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Forskolin Inhibits Insulin-Stimulated Glucose Transport in Rat Adipose Cells by a Direct Interaction with the Glucose Transporter

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SUMMARY

The mechanism of the inhibitory action of forskolin, a plant-derived stimulator of adenylate cyclase, on glucose transport in rat adipose cells was studied. Lipolysis (glycerol release) and glucose transport activity (initial 3-*O*-methylglucose uptake rate) were measured after treatment of intact cells. In isolated plasma membranes, D-glucose transport and glucose-inhibitable binding of cytochalasin B, a specific labeling agent for the glucose transporter, were assayed. Forskolin inhibited insulin-stimulated glucose transport in intact cells at low concentrations which failed to stimulate lipolysis. Furthermore, the adenylate cyclase inhibitor prostaglandin E_2 reduced forskolin-stimulated lipolysis but failed to reverse the transport inhibition. Therefore, the effects of the agent on lipolysis appeared to be dissociable from those on glucose transport. In plasma membrane vesicles, forskolin inhibited D-glucose transport in a competitive manner by an increase in the apparent transport K_m without any detectable change in V_{max}. In parallel to the transport inhibition, the agent inhibited the specific binding of cytochalasin B in both plasma membranes and low density microsomes, which contain the intracellular pool of glucose transporters in insulin-sensitive cells. The K_i of this inhibition (205 nM) was very similar to that of the inhibition of glucose transport in the membrane vesicles (203 nM). It is concluded that forskolin inhibits glucose transport by a direct interaction with the transporter (or a closely related protein) rather than through activation of adenylate cyclase.

Forskolin, a diterpene derived from the Indian plant *Coleus forskohlii*, stimulates adenylate cyclase by a direct interaction with adenylate cyclase (1, 2). The agent is one of the most powerful stimulators of the cyclase, and has thus been widely used to characterize cAMP-dependent mechanisms (3). In adipose tissue, adenylate cyclase stimulators and inhibitors regulate glucose transport in both an inhibitory and a stimulatory way (4–6). Recent evidence has shown that this regulation is achieved independent of insulin's action by a change in the intrinsic activity of the glucose transporter (7). Furthermore, in contrast to lipolysis, the regulation of glucose transport was unlikely to be mediated by cAMP, since alterations in cellular cAMP levels (8) and cAMP-dependent protein kinase activity (9) could be dissociated from the transport alterations. Forskolin appeared to be a promising tool to investigate the underlying mechanisms, since it inhibits glucose transport not only in intact adipose cells, but also in isolated plasma membrane vesicles (4) under conditions where the formation of cAMP is unlikely.

The present study was initiated, therefore, to test a hypothetical direct interaction of forskolin with the glucose transporter or a closely related regulatory protein. With the aid of antilipolytic agents, insulin and PGE_2, the effect of forskolin on lipolysis was dissociated from that on glucose transport. Furthermore, we assumed that the supposed interaction of forskolin with the transporter might change the binding characteristics of cytochalasin B, a well established labeling agent of the glucose transporter. While our work was in progress, an inhibition of cytochalasin B binding by forskolin in human erythrocytes was reported (10). These data and the present results support the view that forskolin and cytochalasin B share a common binding site, and possibly a common mechanism of action on the glucose transporter.

Experimental Procedures

Materials. Forskolin was purchased from Calbiochem-Behring (San Diego, CA). Crystalline porcine insulin was a gift of Dr. R. E. Chance, Eli Lilly and Co. PGE_2 (Minprostin) was obtained from Upjohn GmbH (Heppenheim, Federal Republic of Germany). Crude bacterial collagenase was purchased from Cooper Biochemical; bovine serum albumin (fraction V) was from Reheis Chemical Co.

Glucose transport in cells. Adipose cells were prepared from epididymal fat pads of male Wistar rats according to the method of

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ABBREVIATIONS: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PGE_2, prostaglandin E_2; N_s, stimulatory guanine nucleotide-binding regulatory protein; N_i, inhibiting guanine nucleotide-binding regulatory protein; EDTA, ethylenediaminetetraacetate.

