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Insulin-induced decrease in 5′-nucleotidase activity in skeletal muscle membranes

Amira Klip, Toolsie Ramlal, Andre G. Douen, Elena Burdett, Douglas Young*, Gregory D. Cartee* and John O. Holloszy*

Department of Cell Biology, The Hospital for Sick Children, Toronto, M5G 1X8 Canada and **Department of Medicine, Washington University School of Medicine, St. Louis, MO 63122, USA*

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Insulin releases inositol phosphoglycans from myocytes in culture [(1986) Science 233, 967–972], which display insulinomimetic activity. Because 5′-nucleotidase is anchored to the membrane through inositol-containing phospholipid glycans, we investigated whether insulin could release the enzyme from the membrane. Membranes prepared from hindquarter muscles of rats perfused with insulin showed a 23% decrease in 5′-nucleotidase activity. Isolated membranes from muscle exposed to insulin in vitro also showed a small but reproducible decrease (9%) in 5′-nucleotidase activity relative to unexposed controls. Phospholipase C from *Staphylococcus aureus* released 60% of the membrane-bound 5′-nucleotidase. We propose that insulin may activate an endogenous phospholipase C that cleaves phospholipid-glycan-anchored proteins.

5′-Nucleotidase; Insulin action; Anchored protein; (Skeletal muscle)

1. INTRODUCTION

In skeletal muscle, insulin stimulates glucose transport, oxidation and incorporation into glycogen. Considerable progress has been made in the last two years regarding the identification of water-soluble mediators [1–3] that stimulate glycogen synthase phosphatase [4] and mimic several other actions of insulin [1,5,6] even in intact cells [5]. These purported insulin mediators are inositol-containing phosphoglycans (IPG) produced by phospholipase C (PL-C) hydrolysis of phospholipid-glycans present in BC_3H1 myocytes [1], liver membranes [2] or H35 hepatoma cells [3]. They have been equated to the 'polar head groups' isolated from insulin-treated BC_3H1 myocytes [1] or H35 hepatoma cells [3], which are generated roughly in a 1:1 ratio to diacylglycerol [1].

In cell membranes, IPGs are found in phosphatidylinositol-glycans (phospholipid-glycans) and in phosphatidylinositol-glycan-ethanolamine-proteins (henceforth referred to as phospholipid-glycan-proteins) (review [7]). Membrane proteins anchored to the membrane through phospholipid-glycans include 5′-nucleotidase and alkaline phosphatase (reviews [8,17]). The IPGs released by the action of insulin could arise from hydrolysis of either phospholipid-glycans or phospholipid-glycan-proteins. The nature of the parent compound(s) that are insulin-sensitive has yet to be elucidated. For IPGs to be derived from phospholipid-glycans a single cleavage by a PL-C would suffice. In contrast, for IPGs to be derived from phospholipid-glycan-proteins, a second cleavage by a protease would seem necessary for protein removal (see [9]).

Generation of IPGs can also be induced by addition of PL-C from *Staphylococcus aureus*, suggesting that insulin may activate an endogenous PL-C akin to that from bacteria. Indeed, an insulin-sensitive PL-C activity has been detected in fat cells [10]. In addition to generating IPGs, the

Correspondence address: A. Klip, Department of Cell Biology, The Hospital for Sick Children, Toronto, M5G 1X8 Ontario, Canada

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hypothetical insulin-dependent PL-C could potentially release IPG-ethanolamine proteins. This could change the protein composition of cell membranes. Moreover, the released proteins, if endowed with enzymatic activity, could act at a site distinct from the cell of origin. It is not known whether insulin can release anchored membrane enzymes. This study describes the effect of insulin in situ and in vitro on 5′-nucleotidase activity in skeletal muscle membranes.

