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Insulin increases membrane protein kinase C activity in rat diaphragm

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Calcium/phospholipid-dependent protein kinase activity (protein kinase C) was identified in rat diaphragm membrane and cytosol fractions by means of in vitro phosphorylation either of histones or of a specific 87 kDa protein substrate, combined with phosphopeptide-mapping techniques. Both insulin and tumor-promoting phorbol ester treatment of the diaphragm preparations led to increased protein kinase C activity in the membrane fractions. In contrast to the phorbol ester, however, insulin did not induce a concomitant decrease in cytosolic activity, indicating that translocation of the enzyme had not taken place. Thus, insulin appears to increase specifically membrane protein kinase C activity in rat skeletal muscle, possibly through a mechanism not identical to that induced by phorbol esters.

Insulin; Protein kinase C; 87 kDa protein; (Rat diaphragm, Sarcolenna)

1. INTRODUCTION

Skeletal muscle sarcolemma protein phosphorylation may represent a prominent target for regulatory functions in skeletal muscle. Recent studies have demonstrated a number of sarcolemma phosphoproteins, one of which, a 15 kDa protein, was found to be a target for multisite phosphorylation catalyzed by cyclic AMP-dependent and calcium/phospholipid-dependent protein kinases (protein kinase C) [1,2]. In previous studies, addition of insulin to skeletal muscle sarcolemma in vitro was found to increase phosphorylation of a similar 15 kDa protein [3], apparently through GTP-dependent stimulation of a serine-specific protein kinase which also phosphorylated exogenous histones [4]. More recent work has shown that insulin also stimulates phosphorylation of the β-subunit of the insulin receptor in intact cells on both tyrosine [5-8] and serine [9,10] residues. The insulin-sensitive serine-specific protein kinase(s) which may phosphorylate the 15 kDa protein, the insulin receptor and possibly other membrane proteins remain(s) unidentified. Insulin has previously been found to decrease the activity ratio of cyclic AMP-dependent protein kinase in skeletal muscle [11]. Here, we have analysed the effects of insulin on protein kinase C activity in intact skeletal muscle. Protein kinase activities have therefore been analysed in various subcellular fractions derived from insulin- or phorbol ester-treated [12,13] rat diaphragm preparations, using different substrate

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammoniol]-1-propanesulfonic acid; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMA, phorbol 12-myristyl 13-acetate.

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proteins and peptide-mapping techniques. This has allowed us to identify a pool of membrane-associated protein kinase C which appears to be specifically activated by treatment of the intact preparation with insulin.

2. MATERIALS AND METHODS

2.1. *Materials*

Highly purified pig insulin was a gift from Hagedorn Research Laboratory (Gentofte, Denmark). Histone H2B was from Boehringer, Mannheim. Histone III-S, trypsin, PMA (phorbol 12-myristyl 13-acetate), 8-bromo cyclic AMP, CHAPS, aprotinin, leupeptin, antipain and phosphatidylserine (prepared as in [14]) were from Sigma, thermolysin from Calbiochem, while *Staphylococcus aureus* V8 protease was from Miles Biochemicals. The heat-stable protein inhibitor of cyclic AMP-dependent protein kinase (Walsh inhibitor) from rabbit skeletal muscle was a gift from Dr A.C. Nairn (The Rockefeller University). The 87 kDa protein and protein kinase C, both from bovine brain, were purified as described [15]. SDS-PAGE was performed as in [14], using gels containing a 7–15% gradient of acrylamide.

2.2. *Incubation and isolation of subcellular fractions from rat diaphragm*

Intact rat diaphragms from male rats (150 g body wt, fasted overnight) were dissected and preincubated for 30 min at 30°C in oxygenated Krebs-Ringer bicarbonate medium with 5 mM glucose. Insulin (final concentration 150 nM) or PMA (final concentration 100 nM) were added, and the incubations continued for 5 or 10 min. The diaphragms were then rapidly removed, frozen and stored in liquid nitrogen. They were homogenized (1:10) in an ice-cold medium containing 0.25 M sucrose, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, leupeptin (10 μg/ml), antipain (10 μg/ml) and aprotinin (50 KIU/ml) in 25 mM Tris-HCl (pH 7.4) in an Ultra-Turrax homogenizer. The homogenate was centrifuged for 20 min at 10000 × *g*, the pellet discarded, and aliquots of the crude extract stored on ice for later analysis. Another aliquot of the crude extract was centrifuged for 100000 × *g* for 45 min, and the supernatant was stored on ice for assay of cytosol protein kinase activity, while the crude membrane pellet was extracted with CHAPS (0.5%, w/v) on ice for 30 min followed by a second centrifugation at 100000 × *g* for 45 min. The final supernatant contained all measurable particulate protein kinase C activity, and was used for determination of membrane activity.