Rodbell (11) with minor modifications. Cells were incubated in a Krebs-Ringer Hepes buffer (12) with the agents under investigation, and glucose transport activity was determined with the aid of the nonmetabolizable hexose, 3-*O*-methylglucose (13). Samples of 200 μl containing approximately 400,000 cells were added to 5 μl of buffer containing 0.6 μCi of [^{3}H]methylglucose (final concentration 0.05 mM). The uptake was stopped by addition of 9 ml of ice-cold phloretin solution (1 mM). Accurate timing (usually 3 sec) was achieved by using a metronome set at half-second beats. After addition of silicone oil, the samples were centrifuged, the cell layer was removed, and cell-associated radioactivity was determined. Equilibrium uptake was determined with samples incubated for 60 min with methylglucose, and initial velocities were calculated from the uptake values U_t and U_{max} as previously described (14, 15).

Preparation of membrane fractions. Isolated adipose cells obtained from 16–45 rats were incubated for 25 min in the presence of insulin (10 nM). The cells were washed with homogenization buffer (10 mM Tris, 1 mM EDTA, 250 mM sucrose) and homogenized with a Potter-Elvehjem grinder. Plasma membranes and low density microsomes were prepared by differential centrifugation as described (15, 16).

Glucose transport in plasma membranes. Glucose transport in plasma membrane vesicles was determined under equilibrium exchange conditions using a filtration assay as previously described in detail (7). Samples (20–40 μg of membrane protein) were incubated in 30 μl of buffer containing equal concentrations of D-glucose and L-glucose (0.1 mM in all experiments except in the kinetic studies) at 22° for at least 30 min, and were pulsed for 2.5 sec with 30 μl of the same medium containing approximately 1 μCi of D-[U-^{14}C]glucose and L-[^{3}H]glucose. Uptake was stopped with a 15-fold dilution of ice-cold incubation medium containing 0.133 mM phloretin, and membranes were separated from buffer by filtration. Initial velocities were calculated after correction for L-glucose uptake as described previously (14, 15).

Assay of cytochalasin B binding. Cytochalasin B binding was determined in the presence or absence of forskolin as indicated with the aid of a previously described binding assay (17). The assay was carried out in the presence of 2 μM cytochalasin E in order to reduce binding to sites other than the glucose transporter (17), and samples containing 400 mM D-glucose were included to correct for nonspecific binding. Scatchard plots were constructed, and curves representing nonspecific binding (presence of D-glucose) were subtracted from the total binding according to the method of Rosenthal (18). This procedure routinely yields the linear Scatchard plots indicating a single binding site of cytochalasin B at the glucose transporter. In some experiments (see Fig. 3) one cytochalasin B concentration (40 nM) and varying forskolin concentrations were used, and the data were corrected for nonspecific binding as measured in the presence of 400 mM D-glucose.

Measurement of lipolysis. Lipolysis was assessed by measurement of the glycerol content of incubation media after separation from cells. Glycerol was determined enzymatically without deproteinization by the glycerolkinase method (19).

Calculations. K_I values of the inhibition of glucose transport were calculated from the abscissa intercepts of double reciprocal plots according to the method of Dixon and Webb (20). The K_I values of inhibition of cytochalasin B binding were calculated with the equation of Cheng and Prusoff (21) from forskolin concentrations inhibiting 50% of the cytochalasin B binding as determined with concentration response curves (see Fig. 3, *upper panel*). The K_D of cytochalasin B binding (100 nM) which is required for the calculation was measured in separate experiments. Statistical significance was tested with a paired *t* test and was accepted at the $p \leq 0.05$ level.

