Biochem. J. (1987) 242, 131–136 (Printed in Great Britain)

Protein kinase C is not required for insulin stimulation of hexose uptake in muscle cells in culture

Amira KLIP* and Toolsie RAMLAL
Division of Neurology, The Hospital for Sick Children, and the Departments of Biochemistry and Pediatrics, University of Toronto, Toronto, Ontario M5G 1X8, Canada

The L6 skeletal muscle cell line has been identified as a suitable model to study the action of insulin on glucose uptake in muscle [Klip, Li & Logan (1984) Am. J. Physiol. **247**, E291–E296]. The signals that transfer information from occupied insulin receptors to glucose transporters remain unknown. Here we report that activation of protein kinase C by exogenous phorbol esters results in stimulation of glucose uptake. Protein C kinase activity was induced to migrate from the cytosolic fraction to the microsomal fraction after 40 min of exposure of intact cells to 4β-phorbol 12,13-dibutyrate. In contrast, incubation with insulin did not alter the subcellular distribution of the kinase. Prolonged preincubation of L6 cells with phorbol esters resulted in depletion of kinase C activity, whereas neither the basal rate of glucose uptake nor its stimulation by insulin were affected. This suggests that protein kinase C is expressed in L6 cells, and that insulin stimulation of hexose transport does not involve protein kinase C.

INTRODUCTION

Stimulation of glucose uptake into muscle is one of the better-recognized functions of insulin. A current hypothesis states that a possible mechanism to account for this stimulation is the recruitment of glucose transporters to the cell surface from intracellular membrane stores [1,2]. The signal(s) involved in the transduction of information from the occupied insulin receptors to the glucose transporters remain unknown.

We have demonstrated that the differentiating L6 skeletal muscle cell line is a suitable system to study the stimulation of glucose transport by insulin and the possible mediators of hormone action in muscle [3]. After myoblast fusion into myotubes, the cells become responsive to insulin, and an elevation in the uptake of non-metabolizable hexoses is observed within minutes of addition of the hormone. In these cells, we showed that insulin does not alter the level of cytoplasmic free Ca^{2+}, and that chelation of intracellular Ca^{2+} does not prevent stimulation of glucose transport by the hormone [4]. Furthermore, although insulin caused an early alkalinization of the cytoplasm, this change in pH_i was not essential for stimulation of glucose transport [5]. Hence, neither cytoplasmic Ca^{2+} nor H^+ appear to be the mediators of insulin stimulation of glucose uptake.

Phorbol esters such as TPA and PDB are exogenous activators of protein kinase C. Activation is caused by migration of protein kinase C activity from the cytosol to the plasma membrane in lymphoid cells [6]. Recently, it was reported that prolonged incubation of fibroblasts with phorbol esters causes depletion of protein kinase C, i.e., a decrease in the number of phorbol ester binding sites [7] and in protein kinase C activity [8]. One of the multiple responses of lymphoid cells [9] and fibroblasts [10,11] to TPA is the stimulation of hexose uptake. Hence, phorbol esters are 'insulin-like' agents, insofar as they stimulate glucose uptake. It was therefore possible that stimulation of glucose uptake by insulin could be mediated by stimulation of protein kinase C, through production of the endogenous activator diacylglycerol. In fact, insulin has been reported to increase the levels of diacylglycerol in BC3H-1 mouse myocytes [12]. Diacylglycerol originates from the hydrolysis of phospholipids catalysed by phospholipase C, and this enzyme is elevated in homogenates of insulin-treated fat cells [13].

In the present paper we report that phorbol esters that activate protein kinase C can stimulate glucose uptake in L6 muscle cells. We further compare the ability of PDB and insulin to induce migration of protein kinase C from the cytosol to the membrane. Finally, depletion of protein kinase C is caused by prolonged incubation with PDB, and the response to insulin of glucose transport is measured under these conditions.

MATERIALS AND METHODS

Materials

Culture media and sera were from Gibco. Porcine insulin, phorbol esters, phosphatidylserine, histone type III-S, cytochalasin B, 2-deoxyglucose and ATP were from Sigma, [γ-^{32}P]ATP and 2-deoxy[^{3}H]glucose were from ICN.