2.3 *Standard phosphorylation assay*

Aliquots from the subcellular fractions (5–10 μg protein) were incubated in a volume of 100 μl containing (final concentrations) 10 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 50 μM [γ-^{32}P]ATP, 1 mM DTT, leupeptin (10 μg/ml), Walsh inhibitor (2 μM), 0.1% CHAPS (w/v) and 25 mM Tris-HCl (pH 7.4), in the absence or presence of 1.25 mM $CaCl_2$ and phosphatidylserine (40 μg/ml) as indicated. 50 μg histone H2B or histone III-S, or 0.5–1 μg 87 kDa protein, were added as substrates, the tubes were preincubated at 30°C for 30 s, and the reactions were initiated by addition of ATP. After 30–120 s incubation at 30°C, the reactions were terminated by addition of an SDS-containing 'stop solution' [14], followed by boiling for 2 min. Phosphoproteins were separated by SDS-PAGE, visualized by autoradiography, and phosphorylation was quantitated by liquid scintillation counting of the excised gel pieces [14]. Protein kinase C

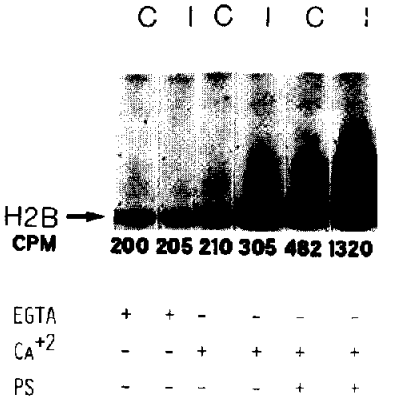

Fig.1. Autoradiogram showing increased activity of protein kinase C in extracts of rat diaphragm incubated with insulin. Histone H2B was phosphorylated, using crude extracts (5 μg protein) of untreated (C) or insulin-treated (I) rat diaphragms as enzyme source, in the absence or presence of $CaCl_2$ (0.25 mM free) and phosphatidylserine (PS, 40 μg/ml). cpm: ^{32}P incorporated into the histone band under the different conditions. Results from a representative experiment are shown.

activity was calculated as the difference between the activity found in the presence of Ca^{2+} and phosphatidylserine and that observed in the presence of EGTA alone. Enzyme activity was linear with respect to incubation time and amount of protein added under the conditions employed. Addition of diolein (4 μg/ml) did not stimulate protein kinase activity under the conditions used, as also seen in other crude systems [14].

Peptide mapping with incomplete proteolysis, using *S. aureus* V8 protease (10 μg), or with limit digestion, using trypsin or thermolysin (100 μg/ml for both) was performed essentially as in [14,15]. Protein was analysed by a dye-binding method [16].

3. RESULTS

Considerable calcium/phospholipid-dependent protein kinase (protein kinase C) activity was found in different fractions from rat skeletal muscle, with the highest specific activity seen in the sarcolemma, followed by the crude sarcoplasmic reticulum, cytosol and contractile protein pellet (not shown). Fig.1 shows results from an experiment where crude extracts, consisting of a 10000 × *g* supernatant and thus containing most of the sarcolemma and sarcoplasmic reticulum together with the cytosol, were assayed for kinase activity with histone H2B as the substrate. Addition of calcium and phospholipid to extracts of control

