the presence of 5 μM colchicin (Table 1). Basal MeAIB uptake was not inhibited at 0.5 μM colchicin (data not shown).

In skeletal muscle, system A transport activity can be activated by adaptive regulation, that is, by amino acid starvation (Guidotti et al., 1975; Le Marchand-Brustel et al., 1982; Logan et al., 1985; Gumà et al., 1988). Therefore, we investigated whether the effect of colchicin on basal MeAIB uptake was a consequence of a blockade of adaptive regulation. To that end, EDL muscles were incubated for increasing periods of time (90 or 180 min) in the absence of amino acids in the medium and MeAIB uptake was assayed thereafter. Basal MeAIB uptake was significantly stimulated by increasing the total incubation time from 90 to 180 min (Table 2). Under these conditions, colchicin partially prevented such an increase and in fact, differences in MeAIB uptake between muscles incubated for 90 or 180 min in the presence of the blocker were insignificant (Table 2). Similar data were obtained when muscles were incubated in the presence of insulin. Thus, under these conditions a 56% increase in MeAIB uptake by EDL muscle was observed by increasing the total incubation time from 90 to 180 min (Table 2). However, in the presence of colchicin, the increase due to adaptive regulation was only 17% (Table 2). Again, under none of these conditions was the insulin effect blocked by the presence of colchicin (Table 2).

To further determine whether there is a relationship between the dependence of system A up-regulation on protein synthesis and on microtubular function, we investigated the stimulatory effect of insulin on system A in hepatocytes, which is a protein synthesis-dependent process (Fehlman et al., 1979). To that end, hepatocytes were incubated in the presence of insulin for 120 min and transport of the specific analog MeAIB was estimated. Under these conditions, insulin caused a two-fold increase in MeAIB transport (Table 3). Na^+-independent MeAIB uptake was 0.22 ± 0.02 nmol/10^6 cells/10 min -it only accounted for 18% of total MeAIB uptake, in agreement with previous observations (Kilberg et al., 1981)- and it was not influenced by insulin. When colchicin was present 30 min before insulin addition, the stimulation caused by insulin was largely prevented (Table 3). This is in keeping with a previous report indicating that colchicin blocks the activation of insulin and glucagon on α-aminoisobutyric acid (AIB) transport by isolated rat hepatocytes (Prentki et al., 1981).

Effect of cytochalasin D on MeAIB uptake by muscle.

It has been previously reported that in KB cells (a cell line derived from human epidermoid carcinoma), AIB uptake stimulated by insulin is inhibited by pretreatment with cytochalasin D, a blocker of microfilament function (Goshima et al., 1984). Based on this report and in order to further investigate the mechanisms by which insulin stimulates system A in skeletal muscle, we tested the effect of 50 µM cytochalasin D on MeAIB uptake by EDL muscle. This concentration is high enough to exert maximal inhibitory effects in cultured cells or tissues (Goshima et al., 1984; Huleux et al., 1989). Data are shown in Table 4. Under basal conditions, insulin caused a 2-fold increase in the rate of MeAIB uptake. However, neither basal nor insulin-stimulated MeAIB uptake by muscle was altered to any extent by the presence of cytochalasin D. Furthermore, 50 µM cytochalasin D did not alter either intracellular muscle concentration of ATP (3.2 and 3.1 µmol/g in control and cytochalasin D-treated groups, respectively) or creatine phosphate (17.1 and 18.9 µmol/g in control and cytochalasin D-treated groups, respectively).

Effect of colchicin and cytochalasin D on insulin-stimulated 3-O-methylglucose uptake by EDL muscle.

Insulin is known to stimulate glucose uptake and glucose transporter translocation in skeletal muscle (Wardzala & Jeanrenaud, 1981, 1983; Klip et al., 1987; Hirshman et al., 1990). To address whether insulin-induced stimulation of glucose transport was dependent on microtubule or microfilament function, in a further set of experiments we investigated the effect of insulin on 3-O-methylglucose uptake by EDL muscles previously incubated in the presence of 5 μM colchicin or 50 μM cytochalasin D (Table 5). Under control conditions, insulin caused a 125% increase in 3-O-methylglucose uptake by muscle. Both basal and insulin-stimulated 3-O-methylglucose uptakes were unaltered by the presence of colchicin or cytochalasin D (Table 5).

DISCUSSION.

Our results demonstrate that adaptive regulation of system A transport activity is sensitive to blockade of microtubule function, in contrast to the stimulatory effect of insulin which is clearly insensitive to colchicin. This is parallel to the differences previously reported regarding the sensitivity of insulin or adaptive regulation to blockers of protein synthesis. Thus, whereas amino acid deprivation causes the activation of system A transport activity by a process which is blocked by inhibition of protein synthesis (Kelley & Potter, 1978; Handlogten et al., 1982), insulin effect in muscle is independent of protein synthesis (Elsas et al., 1968; Le Marchand-Brustel et al., 1982; Gumà et al., 1988). These results further demonstrate that insulin causes the activation of system A transport activity in muscle and liver by completely different mechanisms. Thus, insulin activates system A in rat hepatocytes by a process dependent on protein and RNA synthesis and on microtubular function (Fehlmann et al., 1979; Prentki et al., 1981). In contrast, insulin stimulates system A in muscle by a process independent of protein synthesis (Elsas et al., 1968; Le Marchand-Brustel et al., 1982; Gumà et al., 1988) and microtubular function.

Up-regulation of system A provoked in muscle by adaptive regulation and in hepatocytes by insulin or glucagon share a common property, that

is, the dependence to transcription, protein synthesis and microtubular function. Regarding the stimulatory effect of glucagon on system A transport activity in hepatocytes, it has been reported a) that this effect is a consequence of a greater availability of the corresponding mRNA (Palacín et al., 1990; Tarnuzzer et al., 1990), and b) that the enhanced transport activity can be recovered in proteoliposomes generated from hepatocytes plasma membranes (Bracy et al., 1987). In all, these data support the proposal that glucagon regulates the hepatic system A gene resulting in the novo synthesis of carriers followed by their transport to the plasma membrane. In this regard, it might be proposed that the process by which carriers move from Golgi apparatus to plasma membrane relies upon an intact microtubular function. Colchicin also prevents the increased capacity for amino acid transport of concanavalin A-activated lymphocytes during the replicative phase (Greene et al., 1976), of proliferating hepatocytes after partial hepatectomy (Walker et al., 1978) or of Morris hepatoma cells (Tauber & Reutter, 1980). Therefore, one may postulate that when activation of amino acid transport is due to the arrival of newly synthesized carriers to the plasma membrane, the vectorial movement of vesicles containing system A or other amino acid carriers towards the cell surface is dependent to microtubular function. It is unlikely that microtubules play a role in the transfer of intracellular information triggered by such different signals as insulin, hepatectomy, transformation or amino acid starvation.

In this study, we present evidence that microfilaments are not involved in the up-regulation of system A by insulin or adaptive regulation in skeletal muscle. In this regard, insulin-stimulated system A transport activity is blocked in KB cells by pretreatment with cytochalasin D (Goshima et al., 1984), perhaps due to the inhibition of insulin-induced formation of ruffling membranes. However, this may not be relevant to muscle since muscle cells do not show ruffling movements. For all the above, we favor the view that the mechanisms involved in the modulation of system A differ widely according to cell type.

Based on data obtained in isolated rat adipocytes (Haring et al., 1979) and in our own from the incubated skeletal muscle, we conclude that insulin-activated glucose transport is not dependent on microtubule

or microfilament function. That suggests that blockers of microtubules or microfilaments do not inhibit insulin-mediated glucose transporter translocation. The independence of insulin-stimulated glucose transporter translocation and microtubule or microfilament function, might be due to the fact that transporter-containing intracellular vesicles are located near the cell surface.

We have previously suggested that insulin rapidly stimulates system A transport activity either by stimulating the intrinsic activity of A carriers or by promoting translocation of carriers previously stored in an intracellular location. Based on the previous reasoning, the independence of insulin-stimulated system A in skeletal muscle of microtubule or microfilament function does not rule out a possible insulin-induced carrier translocation.

In summary, our results demonstrate the existence of significant differences between up-regulation of system A due to synthesis of new carriers -adaptive regulation in skeletal muscle or insulin effect in hepatocytes- and amino acid stimulation due to a rapid process independent of protein synthesis -insulin effect in skeletal muscle. Thus, up-regulation of system A dependent of protein synthesis -probably due to synthesis of new carriers- is sensitive to inhibitors of microtubular function, whereas up-regulation independent of protein synthesis is unsensitive to such inhibitors. Data are consistent with colchicin preventing the arrival of newly synthesized A carriers to the cell surface both in response to adaptive regulation in muscle and in response to insulin in the hepatocyte. Furthermore, our data also reveal that inhibitors of microfilaments or microtubules do not block insulin-stimulated glucose transport. Therefore, it is highly unlikely that microtubules or microfilaments are involved in translocation of glucose transporters from an intracellular site to surface membranes in skeletal muscle. This suggests a possible insulin-modulated translocation of amino acid or glucose carriers previously located near the surface membranes in muscle.

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Table 1

EFFECT OF COLCHICIN ON MeAIB UPTAKE BY EDL MUSCLE

	Basal	Insulin	Effect of Insulin
	(nmol MeAIB/g/ 30 min)		(% of increase over basal)
Control	27.5±2.2	41.9±3.5 +	52
Colchicin 5μM, 120 min	20.8±2.6	37.7±4.3 +	81
Colchicin 5 μM, 180 min	17.4±1.7 *	34.5±2.9 +	98

Results are means ± SE for 21-23 observations per group. EDL muscles were incubated for 180 min in the absence or presence of 100 nM insulin for the last 60 min of incubation. Muscles were incubated in the absence or presence of 5 μM colchicin, which was added either throughout the whole incubation period or during the last 120 min of incubation. MeAIB uptake was determined during the last 30 min of incubation. * indicates a significant difference between control and colchicin groups, at $P < 0.05$. + indicates a significant difference between basal and insulin groups, at $P < 0.05$.

Table 2

EFFECT OF COLCHICIN ON ADAPTIVE REGULATION OF SYSTEM A BY EDL MUSCLE

Insulin (nM)	Duration of experiment (min)	Control (nmol MeAIB/g/ 30 min)	Colchicin (nmol MeAIB/g/ 30 min)
0	90	25.9±1.6	21.0±1.4
0	180	34.2±1.8	25.5±1.9 *
100	90	39.1±3.9 +	38.5±4.2 +
100	180	60.9±3.1 +	45.0±2.5 * +

Results are means ± SE for 9-11 observations per group. EDL muscles were incubated for 90 or 180 min in the absence or presence of 100 nM insulin for the last 60 min of incubation. Muscles were incubated in the absence or presence of 5 μM colchicin, which when present was added throughout the whole incubation period. MeAIB uptake was determined during the last 30 min of incubation. * indicates a significant difference between control and colchicin groups, at $P < 0.05$. + indicates a significant difference between basal and insulin groups, at $P < 0.05$.

Table 3

EFFECT OF COLCHICIN ON MeAIB UPTAKE BY ISOLATED RAT HEPATOCYTES

	Basal	Insulin		Effect of Insulin
	(nmol MeAIB/10^6. 10 min)			(% of increase over basal)
Control	1.22±0.18	2.54±0.13	+	108
Colchicin 0.5μM, 120 min	1.39±0.22	1.78±0.07	*	28

Isolated rat hepatocytes were incubated for 180 min in the absence or presence of 100 nM insulin and 0.5 μM colchicin. Cells were then incubated in the presence of [^{14}C]-MeAIB (0.1 mM, 0.125 μCi) for 10 min. * indicates a significant difference between control and colchicin groups, at $P < 0.05$. + indicates a significant difference between basal and insulin groups, at $P < 0.05$. Each point is the mean of three different experiments performed in triplicate.

Table 4

EFFECT OF CYTOCHALASIN D ON MeAIB UPTAKE BY EDL MUSCLE

	Control	Cytochalasin D
	(nmol MeAIB/g/ 30 min)	
Basal	19.3±2.2	23.6±2.5
Insulin	39.8±2.8 *	36.2±1.7 *

Results are means ± SE for 6-8 observations per group. EDL muscles were incubated for 180 min in the absence or presence of 100 nM insulin during the last 60 min of incubation. Muscles were incubated in the absence or presence of 50 μM cytochalasin D, which when present was added during the whole incubation period. MeAIB uptake was determined during the last 30 min of incubation. * indicates a significant difference between basal and insulin groups, at $P < 0.05$. Differences between control and cytochalasin D groups were insignificant.

Table 5

EFFECT OF COLCHICIN AND CYTOCHALASIN D ON 3-O-METHYLGLUCOSE UPTAKE BY EDL MUSCLE

	Basal	Insulin
	(nmol 3-O-methylglucose/g/30 min)	
Control	7.8±0.5	17.6±1.0 *
Colchicin	8.7±1.1	17.7±1.0 *
Cytochalasin D	8.6±0.9	16.3±1.9 *

Results are means ± SE for 5-10 observations per group. EDL muscles were incubated for 180 min in the absence or presence of 100 nM insulin during the last 60 min of incubation. Muscles were incubated in the absence or presence of 5 μM colchicin or 50 μM cytochalasin D, which were added during the whole incubation period. 3-O-methylglucose uptake was determined during the last 30 min of incubation. * indicates a significant difference between basal and insulin groups, at $P < 0.05$. Differences between control and experimental groups were insignificant.