Cell cultures

A high-fusion clone of the L6 muscle cell line originally developed by Yaffe [14] was selected by Dr. G. Cates, University of Western Ontario. These cells undergo myogenesis *in vitro* and retain many of the properties of skeletal muscle [15]. The cells were grown in monolayers in α-Minimal Essential Medium containing 2% (v/v) fetal calf serum in an atmosphere of

Abbreviations used: TPA, 12-*O*-tetradecanoyl-β-phorbol 13-acetate (=4β-phorbol 12-myristate 13-acetate); PDB, 4β-phorbol 12,13-dibutyrate.

* To whom correspondence and reprint requests should be addressed, at: the Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada.

CO_2/air (1:19) at 37 °C. The cells were passaged before confluence (< 20 passages) but were allowed to reach confluence, alignment and fusion prior to determinations of glucose uptake or protein kinase C activity.

2-Deoxyglucose uptake

The cells were deprived of serum and incubated in Minimal Essential Medium containing 25 mM-glucose for 5 h at 37 °C prior to incubation with insulin or phorbol esters (times and concentrations indicated in each Figure). Cells were rinsed twice with glucose-free Hepes-buffered saline solution, followed by determinations of transport of 2-deoxy[^{3}H]glucose (0.1 mM) for 10 min in the same solution. We have previously shown that under these conditions hexose transport is the rate-limiting step of uptake [3,16]. Transport was terminated by aspiration of the radioactive solution followed by three rapid wash steps with cold saline solution. Radioactivity inside the cells was measured after disruption with 0.05 M-NaOH. Non-carrier-mediated hexose uptake was determined in parallel samples in the presence of 5 μM-cytochalasin B, and was subtracted from all measurements reported. Protein was determined by the method of Bradford [17]. All experiments were performed at least three times, and within each experiment triplicate determinations were performed. Results are expressed as the mean ± S.E.M. of (n) measurements.

Protein kinase C in cell homogenates and subcellular fractions

For measurements of the protein kinase C activity in total cell homogenates, cells were trypsinized off the substratum and allowed to recover for 40 min in the presence of 2% fetal calf serum. The cells were then serum-depleted for 5 h. Following this procedure L6 cells retained the capacity to respond to insulin: 2-deoxyglucose (0.01 mM) uptake was 16.6 ± 0.2 pmol/min per mg in the absence of insulin, and 23.2 ± 0.3 (n = 4) in the presence (40 min) of the hormone (5 min uptake, determined by centrifugation through dibutyl phthalate/oil as described earlier [16]). Response to insulin in trypsinized cells is not uncommon. We have observed insulin stimulation of Na^+/H^+ exchange (such as that described in [5]) in trypsinized L6 cells (A. Klip & T. Ramlal, unpublished work). In trypsin/EDTA-treated CHO or IM9 cells, insulin binding was unaltered relative to untreated cells [33]. For protein kinase C determinations, the cells were exposed to insulin or the indicated phorbol ester for the specified time periods. Cells were then lysed by resuspension in Triton solution (0.1 mM-EDTA, 1 mM-EGTA, 0.5% Triton X-100, 0.5 mM-phenylmethanesulphonyl fluoride, 10 mM-2-mercaptoethanol and 20 mM-Tris/HCl, pH 7.5). The suspension was homogenized by 20 strokes in a Dounce-type homogenizer, and then centrifuged at 4 °C for 30 min at 48000 g. Protein kinase C activity was measured in this fraction as described below.

For measurements of the subcellular distribution of protein kinase C, cells were suspended by trypsinization and exposed to 1 μM-PDB or insulin for 40 min. They were then lysed by osmotic shock, followed by freeze–thawing and vortex-mixing. The homogenates were centrifuged at 48000 g for 30 min. The supernatant is referred to as the cytosolic fraction. The pellet was solubilized in Triton solution and centrifuged at 15000 g for 10 min. The supernatant of this material represents the membrane fraction. The protein kinase C activity of both the cytosolic and the membrane fractions was measured as described next.