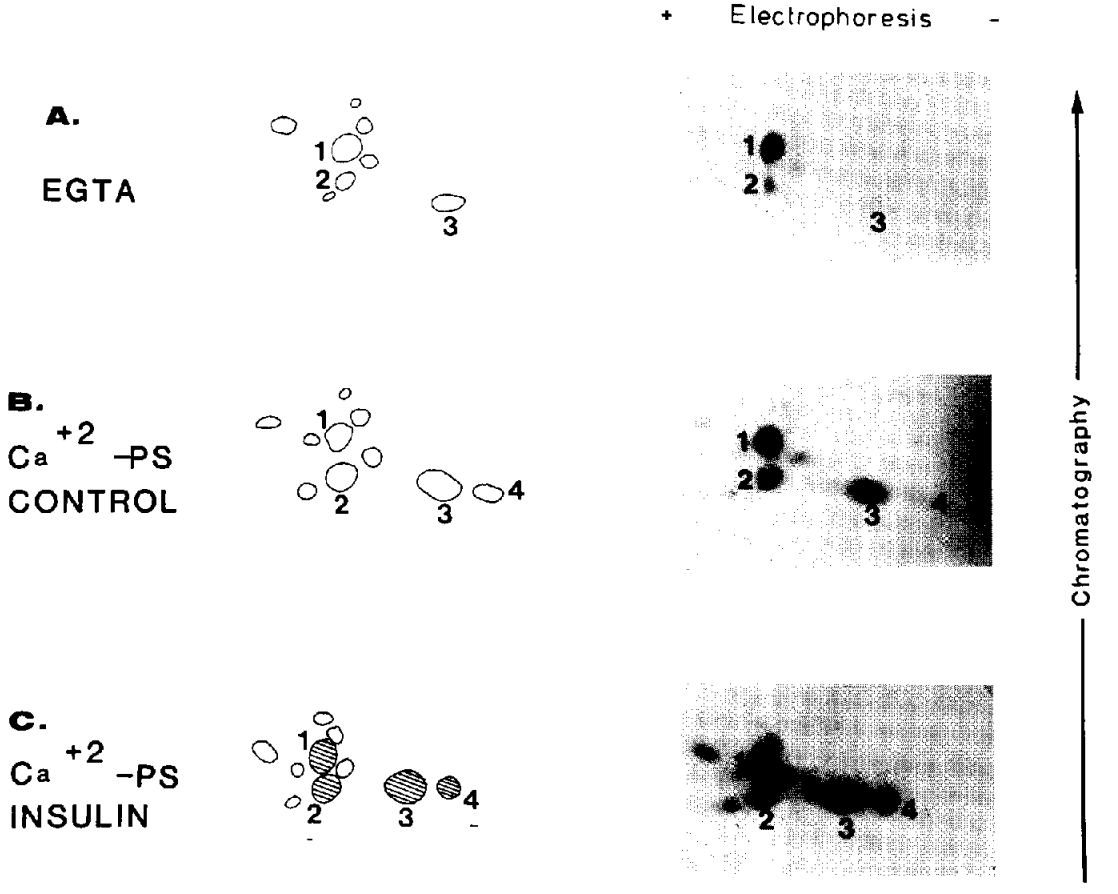

Fig.2. Autoradiogram showing phosphopeptide map of histone H2B phosphorylated by extracts from untreated (control) or insulin-treated (insulin) rat diaphragms. The phosphorylated histone H2B bands shown in fig.1 were excised and subjected to limit digestion by trypsin, followed by two-dimensional separation. (Left) Schematic localization of phosphopeptides upon two-dimensional separation. Numbers indicate the four major phosphopeptides (hatched areas) phosphorylated by the insulin-sensitive protein kinase.

diaphragms (no insulin) more than doubled phosphorylation of the substrate under the conditions employed. By comparison with the control, extracts from insulin-treated diaphragms displayed a 2-fold increase in calcium/phospholipid-dependent histone kinase activity, without any increase in the histone kinase activity seen in the absence or presence of calcium alone. Results from six similar, separate experiments showed that extracts from insulin-treated diaphragms expressed 106 ± 12% of Ca^{2+}-independent, 137 ± 16% ($p = 0.05$) of Ca^{2+}-dependent and 201 ± 32% ($p < 0.01$) of Ca^{2+}/phospholipid-dependent histone kinase activities when compared to activities in extracts from control diaphragms (not shown). Similar results were obtained with histone III-S (not shown). Analysis of cyclic AMP-dependent protein kinase in these extracts (not shown) showed that insulin had decreased the intracellular activity ratio of this enzyme by 20–30%, in agreement with previous results [11].