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(Manuscrit sotmés a revisió al "Biochemical Journal")

Gumà, A., Viñals, F., Palacín, M., Testar, X., Zorzano, A.; Regulation of system A amino acid transport activity by phospholipase C, pertussis toxin and cAMP-inducing agents in skeletal muscle. Modulation of insulin action.

REGULATION OF SYSTEM A AMINO ACID TRANSPORT ACTIVITY BY PHOSPHOLIPASE C, PERTUSSIS TOXIN AND cAMP-INDUCING AGENTS IN SKELETAL MUSCLE. MODULATION OF INSULIN ACTION.

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ABSTRACT.

1. The interaction between pertussis toxin-sensitive G-proteins, diacylglycerol- or cAMP-inducing agents and System A transport activity in skeletal muscle is unknown. Thus, the present study was designed to investigate the effect of phospholipase C, pertussis toxin and different compounds known to promote synthesis of cAMP on System A and glucose transport activities under basal and insulin-stimulated conditions in the incubated muscle. 2. Phospholipase C caused a marked stimulation on alpha-(methyl)aminoisobutyric acid (MeAIB) and 3-0-methylglucose uptake by the incubated muscle. In contrast, the activatory effects of insulin on System A transport activity was largely inhibited by phospholipase C. 3. The effects of phospholipase C on transport processes differ from the effects provoked by phorbol esters (TPA), and therefore are not just a consequence of TPA-sensitive protein kinase C activation. In addition, the effect of phospholipase C on MeAIB uptake was not affected by the presence of verapamil, an inhibitor of voltage-dependent Ca^{2+} channels or by dantrolene-induced inhibition of Ca^{2+} release from the sarcoplasmic reticulum. 4. Pertussis toxin did not modify the uptake of MeAIB or 3-0-methylglucose under basal or insulin-stimulated states. 5. Agents such as isoproterenol, cholera toxin or forskolin, known cAMP inducers, did not alter basal or insulin-stimulated MeAIB uptake. Although basal 3-0-methylglucose uptake was decreased by isoproterenol, this agonist did not affect maximal stimulation of 3-0-methylglucose uptake in the presence of insulin. 6. Our data indicate that System A transport activity is activated by phospholipase C in skeletal muscle, and this effect is not due to diacylglycerol-mediated activation of protein kinase C. The effect of insulin on System A transport activity, is reduced in the presence of phospholipase C, which is mimicked by TPA, suggesting the mediation of protein kinase C. Based on the lack of effect of pertussis toxin or cAMP-inducing agents on insulin-stimulated System A, we conclude that this effect of insulin is not mediated by pertussis toxin-sensitive G-proteins and that cAMP-dependent protein kinase does not

cause any generalized blockade of insulin action in skeletal muscle, in contrast to what has been reported in other cell types.

INTRODUCTION.

The System A carrier is a plasma membrane-bound activity which translocates short polar, straight chain amino acids, including the non metabolizable analog 2-(methylamino)isobutyric acid. System A transport activity is subjected to hormonal regulation, trans-inhibition, and adaptive regulation in a variety of cell types (1-3). In skeletal muscle, System A transport activity is stimulated in response to amino acid starvation -adaptive regulation- (4, 5) by a mechanism that requires protein synthesis and unaltered microtubular function (6). On the other hand, System A transport activity is rapidly activated in skeletal muscle by insulin (7, 8), acute exercise (9, 10), vanadate or raising of intracellular pH (11). The effect of insulin on System A transport activity in muscle is characterized by its independence of protein synthesis (8, 12), microtubular function (6) and the sodium electrochemical gradient (8, 10), and it does not involve any modification of muscle intracellular pH (11). The scarcity of information regarding the mechanisms by which System A is modulated, is based on the lack of information both at the protein and at the mRNA levels.

There is also paucity of information on the mechanisms by which insulin-induced activation of System A transport activity is regulated in skeletal muscle. In this regard, insulin effect on System A transport activity is markedly reduced in skeletal muscle by prior treatment with phorbol esters (13) under conditions which do not modify tyrosine kinase activity of insulin receptors or insulin effect on glucose transport (13). That allows the proposal that activation of TPA-sensitive protein kinase C isoforms must be involved in the regulation of some specific effects of insulin in skeletal muscle. No information is currently available regarding the impact of the activation of cAMP-dependent protein kinase in muscle on basal or insulin-stimulated System A transport activity in muscle and contradictory findings have been reported regarding the effect of adrenergic agonists on glucose transport by muscle (14-17).

It has recently reported that phospholipase C rapidly activates glucose transport in the incubated rat muscle (19-21). Based on the parallelism previously detected under a variety of conditions between the regulation of glucose utilization and System A transport activity in skeletal muscle (9, 10, 18), in this communication we have investigated whether phospholipase C also activates System A in muscle. In addition, we have attempted to define further possible mechanisms of modification of System A transport activity in skeletal muscle. To that end, we have tested the effects of pertussis toxin or cAMP-inducing agents on the modulation of insulin-stimulated System A transport activity. In parallel, we have investigated the effects of these agents on glucose transport activity by the incubated muscle.

MATERIALS AND METHODS.

Materials.

Porcine monocomponent insulin was a gift from Mr. T.L. Jeatran, Eli Lilly & Co. 1-^{14}C]-2-(methylamino)isobutyric acid, [^{14}C]-sorbitol, [^{3}H]-mannitol and [^{14}C]-3-O-methylglucose were obtained from New England Nuclear. Bovine serum albumin (fraction V albumin, fatty acid free), phospholipase C (Type XIV from C perfringens), pertussis toxin, cholera toxin, isoproterenol, dantrolene, verapamil, 12-0-tetradecanoyl-13-acetate and most commonly used chemicals were from Sigma.

Animals and dissection procedures.

Male Wistar rats (50-60 g), obtained from our own colony were used. The rats were fed on Purina Laboratory chow ad libitum. Animals were housed in animal quarters maintained at 22°C with a 12 h-light/12 h-dark cycle. The dissection and isolation of the extensor digitorum longus and soleus muscle was carried out under anesthesia with pentobarbital (5-7 mg/100 g body wt., intraperitoneally) as described previously (22). The isolated muscle was fixed to a stainless-steel clip in order to maintain the muscle under slight tension (approximating the resting length) during the incubation. Such muscles (20-30 mg weight) are able to maintain normal ATP and creatine phosphate concentrations during a 3 h incubation.

Incubations.

Muscles were incubated in a shaking incubator at 37°C for 3 h in 3 ml of Krebs-Henseleit buffer, pH 7.4 containing 5 mM glucose, 0.20% bovine serum albumin and 20 mM Hepes. After addition of the muscles to the vials, they were stoppered and placed in a Dubnoff metabolic shaker set at 37°C and a shaking rate of 60 cycles/min. Vials were gassed with 95% O_2 and 5% CO_2 throughout the incubation period. The incubation medium was kept for no longer than 90 min, and during

prolonged incubations it was renewed every 90 min. At different times, phospholipase C, insulin as well as several drugs such as 12-0-tetradecanoyl-13-acetate, verapamil, dantrolene, isoproterenol, cholera toxin, pertussis toxin or forskolin were added to the incubation medium (see details in Figure legends). Experimental series were performed by comparing biological activity of one muscle to the contralateral one from the same rat (paired muscles).

Measurement of amino acid and glucose uptake and by muscle.

Amino acid uptake by system A was measured in the incubated muscle using the non-metabolizable amino acid analog 2-(methylamino)isobutyric acid (MeAIB). Following the incubation with the above mentioned agents, muscles were transferred to vials with 1.5 ml of Krebs-Henseleit buffer, pH 7.4 containing 5 mM glucose, 0.20% bovine serum albumin, 20 mM Hepes and 0.1 mM [1-^{14}C]-2-(methylamino)isobutyric acid (800 μCi/mmol), 1 mM [^{3}H]-mannitol (330 μCi/mmol) and the diferent modulators at the same concentrations as for the preceeding incubation period. The vials were stoppered and incubated at 37°C in a shaking incubator for 30 min. The gas phase in the vials was 95% O_2 and 5% CO_2. In experiments designed to measure 3-O-methylglucose uptake, muscles were incubated in Krebs-Henseleit buffer containing 2 mM pyruvate instead of glucose, and for the last 30 min of incubation, the medium contained 0.1 mM [^{14}C]-3-O-methylglucose (800 μCi/mmol) and 1 mM [^{3}H]-mannitol (330 μCi/mmol). Following incubation, muscles were digested in 0.25 ml of 0.5 M Protosol tissue solubilizer (Dupont) and radioactivity of muscle digests and aliquots of the incubation media was counted. The amount of each isotope present in the samples was determined and this information was used to calculate the extracellular space. In some experiments, the extracellular space was determined by using [^{14}C]-sorbitol as a probe. Intracellular concentration of ^{14}C-amino acid analog or [^{14}C]-3-O-methylglucose was calculated by subtracting its amount in the extracellular space from the total label found in tissue, as previously reported

(18). In some experimental series, lactate release to the incubation media was measured for the last 30 min of incubation as in (23) or glycogen concentration was measured in muscles after various times of incubation as previously reported (23). Student's t test for paired data was used for statistical analysis.

RESULTS.

Effects of phospholipase C on alpha-(methyl)aminoisobutyric acid (MeAIB) and 3-0-methylglucose uptake by the incubated muscle.

To determine whether phospholipase C alters system A transport activity we incubated extensor digitorum longus muscles in the presence of 0.1 U/ml phospholipase C from Clostridium perfringens for 90 min. This concentration of phospholipase C did not affect mannitol distribution space (0.26±0.01 and 0.30±0.01 ml/g, in control and phospholipase C groups, respectively) or sorbitol distribution space (0.31±0.01 and 0.33±0.02 ml/g, in control and phospholipase C groups, respectively). However, higher concentrations of phospholipase C caused a marked increase in mannitol distribution space (data not shown) and therefore such high concentrations were avoided.

Phospholipase C (0.1 U/ml) caused a marked stimulation of 3-0-methylglucose uptake (Table 1), in keeping with previous observations (20, 21). In fact, the stimulatory effect of phospholipase C was quantitatively as important as the one caused by a supramaximal concentration of insulin (Figure 1); however, no additive effects of phospholipase C and insulin were detected, also in keeping with previous observations (21). Under these conditions, phospholipase C caused the stimulation of System A transport activity by the incubated muscle, as assessed by MeAIB uptake (Table 1). The effect of phospholipase C on System A (44% increase) was lower than the effect provoked by insulin (103% increase). Furthermore, in the presence of phospholipase C, subsequent addition of insulin did not cause any further stimulation of MeAIB uptake (Table 1), indicating a blockade of insulin action. This agrees with the report by Henriksen et al. (20) that phospholipase C and insulin, both a submaximal concentrations, did not display additive effects on 3-0-methylglucose uptake.

Phospholipase C increases the concentration of diacylglycerol in muscle, and therefore this might activate protein kinase C activity. However, the effect of phospholipase C on System A activity was unlikely only due to the stimulation of

protein kinase C. Supporting this view we found that incubation of muscles in the presence of TPA did not cause any significant stimulation ol MeAIB uptake (Table 2). TPA caused a marked inhibition of insulin-stimulated MeAIB uptake by muscle, although the extent of the inhibition was not so great as the effect displayed by phospholipase C (Table 1). Further support to the concept that the effects of phospholipase C are not only due to activation of protein kinase C comes from experiments in which 3-0-methylglucose uptake was measured (Table 2). Thus, basal 3-0-methylglucose uptake was somewhat stimulated by TPA but to a much lesser extent than insulin, in contrast to what found for phospholipase C (Table 2).

There is some discrepancy regarding the mechanism by which phospholipase C stimulates glucose transport in skeletal muscle, and whereas some authors have reported that the activation is substantially inhibited by blockade of Ca^{2+} release from sarcoplamic reticulum (20), Sowell et al. (24) have failed to reproduce these findings. Therefore, in a further step we investigated whether the stimulatory effect of phospholipase C on System A transport activity was sensitive to modifiers of Ca^{2+} cell economy. Inhibition of voltage-dependent Ca^{2+} channels, by addition of verapamil, caused a decrease of MeAIB uptake (approximately 20% decrease) both in the absence or presence of insulin or phospholipase C (Table 3). However, verapamil did not alter the stimulation of MeAIB uptake in response to insulin or phospholipase C (Table 3). The dependence of phospholipase C action on Ca^{2+} release from sarcoplasmic reticulum was next investigated using dantrolene, an inhibitor of Ca^{2+} release from sarcoplasmic reticulum (Table 4). This drug did not cause any inhibition of insulin- or phospholipase C-stimulated MeAIB uptake by the incubated muscle (Table 4).

Effects of pertussis toxin on MeAIB and 3-0-methylglucose uptake by skeletal muscle.

Previous reports have suggested that some effects of insulin are sensitive to treatment with pertussis toxin (25, 26). Therefore, we attempted to determine

whether insulin-stimulated MeAIB or 3-0-methylglucose uptake by muscle was inhibited by prior incubation in the presence of this bacterial toxin.