Protein kinase C activity was measured by a modification of the methods of Kraft & Anderson [6] and of Friedman *et al.* [18]. Briefly, fractions were passed through a DE-52 (DEAE-cellulose) column to remove endogenous lipid, diacylglycerol, kinase inhibitors and unrelated kinases. Protein kinase C was eluted with 70 mM-NaCl [18]. The activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone type III-S. The assays were performed for 6 min at 35 °C with 5–20 μg of protein in a total volume of 250 μl of medium containing 0.5–1.0 μCi of [γ-^{32}P]ATP, 50 μM-ATP, 20 mM-magnesium acetate, 1 mM-$CaCl_2$, 50 μg of histone and 20 mM-Tris/HCl, pH 7.5. The determinations were performed in quadruplicate in the presence and absence of TPA (0.1 μM final concn.) and phosphatidylserine (24 μg/assay), to define the protein kinase C-mediated phosphorylation. The reaction was terminated by addition of 1 ml of ice-cold 25% (w/v) trichloroacetic acid. The precipitated histone was then separated by filtration on 0.45 μm HA Millipore filters, followed by three washes, each with 3 ml of 5% (w/v) trichloroacetic acid. The filters were counted by liquid scintillation.

Table 1. Effect of insulin and phorbol derivatives on hexose uptake

Incubations with the agents were for 40 min at 37 °C, prior to determination of hexose uptake. Results are the mean ± S.E.M. of 4–16 determinations.

Agent	2-Deoxyglucose uptake (pmol/min per mg of protein)
None	70 ± 3
Insulin (0.5 μM)	150 ± 7
TPA (1 μM)	108 ± 8
α-Phorbol 12,13-didecanoate (1 μM)	60 ± 6
α-Phorbol (1 μM)	59 ± 5

RESULTS

Stimulation of hexose uptake by phorbol esters

Table 1 shows the effect of several phorbol esters (TPA, α-phorbol 12,13-didecanoate and α-phorbol, at 200 nM for 40 min) on hexose uptake. Of the three, only TPA is an activator of protein kinase C [19] and only this phorbol derivative significantly stimulated hexose uptake. Another potent activator of protein kinase C is PDB. Fig. 1 shows a dose–response curve of the stimulation of hexose uptake after 40 min of exposure to PDB. Maximal stimulation (46%) was achieved with 100 nM-PDB. At a concentration of 65 nM, PDB caused half-maximal stimulation. The maximal stimulation caused by PDB was consistently lower than that caused by insulin (see Table 1). We have previously determined that maximal stimulation by insulin is observed at 100 nM ([3,5] and Table 2). Table 2 shows the combined effects of PDB and insulin, at submaximal and maximal

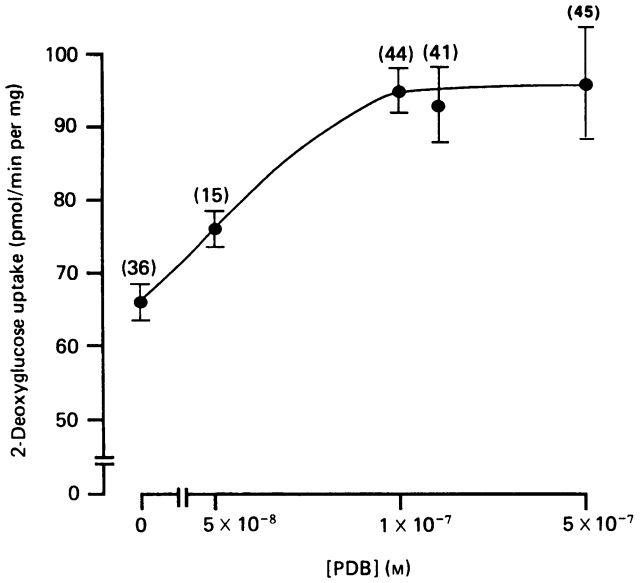

Fig. 1. Effect of PDB on hexose uptake in L6 cells

Cells were incubated with the indicated concentrations of PDB for 40 min prior to 2-deoxyglucose uptake determinations. The points are the mean ± S.E.M. of (*n*) determinations.

