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 μ M) 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-stimulated 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 [^3H]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, 4 β -phorbol 13 α -monoacetate; EDL, extensor digitorum longus; MeAIB, α -(methyl)aminoisobutyric acid; H-7, 1-(5-isoquinolinesulphonyl)-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]moniodoinsulin was obtained from New England Nuclear and [^{125}I -Tyr B26]moniodoinsulin 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, 4 β -phorbol 13 α -monoacetate (MA), polymyxin B, 1-(5-isoquinolinesulphonyl)-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 $\mu\text{Ci}/\text{mmol}$), 1 mM-[^3H]mannitol (330 $\mu\text{Ci}/\text{mmol}$) 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-[1- ^{14}C]3-*O*-methylglucose (800 $\mu\text{Ci}/\text{mmol}$) and 1 mM-[^3H]mannitol (330 $\mu\text{Ci}/\text{mmol}$). 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 [^3H]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]moniodoinsulin 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, 0.2% 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 150 000 *g* for 90 min at 4 °C. The 150 000 *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 20 000 c.p.m. of [^{125}I -Tyr A14]moniodoinsulin ($\approx$ 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]moniodoinsulin.

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),

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

Results are means $\pm$ 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	$23.3 \pm 2.0^*$	19.2 ± 1.7
MA, 90 min	27.8 ± 2.8	29.7 ± 2.9	—

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.

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

Results are means $\pm$ 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	$31.8 \pm 2.5^*$

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

Results are means $\pm$ 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 μ M-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 $\pm$ 1.3	32.3 $\pm$ 3.6*	83
TPA	19.3 $\pm$ 1.9	28.8 $\pm$ 3.4*	49
Polymyxin B	19.3 $\pm$ 2.1	25.8 $\pm$ 3.3	34
TPA + polymyxin B	13.5 $\pm$ 0.9	14.8 $\pm$ 0.7	10

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

Results are means $\pm$ 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 μ M-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 $\pm$ 1.2	34.4 $\pm$ 3.5*	107
TPA	18.6 $\pm$ 2.0	26.1 $\pm$ 3.1*	40
H-7	21.7 $\pm$ 2.5	34.7 $\pm$ 2.7*	60
TPA + H-7	14.2 $\pm$ 1.6	18.0 $\pm$ 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 μ M) 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 μ M-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 $\pm$ 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 μ M-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 $\pm$ 0.3	9.8 $\pm$ 0.5*
Insulin	18.8 $\pm$ 0.8†	19.6 $\pm$ 0.7†

(b) Lactate production

	Lactate (μ mol/h per g of muscle)	
	Control	TPA
Basal	8.4 $\pm$ 0.7	11.5 $\pm$ 0.6*
Insulin	11.9 $\pm$ 0.6†	14.4 $\pm$ 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 μ M, 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 $\pm$ 0.03 and 0.37 $\pm$ 0.02 μ g/mg of muscle in control and TPA-treated muscles respectively). In addition, no significant differences in insulin binding were detected, either when expressed per μ g 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 $\pm$ 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 μ M-TPA. To assess insulin binding, the muscles were then incubated with 30 pM-[¹²⁵I-Tyr^{A14}]moniodoinsulin at 21 °C for 3 h in the absence or presence of 1.5 μ M-insulin. The [¹²⁵I]-insulin specifically bound was determined by subtracting the binding observed in the presence of 1.5 μ M-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 μ l) was incubated for 1 h at 22 °C in buffer containing 30 mM-Hepes, 0.2% BSA, 100 units of bacitracin/ml and [¹²⁵I-Tyr^{A14}]moniodoinsulin, in the absence or presence of 1 μ M-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 $\pm$ 1.7	6.5 $\pm$ 2.1
(b) Purified insulin receptor fmol/ μ g of protein	0.26 $\pm$ 0.06	0.22 $\pm$ 0.06
fmol/mg of tissue	0.11 $\pm$ 0.03	0.08 $\pm$ 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 [¹²⁵I]-insulin to the insulin receptor. Partially purified receptor (10 μ l) was incubated at 22 °C for 60 min in 30 mM-Hepes containing 0.5 nM-[¹²⁵I-Tyr^{B26}]insulin in the absence or presence of 1 μ M 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 -³²P]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 μ l) 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 -³²P]ATP (50 μ M) 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 ³²P 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 ³²P 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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