Results

Fig. 1 shows the concentration dependencies of the effects of forskolin on glucose transport (Fig. 1, *upper panel*) and on lipolysis (Fig. 1, *lower panel*) in isolated rat adipose cells. At

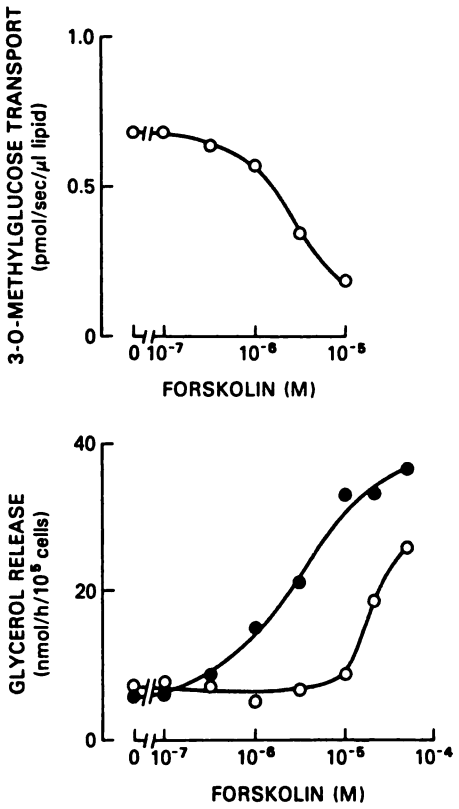

Fig. 1. Effects of forskolin on glucose transport (*upper panel*) and lipolysis (*lower panel*). Cells were incubated in the presence (○) or absence (●) of insulin (4.8 ng/ml) for 30 min, and the indicated concentrations of forskolin were added. After another 30 min of incubation, transport was measured as described in Experimental Procedures. Glycerol was assayed in the incubation medium of separate samples run in parallel. The data represent the means of duplicates of one representative experiment which was repeated four times.

concentrations between 10^{-6} and 10^{-5} M, forskolin produced a marked inhibition of the glucose transport, which had been stimulated by exposure of cells to insulin (Fig. 1, *lower panel*). In contrast, as can be seen in the *upper panel* of Fig. 1, forskolin failed to stimulate lipolysis in this concentration range, if insulin was present (Fig. 1, *open circles*) which shifted the concentration-response curve by about 1 order of magnitude. Thus, the addition of the hormone provided conditions under which forskolin's effect on glucose transport was strikingly dissociated from that on lipolysis.

In order to further establish the observed dissociation of lipolysis and glucose transport, we used PGE_2 as an agent inhibiting lipolysis by a mechanism different from that of insulin. Fig. 2 shows that the agent failed to reverse the inhibitory effect of forskolin on glucose transport (Fig. 2, *upper panel*). The concentration response curves in the presence and absence of PGE_2 were essentially identical. In contrast, the prostaglandin inhibited the stimulatory effect of forskolin on lipolysis. The inhibition was statistically significant at forskolin concentrations of 0.5 and 1 μM. This effect resembled the inhibitory action of insulin, although the absolute inhibitory effect of the prostaglandin at half-maximally stimulating forskolin concentrations (30%) was much smaller than that of insulin (complete inhibition of lipolysis). It should be noted that the magnitudes of the responses of both transport and lipolysis differed largely between the two experimental series

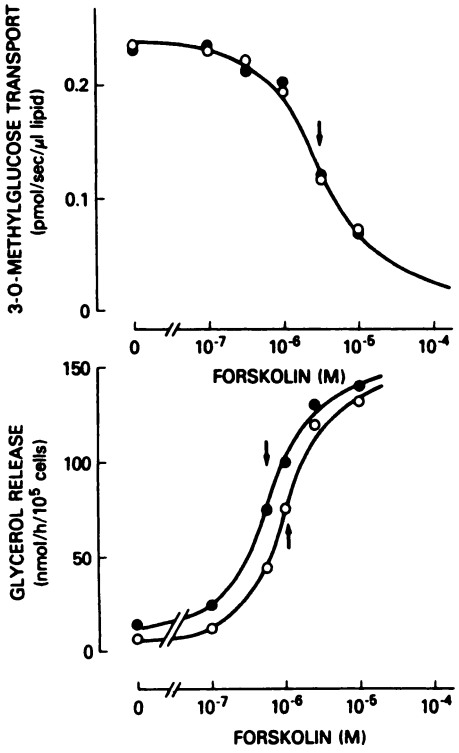

Fig. 2. Effects of PGE_2 on the inhibitory action of forskolin on glucose transport (*upper panel*), and on the stimulatory action on lipolysis (*lower panel*), in isolated rat adipose cells. Cells were incubated with (*upper panel*) or without (*lower panel*) insulin for 30 min. PGE_2 (0.1 μg/ml, ○) and forskolin as indicated were added, and transport or glycerol release was assayed after another 30 min. ●, controls incubated in the absence of PGE_2; ↑, ↓, half-maximally effective forskolin concentrations. The data represent means of four different experiments.