Table 2. Effect of simultaneous addition of insulin and phorbol ester

Incubations with the agents were for 40 min at 37 °C, followed by determination of hexose uptake. Results are the mean ± S.E.M. for two experiments, each performed in quadruplicate.

Agent	2-Deoxyglucose uptake (pmol/min per mg of protein)	(% stimulation over basal)
None	71 ± 1	
Insulin (0.05 μM)	117 ± 4	65
(0.2 μM)	144 ± 8	103
(1.0 μM)	142 ± 5	100
PDB (0.05 μM)	87 ± 6	23
(0.2 μM)	100 ± 1	41
(0.5 μM)	107 ± 5	51
Insulin + PDB (each 0.05 μM)	131 ± 3	85
Insulin + PDB (each 0.2 μM)	148 ± 11	108
Insulin (1.0 μM) + PDB (0.5 μM)	158 ± 16	122

concentrations. It is observed that the two agents give additive stimulation when tested at submaximal concentrations, but maximal concentrations do not produce stimulation above that seen with insulin alone.

Migration of protein kinase C

Fig. 2 shows the distribution of kinase C activity in the cytosolic and membrane fractions of L6 cells pretreated with PDB for 40 min. Protein kinase C is defined as the phospholipid- and TPA-dependent fraction of the total

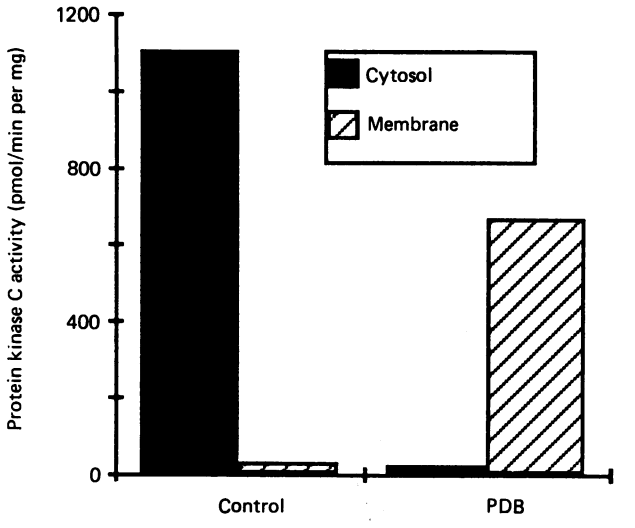

Fig. 2. Subcellular migration of protein kinase C activity caused by PDB

Cells were exposed to 1 μM-PDB for 40 min, followed by separation of subcellular fractions in which protein kinase C activity was determined after passage through a DE-52 column (see the Materials and methods section). Only the protein kinase C component (phospholipid- and TPA-dependent) of the total kinase activity is plotted. Cytosol, control cells: total kinase activity 1223 ± 53 pmol/min per mg, phospholipid- and TPA-independent activity 120 ± 9 pmol/min per mg. Membrane fraction, control cells: total kinase activity 126 ± 13 pmol/min per mg, phospholipid- and TPA-independent activity 132 ± 18 pmol/min per mg. Cytosol, PDB-treated cells: total kinase activity 104 ± 4 pmol/min per mg, phospholipid- and TPA-independent activity 140 ± 21 pmol/min per mg. Membrane fraction, PDB-treated cells: total kinase activity 895 ± 25 pmol/min per mg, phospholipid- and TPA-independent activity 236 ± 23 pmol/min per mg.

kinase activity, measured in the presence of 1 mM-$CaCl_2$. The Figure shows the value of the protein kinase C activity only. In control cells, substantial protein kinase C activity was detected in the cytosolic fraction but not in the membrane fraction. The reverse was true in PDB-treated cells. These results are consistent with a PDB-induced migration of the kinase from the cytosol to the membrane.

Fig. 3 shows the effect of insulin on the distribution of protein kinase C in the cytosolic and membrane fractions. As in the case of the experiment illustrated in Fig. 2, the protein kinase C activity in the control cells was found predominantly in the cytosol. However, unlike the effect of PDB observed in Fig. 2, insulin did not decrease the amount of protein kinase C activity in the cytosol, nor did it increase this activity in the membrane fraction. Therefore, insulin does not appear to induce a migration of protein kinase C from the cytosol to the membrane in L6 cells.