The insulin effect on calcium/phospholipid-dependent histone phosphorylation was further characterized by peptide mapping (fig.2). Following limit digestion with trypsin, four major histone H2B peptides were found to be strongly phosphorylated by the diaphragm extract upon addition of calcium/phosphatidylserine, and all four major plus some minor phosphopeptides were more highly phosphorylated by the insulin-treated extracts. In three separate experiments, radioactivity was determined by scintillation counting of the excised major spots. These experiments showed that labeling of peptide 1 (fig.2) increased from (mean) 84 cpm (control diaphragm, histone phosphorylated without Ca^{2+} buffer) to 140 cpm (control diaphragm, histone phosphorylated with Ca^{2+}/phospholipid-containing buffer) to 222 cpm (insulin-

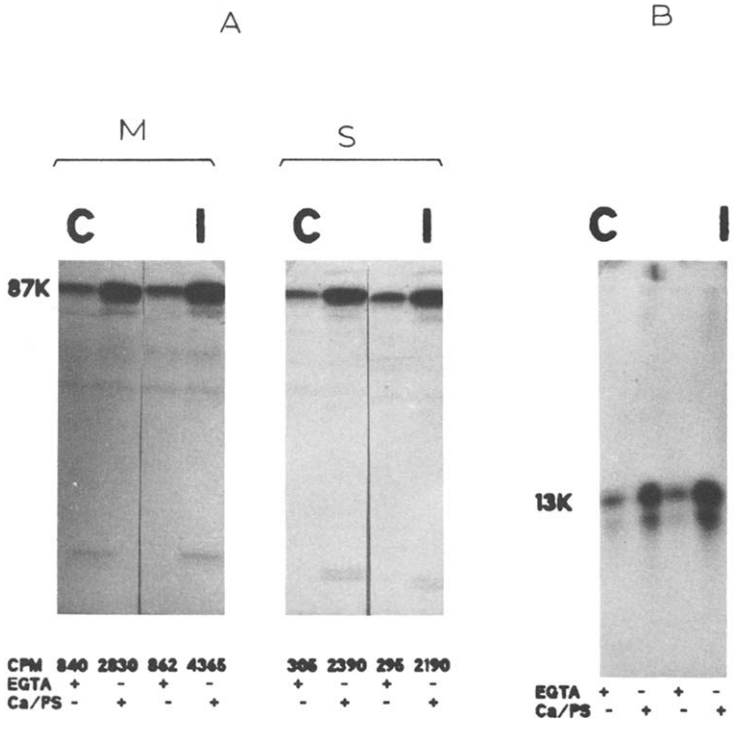

Fig.3. Autoradiogram showing insulin stimulation of 87 kDa protein kinase activity in membranes from rat diaphragm. Membranes (M, 2 μg protein) or cytosol (S, 5 μg protein) fractions from untreated (C) or insulin-treated (I) diaphragms were used as sources for protein kinase C. (A) The 87 kDa protein was phosphorylated, separated by SDS-PAGE and analysed as described in the text. cpm: ^{32}P incorporated into the 87 kDa band under the different conditions, measured by Cerenkov counting. Results from a typical experiment, which was repeated three times with similar results, are shown. (B) Incomplete proteolytic digestion of phosphorylated 87 kDa protein. The phosphoprotein bands visualized in A were excised and subjected to peptide mapping with *S. aureus* V8 protease (10 μg/sample) as described in the text.

treated diaphragm, histone phosphorylated with Ca^{2+}/phospholipid-containing buffer). Peptide 2 increased from 20 to 52 to 92 cpm, peptide 3 increased from 22 to 45 to 242 cpm, while peptide 4 increased from undetectable radioactivity to 20 to 70 cpm under the same conditions. Thus, these results indicate both that histone H2B is a multisite substrate for a calcium/phospholipid-dependent protein kinase in crude extracts from rat diaphragm, and that this kinase shows a higher activity following insulin treatment of the diaphragm preparations.

