Inhibition by forskolin of insulin-stimulated glucose transport in L6 muscle cells

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The cardioactive diterpene forskolin is a known activator of adenylate cyclase, but recently a specific interaction of this compound with the glucose transporter has been identified that results in the inhibition of glucose transport in several human and rat cell types. We have compared the sensitivity of basal and insulin-stimulated hexose transport to inhibition by forskolin in skeletal muscle cells of the L6 line. Forskolin completely inhibited both basal and insulin-stimulated hexose transport when present during the transport assay. The inhibition of basal transport was completely reversible upon removal of the diterpene. In contrast, insulin-stimulated hexose transport did not recover, and basal transport levels were attained instead. This effect of inhibiting (or reversing) the insulin-stimulated fraction of transport is a novel effect of the diterpene. Forskolin treatment also inhibited the stimulated fraction of transport when the stimulus was by 4β -phorbol 12,13-dibutyrate, reversing back to basal levels. Half-maximal inhibition of the above-basal insulin-stimulated transport was achieved with 35–50 μM -forskolin, and maximal inhibition with 100 μM . Forskolin did not inhibit ^{125}I -insulin binding under conditions where it caused significant inhibition of insulin-stimulated hexose transport. Forskolin significantly elevated the cyclic AMP levels in the cells; however its inhibitory effect on the above basal, insulin-stimulated fraction of hexose transport was not mediated by cyclic AMP since: (i) 8-bromo cyclic AMP and cholera toxin did not mimic this effect of the diterpene, (ii) significant decreases in cyclic AMP levels caused by 2',3'-dideoxyadenosine in the presence of forskolin did not prevent inhibition of insulin-stimulated hexose transport, (iii) isobutylmethylxanthine did not potentiate forskolin effects on glucose transport but did potentiate the elevation in cyclic AMP, and (iv) 1,9-dideoxyforskolin, which does not activate adenylate cyclase, inhibited hexose transport analogously to forskolin. We conclude that forskolin can selectively inhibit the insulin- and phorbol ester-stimulated fraction of hexose transport under conditions where basal transport is unimpaired. The results are compatible with the suggestions that glucose transporters operating in the stimulated state (insulin or phorbol ester-stimulated) differ in their sensitivity to forskolin from transporters operating in the basal state, or, alternatively, that a forskolin-sensitive signal maintains the stimulated transport rate.

INTRODUCTION

Insulin stimulates glucose transport into muscle and fat cells, and it has been postulated that at least part of the underlying mechanism is a recruitment of glucose transporters to the cell surface from intracellular stores [1,2]. Supporting evidence for this hypothesis comes from determinations of the number of glucose transporters [1] and glucose transport [2] in isolated plasma and intracellular membranes. Definitive testing of the recruitment hypothesis requires the identification of the increase in glucose transporters on the surface of intact cells. If the newly recruited transporters had different chemical properties, it would be possible to detect recruitment without cell disruption.

In muscle cells, both basal and insulin-stimulated transport are equally sensitive to inhibition by the mould metabolite cytochalasin B [3], with a K_i of 0.3 μM . Forskolin, an activator of adenylate cyclase [4], has recently been shown to inhibit glucose transport in rat

adipocytes [5,6] and cardiocytes [7], and in human erythrocytes [8] and platelets [9]. In the case of adipocytes [6] and cardiocytes [7] the insulin-stimulated transport was also inhibited by the continuous presence of forskolin in the transport assay. Forskolin inhibition of glucose transport in human erythrocytes is readily reversible upon removal of the diterpene [8]. Forskolin also inhibits cytochalasin B binding to isolated membranes from erythrocytes [8] or adipocytes [6], leading to the postulate that it interacts directly with the glucose transporter. More recent evidence shows that indeed the glucose transporter of red cells can be photolabelled with [^3H]forskolin and this reaction is also prevented by cytochalasin B [10].