Incubation of muscles in the presence of concentrations of pertussis toxin as high as 200 ng/ml for 90 min did not cause any significant modification of either basal or insulin-stimulated MeAIB uptake (Table 5). Pertussis toxin caused an increase in the basal rate of 3-0-methylglucose uptake by muscle (Table 6); however, differences disappeared in the presence of insulin (Table 6). The effect of pertussis toxin on basal glucose transport was not mimicked by incubation of muscles in the presence of phentolamine, an alpha-adrenergic antagonist (Table 6).

Effect of cAMP-inducing agents on MeAIB and 3-0-methylglucose uptake by skeletal muscle.

We next determined whether agents that increase intracellular levels of cAMP modulate amino acid and glucose transport by muscle, or whether they compromise insulin action on these membrane processes. To that end, we investigated the effect of isoproterenol, cholera toxin or forskolin on MeAIB and 3-0-methylglucose uptake by the incubated muscle. In a preliminary set of experiments we observed that muscle incubation in the presence of isoproterenol, cholera toxin or forskolin caused a significant drop in muscle glycogen content (Table 7), which agrees with the activation of glycogen phosphorylase activity in the presence of high cAMP concentrations. Furthermore, isoproterenol and forskolin stimulated the rate of muscle lactate production, also indicating that these agents were indeed enhancing cAMP levels. The effect of forskolin on lactate production was not tested, since forskolin is also known to inhibit glucose transport activity (27).

Incubation in the presence of isoproterenol for 90 or 180 min did not modify basal or insulin-stimulated MeAIB uptake (Table 8). This lack of effect was not consequence of interference due to the presence of alpha-adrenergic agonists or

adenosine in the incubation medium. Thus, no effects of isoproterenol on MeAIB were detected in the presence of phentolamine (an alpha-adrenergic antagonist) or in the presence of adenosine deaminase (Table 8). Furthermore, the incubation of muscles in the presence of cholera toxin or forskolin, under conditions that allowed activation of glycogen degradation and acceleration of lactate production (Table 7) did not modify basal or insulin-stimulated MeAIB uptake by muscle (Table 9).

Incubation with isoproterenol for 180 min caused a decrease in the basal uptake of 3-0-methylglucose by the incubated muscle (Table 10), although the uptake maximally stimulated by insulin was not modified (Table 10). However, this effect was not observed when cholera toxin was added to the medium, and in fact, no significant changes in basal or insulin-stimulated 3-0-methylglucose uptake were detected (Table 10).

DISCUSSION.

The results of the present study demonstrate that phospholipase C stimulates two transport processes that are also activated by insulin in skeletal muscle, i.e., System A as well as glucose transport activities. Based on the fact that phospholipase C and insulin share in common their capacity to significantly increase the 1,2-diacylglycerol content in several cell types or tissues (21, 24, 28-30), that has allowed to propose that diacylglycerol might be an intracellular mediator of insulin, perhaps contributing to some extent to the activation of transport processes. However, further experimental work is required to demonstrate this concept.

In fact, the mechanism by which phospholipase C exerts its biological actions is unknown. There is some controversy on whether phospholipase C stimulates glucose transport in skeletal muscle through modifications of intracellular Ca^{2+}. Thus, whereas some investigators have reported that phospholipase C effect on glucose transport is inhibited by blocking Ca^{2+} release from sarcoplasmic reticulum (20), other have not reproduced this finding (24). In the present study, we have substantiated that the stimulatory effect of phospholipase C on System A transport activity is independent of voltage-sensitive Ca^{2+} channels or of Ca^{2+} release from sarcoplasmic reticulum.

Phospholipase C might also mediate its effects on glucose or amino acid transport, via diacylglycerol-induced activation of certain isoforms of protein kinase C (21). We have found that phorbol esters, known activators of protein kinase C, do not mimic all the effects of phospholipase C in skeletal muscle. Thus, the activatory effect of phorbol esters on glucose transport is very limited compared to the effect of phospholipase C. Furthermore, whereas phorbol esters do not activate System A transport activity (13), phospholipase C exerts a marked activation on the activity of this amino acid transport system. In contrast, some effects of phorbol esters and phospholipase C are similar. Thus, both treatments lead to inhibition of insulin-stimulated System A transport activity (13).

In all, our data allow us to propose that phospholipase C mediates some of its effects via an increase in cellular content of diacylglycerol and further activation of protein kinase C activity, whereas some other effects are unrelated to activation of protein kinase C. Whether the other effects of phospholipase C are due to modifications of biophysical properties of plasma membrane or to unknown effects of diacylglycerol (independent of protein kinase C activation), remains to be determined. Supporting this latter contention, diacylglycerol has been shown to activate glucose transport in isolated rat adipocytes by a mechanism which does not involve activation of protein kinase C and is not related to translocation of glucose transporters (31).

In any case, it should be pointed out that phospholipase C does not completely mimic the effect of insulin stimulating glucose transport. Thus, phospholipase C does not translocates glucose carriers from an intracellular site to the plasma membrane (19), in contrast to the rapid translocation of GLUT-4 and GLUT-1 glucose transporters induced by insulin in isolated rat adipocytes (32).

There is certain evidence suggesting some interaction between insulin and G-proteins. This is based on the following findings, a) some effects of insulin are sensitive to pertussis toxin, an agent which ADP-ribosylates and inactivates some G-proteins, in several cell types (26, 33-36), b) G-proteins undergo tyrosine phosphorylation by the purified insulin receptors in solubilized preparations and in phospholipid vesicles (37-39), c) insulin inhibits pertussis toxin-catalyzed ADP-ribosylation of G-proteins (40), and d) there is a loss of functional Gi in insulin-resistant states (41). In our study, we have provided evidence that pertussis toxin treatment of incubated muscles, does not alter the effect of insulin stimulating glucose and System A transport activities. This agrees with previous observations performed in isolated cardiac myocytes, in which pertussis toxin did not alter insulin-stimulated glucose transport (42) and suggests that the effect of insulin stimulating glucose transport in muscle is not mediated by a pertussis toxin-sensitive G-protein. However, that does not excludes the involvement of

GTP binding proteins in the signal transduction of insulin stimulating glucose transport, and in this regard, it has been recently reported that nonhydrolyzable GTP analogs induce translocation of GLUT-4 glucose transporters in isolated rat adipocytes (43).

Agents such as isoproterenol, forskolin or cholera toxin, that cause increase in the intracellular concentration of cAMP, and subsequent activation of glycogen degradation and glycolysis in skeletal muscle, did not alter basal transport activity of System A. This is opposed to what occurs in other cell types, such as in the hepatocyte, where hormones that induce cAMP levels or cAMP analogs cause a marked activation of System A transport activity, by mechanisms that are dependent on microtubulat function, RNA and protein synthesis (44, 45). Therefore, we conclude that the mechanisms linking cAMP induction to activation of System A transport activity in the hepatocyte are not active in skeletal muscle.

Furthermore, it has been previously reported that an increase in the intracellular concentration of cAMP negatively modulates insulin action in a variety of cell types. Thus, some cell types respond to increased cAMP levels by inhibiting insulin receptor kinase activity (46, 47) and, in fact, cAMP-dependent protein kinase activity has been found to phosphorylate and to inhibit tyrosine kinase activity of purified insulin receptors (48, 49). In this study, we have found that incubation of muscles in the presence of cAMP-inducing agents does not cause any modification of insulin-stimulated glucose or amino acid uptake. The lack of effect of insulin-induced stimulation of 3-0-methylglucose transport agrees with previous observations performed in perfused muscle and in the incubated muscle preparation (16, 17). Therefore, the inhibitory effects of catecholamines or ß-adrenergic agonists on insulin-stimulated glucose uptake or glycogen synthesis, previously reported in muscle (16, 17, 50), must be specifically due to the effect of catecholamines increasing the intracellular concentration of glucose-6-phosphate subsequent to the activation of glycogen

breakdown. Based on all the above considerations, we propose a) that it is unlikely that elevated concentrations of cAMP lead to inhibition of tyrosine kinase activity of the insulin receptor and generalized inhibition of insulin action in muscle, and b) that the pathway/s involved in insulin-induced stimulation of glucose or amino acid transport processes are not regulated by increasing cAMP concentrations in skeletal muscle.

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Table 1

EFFECT OF PHOSPHOLIPASE C ON MeAIB AND 3-0-METHYLGLUCOSE UPTAKE BY MUSCLE

A) MeAIB UPTAKE

	CONTROL	PHOSPHOLIPASE C
	(nmol MeAIB./g/30 min)	
BASAL	11.7±0.9	16.8±1.1 *
INSULIN	23.8±2.9 +	16.4±1.2 *

B) 3-0-METHYLGLUCOSE UPTAKE

	CONTROL	PHOSPHOLIPASE C
	(nmol 3-0-methylglucose/g/30 min)	
BASAL	7.8±1.1	13.1±0.8 *
INSULIN	13.4±0.6 +	14.3±0.6

Results are means±SEM of 6 to 9 observations per group. EDL muscles were incubated in the absence or presence of 0.1 U/ml of phospholipase C (for 90 min) or 100 nM insulin (for 60 min). *, indicates a significant difference between control and phospholipase C groups, at $P< 0.05$. +, indicates a significant difference between basal and insulin groups, at $P< 0.05$.

Table 2

EFFECT OF PHORBOL ESTERS ON MeAIB AND 3-0-METHYLGLUCOSE UPTAKE BY MUSCLE

A) MeAIB UPTAKE

	CONTROL	TPA
	(nmol MeAIB/g/30 min)	
BASAL	23.4±3.2	24.5±2.4
INSULIN	53.2±2.1 +	42.6±3.2 *+

B) 3-0-METHYLGLUCOSE UPTAKE

	CONTROL	TPA
	(nmol 3-0-methylglucose/g/30 min)	
BASAL	6.9±0.3	9.8±0.5 *
INSULIN	18.8±0.8 +	19.6±0.7 *+

Results are means±SEM of 6 to 10 observations per group. EDL muscles were incubated in the absence or presence of 0.5 μM 12-0-tetradecanoyl-13-acetate (TPA) (for 90 min) or 100 nM insulin (for 60 min). *, indicates a significant difference between control and TPA groups, at $P< 0.05$. +, indicates a significant difference between basal and insulin groups, at $P< 0.05$.

Table 3

EFFECT OF VERAPAMIL ON PHOSPHOLIPASE C- AND INSULIN-STIMULATED MeAIB UPTAKE BY MUSCLE.

A)	CONTROL	PHOSPHOLIPASE C	% effect
	(nmol MeAIB/g/30 min)		
BASAL	17.1±1.9	25.3±3.4 *	48
VERAPAMIL	13.1±2.3	20.3±5.4 *	55

B)	CONTROL	INSULIN	% effect
	(nmol MeAIB/g/30 min)		
BASAL	15.5±0.9	33.1±2.4 *	114
VERAPAMIL	11.9±0.8	27.4±2.8 *	130

Results are means±SEM of 4 to 8 observations per group. Muscles were incubated in the absence or presence of phospholipase C (0.1 U/ml, 90 min), insulin (100 nM, 60 min) and verapamil (0.1 mM, 90 min). *, indicates a significant difference between control and phospholipase C groups or control and insulin groups, at $P< 0.05$. +, indicates a significant difference between basal and verapamil groups, at $P< 0.05$.

Table 4

EFFECT OF DANTROLENE ON PHOSPHOLIPASE C- AND INSULIN-STIMULATED MeAIB UPTAKE BY MUSCLE.

A)	CONTROL	PHOSPHOLIPASE C	% effect
	(nmol MeAIB/g/30 min)		
BASAL	19.6±1.9	27.9±2.7 *	42
DANTROLENE	15.5±1.8	24.7±1.5 *	59

B)	CONTROL	INSULIN	% effect
	(nmol MeAIB/g/30 min)		
BASAL	16.7±1.6	35.2±3.4 *	111
DANTROLENE	18.2±1.1	37.2±2.6 *	109

Results are means±SEM of 5 to 8 observations per group. Muscles were incubated in the absence or presence of phospholipase C (0.1 U/ml, 90 min), insulin (100 nM, 60 min) and dantrolene (15 μM, 90 min). *, indicates a significant difference between control and phospholipase C groups or control and insulin groups, at $P < 0.05$. Differences between basal and dantrolene groups were insignificant.

Table 5

EFFECT OF PERTUSSIS TOXIN ON BASAL AND INSULIN-STIMULATED MeAIB UPTAKE BY MUSCLE.

	BASAL	INSULIN	% effect of insulin
	(nmol MeAIB/g/30 min)		
CONTROL	33.6±3.7	52.1±4.9 *	55
100 ng/ml PERTUSSIS TOXIN	33.2±3.9	55.2±5.4 *	66
200 ng/ml PERTUSSIS TOXIN	31.0±3.0	49.9±4.1 *	61

Results are means±SEM of 9 to 10 observations per group. Muscles were incubated in the absence or presence of insulin (100 nM, 60 min) or pertussis toxin (100 or 200 ng/ml, 90 min). *, indicates a significant difference between basal and insulin groups, at $P < 0.05$. Differences between basal and pertussis toxin-treated groups were insignificant.