The crude extract described above contained both membrane fragments and cytosol. We therefore analysed the insulin effect in the separated fractions. Since both histone H2B and histone III-S can be phosphorylated by a number of protein kinases, a brain enriched 87 kDa protein which appears to be a specific substrate for protein kinase C [14,15,17] was used as substrate in these experiments. Fig.3 and table 1 show that membrane extracts from rat diaphragm phosphorylated the 87 kDa protein in a manner dependent upon calcium and phosphatidylserine. This activity was increased following incubation of the diaphragm with insulin. In contrast, incubation of the 87 kDa protein with rat diaphragm cytosol showed lower specific protein kinase C activity in this fraction,

Table 1

Stimulation of protein kinase C activity in membrane fractions of rat diaphragm by insulin and PMA

Treatment	Subcellular fraction			
	Membrane (Phosphorylation buffer)		Cytosol	
	EGTA	Ca^{2+}/PS	EGTA	Ca^{2+}/PS
Control	24 ± 2	103 ± 6	12 ± 2	27 ± 3
Insulin	25 ± 2	156 ± 7[a]	13 ± 2	28 ± 3
Control		97 ± 5		29 ± 2
PMA		175 ± 8[a]		21 ± 2[b]

Activities are given as (cpm/min per mg protein) × 10^{-3} (mean ± SE, $n = 3$). Purified 87 kDa protein (0.5 μg) was incubated with membrane or cytosol extracts (5 μg protein) prepared from rat diaphragms previously incubated in the absence or presence of either insulin or PMA, and analysed as described in the text. [a] $p<0.01$; [b] $p<0.05$; compared with values obtained from control diaphragms (*t*-test)

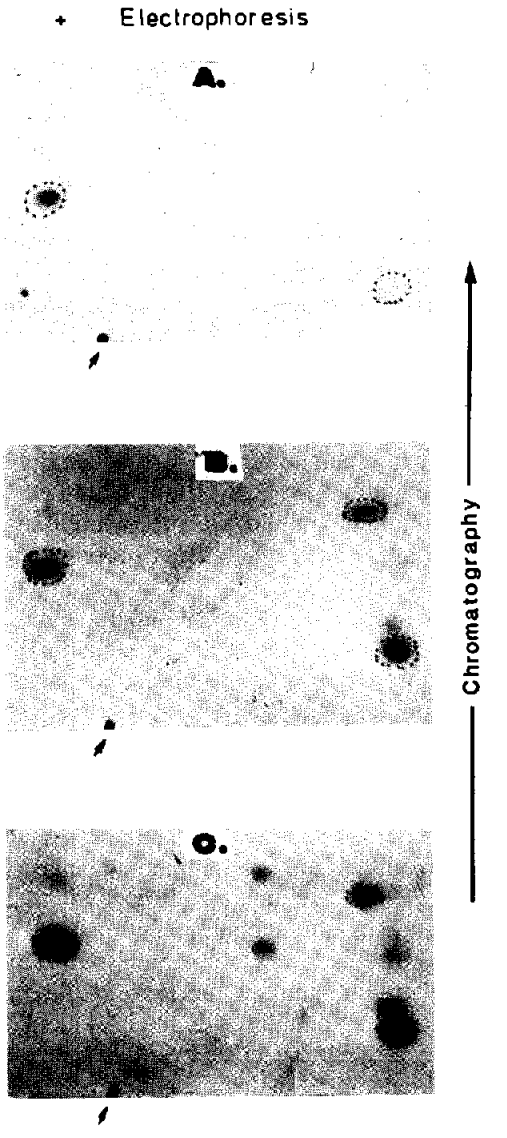

Fig.4. Autoradiogram showing phosphopeptides generated by limit digestion of the 87 kDa protein phosphorylated by membrane fractions from rat diaphragm. The 13 kDa phosphopeptides generated by incomplete proteolysis of the 87 kDa band (fig.3B) were subjected to thermolysin digestion, and analysed as described in the legend to fig.2. (A) Rat diaphragm incubated in the absence or presence of insulin; 87 kDa protein phosphorylated in the presence of EGTA. (B) Rat diaphragm incubated in the absence of insulin; 87 kDa protein phosphorylated in the presence of Ca^{2+}/phosphatidylserine. (C) Rat diaphragm incubated in the presence of insulin; 87 kDa protein phosphorylated as in B. Arrows indicate origin of two-dimensional separation. The major phosphopeptides are outlined.

and pretreatment with insulin had no effect on this activity (fig.3, table 1).