2. MATERIALS AND METHODS

2.1. *Animals and perfused hindlimb preparation*

Male Sprague-Dawley rats weighing 250–350 g were fasted overnight prior to experiments, and surgical preparation of the hindquarter with placement of catheters in the abdominal aorta and inferior vena cava was performed essentially as in [11,12]. Hindlimbs were perfused with 50 ml oxygenated Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 4 g/100 ml bovine serum albumin and 8 mM glucose, followed by 20 min perfusion with 0 or 1000 μU/ml porcine insulin (20 ml/min). An approx. 5-fold increase in the rate of glucose uptake (arterial–venous difference) was observed in response to insulin. Immediately after perfusion the muscles of both hindlimbs were dissected out, trimmed of fat and connective tissue while kept on ice, frozen in liquid N_2 and kept at -70°C until used for preparation of membranes.

2.2. *Membrane preparation and enzyme activities*

Muscle membrane isolation was carried out exactly as described in [12] by differential centrifugation and separation on sucrose gradients. The membranes isolated on top of 25% sucrose were washed by dilution in 10 mM $NaHCO_3$ (pH 7.0), 0.25 M sucrose, 5 mm NaN_3, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 190000 × g for 60 min. The membranes were resuspended in medium of the same composition and stored frozen at -120°C. This fraction was previously shown to contain purified plasma membranes [12], based on its enrichment in the plasma membrane markers phosphodiesterase I and Mg^{2+}-ATPase (>3-fold increase over crude membranes) and absence of the sarcoplasmic reticulum marker Ca^{2+}-dependent ATPase [12]. Protein content was determined by the dye-binding procedure of Bradford [13]. Liver membranes were prepared by a modification of the method of Song et al. [14].

5′-Nucleotidase was assayed as described by Song and Bodansky [15]. Briefly, 25 μg protein were incubated with 5 mM AMP in 0.1 M Tris-HCl (pH 8.3) containing 10 mM $MgCl_2$ for 30 min at 37°C. Phosphate production was measured by reaction with ammonium molybdate and $FeSO_4$. Plasma membrane Mg^{2+}-ATPase was assayed as in [16]; alkaline phosphatase (pH 10.3) was assayed as in [17] using bis(*p*-nitrophenyl) phosphate as substrate. Adenyl cyclase was measured as in [18].

Insulin action in vitro: membranes (1 mg protein) were incubated in a final volume of 25 ml without or with 1 μM insulin in 2 mM $MgCl_2$, 10 mM $NaHCO_3$, 7 mM $CaCl_2$, 1 mM ATP for 30 min at 37°C. During incubation, 1 mM ATP was re-added every 10 min (deemed necessary to maintain activity of the insulin receptor tyrosine kinase). Membranes were then centrifuged at 190000 × g for 60 min and used for determinations of protein, 5′-nucleotidase and alkaline phosphatase.

Treatment with PL-C from *S. aureus* (spec. act. 100 μmol/min per ml towards phosphatidylinositol bisphosphate, kindly provided by Dr M. Low and Dr M. Moscarello): 50 μl membranes in the $NaHCO_3$/sucrose containing PMSF and NaN_3 were mixed with 50 μl of 0.1 M Tris (pH 7.4) containing 2 mM EGTA ± PL-C for 30 min at 37°C. The membranes were then pelleted in an airfuge at maximal velocity for 10 min. The 5′-nucleotidase activities of both complete pellet and supernatant were immediately determined as described above.

3. RESULTS

3.1. *5′-Nucleotidase activity in isolated plasma membranes from muscles of insulin-treated rats*

We have previously shown that perfusion of hindquarter muscles with insulin results in the migration of glucose transporters from an intracellular membrane fraction to the plasma membrane [12]. This was concluded from the observed increase in number of glucose transporters (detected as glucose-protectable cytochalasin B-binding sites) in the isolated plasma membranes, and concomitant decrease in the number of transporters in an isolated fraction containing intracellular membranes. Those observations indicate that changes that occur during perfusion with the hormone can be retained in the isolated membranes. Here, we examine the activity of 5′-nucleotidase in plasma membranes isolated from control and insulin-perfused muscles. The protein yield of the isolated membranes was not significantly different in insulin and control preparations (57 ± 9 and 57 ± 7 μg/g tissue, respectively, each n = 8). Table 1 lists the activity of 5′-nucleotidase in 10 control and 10 membrane preparations. This activity was decreased by an average of 23 ± 6% by the insulin treatment. In 8 of the 10 pairs of membrane preparations, Mg^{2+}-ATPase activity (another plasma membrane marker [12,16]) was also measured (table 1). The average Mg^{2+}-ATPase activity was not significantly different in the control and insulin-treated group. In one set of membranes from control and insulinized animals, forskolin-activated adenyl cyclase activity was also measured. This activity was 9.4 pmol/mg per h in control membranes and