Table 6

EFFECT OF PERTUSSIS TOXIN ON BASAL AND INSULIN-STIMULATED 3-0-METHYLGLUCOSE UPTAKE BY MUSCLE.

	BASAL	INSULIN
	(nmol 3-0-methylglucose/g/30 min)	
CONTROL	7.4±1.2	16.8±2.3 *
100 ng/ml PERTUSSIS TOXIN	10.4±1.3	16.3±2.8 *
PHENTOLAMINE	7.0±1.1	20.1±1.1 *

Results are means±SEM of 6 to 8 observations per group. Muscles were incubated in the absence or presence of insulin (100 nM, 60 min), pertussis toxin (100 ng/ml, 90 min) or phentolamine (12 μM, 180 min). *, indicates a significant difference between basal and insulin groups, at $P < 0.05$. Differences between basal and pertussis toxin-treated groups were insignificant.

Table 7

EFFECT OF ISOPROTERENOL, CHOLERA TOXIN AND FORSKOLIN ON LACTATE PRODUCTION AND GLYCOGEN CONCENTRATION BY MUSCLE.

A) Muscle Glycogen Concentration

	CONTROL	EXPERIMENTAL
	(μmol glucose/g. tissue)	
ISOPROTERENOL	35.7±0.9	28.3±0.7 *
CHOLERA TOXIN	23.5±1.1	19.3±2.4 *
FORSKOLIN	26.8±2.2	21.7±2.7 *

B) Lactate Production

	CONTROL	EXPERIMENTAL
	(μmol lactate/g. tissue/30 min)	
ISOPROTERENOL	1.7±0.2	2.7±0.3 *
CHOLERA TOXIN	2.7±0.2	3.4±0.2 *

Results are means±SEM of 3 to 11 observations per group. EDL muscles were incubated in the absence or presence of isoproterenol (1 μM, 30 min for muscle glycogen measurements or 180 min for lactate production assessment), cholera toxin (10 μg/ml, 180 min) or forskolin (50 μM, 90 min). *, indicates a significant difference between control and experimental groups, at $P < 0.05$.

Table 8

EFFECT OF ISOPROTERENOL ON BASAL AND INSULIN-STIMULATED MeAIB UPTAKE BY MUSCLE.

	BASAL	INSULIN
	(nmol MeAIB/g/30 min)	
CONTROL	18.9±1.3	37.7±2.4 *
ISOPROTERENOL (90 min)	19.4±1.2	39.1±3.1 *
ISOPROTERENOL (180 min)	16.1±2.4	32.0±2.7 *
PHENTOLAMINE	19.9±1.8	33.5±3.4 *
PHENTOLAMINE + ISOPROTERENOL (180 min)	15.6±0.7	33.1±4.5 *
CONTROL	22.2±1.6	36.8±1.8 *
ADENOSINE DEAMINASE	21.7±1.9	39.3±1.6 *
ISOPROTERENOL (180 min)	21.8±4.1	36.2±3.2 *
ISOPROTERENOL (180 min) + ADENOSINE DEAMINASE	18.3±2.6	39.8±2.9 *

Results are means±SEM of 5 to 20 observations per group. Muscles were incubated in the absence or presence of insulin (100 nM, 60 min), isoproterenol (1 μM, 90 or 180 min), phentolamine (12 μM, 180 min), adenosine deaminase (1 U/ml, 180 min). *, indicates a significant difference between basal and insulin groups, at $P < 0.05$. Differences dur to isoproterenol treatment were insignificant.

Table 9

EFFECT OF CHOLERA TOXIN AND FORSKOLIN ON BASAL AND INSULIN-STIMULATED MeAIB UPTAKE BY MUSCLE.

	BASAL	INSULIN
	(nmol MeAIB/g/30 min)	
CONTROL	20.7±1.9	33.7±3.3 *
5 µg/ml CHOLERA TOXIN	23.8±2.1	39.5±3.3 *
10 µg/ml CHOLERA TOXIN	18.6±1.0	32.5±2.6 *
CONTROL	22.7±2.9	33.2±3.1 *
FORSKOLIN	21.5±2.5	36.1±2.9 *

Results are means±SEM of 3 to 9 observations per group. Muscles were incubated in the absence or presence of insulin (100 nM, 60 min), cholera toxin (5 or 10 µg/ml, 180 min) or forskolin (50 µM, 90 min). *, indicates a significant difference between basal and insulin groups, at $P < 0.05$. Differences due to treatment with cholera toxin or forskolin were insignificant.

Table 10

EFFECT OF ISOPROTERENOL OR CHOLERA TOXIN ON BASAL AND INSULIN-STIMULATED 3-0-METHYLGLUCOSE UPTAKE BY MUSCLE.

	BASAL	INSULIN
	(nmol 3-0-methylglucose/g/30 min)	
CONTROL	9.7±1.7	18.1±1.9 *
ISOPROTERENOL	5.8±1.4 +	19.2±2.5 *
CONTROL	6.6±1.6	14.7±3.0 *
CHOLERA TOXIN	6.8±1.1	16.4±0.9 *

Results are means±SEM of 3 to 6 observations per group. Muscles were incubated in the absence or presence of insulin (100 nM, 60 min), isoproterenol (1 μM, 180 min) or cholera toxin (5 μg/ml, 180 min). *, indicates a significant difference between basal and insulin groups, at $P< 0.05$. +, indicates a significant difference between control and isoproterenol groups at $P< 0.05$.

D. Conclusions

1. L'acció de la insulina sobre l'activitat de transport del sistema A, per a aminoàcids neutres, en múscul esquelètic es caracteritza per ser ràpida, independent de síntesis protèica i del gradient electroquímic de Na^+.

2. La insulina provoca increments en la Vmax del transport de l'anàleg aminoacídic i substrat del sistema A, MeAIB, sense alterar la Km, la qual cosa pot interpretar-se bé com a conseqüència d'un increment en el nombre de transportadors a la membrana plasmàtica, o bé per estimulació de l'activitat intrínseca dels transportadors ja presents a la membrana.

3. L'acció de la insulina sobre el sistema A en múscul esquelètic no depén d'una adequada funcionalitat de microtúbuls i de microfilaments, per la qual cosa difereix del mecanisme pel qual la insulina estimula el sistema A al fetge, on aquesta acció és dependent de síntesi proteica. Tanmateix, l'efecte de la insulina sobre el sistema A en múscul difereix de la "regulació adaptativa", que és dependent de síntesi proteica i necessita d'una adequada funcionalitat de microtúbuls i de microfilaments.

4. Diversos autors han proposat que la insulina provoca la translocació de transportadors de glucosa en múscul esquelètic, des d'un locus intracel.lular cap a la membrana plasmàtica. Aquesta translocació, de ser certa, tampoc depén d'una adequada funcionalitat de microtúbuls ni de microfilaments. Per tant si hi ha una exocitosi de vesícules dependent d'insulina, aquesta és independent de l'activitat de microtúbuls i de microfilaments.

5. L'acció de la insulina estimulant l'activitat de transport del sistema A requereix d'una inalterada activitat tirosina quinasa del receptor de la insulina.

6. Els esters de forbol estimulen el transport de glucosa, però no afecten l'activitat de transport del sistema A en múscul esquelètic. Per tant, la proteïna quinasa C no és un mediador de l'acció de la insulina sobre el sistema A en múscul.

7. En la via de transducció de l'acció de la insulina que condueix a l'activació del transport de glucosa i del sistema A, no intervé una proteïna reguladora G sensible a la toxina pertussis.

8. Els esters de forbol regulen negativament l'acció de la insulina estimulant el sistema A en múscul esquelètic. Aquest efecte es selectiu atés que els esters de forbol no modulen l'estimulació del transport de glucosa en resposta a la insulina. Aquesta modulació no esdevé a nivell del receptor de la insulina doncs tant l'activitat d'unió a la insulina com l'activitat tirosina quinasa romanen inalterades per tractaments in vivo amb esters de forbol.

9. Els nostres resultats indiquen que la PMXB, l'H-7 i l'estaurosporina, no solament són inhibidors de la proteïna quinasa C sinò que a més, i de manera independent, inhibeixen algunes de les accions de la insulina. Per tant no resulta adequada la utilització d'aquests inhibidors per a demostrar la participació de la proteïna quinasa C en l'acció de la insulina.

10. Agents que indueixen la producció de cAMP, com són l'isoproterenol, la toxina colèrica o la forskolina, no alteren l'activitat basal del sistema A ni l'estimulada per la insulina en múscul esquelètic. Per tant, l'activació de la proteïna quinasa dependent de cAMP no regula l'acció de la insulina sobre el sistema A.

11. La fosfolipasa C incrementa l'activitat del sistema A en múscul esquelètic i produeix un blocatge total de l'acció de la insulina estimulant aquesta activitat de transport. Aquest efecte de la fosfolipasa C no resulta afectat ni per blocadors de canals de Ca^{2+} de la membrana plasmàtica, ni per blocadors de la sortida de Ca^{2+} del reticle sarcoplàsmic. Aquests resultats permeten suggerir que l'efecte de la fosfolipasa C no depen de modulacions en la concentració de Ca^{2+} intracel.lular.

E. Apèndix: Protocols experimentals.

Índex

pàgina

1. Animals experimentals.

S'han utilitzat rates albines, Rattus norvegicus, de la soca WISTAR, procedents de la colònia de cria de l'estabulari de la Facultat de Biologia (Universitat de Barcelona). L'estudi d'incubació de músculs es va portar a terme amb mascles de 40 a 70 g de pes corporal, pes corresponent a l'estadi de 1 a 7 dies després del deslletament (la fase d'alletament era de 21 dies). L'obtenció d'hepatòcits fou realitzada a partir de mascles de 200-250 g de pes corporal. L'obtenció de vesicules de membrana plasmàtica de fetge de rata fou realitzada a partir de mascles de 200-300 g de pes corporal. Per a la purificació de receptors de la insulina, l'extracció de porcions de musculatura es realitzava en rates de 180 a 200 g de pes corporal.

Les rates sempre s'utilitzaren en condicions d'alimentació i amb lliure accés a la beguda. Les condicions ambientals de l'estabulari eren d'una temperatura de 22+/-1°C, amb alternància de llum-foscor de 12 h cadascuna.

2. Obtenció de músculs soleus i extensor digitorum longus (EDL) de rata.

Les rates eren anestesiades amb pentobarbital sòdic (100 mg/5 ml de ClNa,0.9% -salí-) en una relació de 150 μl per 50 g de pes corporal. L'anestèsia era injectada intraperitonealment, entre 5 i 10 minuts abans de procedir-se a l'extracció dels músculs, moment en què es comprovava l'adequada situació d'alienament de l'animal.

- Extracció del soleus.

Inicialment tallàvem la pell pel turmell i la desplaçàvem fins a l'alçada del genoll, deixant a la vista la musculatura. Amb unes pinces de posició fixa, agafàvem el tendó distal del múscul plantaris, el qual tallàvem, separant tota la massa muscular unida a ell de la resta de musculatura de la zona, tot estirant en direcció a la part posterior del genoll i fins arribar a l'alçada d'aquest. A la part interior del paquet muscular que mantenïem pinçat quedava al descobert el múscul soleus. Aleshores amb unes pinces es feia passar un fil per sota del seu tendó proximal i es lligava aquest abans de procedir a tallar el tendó. Després se separava el múscul de la resta del paquet muscular al qual es trobava unit, tallant els vasos i els nervis que li arribaven per la part posterior. En arribar a l'alçada del tendó distal del soleus i un cop retirada la pinça fixada a ell, es procedia a lligar i tallar aquest, de forma que el múscul quedava isolat i amb un fil a cada extrem. Normalment, abans de tallar els tendons, els fils es lligaven a un filferro, la longitud del qual era aproximadament la mateixa que la del múscul. Això es feia per tal de mantenir la tensió que el múscul tenia in situ, un cop aquest era isolat. Seguidament el múscul es col.locava en un medi tamponat a 7.4 i refredat amb gel, fins al moment de procedir a la seva incubació.

- Extracció de l'EDL

Un cop tallada i desplaçada la pell des del turmell fins a l'alçada del genoll, mantenint el peu de la rata estès, per la part anterior i en posició distal s'observaven dos tendons paral.lels corresponents als músculs tibialis anterior i EDL. Per sobre d'aquests tendons i en posició obliqua se situava l'anomenat lligament anular, el qual, un cop aïllat amb unes pinces, es tallava. Seguidament, s'agafava el tendó més superficial corresponent al del tibialis anterior, i es tallava, estirant aquest múscul en direcció al genoll i tallant-lo en arribar-hi. El tendó distal de l'EDL, un cop aïllat, es lligava amb un fil, fent el mateix pel tendó proximal. Seguidament els fils es nuaven a un filferro de longitud similar a la del múscul in situ i finalment els tendons eren tallats. Com en el cas del soleus, l'EDL ja extret se submergia en un tampó de les mateixes característiques que l'esmentat i es deixava a 0-4°C fins al moment d'iniciar-se la incubació dels músculs, moment que tenia lloc en finalitzar l'extracció de tots els músculs necessaris per a la incubació, al voltant de 45 min des de l'inici de l'extracció.