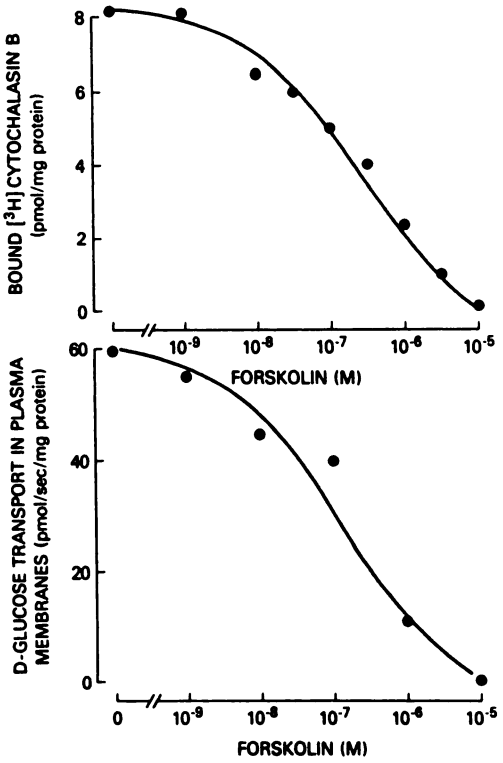

Fig. 3. Concentration dependency of the inhibitory effect of forskolin on glucose transport (*lower panel*) and on glucose-inhibitable cytochalasin B binding (*upper panel*) in plasma membranes prepared from insulin-treated adipose cells. Plasma membranes were incubated for 15 min with the indicated concentrations of forskolin at 22°, and transport was assayed as described in Experimental Procedures. Cytochalasin B binding was assayed in the presence of 40 nM [^{3}H]cytochalasin B and 2 μM cytochalasin E, and was corrected for nonspecific binding as measured with samples incubated in the presence of 400 mM D-glucose. The data represent means of triplicates of a representative experiment.

(Figs. 1 and 2). Seasonal variations may account for these differences, since the experiments were carried out with an interval of several months.

Fig. 3 shows the effects of forskolin on glucose transport (Fig. 3, *lower panel*) and on glucose-inhibitable cytochalasin B binding (Fig. 3, *upper panel*) in plasma membranes isolated from insulin-stimulated adipose cells. Forskolin produced a complete inhibition of D-glucose transport at concentrations which were about 1 order of magnitude lower than those necessary to inhibit glucose transport in intact cells. In parallel, cytochalasin B binding was inhibited by forskolin over the same concentration range. Fig. 3 (*upper panel*) shows binding data corrected for a nonspecific fraction of cytochalasin B binding which was assayed in the presence of 400 mM D-glucose. This nonspecific binding was essentially unaffected by 10 μM forskolin (data not shown), whereas the specific (glucose inhibitable) fraction of the binding was fully inhibited at this forskolin concentration in plasma membranes (Fig. 3, *upper panel*) as well as in low density microsomes (not shown).

A study of the kinetics of the transport inhibition in the plasma membrane vesicles (Fig. 4) revealed that the inhibitory effect of forskolin was mainly, if not exclusively, competitive: the agent increased the transport K_m without any detectable change in its V_{max}. Under the assumption of a purely competitive type of inhibition, a K_I of 203 ± 20 nM (n = 3) for the inhibitory effect of forskolin was calculated from these data.