Table 1

Effect of in situ insulin on 5′-nucleotidase and Mg^{2+}-ATPase activities

	5′-Nucleotidase	Mg^{2+}-ATPase
Control	365 ± 23 (10)	2589 ± 436 (8)
Insulin	273 ± 15 (10)	2451 ± 337 (8)
Δ(Control − insulin)	92 ± 23 (10)	138 ± 351 (8)
p (paired)	<0.005	$\geqslant 0.05$
p (not paired)	<0.001	$\geqslant 0.05$
$(I/C) - 1$	0.23 ± 0.06 (10)	0.06 ± 0.14 (8)

Plasma membranes were prepared from skeletal muscles of control and insulinized animals (see section 2). The activities of 5′-nucleotidase and Mg^{2+}-ATPase were subsequently measured. Results are given in nmol/min per mg protein, expressed as means ± SE of (n) independent membrane preparations, each assayed in triplicate. $(I/C) - 1$ represents the averaged fractional decrease in activity caused by insulin

Table 2

Effect of in vitro insulin on the activities of 5′-nucleotidase and alkaline phosphatase

	5′-Nucleotidase	Alkaline phosphatase
Δ (Control − insulin)	17.3 ± 3.9	570 ± 120
t	4.436	4.75
n	4	4
p	<0.025	<0.025
$(I/C) - 1$	0.092 ± 0.026	0.125 ± 0.043

Plasma membranes were prepared from skeletal muscle of control animals, and then exposed in vitro to 0 or 1 μM insulin (see section 2). The activities of 5′-nucleotidase and alkaline phosphatase were subsequently determined. Results are of n = 4 independent membrane preparations, each assayed in triplicate. Δ, mean of the differences between paired control and insulin-treated samples, in nmol/min per mg protein. The statistical parameters of Student's t-test for paired data are included. $(I/C) - 1$, fractional change in insulin-treated samples relative to controls

19.9 pmol/mg per h in membranes from insulin-treated rats. This activity represented a 1.8- and 2.3-fold enrichment, respectively, relative to crude microsomes. Thus, the insulin-dependent decrease in 5′-nucleotidase is not due to a generalized decrease in other membrane marker enzymes.

3.2. *5′-Nucleotidase activity in membranes from skeletal muscle treated with insulin in vitro*

Table 2 shows the result of the in vitro action of insulin on isolated muscle membranes. Membranes were exposed to the hormone as described in section 2. The activity of 5′-nucleotidase in insulin-treated membranes was decreased by an average of 9.2 ± 2.6% vs that in untreated membranes. In the same membrane preparations, the activity of another phospholipid-glycan-protein, alkaline phosphatase, was also significantly decreased (12.5 ± 4.3%) by insulin. These results confirm the effects of insulin in the perfused muscle, and further suggest that the effect of the hormone of decreasing selective enzymatic activities in the plasma membrane does not require an intact cell. Therefore, presumably all the components necessary to elicit a decrease in 5′-nucleotidase or alkaline phosphatase activities are present in the isolated membranes.

The effect of insulin reported above appears to be tissue-specific. Exposure of isolated membranes from rat liver to insulin failed to elicit a decrease in 5′-nucleotidase activity: 103 ± 3 nmol/min per mg protein in control, and 97 ± 7 nmol/min per mg protein in insulin-treated membranes.