We have demonstrated that L6 cells are a suitable model to study insulin stimulation of hexose transport in skeletal muscle [3]. We report here that forskolin inhibits basal and insulin-stimulated hexose transport in L6 cells when present during the transport assay. In this way, it is confirmed that the diterpene acts on L6 in a similar

Abbreviations used: IBMX, isobutylmethylxanthine; PDB, 4β -phorbol 12,13-dibutyrate; α -MEM, α Minimum Essential Medium; DDA, 2',3'-dideoxyadenosine.

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way as in adipocytes [6] or cardiocytes [7]. However, it is further shown that treatment with forskolin after insulin action, followed by removal of the diterpene prior to the transport assay, results in recovery of basal, but not of insulin-stimulated, rates of transport. This novel observation suggests that there is a selective interference by forskolin with either the insulin-stimulated transporters or with a signal that maintains the insulin-stimulated state. This effect is shown not to be caused by elevations in cyclic AMP levels.

MATERIALS AND METHODS

Materials

Porcine insulin, 2-deoxy-D-glucose, 8-bromo cyclic AMP, cytochalasin B, IBMX, PDB and forskolin were from Sigma Chemical Co. DDA was purchased from Calbiochem. Forskolin stock solutions (100 mM) were made in dimethyl sulphoxide or ethanol and used within a week. The final concentration of solvent never exceeded 5 μ l/ml. Cholera toxin was from List Biologicals. Culture media and sera were from GIBCO. The radioimmunoassay kits for cyclic AMP measurements were from New England Nuclear. 2-Deoxy-D-[3 H]glucose was obtained from ICN. [Tyr^{A14,125}I]Insulin was a kind gift from Drs. C. C. Yip and M. Moule, University of Toronto.

Cell cultures

L6 cells were grown in 24-well plates and allowed to fuse into myotubes as described previously [3,11]. Briefly, myotube cultures were grown in α -MEM containing 2% fetal calf serum, 100 mg of streptomycin and 100 000 units of penicillin/litre, in an atmosphere of CO₂/air (1:19). The medium was changed 24 h before experimental assay.

2-Deoxyglucose uptake

Prior to each experiment, cells were depleted of serum for 5 h at 37 °C in α -MEM supplemented with 25 mM-D-glucose. Insulin (100 nM) or PDB (1 μ M) were then added for 30 min at 37 °C. Forskolin was added to the cells in culture media at 37 °C, after the insulin or PDB treatment, unless otherwise stated. The cells were rinsed three times in Hepes-buffered saline solution (122 mM-NaCl, 4.9 mM-KCl, 2.5 mM-MgSO₄, 1 mM-CaCl₂, 20 mM-Hepes, pH 7.4), and carrier-mediated hexose uptake (cytochalasin B-inhibitable) of 10 μ M-2-deoxy-D-[3 H]glucose in the above solution was measured for 10 min as described previously [11]. We have shown that under the conditions used, initial rates of transport are measured [3,11]. This was followed by rinsing the cells quickly three times with ice-cold Hepes-buffered saline, and radioactivity trapped inside the cells was determined with a scintillation counter after disruption with 0.05 M-NaOH. Non-carrier-mediated uptake was measured in parallel incubations in the presence of 5 μ M-cytochalasin B and it amounted to < 5% of the total uptake. This value was subtracted from the total uptake. Protein was determined by the Bradford method [12], and results are expressed as pmol/min per mg of protein.

¹²⁵I-Insulin binding

¹²⁵I-Insulin (50 nM) binding to monolayers of L6 cells was measured as described earlier [13], for 30 min at 37 °C in α -MEM containing 1% bovine serum albumin (fatty acid-free). Insulin concentrations were adjusted

with non-radioactive insulin. Other conditions are described in the Results section. Non-specific binding was estimated from the binding in the presence of 100 μ M non-radioactive insulin and has been subtracted from the results.