As the Scatchard plots (Fig. 5) of cytochalasin B binding in the presence and absence of forskolin revealed, the interaction

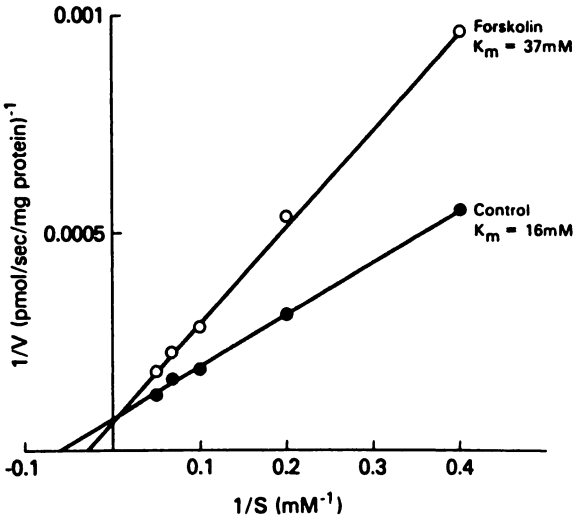

Fig. 4. Substrate dependency (Lineweaver-Burke plot) of the inhibitory effect of forskolin on D-glucose transport in isolated plasma membranes from insulin-treated adipose cells. Plasma membranes were incubated in the presence of the indicated glucose concentrations (equilibrium exchange conditions) with (○) or without (●) 225 nM forskolin for 30 min, and glucose transport was assayed as described. The data represent means of triplicates of a representative experiment.

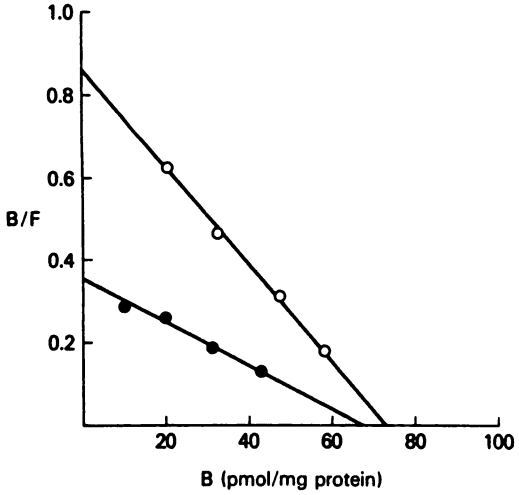

Fig. 5. Scatchard plot of the glucose-inhibitable cytochalasin B binding in low density microsomes from rat adipose cells in the presence (●) or absence (○) of 225 nM forskolin. The data represent means of duplicate samples from a representative experiment.

of these agents with the glucose transporter appeared to be competitive. The agent increased the K_D of the glucose-inhibitable cytochalasin B binding without any significant reduction of the number of sites (R_0). Thus, a competitive type of inhibition was assumed and, according to the method of Cheng and Prusoff (21), a K_I was calculated from these data (205 ± 25 nM, $n = 3$) which was identical to that of the transport inhibition.

Discussion

The above presented data fully dissociate the effect of forskolin on glucose transport from that on lipolysis in intact adipose cells. First, glucose transport was inhibited under conditions under which lipolysis was not stimulated, and second, PGE_2 inhibited the forskolin-stimulated lipolysis but failed to reverse the inhibition of glucose transport. These findings, together with the finding that forskolin inhibited glucose transport in isolated plasma membranes in the absence of ATP, render it unlikely that the effect of forskolin on glucose transport is mediated by cAMP. However, this conclusion requires some further consideration, since lipolysis appears to be a rather indirect parameter for adenylate cyclase activity, cAMP levels, or the activity of the cAMP-dependent protein kinase. According to the comprehensive work of Honnor *et al.* (22–24), the extent of a stimulation of lipolysis is always correlated with the activity ratio of the cAMP-dependent protein kinase (A-kinase), regardless with which agent the stimulatory effect was achieved. The effects of inhibitors operating via N_i can be fully accounted for by a decrease in the A-kinase activity. Insulin, in contrast, is capable only of inhibiting the partially activated A-kinase. Above a certain level of A-kinase stimulation, insulin operates via different mechanisms, possibly by activation of a phosphatase (24). It can be argued, therefore, that the dissociation of lipolysis from glucose transport in the presence of insulin does not necessarily imply a dissociation of A-kinase activity and, thereby, cAMP levels, from glucose transport. However, in that case one would have to expect at least a partial reversal of the inhibition of glucose transport by PGE_2, which operates via N_i and should therefore reduce cAMP levels and A-kinase activity.