Depletion of protein kinase C by prolonged exposure to phorbol esters

Incubation of L6 cells with either TPA or PDB for long time periods resulted in a progressive decrease in the activity of protein kinase C in total cell homogenates

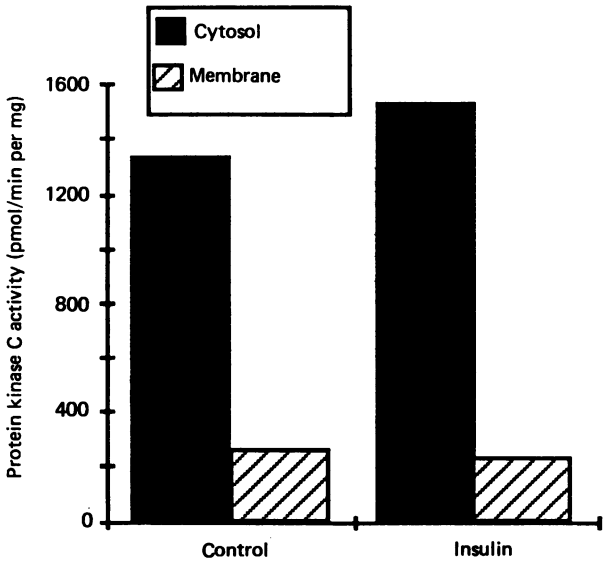

Fig. 3. Effect of insulin on the subcellular distribution of protein kinase C

Cells were exposed to 1 μM-insulin for 40 min, followed by separation of subcellular fractions in which protein kinase C activity was determined, after partial purification of the enzyme in DE-52 columns (see the Materials and methods section). Only the protein kinase C component (phospholipid- and TPA-dependent) of the total kinase activity is plotted. Cytosol, control cells: total kinase activity 1451 ± 61 pmol/min per mg, phospholipid- and TPA-independent kinase activity 117 ± 3 pmol/min per mg. Membrane fraction, control cells: total kinase activity 429 ± 17 pmol/min per mg, phospholipid- and TPA-independent activity 188 ± 24 pmol/min per mg. Cytosol, insulin-treated cells: total kinase activity 1681 ± 57 pmol/min per mg, phospholipid- and TPA-independent activity 147 ± 14 pmol/min per mg. Membrane fraction, insulin-treated cells: total kinase activity 387 ± 11 pmol/min per mg, phospholipid- and TPA-independent activity 154 ± 12 pmol/min per mg.

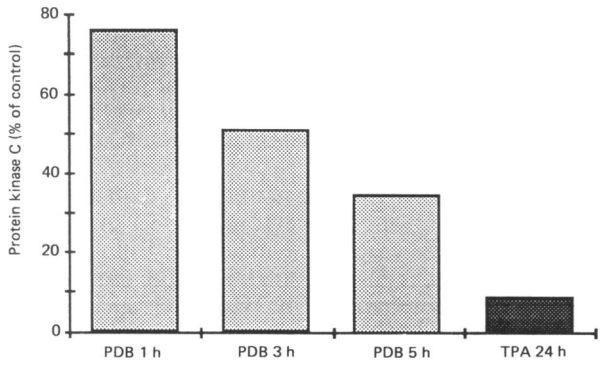

Fig. 4. Depletion of protein kinase C activity by long-term incubation with phorbol esters

Cells were exposed to phorbol esters, then lysed with Triton solution, and protein kinase C activity was determined in the complete cell extract, after purification of the activity through DE-52 columns (see the Materials and methods section). The protein kinase C activity (phospholipid- and TPA-sensitive) is plotted as a function of the total activity in paired control cells incubated for the same time period but without phorbol ester.

Table 3. Effect of prolonged preincubation with phorbol ester on hexose uptake

The cells were washed with minimal essential medium between the preincubation and the incubation. 2-Deoxyglucose uptake was determined after the incubation period as described under 'Materials and methods'. Results are the mean of four determinations.