3. Assaigs de la captació de l'àcid α-(metil)aminoisobutíric (MeAIB) i de la 3-O metilglucosa (MeGlu), a múscul esquelètic.

3.1. Període d'incubació "Pre-Captació".

Aquesta fase tenia com a objectiu el tractament dels músculs amb els compostos que eren objecte d'estudi; els tipus de compostos, la concentració i el temps d'acció s'especifiquen a les gràfiques o taules on s'expressen els resultats obtinguts. La durada d'aquest període, en general, era de 150 minuts, presentant dues fases, una de 60 minuts i l'altre de 90 minuts, intercanviables en funció de les necessitats experimentals, i que tenia com a objectiu renovar el medi d'incubació dels músculs tractats. Aquest medi consistia en un tampó Krebs-Hepes que presentava la següent composició:

Solucions stock:	Concentracions finals:
NaCl (32 g/200 ml).....7.5 ml	(7.47 mg/ml)
KCl (1.765 g/50 ml)	(0.353 mg/ml)
$MgSO_4.7H_2O$ (1.46 g/50 ml)	(0.29 mg/ml)
KH_2PO_4 (0.806 g/50 ml) .....1.5 ml	(0.161 mg/ml)
$CaCl_2.2H_2O$ (3.73 g/100 ml)....1.5 ml	(0.373 mg/ml)
Hepes.....0.7148 g	(20 mM)

gasejat durant 20 min amb carbogen (O_2:CO_2, 95:5) i portat a pH 7.4 amb NaOH, 5N, prèviament a enrasar-lo a 150 ml amb aigua destil.lada.

Aquest tampó era suplementat amb Albúmina Sèrica Bovina lliure d'àcids grassos (BSA-FFA) al 10% fins a obtenir una concentració del 0.2% (3 ml / 150 ml). Aquesta albúmina havia estat prèviament dialitzada, filtrada i conservada en alíquotes a -20°C. En estudis de la captació de MeAIB, addicionàvem al tampó, com a substrat energètic, glucosa 0.5 M fins assolir una concentració de 5 mM (1.5 ml/150 ml), la qual també havia estat diluïda i conservada en alíquotes a -20°C. Tant l'albúmina com la glucosa estaven diluïdes en el mateix tampó que es feia servir per a la incubació. Per aquelles incubacions on s'estudiava la captació de MeGlu, el substrat energètic utilitzat era l'àcid pirúvic (33 mg / 150 ml).

Previ a la fase d'extracció dels teixits, es preparaven els medis d'incubació en vials de cintil.lació, corresponentment condimentats, restant a 4°C fins al moment de donar inici a la incubació dels músculs.

Els músculs, immediatament després de la seva extracció, eren submergits en el tampó Krebs-Hepes enriquit, a 0-4°C fins al moment d'iniciar-se la incubació (període que oscil.lava entre els 5 i els 45 min) i mantenint-se aparellats segons la seva procedència.

Les incubacions es duien a terme en un bany Dubnoff termostatitzat a 37°C i amb agitació unidireccional constant (45 r.p.m). Els vials eren gasejats amb carbogen durant tota la incubació.

3.2. Període d'incubació "Captació".

Tret dels experiments previs on es van definir les condicions de linialitat al llarg del temps de la captació del substrat en qüestió, aquest període fou sempre de 30 minuts, tant per a les captacions de MeAIB com per a les de MeGlu.

Els medis d'incubació tenien la mateixa composició als del període precedent a excepció del fet que aquests tenien una barreja radiactiva amb el substrat de la captació i amb un marcador de l'espai extracel.lular:

per a la captació de MeAIB,

àcid alfa-(1-^{14}C)-metilaminoisobutíric...0.125 μCi
àcid alfa-metilaminoisobutíric.....0.1 mM
D-(1-^{3}H(N))-manitol.....0.5 μCi
D-manitol.....1 mM

Activitat específica del MeAIB.....800 μCi/mmol
" " " manitol.....330 μCi/mmol,
(En un volum final de 1.5 ml per vial d'incubació).

per a la captació de MeGlu,

s'utilitzaven les mateixes activitats específiques que pel MeAIB i el marcador, essent el substrat de la captació el 3-O-(metil-^{14}C)-D-Glucosa.

3.3. Tractament de les mostres incubades.

Finalitzada la incubació, els músculs eren retirats del vial , rentats lleugerament amb salí i ràpidament congelats amb N_2 líquid. Seguidament es procedia a pesar les mostres (pes promig: 15-30 mg) i a introduir-les en tubs pyrex de 8 ml amb taps de rosca, que contenien 250 μl d'un disolvent orgànic (Protosol, de New England Nuclear). Aquests tubs eren escalfats a 45°C durant 2-3 h amb agitació constant (50-60 r.p.m.). Quan assolíem la total digestió del múscul, afegíem 250 μl de toluè per diluir la mostra, i 300 μl d'aigua oxigenada (30%) per decolorar-la. Així restaven 10 min més a la mateixa temperatura. Seguidament acidificàvem suaument la mostra tot afegint 150 μl d'àcid acètic, 25% i finalment addicionàvem 250 μl d'aigua destil.lada per aconseguir una total transparència en presència del líquid de cintil.lació. Aquest s'addicionava inicialment en una quantitat de 5 ml al tub, seguit una agitació forta fins assolir l'homogeneïtat del seu contingut. Aquest s'abocava en un vial de cintil.lació per seguidament rentar el tub amb 5 ml de líquid de cintil.lació que igualment, després d'ésser agitat, era passat al vial en qüestió.

Alhora que es processaven els músculs, també recollíem una alíquota (50 ul) del medi d'incubació per tal de tenir referència dels comptes totals fets servir en la captació. Aquesta alíquota es col.locava en un vial de cintil.lació juntament amb 300 μl d'aigua destil.lada i 6 ml de líquid de cintil.lació.

Tant les mostres procedents dels músculs com les procedents dels medis, eren portades a un comptador de radiació β, Packard, on sota un protocol programat per a doble comptatge (^{3}H-^{14}C), obtenïem les dpm de cada isòtop.

3.4. Càlculs.

Per tal de convertir les dpm en una mesura de la captació del substrat emprat, realitzàvem els següents càlculs:

$$\text{nmols MeAIB/g múscul} = \frac{(\text{dpm } ^{14}\text{C/g múscul}) - ((\text{dpm } ^{14}\text{C/ml medi}) \times (\text{ml d'EE/g múscul}))}{(\text{dpm } ^{14}\text{C}/\mu\text{mol MeAIB en el medi})}$$

$$\text{EE, espai extracel.lular (ml/g múscul)} = \frac{(\text{dpm } ^{3}\text{H/g múscul})}{(\text{dpm } ^{3}\text{H/ml medi})}$$

Finalment expressem la captació com a: nmols MeAIB / g múscul x 30 min d'incubació

4. Obtenció d'hepatòcits aïllats de rata i assaig de la captació de MeAIB.

4.1. Perfusió del fetge i obtenció dels hepatòcits.

Se seguí la metodologia emprada per Clark et al.(1972) i posteriorment modificada per Katz et al.(1975).

La rata era anestesiada per injecció intraperitoneal de pentobarbital sòdic en una dosi de 60 mg/Kg de pes corporal. Un cop anestesiat l'animal es practicava una laparotomia en V des de la línia alba inferior fins a la caixa toràcica. La paret abdominal es retirava amunt, sobre la caixa toràcica, i se subjectava amb una pinça hemostàtica. Es col.locaven dos fils al voltant de la vena porta sobre l'espai desprovist de greix i un altre al voltant de la vena cava posterior sobre el ronyó dret. S'encanulava la vena porta amb una cànula de plàstic de 14 gauges i amb flux gota a gota. El flux del perfundit s'aconseguia per pressió hidrostàtica (l'embut de decantació del tampó de perfusió estava aproximadament a un metre d'alçada sobre el fetge). Ràpidament es lligava el primer fil (distal al fetge) de la vena porta per fixar la cànula. Es seccionava la vena cava posterior, per sota del ronyó dret, amb tisores, de manera que el perfundit fluïa lliurement i el fetge perdia ràpidament el seu color característic. A continuació es lligava el segon fil de la vena porta per assegurar la cànula. Llavors el flux del perfundit s'incrementava a 30-40 ml/min. S'utilitzaven uns 150 ml d'un medi de perfusió que tenia la següent composició (tampó A),

"tampó Krebs-Henseleit bicarbonat lliure de Ca2+"

NaCl, 118 mM
KCl, 4.75 mM
NaHCO3, 25 mM
KH2PO4, 1.18 mM
MgSO4, 1.18 mM

contenint àcid pirúvic, 5 mM i alfa-D-glucosa, 5 mM i suplementat amb EGTA, 0.5 mM, pH 7.4. Aquest tampó es mantenia a 37°C i amb gaseig de carbogen continu.

Mentre es realitzava aquesta perfusió, s'anava separant el fetge de l'animal i aquest es transferia a una cambra termostatitzada a 37°C. La perfusió dins la cambra era a circuit tancat fent passar 225 ml del tampó A al que s'havia afegit 0.31 mg/ml de col.lagenasa i 5 μmols de $CaCl_2$ per quelar el EGTA residual. La perfusió es deixava progressar durant uns 30-40 min, després dels quals es comprovava si el fetge ja estava digerit. Dins la cambra el líquid de perfusió era impulsat per una bomba peristàltica (Harvard) i la velocitat del flux es fixava aproximadament a 30-60 ml/min.

Quan el fetge ja estava digerit, es treia de la cambra de perfusió i es rentava perfundint uns 150-200 ml de tampó B, el qual tenia la mateixa composició de sals que el tampó A però se li havia addicionat $CaCl_2$, 2.5 mM i no hi havia substrats energètics. Posteriorment el fetge es disgregava mecànicament per la superfície amb espàtules de plàstic en un recipient que contenia tampó B. La suspensió original de cèl.lules aconseguides es filtrava amb nylon i es centrifugava a t^a ambient a 600xg (un cop s'arribava a aquesta velocitat, la centrífuga es deixava parar sense utilitzar el fre). Es descartava el sobrenedant i es resuspenia el precipitat en el mateix tampó. Es repetia aquest procés dos cops més. Al final les cèl.lules precipitades es resuspenien segons una dilució 1:100 en el tampó B, per tal d'aconseguir una densitat aproximada de 1,000.000 cèl.lules / ml. El volum de cèl.lules empaquetades que s'obtenia era aproximadament de 4-5 ml.

Abans de realitzar les incubacions d'aquests hepatòcits, calia verificar la viabilitat de les cèl.lules. Per això, vàrem fer servir la tècnica d'exclusió del colorant vital Blau de Tripà (en solució col.loidal, tinció no específica) (Howard & Pesh, 1968). Aquest colorant penetra dins de les cèl.lules afectades o mortes, tenyint de blau els citoplasmes i de forma més intensa, els seus nuclis. Per contra, les cèl.lules intactes són capaces d'excloure aquest colorant si la relació ATP/ADP, índex de la integritat de les cèl.lules, és l'adequada.

Aquest colorant es preparava de la següent manera (segons Miralpeix, 1986),
es dissolien en 95 ml d'aigua destil.lada,

Blau de Tripà, 0.4 g
NaCl, 0.81 g
KH_2PO_4, 0.66 g, seguint aquest ordre.

Es portava a ebullició, es deixava refredar i s'ajustava el pH a 7.2-7.3 (amb NaOH, 1N).

S'enrasava a 100 ml amb aigua destil.lada.

Per a conservar-ho, es dissolien 0.05 g de 4-hidroximetil-benzoat i es preservava de la llum.

Per tal de realitzar la prova de viabilitat de les cèl.lules, es posaven 100 μl de colorant i 50 μl de la suspensió de cèl.lules. Després d'agitar suaument, agafàvem una mostra de 100 μl i l'introduíem en una cambra de comptatge de Neubauer. Fèiem l'observació en un microscopi òptic a 100 augments. La viabilitat ve determinada pel nombre de cèl.lules intactes per cada 100 cèl.lules totals. Unicament utilitzàvem les preparacions que presentaven una viabilitat superior al 80%.

4.2. Incubació dels hepatòcits.

Inicialment el volum total d'hepatòcits necessaris per realitzar l'assaig era incubat durant 30 min en un bany termostatitzat a 37°C i amb agitació orbital d'una intensitat aproximadament de 80-100 cicles/min, velocitat necessària per evitar que les cèl.lules en suspensió sedimentessin. Ja des del principi de la incubació els hepatòcits es trobaven en un medi "enriquit" preparat a partir del tampó B (veure apartat c.2), al qual li addicionàvem albúmina sèrica bovina (dialitzada i lliure d'àcids grassos), 1% i bacitracina, 0.8 mg/ml.

Durant tota la incubació, els recipients que contenien les suspensions d'hepatòcits eren conectats a un sistema de gaseig de carbogen.

La relació entre el volum de cèl.lules incubades i el volum del matrau utilitzat per a la seva incubació, fou sempre de 1:10 respectivament. Aquests recipients eren tapats amb taps de goma a través dels quals es conectava el ja esmentat sistema de gasseig.