Cyclic AMP determination

Cyclic AMP accumulation in cells was determined in parallel wells, under conditions identical to those used for hexose transport. Incubations were terminated by aspiration of the medium and addition to the cells of ice-cold 0.01 M-HCl which was used to extract the cyclic AMP as previously described [14,15]. Cyclic AMP was measured by radioimmunoassay in the cell extracts using corresponding concentrations of 0.01 M-HCl in the standards, as previously described [14,15].

RESULTS

Effect of forskolin on basal and insulin-stimulated hexose uptake

Fig. 1 shows that forskolin (100 μ M) present during the 10 min transport assay completely inhibited hexose uptake. This inhibition was observed in both basal and insulin-stimulated cells (i.e. cells treated with insulin for 30 min followed by exposure to forskolin during the transport assay). Exposure of basal (insulin-untreated) cells to forskolin for 15 min followed by removal of the diterpene by three rinses resulted in complete recovery of the basal rate of transport. In contrast, when insulin-pretreated cells were exposed for 15 min to forskolin (followed by removal of the diterpene) prior to the

Fig. 1. Effect of forskolin on basal and insulin-stimulated hexose transport

L6 myotubes were depleted of serum for 5 h as described under Methods, and then incubated without or with insulin (0.1 μ M) for 30 min. The cells were then rinsed twice in glucose-free and insulin-free Hepes-buffered saline solution, and transport of 2-deoxy[3 H]glucose (10 μ M) was determined for 10 min in the absence (cross-hatched bars) or presence (solid bars) of 100 μ M-forskolin. In the third and fourth experimental conditions (stippled and open bars) the cells were incubated with or without 100 μ M-forskolin, respectively, for 15 min following the $\pm$ insulin incubation. This was followed by three washes in forskolin-free solution, followed by hexose transport determination in the absence of the diterpene. Results are the mean $\pm$ S.E. of four to six independent experiments, each performed in quadruplicate.

Fig. 2. Concentration dependence of the inhibition of insulin-stimulated and phorbol ester-stimulated hexose transport by forskolin

Serum-depleted cells were treated without other agents (■), with $0.1 \mu\text{M}$ -insulin (□) or with $1 \mu\text{M}$ -PDB (△) for 30 min, followed by three rinse steps in insulin-free solution and immediate exposure to the indicated concentrations of forskolin for 15 min. (The samples incubated with 'zero' forskolin were equally incubated for 15 min). The diterpene was washed away and hexose transport was determined in Hepes-buffered saline solution. Results are the mean $\pm$ S.E. of two (insulin) or three (PDB) independent experiments, each performed in quadruplicate.

transport assay, the insulin-stimulated hexose transport was lost. Instead, transport recovered only to the basal rate (Fig. 1). In the absence of forskolin (15 min of 'wash only') only a small decrease in insulin-stimulation was observed (open bar, Fig. 1). Therefore, the return to basal levels of transport caused by forskolin cannot be due to removal of insulin in this time period. The experimental conditions required to observe the differential recoveries of basal and insulin-stimulated transport rates therefore required the removal of the diterpene prior to assaying hexose uptake. Addition of dimethyl sulphoxide alone did not reproduce any of the above effects of forskolin (not shown). Unless otherwise stated, in all subsequent experiments forskolin was added after 30 min stimulation with insulin, and was removed prior to assaying hexose transport.

Fig. 2 shows the forskolin concentration dependence for inhibition of the insulin-stimulated fraction of hexose transport. Half-maximal inhibition was obtained at approx. $50 \mu\text{M}$ -forskolin, and maximal inhibition (reaching basal transport levels) at $100 \mu\text{M}$. Forskolin also inhibited stimulated transport produced by agents other than insulin. We have previously shown that phorbol esters such as PDB stimulate hexose uptake in L6 cells [11]. Fig. 1 shows that this stimulation was also reversed by subsequent treatment with forskolin. The dose-dependence of this inhibition was similar to that of inhibition of insulin-stimulated transport.