Preincubation (5 h)	Incubation (40 min) ...	2-Deoxyglucose uptake (pmol/min per mg of protein) −PDB	+PDB
No additions		66 ± 3	93 ± 5
+PDB		53 ± 1	49 ± 2

Table 4. Effect of prolonged incubation with phorbol esters on insulin action

The cells were rinsed with culture medium between the preincubation and the incubation. Results are the mean of 16 determinations.

Preincubation	Incubation (40 min) ...	2-Deoxyglucose uptake (pmol/min per mg of protein) −Insulin	+Insulin
No additions, 5 h		68 ± 6	136 ± 6
PDB, 5 h		71 ± 2	137 ± 4
No additions, 24 h		80 ± 3	130 ± 3
TPA, 24 h		94 ± 1	146 ± 1

(Fig. 4). Maximal inhibition was observed after 24 h, but 50% inhibition occurred in 5 h. The decrease in protein kinase C activity was largely the result of a decrease in the protein kinase C component of the total kinase activity, rather than the result of an increase in the phospholipid- and TPA-independent component. For example, in total extracts from cells exposed to PDB for 5 h, after passage through the DE-52 column, the total kinase activity was 673 ± 37 pmol/min per mg, and the phospholipid- and TPA-independent activity was 133 ± 12 pmol/min per mg. In control cells, the total protein kinase activity was 1617 ± 57 pmol/min per mg, and the phospholipid- and TPA-independent activity was 83 ± 4 pmol/min per mg. These results indicate that the activity of protein kinase C in total cell extracts decreases progressively with prolonged incubation with phorbol esters.

Effect of depletion of protein kinase C on hexose uptake

The effect of depletion of protein kinase C on hexose uptake was tested by measuring 2-deoxyglucose uptake after preincubation with 1 μM-PDB for 5 h (Table 3). Following the preincubation, the cells were washed three times and exposed to either control medium or PDB-containing medium for 40 min. Hexose uptake was subsequently measured. The rate of uptake in cells preincubated with PDB was only slightly lower compared with cells preincubated in control medium (this decrease

was not always observed; see Table 4). Importantly, after a 5 h preincubation with PDB followed by repeated washing, PDB was no longer effective in stimulating hexose uptake, consistent with the down-regulation of protein kinase C caused by the preincubation.

Table 4 shows the result of prolonged incubation with phorbol esters on the stimulation of hexose uptake by insulin. After 5 h of preincubation with PDB, the cells were still sensitive to insulin. The stimulation caused by insulin was 100% in untreated cells and 93% in cells pretreated with PDB. Incubation in minimal essential medium for 24 h resulted in a somewhat lower stimulation by insulin (63%). A small but significant increase above this value was also observed in TPA-treated versus control cells after 24 h. This may be due to the fact that TPA is not efficiently removed by washing the cells with fresh solution. Nonetheless, the presence of TPA during the preincubation period did not prevent significantly the subsequent stimulation of transport by insulin (55%).

DISCUSSION

Given that stimulation of hexose uptake is a consequence of insulin binding to its receptor at the cell surface, it is generally assumed that intracellular signals must mediate the transfer of information from the hormone–receptor complex to intracellular and/or membrane targets. A variety of agents have been proposed to mediate insulin action in muscle [20], notably cytoplasmic Ca^{2+} [21], the intracellular pH [22], the transmembrane potential [23], the tyrosine kinase activity of the insulin receptor [24], and protein kinase C [25] among others. However, direct proof of the involvement of these pathways in insulin stimulation of hexose uptake was lacking until recently. Using the L6 cell line as a model of skeletal muscle, we have shown that insulin does not cause a change in cytoplasmic Ca^{2+} and that chelation of intracellular Ca^{2+} by quin2 in Ca^{2+}-free media does not prevent stimulation of hexose uptake by the hormone [4]. Moreover, although insulin caused a rapid cytoplasmic alkalinization in these cells, the change in cytoplasmic pH was insufficient to produce stimulation of glucose transport, and prevention of the insulin-mediated cytoplasmic pH change did not prevent the increase in glucose uptake [5]. Recently, we have shown that in K^+-depolarized L6 cells, insulin is still capable of stimulating hexose uptake [26]. Hence, neither changes in cytoplasmic Ca^{2+}, intracellular pH nor the transmembrane potential appear to be required for the insulin-mediated elevation in glucose transport. The role of the insulin receptor tyrosine kinase in signal transduction has not been determined, but it is intriguing that certain conditions that result in the loss of response of sugar uptake to insulin (such as in skeletal muscle of the obese mouse) are accompanied by impairment in the insulin receptor kinase activity [27], whereas in other conditions (such as muscle denervation) loss of stimulation of glucose uptake by insulin occurs in spite of the presence of a healthy insulin receptor tyrosine kinase activity [28]