Després d'aquests 30 min inicials, les cèl.lules eren transferides a diferents vials, cadascun dels quals ja contenia el tractament específic que l'assaig requerís. Així, normalment, la insulina, 100 nM, s'addicionava al principi d'aquest segon període d'incubació, el qual tenia una durada total de 2 h. Al final d'aquest procés de tractament de les cèl.lules, s'iniciava la fase de "captació", la qual tenia una durada de 10 min. Després de finalitzar cada fase d'incubació, les cèl.lules eren precipitades i resuspeses en medi fresc. Del volum de cèl.lules en suspensió d'un matrau de la fase anterior, s'extreien tres alíquotes de 1.35 ml cadascuna que s'incubaven en presència d'una "barreja radiactiva" formada per MeAIB, 0.1 mM (800 μCi/mmol) i manitol, 1mM (330 μCi/mmol) (veure l'apartat b.2). Aquesta fase s'aturava als 10 min recollint 750 μl de suspensió de cèl.lules, per matrau, i col.locant-les en vials eppendorfs sobre 400 μl d'una barreja d'olis (dinonylphthalate:dibutylphthalate, 1:3, v:v, qualitat Fluka), ràpidament seguit d'una centrifugació en microfuga calibrada a velocitat màxima (12.000 x g) durant 20 s.

Fruit d'aquesta centrifugació era la separació de les cèl.lules (que queden al fons del tub) respecte al medi, que resta surant damunt la barreja d'olis.

4.3. Tractament de les mostres incubades.

El medi el recollíem sense tocar la barreja d'olis. Una alíquota de 50 μl era portada a comptar. D'altra banda, afegíem als tubs eppendorfs salí fins a reomplir-los, per tal de netejar el tub de la radiació adherida i seguidament el retiràvem, juntament amb la barreja d'olis. Les parets del tub eren eixugades amb cotó fluix i les cèl.lules eren solubilitzades per addició de 100 μl de SDS 10% durant 2-3 h a temperatura ambient. Passat aquest temps els tubs eren totalment omplerts amb líquid de cintil.lació i col.locats en vials de cintil.lació per ésser portats a comptar a un comptador de radiació β (veure apartat b.3).

Es realitzaren "blancs" que consistien en medis d'incubació de la fase de captació, en absència de cèl.lules. Aquests mai excediren el 5% del total de la radiactivitat, que en cada experiment era avaluada.

4.4 Càlculs:

a = dpm ^{14}C (pellet) / 10^6 cèl.lules
b = dpm ^{14}C / ml de medi

(a / b) x 100 nmols MeAIB/ml de medi = nmols MeAIB / 10^6 cèl.lules x 10 min de captació

El número de cèl.lules s'obtenia per comptatge en microscopi òptic a 100 augments a la càmbra de Neubauer i seguint la següent relació, fruit de proves precedents,
nº de cèl.lules per requadre de 16 quadrets (promig) = A
volum total = 150 μl (100 μl colorant + 50 μl suspensió de cèl.lules)

A x (150 μl totals / 50 μl suspensió de cèl.lules) x 10.000 = A x 30.000 cèl.lules / ml.

Tanmateix s'observà que la relació entre el nombre de cèl.lules i el pes humit era la següent:
1,000.000 cèl.lules pesen aprox. 10 mg.

5. Assaig de la capacitat d'unió de la insulina al seu receptor en músculs incubats (assaig de "binding").

Els assaigs de "binding" foren realitzats a 21°C segons la tècnica descrita per Le Marchand-Brustel i col. al 1978 (Le Marchand-Brustel et al., 1978). Músculs EDL de 20-30 mg foren inicialment incubats durant 30 min en presència o absència del tractament adequat, en 3 ml del tampó normalment utilitzat en les incubacions de músculs (tampó Krebs Hepes, 20 mM, pH 7.4, enriquit amb 0.2% d'albúmina i 5 mM de glucosa). Posteriorment els músculs entraven en contacte amb 30 pM-[^{125}I-tyrA14]monoiode insulina, durant 3 h. Simultàniament incubàvem músculs que a més de la insulina marcada tenien insulina freda en una concentració de 1.5 μM. Els primers ens donaran la "unió total", mentre que els segons ens donaran la "unió no-específica". Si a la unió total se li resta la unió no-específica obtenim la "unió específica". En les nostres condicions experimentals el valor de "unió no-específica" era aproximadament del 30% respecte al de "unió total".

Finalitzada la incubació amb la insulina, els músculs eren sotmesos a 5 rentats de 5 min cadascun amb salí (NaCl, 0.9%) fred, que contenia 0.25% d'albúmina bovina (RIA grade, Sigma). Aquests rentats es feien a 0-4°C. La radiactivitat era quantificada en un comptador Packard per radiació gamma.

La degradació de la ^{125}I-Insulina era detectada per l'increment de radiactivitat en el sobrenedant de l'àcid tricloroacètic, a partir del medi d'incubació. Aquesta degradació era habitualment del 5-10%.

6. Purificació parcial de receptors de la insulina procedents de múscul esquelètic.

6.1. Homogeneïtzació i ultracentrifugació.

Per procedir a la purificació de receptors es requeria de pools 10-15 músculs, prèviament incubats, amb pes total aproximat de 200-300 mg de teixit.

Els músculs es conservaven en N_2 líquid des del moment final de la incubació fins a l'inici de l'homogeneïtzació. Per tal de procedir a aquest pas, els músculs eren pesats i ràpidament introduïts en un tub que contenia 2 ml de "tampó d'homogeneïtzació". Aquests eren els seus components,
-() = concentracions finals-

233 μl Hepes, 1 M, pH 7.4 (25 mM)
400 μl EDTA, 100 mM (4 mM)
400 μl EGTA, 100 mM (4 mM)

Inhibidors de proteases:

340 μl Aprotinina, 1 U d'inhibidor de cal.licreïna / ml
10 μl Fenilmetilsulfonil fluoride (PMSF), 2 M (2 mM)
10 μl Leupeptina, 2 mM (2 μM)
20 μl Pepstatina, 1 mM (2 μM)
14.3 mg Bacitracina (1 mM)
39 mg Benzamidina (25 mM)

Inhibidors de fosfatases:
1 ml Fluorur sòdic, 100 mM (10 mM)
100 μl Ortovanadat sòdic, 10 mM (0.1 mM)
(1 ml Pirofosfat sòdic, 100 mM (10 mM))

per a un volum total de 10 ml (pH = 7.4).

L'homogeneïtzació es realitzava amb el trencament mecànic d'aquests teixits mitjançant un homogeneïtzador tipus Politron. Aquest procés i els que esdevenien fins a l'obtenció dels receptors parcialment purificats es realitzaven a 4-8°C.

Posteriorment al trencament del teixit s'afegia un detergent, el Tritó X-100 (10%), fins a assolir una concentració de l'1% i es deixava l'homogeneitzat en agitació constant durant 60-90 min.

Passat aquest temps, s'ultracentrifugava la mostra en tubs de policarbonat destapats, a 143000xg durant 90 min i es recollia el sobrenedant obtingut, aproximadament 2 ml.

Quan no calia incubar prèviament els músculs, per a purificar receptors s'obtenien porcions de musculatura de les extremitats posteriors de les rates. Aquestes porcions contenien una barreja de músculs de fibra blanca i vermella (soleus, quadríceps i gastrocnèmius, essencialment). Posteriorment a la disseció i congelació en N_2 líquid, els músculs es pulveritzaven en morters plens de N_2 líquid, per a seguidament entrar en contacte amb el "tampó d'homogeneïtzació" (sense inhibidors de fosfatases), en una proporció de 4 g múscul / 10 ml de tampó; l'homogeneïtzació es completava en passar aquestes mostres pel politron. Els procés seguia igual que pels músculs incubats amb l'única excepció que els tubs de policarbonat amb els que s'ultracentrifugava eren tapats amb taps de rosca de tungsté. Es recollien 6 ml de sobrenedant.

6.2. Cromatografia d'afinitat en aglutinina de germen de blat.

El sobrenedant recollit es passa per columnes de boletes d'agarosa que tenen enllaçades, convalentment, aglutinina de germen de blat, compost que presenta alta afinitat pel àcid siàlic de les glicoproteïnes, tenint el receptor de la insulina aquestes característiques (Per a 2 ml de sobrenedant el volum de columna era de 0.2 ml i la mostra es passava durant 30 min per la columna, mentre que per a 6 ml de sobrenedant, es reciclava 3 vegades la mostra per una columna de 2 ml -es manté una relació aproximada de 1.5-2 g múscul/ ml de columna-). Seguidament, la columna es renta amb un tampó que conté hepes, 25 mM i tritó X-100, 0.1% (quan volem preservar la mostra de l'acció de les fosfatases, s'addiciona pirofosfat sòdic, 10 mM), pH 7.4. Aquest rentat es fa amb un volum de tampó 100 vegades superior al volum de la columna. Finalment es passa per la columna el tampó d'el.lució (1 ml per a columnes de 0.2 ml i 7 ml per a columnes de 2 ml) que presenta una composició similar al de rentat però amb l'addició de N-acetilglucosamina, 0.3 M, la qual desplaça les glicoproteïnes de la columna. Les alíquotes recollides eren segons l'ordre d'el.lució, 100-300-200-200 μl (columnes de 0.2 ml), trobant-se la major part de glicoproteïnes en la fracció de 300 μl i de 1-2-2-2 ml (columnes de 2 ml) trobant-se la major part de glicoproteïnes en la primera fracció de 2 ml.

6.3. Valoracions:

6.3.1. *Assaig de la concentració de proteïnes.*

Vàrem utilitzar el mètode de Bradford, basat en el canvi de color del blau brillant de Comassie en resposta a diferents concentracions de proteïna (Bradford, 1976). En una solució àcida, el blau

brillant de Comassie quan es lliga a proteïnes canvia el màxim d'absorbància de 495 nm a 595 nm.

Reactius:

- Reactiu per l'assaig de proteïna de la casa Bio-Rad diluït 5 vegades en aigua destil.lada i filtrat.
- Tampó fosfat pH 7.4: dihidrogenfosfat sòdic, 50 mM, hidrogenfosfat sòdic, 50 mM.
- solució de gamma-globulina bovina 0.1% en tampó fosfat.

Procediment:

Es feien reaccionar 50 μl de mostra amb 2.5 ml del reactiu per l'assaig de proteïna Bio-Rad, diluït. Passats 5-10 min fèiem les lectures d'absorbància a 595 nm.

Paral.lelament a partir de la solució de gamma-globulina al 0.1% preparàvem una patró que va de 0 a 25 μg de proteïna per tub (25 μl), a la que li addicionavem 2.5 ml del reactiu per l'assaig de proteïna Bio-Rad, diluït. La lectura espectrofotomètrica es fa alhora que la de les mostres a 595 nm.

6.3.2. *Assaig de la capacitat d'unió de la insulina a l'eluït (Assaig de binding).*

Per a cada preparació de receptors fèiem un assaig inicial d'unió per conèixer la seva activitat d'unió a la insulina.

En aquests experiments incubàvem els receptors amb una concentració traça (50-100 pM) d'insulina radiactiva sense afegir o afegint insulina freda en excés (1 μM). Així teníem dades de la unió total i de la inespecífica respectivament. Obteníem la unió específica restant la inespecífica de la total.

Solucions de treball:

- Tampó de "binding", pH 7.6: 3.75 mg de Bacitracina, 25 mg d'albumina RIA grade i portat a 25 ml amb hepes 30 mM.
- Tampó fosfat, pH 7.4: Dihidrogenfosfat sòdic, 50 mM, Hidrogenfosfat sòdic, 50 mM.
- Solució de gamma-globulina, 0.1%, en tampó fosfat, pH 7.4.
- Solució de polietilenglicol 6000, 25%, en tampó fosfat diluït 5 vegades, pH 7.4.
- Solució mare d'insulina (Eli Lilly & Company), 2 mg/ml, en HCl, 5 mM.
- Solució d'insulina, 10 μM: Portem 28 μl de la solució mare d'insulina a 1 ml amb tampó de "binding".
- Solució d'insulina-^{125}I: Diluïm 100 vegades la insulina-(I125-tyrA14), (New England Nuclear, NEX-196) en tampó de "binding" (aprox. 20000 cpm en 20 ul).

Procediment:

Afegim a tubs eppendorf,

- 20 μl d'insulina-I125 (aprox. 20000 cpm)
- 20 μl d'insulina freda, 10 uM, o 20 μl de tampó de "binding".
- 140 μl de tampó de "binding".
- 20 μl de receptors parcialment purificats (eluït de la columna).

Deixem incubar 1 h a t^a ambient i afegim sequencialment,

- 500 μl de gamma-globulina, 0.1%
- 500 μl de polietilenglicol, 25%

(prèviament refredats en gel i mantenint aquest ordre).

Agitar, a fons, les mostres i deixar-les en gel un temps mínim de 45 min.

Centrifuguem les mostres en una microfuga (Heraeus) a 13.000 rpm durant 10 min i portem a comptar els precipitats, tallant la part inferior dels tubs eppendorf, en un comptador Packard de radiació gamma.