The sensitivity to forskolin was also determined under conditions where the diterpene is present in the transport assay. In these conditions, half-maximal inhibition of basal transport was achieved with $5.23 \pm 0.51 \mu\text{M}$ -forskolin ($n = 10$). The sensitivity to forskolin of insulin-stimulated transport when the diterpene was present during the transport assay was also determined. This was

Fig. 3. Time course of inhibition of insulin-stimulated transport by forskolin

Cells were incubated with or without insulin ($0.1 \mu\text{M}$) for 30 min, followed by three rinse steps and exposure to $100 \mu\text{M}$ -forskolin for the indicated time periods. The cells were rinsed twice in Hepes-buffered saline solution and hexose transport was determined. Results are expressed as the ratio of transport in cells pretreated with insulin relative to unstimulated (basal) cells, each experimental pair exposed to forskolin for the same time. The points are the mean $\pm$ S.E. of three independent experiments, each performed in quadruplicate.

achieved by subtracting the forskolin dose-response curve of basal transport from the dose-response curve of transport in insulin-treated cells (since the latter curve is considered to be the sum of the basal and the stimulated transport). Half-maximal inhibition of this insulin-sensitive fraction of transport was observed with $4.45 \pm 0.64 \mu\text{M}$ -forskolin ($n = 10$).

The above results indicate that the direct inhibition of transport by forskolin (i.e. forskolin present in the transport assay) is about 10 times more sensitive to the drug than is the reversal of the stimulated rate of transport to basal levels (i.e. treatment with forskolin prior to transport assay in the absence of the diterpene).

Inhibition of hormone-stimulated transport back to basal levels by forskolin treatment was rapid, with 75% inhibition observed after only 5 min of exposure to the diterpene (Fig. 3). Shorter exposures were not tested.

In order to test whether forskolin could prevent insulin stimulation of hexose uptake, the diterpene was added for 15 min and then removed prior to addition of the hormone. Table 1 shows that under these conditions insulin action was unaffected, and no inhibition of transport was observed. Thus, the inhibitory action of forskolin is not one of preventing insulin action but rather one of either reversing the hormone action or of inhibiting the insulin-stimulated transporters.

Effect of forskolin on ^{125}I -insulin binding

The effect of forskolin on ^{125}I -insulin binding was tested according to two protocols: (i) addition of $100 \mu\text{M}$ -

Table 1. Effect of forskolin prior to or during insulin action

Cells were serum depleted for 5 h. Hexose transport was determined after preincubation in the indicated conditions. Concentrations of agents: 0.1 μ M-insulin, 100 μ M-forskolin. Each addition was followed by three quick rinses, and finally determination of hexose uptake was described under Methods (in the absence of either insulin or forskolin). Results are in pmol/min per mg of protein, expressed as the mean $\pm$ S.E. of at least three determinations.

Treatment	Transport
Basal	10.8 $\pm$ 0.2
Insulin 30 min $\rightarrow$ MEM 15 min	19.8 $\pm$ 1.0
Insulin 30 min $\rightarrow$ forskolin 15 min	12.2 $\pm$ 1.0
Forskolin 15 min $\rightarrow$ insulin 30 min	21.0 $\pm$ 2.0

forskolin during the 30 min incubation of the cells with 50 nM (radioactive plus unlabelled) insulin, or (ii) 30 min preincubation with 50 nM-insulin followed by rinsing and 15 min incubation with 100 μ M-forskolin. This second condition exactly parallels that producing inhibition by forskolin of insulin-stimulated hexose transport. Control experiments included similar incubations in the absence of the diterpene. The results indicated that: (i) forskolin present during the incubation with insulin did not decrease, and in fact increased, 125 I-insulin binding (0.64 $\pm$ 0.08 pmol/mg of protein in the absence and 1.14 $\pm$ 0.20 pmol/mg in the presence of forskolin, $n = 4$) and (ii) preincubation of cells with 125 I-insulin followed by medium change and a 15 min incubation in insulin-free, 1% bovine serum albumin-containing α -MEM at 37 $^{\circ}$ C resulted in significant loss of radioactivity from the cells (to 0.25 $\pm$ 0.04 pmol/mg of protein). This residual binding was not affected by the presence of 100 μ M-forskolin during the 15 min post-insulin incubation (0.19 $\pm$ 0.05 pmol/mg). (The radioactivity lost from the cells during the 15 min incubation, without or with forskolin, was recovered in the incubation medium.)