3.3. *Effect of phospholipase C of* S. aureus *on 5′-nucleotidase activity*

The small but significant decrease in 5′-nucleotidase activity caused by insulin could be due to release of anchored enzyme. To calculate the maximum amount of enzyme that is anchored through phospholipid-glycans, membranes from control skeletal muscle were treated with PL-C from *S. aureus*. When 30 μg membrane protein were thus treated and centrifuged, 5′-nucleotidase activity in the pellet decreases by 60% (from 319 to 124 nmol/30 min). The activity lost was nearly quantitatively recovered in the supernatant (225 nmol/30 min in supernatants of PL-C-treated vs 28 nmol/30 min in those of untreated membranes).

4. DISCUSSION

4.1. *Possible mechanisms of insulin-mediated decrease in 5′-nucleotidase activity*

The results of this study indicate that in situ treatment with insulin results in a 23% loss of

5′-nucleotidase activity in plasma membranes isolated from skeletal muscles of the rat hindquarter. This effect is mimicked to some extent by incubating control membranes in the presence of insulin in vitro (9% decrease in enzyme activity). 60% of 5′-nucleotidase activity can also be released from muscle membranes by exogenous PL-C. Therefore, assuming that the insulin-mediated decrease in 5′-nucleotidase activity results from hormonal activation of an endogenous PL-C, it can be calculated that insulin releases 42% in situ and 15% in vitro of the total releasable 5′-nucleotidase (i.e. of the total activity releasable by exogenous PL-C).

An alternative explanation might be that insulin inhibits the membrane-bound 5′-nucleotidase. To establish whether the hormone induces release of enzyme from the membranes, one needs to test the aqueous medium for 5′-nucleotidase activity. Unfortunately, this cannot be done in the in situ perfusion experiments. Also, the activity of this enzyme in the supernatant of in vitro insulin-treated membranes could not be assessed due to interference by ATP with the 5′-nucleotidase assay. Therefore, at present, the suggestion that the insulin-dependent decrease in activity of this enzyme is mediated by an endogenous PL-C relies on the analogy of the hormonal effect to the effect of exogenous PL-C.

To our knowledge, there are no reports that demonstrate an insulin-dependent release of anchored proteins through the activation of endogenous PL-C. However, circumstantial evidence suggesting this possibility has been recently published: Levy et al. [19] reported a decrease in the total activity of alkaline phosphatase in rat osteoblastic cells after chronic (24 h) insulin treatment. Similarly, the content of heparan sulfate proteoglycan, another anchored protein, was lowered by exposure of cells to insulin for 12 h [20]. The nature of the association of that protein with the membrane, however, remains controversial [21]. It was suggested recently that alkaline phosphatase may be released from intact cells by insulin through the activation of an unidentified cellular protease [22]. We believe that the decrease in membrane-bound 5′-nucleotidase activity reported in our study is not the exclusive result of proteolysis, since PMSF, a serine protease inhibitor, was present during all incubations.

4.2. *Significance of the insulin-dependent decrease in 5′-nucleotidase activity*

The present results indicate that in rat skeletal muscle membranes there is an insulin-dependent decrease in the activity of 5′-nucleotidase. The physiological significance of this finding remains to be determined. However, it is intriguing that the levels of 5′-nucleotidase are 3-fold higher in adipocytes from diabetic (streptozotocin-treated) rats than in control animals, and that this activity decreases to close to normal values following incubation of intact adipocytes with insulin or following in vivo insulin treatment of diabetic animals [23]. Thus, it is tempting to suggest that insulin decreases the level of membrane-bound 5′-nucleotidase in vivo, and that this hormonal regulation of the enzyme may be abnormal in insulin-deficient states (e.g. diabetes).

In summary, we have shown that insulin decreases 5′-nucleotidase activity in plasma membranes from insulin-treated muscle, as well as in plasma membranes exposed in vitro to the hormone. If the decrease is due to the liberation of anchored protein molecules this would constitute evidence that insulin can activate the hydrolysis of phospholipid-glycan-proteins. In addition, the results suggest caution in the use of 5′-nucleotidase as a plasma membrane marker, since different metabolic conditions may result in loss of the enzyme from otherwise pure plasma membranes.

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