Les diferències entre els comptes dels "pellets" de mostres que no contenen insulina freda i els dels que en contenen, és la unió específica. La unió específica l'expressem com a percentatge de la radiactivitat unida específicament respecte la total i com a percentatge de la radiactivitat unida específicament respecte la total per ug de proteïna (prèvia valoració de proteïnes).

7. Assaig d'entrecreuament de la insulina al seu receptor ("Affinity Cross-Linking").

Aquest assaig consisteix en generar un complexe covalent entre la insulina-^{125}I i el seu receptor; això s'aconsegueix amb reactius bifuncionals capaços de generar enllaços amida amb grups N-amino terminals o de lisines, tals com el disuccimidil suberat (DSS) (Pilch i Czech, 1979 i 1980).

Assaig en tubs eppendorf:

- 10 μl d'insulina freda, 10 μM, o 10 μl de tampó hepes, 30 mM, pH 7.6.
- 30 μl d'insulina-^{125}I (Insulina-^{125}-tyrB26, Amersham, IM.167)

Solució mare : 10 μCi (liofilitzada) / 200 μl d'aigua
25 μl de la solució mare / 500 μl tampó hepes
(0.5 nM d'insulina-^{125}I per vial d'assaig).

- 50 μl de tampó hepes, 30 mM, pH 7.6.
- 20 μl de receptors (eluït de la columna de WGA).

Aquesta barreja es deixa incubar 30 min a t^a ambient.

Transcorregut aquest temps posem les mostres en gel durant 5 min. Afegim 2 μl de la solució de DSS (conc. final, 1 mM). La solució de DSS es prepara just abans d'utilitzar-la: 4 mg DSS / 1 ml DMSO. Passats 15 min aturem la reacció afegint 50 μl de LSB (Laemmli Sample Buffer)x2 amb o sense DTT (Ditiotreitol), depenent de com vulguem córrer les mostres en l'electroforesi en gels de SDS-poliacrilamida.

Composició del LSBx2, per 20 ml:

8 ml de tampó Tris 0.5 M pH 6.8
4 ml de SDS, 20%
8 ml de Glicerol
un polsim de blau de bromofenol
(616 mg DTT)

8. Assaigs de l'activitat tirosina quinasa del receptor de la insulina.

8.1. Autofosforilació.

Incubem durant 30 min a t^a ambient, tubs eppendorf que contenen:

- 10 μl de "solució de cations":

Clorur de manganès, 40 mM
Clorur de magnesi, 100 mM
en tampó hepes, 30 mM, pH 7.6.

- 10 μl de diferents solucions d'insulina (1 nM, 10 nM, 100 nM, 1 μM, en tampó hepes, 30 mM, pH7.6),
o 10 μl de tampó hepes, 30 mM, pH 7.6.
- 30 μl de tampó hepes, 30 mM, pH 7.6.
- 20 μl de receptors (eluït de la columna de WGA).

Seguidament afegim 30 μl de solució d'ATP, i incubem 4 min més.

Solució d'ATP:

- 100 μl ATP no marcat (9 mg / 10 ml tampó hepes, 30 mM, pH 7.6; conc. final 1.5 mM)
- 10 μl ATP-gamma-32P: Obtingut a partir d'àcid ortofosfòric 32P, 2 mCi, lliure d'àcid i en un volum de 50 μl, NEN, que es posa en contacte durant 1 h a t^a ambient, amb el Kit gamma-prep A de Promega BioTec -50 μl/tub-. La reacció de formació de l'ATP s'atura escalfant a 95°C durant 5 min. La solució obtinguda es divideix en alíquotes de 10 μl cadascuna -200 μCi- i es congelen a -20°C. L'eficàcia de la síntesi es comprova per la quantitat de radiactivitat que queda al sobrenedant en presència de carbó actiu. (Eficiències del 80-95% de síntesi eren assolides).
- 890 μl tampó hepes, 30 mM, pH 7.6.

La reacció s'atura addicionant 50 μl de LSBx2 amb DTT.

8.2. Assaig de l'activitat tirosina quinasa per a substrats exògens.

Incubem en tubs eppendorf durant 1 h a t^a ambient:

- 10 μl de "solució de cations":
  Acetat de magnesi, 500 mM
  (Clorur de manganès, 40 mM)
  en tampó hepes, 30 mM, pH 7.6.
- 10 μl de diferents solucions d'insulina (veure apartat h.1.)
  o 10 μl de tampó hepes, 30 mM, pH 7.6.
- 40 μl de tampó hepes, 30 mM, pH 7.6
- 10 μl de receptors (eluït de la columna de WGA).

Els "blancs" tan sols diferien de la resta per la manca de receptors.

Afegim 30 μl de la solució d'ATP (veure apartat h.1) i incubem durant 10 min a t^a ambient.

Afegim 20 μl d'una solució d'un pèptid sintètic, el poli Glu-Tyr (4:1), que fa la funció de substrat exogen (solució mare: 3 mg / 2 ml tampó hepes, 30 mM, pH 7.6).

Deixem progressar la incubació 30 min més, transcorreguts els quals, l'aturem pipetejant 100 μl de la mostra sobre quadrats de 2x2 cm de paper Whatmann 3MM que ràpidament són submergits en una solució de rentat (àcid tricloroacètic -TCA-, 10% i pirofosfat sòdic, 10 mM). Es feien cinc rentats, cadascun de 30 min i finalment els papers es deixaven assecar sobre paper de filtre, i introduïts en vials de cintil.lació, es portaven a comptar, juntament amb un control dels comptes totals, a un comptador Packard de radiació beta (Comptatge de la radiació Cerenkov).

Els resultats s'expressen com a pmols de fosfat incorporat al copolimer, per fmol de receptor, en 30 min d'incubació.

9. Electroforesi en gels de SDS-poliacrilamida.

Tant per mostres obtingudes per assaigs de Cross-Linking com les obtingudes per autofosforilació, requerim d'un mètode per visualitzar el receptor de la insulina, ja sigui en la seva forma tetramèrica ($\alpha_2\beta_2$, assaigs en absència de DTT) o tan sols com a subunitat β (assaigs d'autofosforilació) o alfa (assaigs de Cross-Linking, +DTT). El mètode emprat és una electroforesi en gels de poliacilamida on les mostres han estat previament tractades amb SDS (gràcies a la presència d'aquest compost en el tampó de LSB, veure apartat g).

L'equip d'electroforesi és el protean 2 de Bio-Rad i tots els reactius emprats per a l'electroforesi són també BioRad.

El gruix del gel era d'1.5 o 3 mm en funció de la quantitat de mostra que ens interesses carregar. Els percentatges d'acrilamida eren del 5% quan treballàvem en condicions no reductores (-DTT) o del 7.5% quan treballàvem en condicions reductores (+DTT).

9.1. Preparació dels gels de correguda (Running Gels).

Per preparar un gel de 7.5% d'acrilamida-bisacrilamida i de 1.5 mm de gruix, barregem:

- 7.5 ml de solució d'acrilamida:bisacrilamida (30:0.8%), filtrada i preservada de la llum a 4°C.
- 7.5 ml de tampó Tris 1.5 M pH 8.8.
- 135 μl de solució de persulfat d'amoni al 10% (conservat a -20°C).

Tot això és portat a 30 ml amb aigua destil.lada i s'afegeix 10 μl de Temed (agent polimeritzador, a l'igual que el persulfat d'amoni). Seguidament aboquem la barreja entre els vidres de motllura del gel i amb molta cura afegim aigua destil.lada damunt la barreja, per tal que la polimerització a la part superior del gel sigui correcta. Deixem polimeritzar el gel com a mínim 1 h.

9.2. Preparació dels gels d'empaquetament (Stacking Gels).

Per a un gel d'1.5 mm de gruix, barregem:
- 1.1 ml de la solució d'acrilamida-bisacrilamida (veure el apartat i.1).
- 2.5 ml del tampó Tris 0.5 M, pH 6.8.
- 6.2 ml d'aigua destil.lada.
- 100 μl de SDS, 10%.
- 100 μl de persulfat d'amoni, 10%.
- 10 μl de Temed.

Abans d'afegir el Temed, aboquem l'aigua que havíem col.locat damunt el gel de correguda. Ja amb el Temed addicionat, aboquem ràpidament la barreja en la motllura, damunt el gel de correguda i posem un "pinta" per formar els pouets on dipositarem les mostres que hauran de córrer en el gel (les pintes utilitzades han estat de 10 pouets).

9.3. Càrrega de les mostres i correguda de l'electroforesi.

Abans de carregar les mostres, les bullim a 95°C durant 5 min. Carreguem entre 80-100 μl de mostra per pouet. Cada gel conté almenys en un carril, una estandard de pesos moleculars. La utilitzada és de Bio-Rad (161 0303) que conté:

Proteïna:	Pes Molecular x 1000:
miosina de múscul esquelètic de conill	200
beta-galactosidasa d'E. coli	116.25
fosforilasa b de múscul de conill	97.4
albúmina sèrica bovina	66.2
ovoalbúmina d'ou de gallina	42.699

Aquesta estandard ve en un volum de 200 μl, 100 μl dels quals els diluïm en 4 ml de LSB+DTTx1 i els altres 100 els diluïm en 4 ml de LSB-DTTx1. Es fan alíquotes de 90 μl que es congelen a -20°C.

Els pouets que en principi quedarien buits, s'omplen amb LSB+DTTx2.

El sistema d'electroforesi es connecta a una font de corrent d'amperatge variable, de manera que per córrer un sol gel apliquem una intensitat de corrent de 10 mA, mentre que per a dos gels, 20 mA. La correguda té una durada aproximada de 15 hores i s'atura abans que el front surti del gel.

9.4. Processament del gel.

- Tinció.

Aquest procés es realitzava submergint el gel (al que ja se li ha eliminat el gel d'empaquetament) en una solució de tinció de blau de Comassie (per 4 l: 1 l d'isopropanol, 300 ml d'àcid acètic glacial, 2.7 l d'aigua destil.lada i 2 g de brillant blue R) i deixant-lo a t^a ambient o a 37°C (segons la rapidesa de tinció que desitgem) en agitació orbital constant. El procés finalitza quan el gel té el mateix color que la solució de tinció.

- Destinció.

Amb aquest procés pretenem deslligar del gel tot aquell tint que no està unit a proteïna.

Submergim el gel en la solució destenyidora (per 4 l: 300 ml d'isopropanol, 300 ml d'àcid acètic i 3.4 l d'aigua destil.lada) i el mantenim en agitació suau fins que comencin a contrastar amb claredat les marques de l'estàndard de pesos moleculars. (Podem reciclar la solució destenyidora, filtrant-la amb carbó actiu).

- Eixugat del gel.

Col.loquem el gel en aigua destil.lada, on el posarem en contacte amb un suport de paper Whatmann 3MM. Seguidament posem el gel en un eixugador de gels, connectat a una bomba de buit, durant 1 h 30 min a 80°C i amb un cicle d'escalfament adequat pel percentatge d'acrilamida-bisacrilamida del gel.

És convenient marcar el paper-suport amb algun compost marcat radiactivament i dissolt en LSB.

10. Autoradiografies.

Per l'autoradiografia posem en contacte film Kodak X-Omat AR amb el gel ja eixugat. El film, de l'altra banda, estarà amb contacte amb una pantalla intensificadora Cronex Lightening Plus (Dupont). Per exposar el film utilitzem unes cassettes de cartró hermètiques de Kodak.

El film s'exposa a -80°C durant 1-3 dies depenent de la intensitat del senyal emès.

El revelat del film es fa segons mètodes convencionals:

- 2 min en el líquid revelador.
- 30 s en el bany d'atur.
- 5 min en el líquid fixador.

(Aquests líquids són de AGFA)

Després del revelat es renta el film amb aigua corrent i seguidament es deixa eixugar.

La quantificació dels autoradiogrames fou realitzada per densitometria d'escandallatge dintre d'un rang de resposta linial.

11. Assaig del transport d'alanina en vesícules de membrana plasmàtica de fetge de rata.

11.1. Obtenció de les vesícules.

Aquesta tècnica fou posada a punt per Van Amelsvoort i col. (1978) i modificada per Pastor-Anglada i col. (1987).