Effect of forskolin on cyclic AMP levels

Forskolin is a potent activator of adenylate cyclase (see [4]), but the inhibition of glucose transport by forskolin (when the diterpene is present in the transport assay) has been shown to be independent of the stimulation of adenylate cyclase [6,8,10,19]. In fact, the competitive nature of the inhibition by forskolin of cytochalasin B binding [6,8] points to a direct interaction of the diterpene with the transporter. The results presented in this study, however, show a persistent inhibition only of the insulin-stimulated fraction of transport after treatment with forskolin. This action of the diterpene could be due either to a direct interaction with transporters or to interference with a signal that maintains the stimulated rate of hexose uptake. This putative forskolin-sensitive signal could presumably involve modulation of cyclic AMP levels. In order to test this possibility, other agents that are known to increase cyclic AMP levels were tested on insulin-stimulated (19.8 $\pm$ 1.0 pmol/min per mg of protein) and on basal (10.7 $\pm$ 1.0 pmol/min per mg of protein) hexose transport. Addition of 8-bromo cyclic AMP (0.5 mM for 15 min after insulin treatment) did not inhibit insulin-stimulated hexose transport (19.3 $\pm$ 0.8 pmol/min per mg

of protein) and even caused a small but significant increase in basal transport rates (13.3 $\pm$ 0.2 pmol/min per mg of protein). Cholera toxin (15 μ g/ml for 15 min after insulin treatment) had a small inhibitory action on insulin-stimulated transport (from 19.8 $\pm$ 0.4 pmol/min per mg of protein to 15.6 $\pm$ 2.8 pmol/min per mg of protein) as well as on basal transport (from 10.8 $\pm$ 0.6 pmol/min per mg of protein to 7.9 $\pm$ 2.1 pmol/min per mg of protein), but the incremental rise in glucose transport caused by insulin was not affected (83% and 97%, without and with cholera toxin, respectively). The above are results of three independent experiments each performed in quadruplicate.

To test further a possible involvement of the activation of adenylate cyclase in the inhibition of glucose uptake by forskolin, a forskolin analogue that is ineffective towards the cyclase was tested. 1,9-Dideoxyforskolin is incapable of activating adenylate cyclase [18], but mimicked forskolin action on glucose transport: when tested directly during the transport assay, 1,9-dideoxyforskolin (100 μ M) completely inhibited both basal transport (from 9.8 $\pm$ 0.1 pmol/min per mg of protein to 0.4 $\pm$ 0.1 pmol/min per mg of protein) and insulin-stimulated transport (from 20.1 $\pm$ 0.2 pmol/min per mg of protein to 1.5 $\pm$ 0.1 pmol/min per mg of protein). A post-insulin incubation with 100 μ M-1,9-dideoxyforskolin (15 min), followed by removal of this agent and transport assay, showed that the drug inhibited the insulin-stimulated transport to near basal levels (to 10.5 $\pm$ 0.5 pmol/min per mg of protein).