In the present paper we tested whether protein kinase C is involved in insulin stimulation of hexose uptake. Observations made in fibroblasts [10,11], lymphoid cells [9], and more recently in BC3H-1 smooth-muscle-like myocytes [25] and rat adipocytes [29] indicate that phorbol esters activate glucose uptake, that is they have an 'insulin-like' effect. As shown in Fig. 1 and Table 1, phorbol esters that stimulate protein kinase C also stimulate hexose uptake in L6 cells. Interestingly, whereas phorbol esters slightly ($< 30\%$) decrease the response of glucose transport to insulin in adipocytes [29], this was not observed in L6 muscle cells (Table 2). It is possible that such a small effect of the phorbol ester would go undetected in L6 cells, given the much larger stimulation of glucose uptake by insulin observed in rat adipocytes than in muscle cells. Alternatively, phorbol esters may interfere differently with the mechanism of action of insulin in the two tissues.

Phorbol esters are exogenous analogues of diacylglycerol, the endogenous activator of protein kinase C [19,30]. Insulin increased the levels of diacylglycerol in BC3H-1 myocytes and therefore it was proposed that insulin may activate glucose uptake by elevating diacylglycerol levels and consequently activating protein kinase C [12]. However, direct measurements of protein kinase C activity in response to insulin were not reported. Activation of protein kinase C by exogenous phorbol esters in fibroblasts [6] and adipocytes [31] is manifested in a migration of the kinase from the cytosol to the membrane, and measurement of this migration is taken as evidence of activation of the protein kinase C signalling pathway [6,31]. Fig. 2 of the present paper demonstrates that PDB induces migration of protein kinase C from the cytosol to the membrane in L6 differentiated muscle cells. However, insulin was unable to cause a similar translocation (Fig. 3). This suggests that the hormone does not activate protein kinase C and hence it is unlikely that it acts through a protein kinase C-mediated signal. Interestingly, a similar inability of insulin to induce migration of protein kinase C from the cytosol to the membrane was recently reported for rat adipocytes [31], confirming the unlikelihood that the hormone acts through the kinase C system.

In order to test further whether protein kinase C could be involved in insulin stimulation of hexose uptake, it was desirable to inhibit or inactivate the enzyme. A procedure to decrease markedly the activity of protein kinase C in fibroblasts was described by Rodriguez-Pena & Rozengurt [8], based on the prolonged incubation of cells with phorbol esters. This depletion of protein kinase C provides a tool to test the involvement of this enzyme in the stimulation of hexose uptake by insulin (see also [32]). As shown in Fig. 4, incubation with phorbol esters for periods from 1 to 24 h resulted in the progressive loss of protein kinase C activity from total cell extracts. More importantly, the disappearance of protein kinase C activity correlated with the inability of the cells to respond further to phorbol esters by stimulation of hexose uptake (Table 2). However, insulin was still fully potent in stimulating hexose uptake (Table 4). These results are interpreted to indicate that inactivation of protein kinase C activity is without effect on stimulation of glucose transport by insulin, and therefore it is suggested that the hormone increases glucose uptake by a mechanism independent of protein kinase C activity. The signal(s) that mediate insulin stimulation of hexose uptake in muscle remain to be elucidated.

We thank Drs. G. Mills and S. Grinstein for valuable discussion, and Dr. G. Cates for selecting the high-fusion L6 cell mutant. We are indebted to D. Walker and E. Mack for

excellent technical assistance. This work was supported by the Medical Research Council of Canada. A.K. is an MRC Scientist.