Identification of protein kinase C in the membrane fraction was further obtained by peptide mapping of the 87 kDa phosphoprotein. As shown in fig.3B, a major 13 kDa phosphopeptide was generated after incomplete proteolysis with *S. aureus* V8 protease. When this 13 kDa phosphopeptide band was subjected to limit digestion with thermolysin, two-dimensional separation of the resulting peptides (fig.4) showed two major and six minor phosphopeptides, all of which showed increased phosphorylation following incubation with membrane extracts derived from insulin-treated diaphragm. These phosphopeptide maps were identical (not shown) to those obtained from purified 87 kDa protein phosphorylated simultaneously (not shown) by purified protein kinase C [15,17].

Tumor-promoting phorbol esters have been reported to be able to mimic endogenous diacylglycerol in binding protein kinase C to membrane phospholipids, thereby activating the enzyme [12,13]. We therefore examined protein kinase C activity after incubation of the diaphragm with PMA. This treatment led to a significant increase in protein kinase C activity in the membrane fraction, concomitant with a significant decrease in activity in the soluble fraction (table 1). This effect was different from that induced by insulin, where protein kinase C activity was increased in the membrane fraction without any decrease in the cytosol. The latter type of response might therefore be caused by a direct effect of insulin on membrane protein kinase C. Such an effect has previously been observed in isolated sarcolemma membranes treated with insulin [18].

4. DISCUSSION

This work has demonstrated a protein kinase activity in both particulate and soluble fractions from rat diaphragm preparations which could be identified as protein kinase C by various criteria, including calcium and phospholipid dependency, phosphopeptide maps of identified substrates, and comparison with results obtained using purified brain protein kinase C. The results using the 87 kDa protein as substrate are particularly clear, since this protein appears to be a specific substrate for protein kinase C, and is not phosphorylated by either cyclic AMP-, cyclic GMP- or calcium/calmodulin-dependent protein kinases [14,15,17,19].

Our data further indicate that the membrane-associated form of this protein kinase is regulated by insulin. Previous work has failed to demonstrate insulin effects on protein kinase C in both adipocytes [20], 3T3-L adipocytes [21] or 3T3-L1 fibroblasts [22]. However, insulin appears to increase protein kinase C activity in both membranes and cytosolic fractions in BCH3H-1 myocytes [23]. Somewhat different results have been obtained in the present work on intact diaphragms, since we observed that treatment with insulin increased protein kinase C activity in the membranes, but had no apparent effect on the cytosolic activity. In contrast, protein kinase C was increased in the membrane and decreased in the cytosol after incubation of the diaphragm with phorbol ester, suggesting that translocation had taken place. Such an effect of phorbol esters has also been demonstrated in many other cell types [13]. Certain agonist-receptor interactions, e.g. gonadotropin-releasing hormone and thyrotropin-releasing hormone [24,25], also promote redistribution of protein kinase C to the membranes, while other hormones (e.g. ACTH) or lectins like concanavalin A may promote either translocation of protein kinase C from membranes to cytosol [26], or a net increase in activity without translocation [27,28]. These results indicate that different modes of activation of the enzyme may be involved under different conditions.

Agonist-mediated activation of protein kinase C has been attributed to activation of phospholipase C, with breakdown of inositol phospholipids and generation of diacylglycerol leading to membrane translocation and activation of the cytosolic enzyme [12]. Insulin has been reported to activate phospholipase C [29] and increase production of diacylglycerol [30,31]. However, since no insulin-induced translocation of protein kinase C was found in our study, this mechanism cannot fully explain the net increase of membrane enzyme activity seen after insulin treatment of the diaphragm. A more complex mechanism may be involved, such as phosphorylation of the enzyme [32], activation of a specific isozymic form of protein kinase C [33], or stable conformational

changes due to insulin-induced association of phospholipids with the enzyme.

The increased activity of protein kinase C in the membrane may be of importance in the transduction of the insulin signal. Activation of endogenous protein kinase C in the membrane may lead to increased phosphorylation of membrane proteins, e.g. the 15 kDa sarcolemma protein [1,2]. The insulin receptor has also recently been reported to be phosphorylated on serine residues by protein kinase C in vitro [34], confirming previous results obtained with phorbol ester application to intact cells [35]. Since serine phosphorylation of the insulin receptor appears to decrease the activity of the receptor both as a tyrosine-specific protein kinase and as a transducer of the insulin signal [35], insulin-induced activation of membrane-bound protein kinase C may be of importance as a control mechanism of the activity of the insulin receptor itself.

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