In order to evaluate directly the potential role of cyclic AMP in mediating the effects of forskolin on insulin-stimulated glucose transport, cyclic AMP accumulation and glucose transport were measured in parallel in the absence and presence of IBMX or DDA. IBMX is known to inhibit cyclic AMP degradation and thereby to augment the accumulation of cyclic AMP caused by low concentrations of forskolin [16]. DDA is known to inhibit cyclic AMP production at the level of the catalytic unit of adenylate cyclase [17]. As shown in Fig. 4, forskolin did indeed stimulate cellular cyclic AMP accumulation, and in the presence of IBMX this effect was markedly augmented. In contrast, addition of IBMX did not alter forskolin-mediated inhibition of insulin-stimulated hexose transport. For example, at 20 μ M-forskolin, cyclic AMP accumulation was increased by IBMX to a level comparable to that achieved with 75 μ M-forskolin alone, yet 20 μ M-forskolin in presence of IBMX was insufficient to inhibit insulin-stimulated hexose transport. Similarly, inhibition of cyclic AMP accumulation also showed lack of correlation with inhibition of insulin-stimulated hexose transport. As shown in Table 2, DDA largely inhibited forskolin-stimulated cyclic AMP accumulation, but this did not prevent inhibition of insulin-stimulated hexose transport by forskolin. The level of cyclic AMP attained with forskolin plus DDA *per se* is not sufficient to cause inhibition of insulin stimulation of hexose transport (see Fig. 4).

DISCUSSION

This study confirms in L6 cells the observation made in adipocytes [5,6] cardiocytes [7] and erythrocytes [8], that forskolin, when present during the transport assay, inhibits glucose transport completely. However, a novel

Fig. 4. Effect of IBMX on cyclic AMP accumulation (a) and insulin-stimulated hexose transport (b)

Cells were incubated without or with $0.1 \mu\text{M}$ -insulin for 30 min, followed by two rinse steps and exposure to the indicated concentrations of forskolin for 15 min in the absence or presence of 1 mM -IBMX. The cells were then rinsed three times and parallel wells were analysed for cyclic AMP accumulation and hexose transport. Results are the mean $\pm$ S.E. of four determinations in one experiment representative of two. Each well was tested for cyclic AMP twice, and the mean of four wells $\pm$ S.E. is indicated. Transport is reported as the ratio of insulin-stimulated transport/basal transport. In (b), $P < 0.001$ for comparison with corresponding sample without IBMX at forskolin concentrations 0 – $50 \mu\text{M}$; $P < 0.002$ at $75 \mu\text{M}$ -forskolin; and $P < 0.03$ at $100 \mu\text{M}$ -forskolin.

effect of forskolin is reported, namely that whereas the basal rate of transport is restored upon removal of forskolin, the insulin-stimulated transport rate is not restored. In insulin-treated cells exposed to forskolin transiently, the basal rate of transport rather than the stimulated rate is displayed. Thus a post-insulin, transient treatment with forskolin leads to complete recovery of basal transport but not of insulin-stimulated hexose transport. To our knowledge, this selective effect of forskolin for the insulin-stimulated fraction of transport has not reported by others because in previous studies the diterpene was always present during the transport assay (see [5–7]). This indicates that treatment with forskolin (followed by a forskolin-free transport assay) either inhibits the insulin-stimulated transporters or interferes with a signal that maintains the stimulated rate of transport.

Table 2. Effect of DDA on cyclic AMP levels and hexose transport

Cells were incubated at 37°C for 30 min without or with $0.1 \mu\text{M}$ -insulin, without or with $100 \mu\text{M}$ -DDA, followed by two rinses and a 15 min incubation with $100 \mu\text{M}$ -forskolin and/or DDA as indicated. After three rinses hexose transport and cyclic AMP accumulation were determined in parallel wells as described under Methods. Results are of four determinations in one experiment representative of two. Each well was tested for cyclic AMP twice, and the mean of four wells $\pm$ S.E. is indicated. * $P < 0.001$ compared to the corresponding sample without DDA.

Forskolin	DDA	Cyclic AMP accumulation (pmol/45 min per mg of protein)	2-Deoxyglucose transport (insulin/basal)
–	–	108 ± 18	2.07
–	+	54 ± 4	2.31
+	–	13420 ± 6351	1.03
+	+	$3680 \pm 63^*$	0.96

Possible mechanisms of forskolin inhibition of insulin-stimulated hexose transport

One can envisage at least four ways by which forskolin could potentially produce inhibition of insulin-stimulated transport: (i) by inhibiting insulin binding, (ii) by inhibiting an insulin-dependent signal, (iii) by increasing cyclic AMP levels or interacting with a G protein, and (iv) by directly and specifically inhibiting the insulin-stimulated transporters.