Les rates eren decapitades i el fetge ràpidament extret, pesat i submergit en un tampó hepes, 10 mM (tampó A), a 4°C. El fetge era homogeneïtzat, suaument, a mà en 60 ml de tampó. Seguidament es filtrava amb niló i es portava a un volum de 180 ml amb el mateix tampó, prèvia addició de 1.8 ml d'EDTA, 100 mM. Una alíquota (2 ml) era congelada en N2 líquid i conservada per a posteriors valoracions. La resta es repartia en 4 tubs, que eren sotmesos a centrifugació (30000xg, 20 min; centrifuga Sorvall model RC 5C amb un rotor angular SA 600). Es descartava el sobrenedant i es resuspenia el pellet amb el tampó ja esmentat suplementat amb EDTA, fins a un volum de 40 ml. Seguidament se centrifugaven a 700xg durant 10 min. Recollíem el sobrenedant i el pellet era resuspès en 5 ml del tampó-A seguit d'una centrifugació idèntica a l'anterior. Novament recollíem el sobrenedant i l'ajuntàvem amb l'anterior, mentre que descartàvem el pellet. Els sobrenedants eren portats a 50 ml, extraient una nova alíquota de 2 ml que ràpidament era congelada en N_2 líquid (fracció I) i conservada per a posteriors valoracions. Aleshores obtenïem dues alíquotes de 24 ml cadascuna, a les que afegíem 3 ml de solució de Percoll isotònic a pH 7.5 (90 ml de Percoll més 10 ml de sacarosa 2.5 M). Després d'una vigorosa agitació, les dues alíquotes eren centrifugades durant 20 min a 30000xg (centrifugació isopícnica en gradient de densitat). Les membranes que se situen en una part del gradient que correspon a una densitat de 1.038 g/ml (Pastor-Anglada i col. 1987), eren recollides amb pipetes pasteur i col.locades en un tub on s'afegia el tampó-A fins a 40 ml. Es centrifugava 30 min a 30000xg, es descartava el sobrenedant i el pellet es resuspenia en 40 ml del mateix tampó per a sotmetre'l a un segon rentat (30000xg, 30 min). El pellet resultant es resuspenia i re-homogeneïtzava en un volum conegut (5-10 ml del tampó A) per obtenir una concentració de proteïna d'uns 4 mg/ml, i després de fer alíquotes, es conservaven congelades a -80°C.

Per tal de determinar l'eficiència en el procés de purificació de membrana plasmàtica, es determinava l'activitat de diferents enzims marcadors de diferents membranes: 5'-nucleotidasa (membrana plasmàtica), glucosa-6-fosfatasa (reticle endoplasmàtic), N-acetil.beta-D-glucosaminidasa (lisossomals), citocrom C oxidasa (mitocondrials).

11.2. Assaig del transport d'alanina.

Per l'assaig del transport total d'alanina, 10 μl de vesícules (20-40 μg de proteïna) eren incubades a t^a ambient en un medi (30 μl) que contenia sacarosa, 0.25 M, $CaCl_2$, 0.2 mM, $MgCl_2$, 10 mM, NaSCN, 100 mM, L-[2,3-^{3}H]-alanina (activitat específica 0.126 μCi/nmol) 0.2 mM, hepes-KOH, 10 mM pH 7.5, a més del tractament objecte de l'estudi. La captació d'alanina era aturada en afegir 1 ml d'un tampó hepes, 10 mM, amb sacarosa, 0.25 M, $CaCl_2$, 0.2 mM, NaCl, 100 mM, pH 7.5, refredat en gel, a les vesícules de membrana, les quals eren immediatament filtrades a través de filtres de nitrocel.lulosa (mida del porus 0.45 μm, diàmetre 25 mm), acoplats a una bomba de buit, de manera que les vesícules quedaven retingudes al filtre. La filtració anava seguida d'un rentat amb 4 ml del mateix tampó fred. El filtre es col.locava en un vial de cintil.lació per tal de comptar la quantitat de radiació beta incorporada a les membranes, que ja contenia 6 ml de líquid de cintil.lació. Per diferenciar el component de radiactivitat deguda al transport sodi-dependent de la deguda al transport sodi independent més la difusió, es realitzava el mateix assaig amb un medi que contenia KSCN en lloc de NaSCN. En aquest cas el tampó per aturar la reacció contenia KCl en lloc de NaCl. Quan es volia esbrinar el percentatge del transport d'alanina sodi dependent degut al sistema A, se suplementava el medi amb àcid alfa-metilaminoisobutíric (MeAIB) a pH 7.5 a fi que la concentració final de MeAIB fos 10 mM. En aquestes condicions s'assumeix que el transport d'alanina, 0.2 mM, queda bloquejat, a través del sistema A. Aleshores, per diferència entre el transport sodi-dependent total i el transport sodi-dependent en presència de MeAIB, hom obté el component degut al sistema A.

Els blancs s'obtenien en filtrar 30 μl del medi d'incubació seguit d'un rentat amb 4 ml del tampó B. La quantitat total de radiactivitat present en l'assaig es valorava en cada experiment tot portant a comptar 10 μl del medi d'incubació (per duplicat). La captació s'expressa en pmols d'alanina retinguts per mg de proteïna. Després de cada aïllament es feia un assaig de transport a 10 s, temps al qual la velocitat del transport és encara aproximadament igual a la inicial. El valor obtingut era comparat amb el valor d'equilibri, determinat als 30 min d'incubació. D'aquesta manera es veia la capacitat concentrativa d'alanina d'una determinada preparació. Aquelles preparacions que entre 10 s i 30 min tenien una capacitat concentrativa inferior a 2 vegades, eren rebutjades.

12. Valoració de diversos metabòlits.

12.1. Valoració de l'alliberament de lactat pel múscul incubat.

Per realitzar valoracions dels nivells de lactat, prèviament havíem de desproteïnitzar i neutralitzar el medi d'incubació, per la qual cosa seguírem el següent protocol,

- Desproteïnització.

Per 1 ml de medi d'incubació (procedent del període de "captació") calia afegir 110 μl d'àcid perclòric (PCA) al 40%. Seguidament centrifugàvem (500xg, 4°C, 20 min). Un volum fixe del sobrenedant obtingut (950 μl) era recollit.

- Neutralització.

Als 950 μl de sobrenedant, addicionàvem 30 μl d'indicador universal (Merck) i basificàvem amb trietanolamina-KOH (6.63 ml de trietanolamina + 13.2 g KOH, portat a 100 ml amb aigua destil.lada) fins a assolir la neutralitat (aprox. 275 μl). Seguidament centrifugàvem en una microfuga Heraeus a t^a ambient (13000 rpm, 5 min). El sobrenedant, ja desproteïnitzat i neutralitzat, era congelat a -20°C.

- Valoració espectrofotomètrica.

Barreja de reacció:
- 35 ml Tris-base, 0.1 M, pH 8.5
- 2.5 ml d'hidròxid d'hidracina

portat a pH 8.5 i enrasat a 50 ml amb aigua destil.lada.
Es retiren 400 μl per diluir la lactat deshidrogenasa (100 ul, Boehringer).

S'afegeixen 25 mg de NAD+.

Patró:
des de 0 a 25 nmols de lactat per tub.
100 μl de lactat de diverses concentracions
1 ml de barreja de reacció.

Mostres:
200 μl de mostra
900 μl de barreja de reacció.

Lectura:
espectrofotomètrica amb una longitud d'ona de 340 nm.
a) Pre-lectura.
b) Afegim 10 μl de LDH per tub. Esperem 30 min.
c) Lectura.

Resultats:
s'expressen com a (nmols de lactat/ml de medi x 30 min).

12.2. Valoració de la concentració de glicogen muscular.

Músculs EDL de 20-30 mg de pes foren utilitzats per realitzar mesures dels nivells de lactat, abans o després de ser incubats. El mètode de valoració es basa en la següent reacció:

HCl HQ G6PDH
glicogen-------->glucosa-------->glucosa-6-P---------->6 fosfoglucolactona
100°C ATP NADP+ + NADPH+H+

Els músculs, un cop obtinguts, són ràpidament congelats en N_2 líquid, pesats i col.locats en un tub de pyrex amb 1 ml de HCl, 1N, que és tapat i posat al bany maria durant dues hores. Després poden conservar-se a 4°C fins a una setmana. Per a la valoració, es prepara la següent barreja de reacció:

per 45 ml,
Tris, 0.5 M, pH 7.4 ..... 41.4 ml
ATP, 100 mM ..... 0.45 ml
$NADP^+$, 1% ..... 2.25 ml
MgCl2, 0.5 M ..... 0.45 ml
G6PDH (de llevat),
5 mg/ml, dil.1/20 ..... 0.45 ml

Patró:
des de 0 a 50 nmols de glucosa per tub.
50 μl de solució de glucosa de diverses concentracions
950 μl de barreja de reacció.

Mostra:
30 μl de mostra (múscul digerit)
970 μl de barreja de reacció

Lectura:
a l'espectrofotòmetre amb una longitud d'ona de 340 nm.
a) Pre-lectura.
b) Addició de 10 μl d'hexoquinasa(HQ), 2 mg/ml, dil.1:10.
Esperem 60 min i 90 min.
c) Lectura després de cadascun d'aquests temps.

Resultats:
s'expressen com a (nmols glucosa/mg múscul).

12.3. Valoració de les concentracions musculars de creatina-fosfat i d'ATP.

Aquestes valoracions es realitzaren a partir de músculs EDL de 20-30 mg de pes, abans o després de ser incubats. El mètode de valoració és fluorimètric i es basa en el descrit per Lamprecht i col., 1977. La reacció que té lloc és la següent:

$$\text{Creatina fosfat} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatina} + \text{ATP}$$

$$\text{Glucosa} + \text{ATP} \xrightarrow[\text{HQ}]{Mg^{2+}} \text{Glucosa-6-P} + \text{ADP}$$

$$\text{Glucosa-6-P} + NADP^{+} \xrightarrow{\text{G6PDH}} \text{6 fosfogluconat} + NADPH+H^{+}$$

Un cop obtinguts els músculs, eren rapidament congelats en N_2 líquid, pesats i homogeneïtzats a 4°C en 2 ml d'àcid perclòric, 6%, amb un homogeneïtzador tipus politron. Seguidament se centrifuga l'homogeneïtzat a 500xg, 4°C, durant 20 min. El sobrenedant obtingut (1.8 ml) és neutralitzat amb trietanolamina-KOH (0.5M-2N) i de nou és centrifugat durant 15 min a les mateixes condicions. Novament recollim el sobrenedant i el congelem a -20°C fins al moment de realitzar la valoració.

Preparació de la barreja de reacció:
Tris, 0.5 M, pH 7.4 ....15 ml (0.15 M)
Glucosa, 0.1 M .....1 ml (2mM)
MgCl2, 0.5 M .....0.2 ml (2 mM)
NADP+ .....25 mg (0.5 mg/ml)
que són portats a 50 ml amb aigua destil.lada.

Patró:
fem una patró per a l'ATP (des de 0 fins a 85 nmols per tub) i una patró per a la creatina-fosfat (des de 0 fins a 85 nmols per tub).
1 ml d'ATP o creatina-fosfat
1 ml de barreja de reacció.

Mostres:
400 μl de mostra (múscul homogeneïtzat)
600 μl d'aigua destil.lada
1 ml de barreja de reacció.

Lectura:
fluorimètrica amb una longitud d'ona d'excitació de 340 nm i amb una longitud d'ona d'emissió de 460 nm.
a) Afegir 10 μl de G6PDH, 5 mg/ml, 1:20 en albúmina bovina,0.05%. Esperar 5-10 min.
b) Lectura (A).
c) Afegir 10 μl de HQ, 2 mg/ml, 1:10 en albúmina bovina, 0.05%. Esperar 15 min.
d) Lectura (B).
B - A = Quantitat d'ATP de la mostra (nmols/tub)
e) Afegir 20 μl de Creatina quinasa, 10 mg/ml, i 100 μl d'ADP, 10 mM. Esperar 30 min.
f) Lectura (C).
C - B = Quantitat de creatina-fosfat de la mostra (nmols/tub)

Resultats:
s'expressen com a (nmols d'ATP o creatina-fosfat/mg múscul)

ABREVIATURES EMPRADES.

ADA = Adenosina deaminasa
ADP = Adenosins bis-fosfat
AIB = àcid α-aminoisobutíric
ATP = Adenosina tri-fosfat
BSA = Albúmina sèrica bovina
BCH = 2-amino-2-norborà-2-àcid carboxílic
BZS = Benzilsuccinat
CDP = Citidina bis-fosfat
cAMP = Adenosina mono-fosfat cíclic
DSS = Dissucimidil suberat
DTT = Ditiotreitol
EDL = Extensor digitorum longus
FFA = Acids grassos lliures
GABA = àcid γ-aminoisobutíric
GPA = 4-amino-1-guanilpiperidina-4-àcid carboxílic
H = Hormona
H-7 = 1-(5-isoquinonilsulfonil)-2-metilpiperazina
IP = Inositol 4-fosfat
IP_2 = Inositol 1,4-bisfosfat
IP_3 = Inositol 1,4,5-trifosfat
Km = Constant de Michaelis
LPL = Lipoproteïna lipasa
LSB = Laemmli Sample Buffer
MA = 4β-forbol 13α-monoacetat
MeAIB = Acid α-(metil)aminoisobutíric
MeGlu = 3-O metilglucosa
PA = Acid fosfatídic
PC = Fosfatidil colina
PDBu = 4β-forbol 12,13-dibutirat (=PDB)
PI = Fosfatidil inositol
PIP = Fosfatidil inositol 4-fosfat
PIP_2 = Fosfatidil inositol 4,5-bisfosfat
PMA = 4β-forbol 12-miristat 13-acetat (=TPA)
PMXB = Polimixina B
POS = Fosfo-oligosacàrid
PQ-A = Proteïna quinasa dependent de cAMP
PQ-C = Proteïna quinasa dependent de Ca^{2+} i fosfolípids
R = Receptor
SDS = Sodium Dodecil Sulfat
TC = Toxina colèrica
TP = Toxina pertussis
TPA = 12-O-tetradecanoil-β-forbol 13-acetat
UV = Ultravioleta
Vmax = Velocitat màxima
WGA = Aglutinina de germen de blat