REFERENCES

1. Wardzala, L. J. & Jeanrenaud, B. (1981) J. Biol. Chem. **256**, 7090–7093
2. Watanabe, T., Smith, M. M., Robinson, F. W. & Kono, T. (1984) J. Biol. Chem. **259**, 13117–13122
3. Klip, A., Li, G. & Logan, W. J. (1984) Am. J. Physiol. **247**, E291–E296
4. Klip, A., Li, G. & Logan, W. J. (1984) Am. J. Physiol. **247**, E297–E304
5. Klip, A., Ramlal, T. & Cragoe, E. J., Jr. (1986) Am. J. Physiol. **250**, C720–C728
6. Kraft, A. S. & Anderson, W. B. (1983) Nature (London) **301**, 621–624
7. Collins, M. K. L. & Rozengurt, E. (1984) J. Cell. Physiol. **118**, 133–142
8. Rodriguez-Pena, A. & Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. **120**, 1053–1059
9. Klip, A., Rothstein, A. & Mack, E. (1984) Biochem. Biophys. Res. Commun. **124**, 14–22
10. Driedger, P. E. & Blumberg, P. M. (1977) Cancer Res. **37**, 3257–3265
11. Lee, L. S. & Weinstein, I. B. (1979) J. Cell Physiol. **99**, 451–460
12. Farese, R. V., Barnes, D. E., Davis, J. S., Standaert, M. L. & Pollet, R. J. (1984) J. Biol. Chem. **256**, 7094–7100
13. Koepfer-Hobelsberg, B. & Wieland, O. H. (1984) Mol. Cell. Endocrinol. **36**, 123–129
14. Yaffe, D. (1968) Proc. Natl. Acad. Sci. U.S.A. **61**, 477–483
15. Shainberg, A., Yagil, G. & Yaffe, D. (1971) Dev. Biol. **25**, 1–9
16. Klip, A., Logan, W. J. & Li, G. (1982) Biochim. Biophys. Acta **687**, 265–280
17. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
18. Friedman, Y., Poleck, T., Henricks, L. & Burke, G. (1985) Biochem. Biophys. Res. Commun. **130**, 971–980
19. Shoyab, M. & Todaro, G. J. (1980) Nature (London) **288**, 451–455
20. Czech, M. P. (1985) Annu. Rev. Physiol. **47**, 357–381
21. Clausen, T. (1980) Cell Calcium **1**, 311–325
22. Moore, R. D. (1983) Biochim. Biophys. Acta **737**, 1–49
23. Zierler, K. & Rogus, E. M. (1980) Am. J. Physiol. **239**, E21–E29
24. Karlsson, F. A. & Kahn, C. R. (1982) Science **215**, 185–187
25. Farese, R. V., Standaert, M. L., Barnes, D. E., Davis, J. S. & Pollet, R. J. (1985) Endocrinology (Baltimore) **116**, 2650–2655
26. Klip, A., Ramlal, T. & Walker, D. (1986) FEBS Lett. **205**, 11–14
27. Le Marchand-Brustel, Y., Gremeaux, T., Ballotti, R. & Van Obberghen, E. (1985) Nature (London) **315**, 676–679
28. Burant, C. F., Treutelaar, M. K. & Buse, M. G. (1986) J. Biol. Chem. **261**, 8985–8993
29. Kirsch, D., Obermaier, B. & Haring, H. U. (1985) Biochem. Biophys. Res. Commun. **128**, 824–832
30. Nishizuka, Y. (1984) Nature (London) **308**, 693–698
31. Glynn, B. P., Colliton, J. W., McDermott, J. M. & Witters, L. E. (1986) Biochem. Biophys. Res. Commun. **135**, 1119–1125
32. Blackshear, P. J., Witters, L. A., Girard, R. R., Kuo, J. F. & Quamo, S. N. (1985) J. Biol. Chem. **260**, 13304–13315
33. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. & Rutter, W. I. (1986) Cell **45**, 721–732

Received 19 May 1986/7 October 1986; accepted 27 October 1986