(i) The effect of forskolin is observed after glucose transport has been stimulated by insulin, suggesting that the diterpene acts at a post-binding step. Moreover, a direct inhibition of insulin binding by forskolin would be insufficient to explain the inhibition of transport stimulated by phorbol ester (see Fig. 2). Finally, ^{125}I -insulin binding under conditions identical to those used in hexose transport studies did not decrease in the presence of forskolin. In fact, opposite to the loss of insulin-stimulated transport, forskolin treatment resulted in an increase in ^{125}I -insulin binding. The cause of such an increase is presently unknown. Hence, the inhibition of insulin-stimulated transport cannot be explained by a loss of hormone binding.

(ii) The signals that mediate stimulation of hexose uptake by insulin remain unknown. Candidates include phosphorylation of an intermediate(s) through the insulin receptor tyrosine kinase [20–22], as well as unknown steps involved in the recruitment of glucose transporters to the cell surface. When specific intermediates for stimulation of hexose transport by insulin are identified, it will become important to test the possible effect of forskolin on their phosphorylation or dephosphorylation. It is also possible that the recycling of glucose transporters between intracellular stores and the cell membrane is a continuous process, the relative rates of which are changed by insulin. In this case, the inhibition by forskolin could occur either by inhibition of recruitment of transporters to the membrane or by increasing their retrieval back to an intracellular location. Although there is no precedent for interference by forskolin in

intracellular traffic, these possibilities remain to be tested.

(iii) The best identified function of forskolin in intact cells is that of stimulating adenylate cyclase and increasing the intracellular levels of cyclic AMP. Stimulation of adenylate cyclase by forskolin has been shown to involve a direct action at the level of the catalytic unit [23,24] and can also cause activation through the stimulatory guanine nucleotide regulatory protein, G_s [23,24]. However, in the current study, the effects of forskolin on cyclic AMP production could be dissociated readily from its effects on glucose transport, suggesting that inhibition of insulin-stimulated hexose transport cannot be explained by an increase in cyclic AMP levels: potentiation by IBMX of the increase in cyclic AMP caused by submaximal doses of forskolin did not potentiate the inhibition of insulin-stimulated transport (Fig. 4), and drastic reduction by DDA of the forskolin-mediated increase in cyclic AMP production did not reduce the inhibition of insulin-stimulated transport (Table 2). Further, 1,9-dideoxyforskolin effectively reproduced the selective inhibition of insulin-stimulated hexose transport.

Of interest, no correlation was found between cyclic AMP-dependent protein kinase activity and regulation of insulin-stimulated hexose transport by β -adrenergic agents in adenosine deaminase-treated rat adipocytes [25]. In these cells, insulin-stimulated transport is partly inhibited by isoproterenol, glucagon or ACTH and is stimulated by prostaglandin E_1 and nicotinic acid. All of these effects were dissociated from the concomitant activation or inhibition of A-kinase activity [25]. Based on these results, as well as on experiments using guanine nucleotide analogues and bacterial toxins in hepatocytes [26] and platelets [27], it has been suggested that G-proteins may participate in insulin signalling. It is conceivable that the effects of forskolin observed in the current study may similarly involve a G-protein, independent of direct effects on adenylate cyclase catalytic activity and cyclic AMP production. It can therefore be proposed that if forskolin inhibits insulin-stimulated transport by interfering with an insulin-dependent signal, this signal must be long-lived (since its inhibition occurs after removal of insulin) and it must be different from a change in cyclic AMP levels.

(iv) As stated above, forskolin interacts directly with the glucose transporters, producing inhibition of both basal and insulin-stimulated transport. These similarities could be responsible for the direct inhibition of transport. The direct inhibition of basal transport is readily reversible (Fig. 1). In contrast, the specific inhibition of the insulin-stimulated fraction of transport persisted after removal of forskolin (Fig. 1), suggesting that a more permanent interaction of the diterpene may take place with the insulin-stimulated transporters.

A specific interaction with the insulin-stimulated transporters could arise only if the latter had different reacting properties than the basal transporters. Although both basal and stimulated transport are equally sensitive to cytochalasin B in L6 cells [3], there is precedent for differences in their sensitivity to other agents. Aiello *et al.* [28] reported that the insulin-activated glucose transport system of adipocytes and/or its environment are more sensitive to dipeptides such as Cbz-Gly-Phe-NH₂, resulting in a selective inhibition of stimulated transport. A preliminary communication indicates different suscepti-

bility to neuraminidase of basal and insulin-stimulated glucose transporters of rat adipocytes [29]. Simpson *et al.* [30] detected a slight but significant difference in the K_d of binding of cytochalasin B to membranes derived from basal and insulin-activated rat adipocytes. More recently, Greco-Perotto *et al.* [31] reported that the Hill coefficient of cytochalasin B binding in plasma membranes from rat adipocytes was increased by insulin pretreatment [31]. Further, Horuk *et al.* [32] demonstrated the existence of heterogeneous species of glucose transporters in human adipocytes and showed that one of these isoforms, with pI 5.6, is exclusive of the plasma membrane. Interestingly, in rat liver (a tissue in which glucose transport is independent of insulin), only one isoform of the transporter was detected, with pI 6.05 [33]. The liver transporter also appears to differ from that in adipocyte membranes by its lower affinity for cytochalasin B [34]. Taken together, the above observations point to a heterogeneity of glucose transporters in different tissues, insulin-responsive and unresponsive, as well as in basal and insulin-stimulated states. Our results would suggest that perhaps binding of [³H]forskolin could be instrumental in characterizing the different isoforms of the transporter.

It has been reported recently that in addition to increasing the V_{max} of transport (consistent with either a change in the transporter's intrinsic activity or in transporter number) insulin also decreases the K_m for transport (consistent with a change in the intrinsic activity of the transporters) [35,36]. If inhibition of insulin-stimulated hexose transport by forskolin occurred by a selective interaction with the insulin-stimulated transporters, this would argue against a sole action of insulin to modify pre-existing transporters. Instead, the inhibition by forskolin would favour the hypothesis that new transporters are recruited to the cell membrane upon insulin action. Moreover, these transporters would appear to have different chemical properties from the basal transporters. The new transporters could have the same or different K_m for transport relative to the basal transporters. Alternatively, forskolin could be potentiating the removal of the glucose transporters from the plasma membrane (like for example to an intracellular membrane pool). In this case, too, it could be postulated that such retrieval of transporters recognizes specifically the fraction of transporters initially recruited by insulin, since the reversal by forskolin recognizes only the insulin-stimulated but not the basal transport. Experiments are underway to distinguish between an inhibition by forskolin of insulin-stimulated transporters or a retrieval from the membrane of these transporters.

An alternative interpretation of the results presented would be that forskolin reverses insulin action by a mechanism unrelated to its interaction with the glucose transporters. In this case, forskolin would be instrumental in identifying the signalling mechanism of the hormone.

In conclusion, it appears that a post-insulin, transient treatment with forskolin does not affect basal transport but reverses the stimulation of transport caused by either insulin or phorbol ester. This inhibitory action is independent of increases in intracellular cyclic AMP, and may occur as a result of a direct interaction with the glucose transporters. If the irreversible inhibition of stimulated transport is the result of a specific interaction with the stimulated glucose transporters, then these molecules must have different chemical or structural

properties from the basal transporters. Alternatively, forskolin could be interacting with a long-lasting signal triggered by pre-exposure to insulin, the role of which would be to maintain an elevated level of transport.

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