A similar dissociation of the effects of xanthines on lipolysis from those on glucose transport has previously been reported (25, 26). In addition, the inhibitory effects of catecholamines have been shown to be independent from cAMP-dependent pathways (8, 9). These data, together with the present findings, support the notion that the mechanisms by which stimulators and inhibitors of lipolysis regulate glucose transport do not involve cAMP.

In other respects the characteristics of the inhibitory effect of forskolin were strikingly different from those of the catecholamines. Isoproterenol predominantly decreased the transport V_{max} with little or no change in K_m in intact cells (5) as well as in membranes from pretreated cells (7). In contrast, the inhibitory effect of forskolin reflects an increase in the transport K_m. Furthermore, forskolin inhibited the insulin-stimulated glucose transport to near basal levels in intact cells as well as in isolated plasma membranes. In contrast, isoproterenol decreased the insulin-stimulated transport by only about 50% in cells or in membranes from pretreated cells (7), and attempts to produce the inhibitory effect of catecholamines in a cell-free system have failed (4).[1] These striking differences suggest that catecholamines and forskolin inhibit glucose transport via distinct mechanisms, as has been proposed previously (4).

Like the effect of forskolin in isolated plasma membranes, the inhibitory effect of cytochalasin B on glucose transport has been reported to be purely competitive (27). The present data demonstrate that forskolin is a competitive inhibitor of cytochalasin B binding in plasma membranes from adipose cells. The striking similarity of the K_I values of glucose transport inhibition and cytochalasin B binding suggests that the agents share a common site of action at the glucose transporter. As has been suggested for cytochalasin B (28), this site of action might be located at the cytoplasmic region of the transporter molecule, binding to which would render the transporter inaccessible to glucose. In addition, more speculative mechanisms might be given involving regulatory proteins associated with the transporter (see below).

Forskolin has been previously shown to inhibit glucose transport in human erythrocytes and platelets independent of its action on adenylate cyclase (10, 29). Similar to the observations presented in the present study, the agent inhibited cytochalasin B binding to erythrocyte membranes (10). Although the effects were observed at μM concentrations of forskolin, and the inhibitory effect on transport appeared to be noncompetitive, the data are compatible with a direct inactivation of the transport system by the agent. In a preliminary report (30), photocatalyzed cross-linking a forskolin to a 45-kDa protein, which probably represents the glucose transporter, has been shown in erythrocyte membranes. This finding is in agreement with the above drawn conclusion, although the K_D of the reaction was about 1 order of magnitude higher than that derived from our data.

Several studies have shown that forskolin may modulate adenylate cyclase by more than one mechanism. In addition to the direct, GTP-independent stimulation requiring μM concentrations of forskolin, at lower concentrations a stimulatory effect emerges which depends on a functional N_s-subunit (27). Accordingly, binding studies have revealed at least two forskolin-binding sites (31, 32). In addition, nM concentrations of

[1] H. G. Joost, unpublished results.

forskolin have recently been reported to inhibit the cyclase in platelets provided it was fully activated through a stimulation mediated by N_s (33). It can be concluded, therefore, that forskolin operates by modifying the binding of regulatory proteins to the cyclase. Recently, a model of transport regulation has been suggested involving the direct interaction of the regulatory proteins N_s and N_i with the transporter (9). It is tempting to speculate, therefore, that the transport inhibition produced by forskolin reflects a modification of the binding of regulatory proteins to the transporter, analogous to that mediating the effects on the cyclase. The failure of PGE_2 to reverse the inhibition does not necessarily contradict this notion, since the site of action of forskolin might be located at the transporter. This speculation would imply, however, that cytochalasin B operates via the same mechanism.

In summary, the present data indicate that forskolin inhibits glucose transport independent of its action on adenylate cyclase. In analogy to cytochalasin B, its most likely site of action is the cytoplasmic region of the transporter. A more speculative mechanism involves the binding of regulatory proteins to hypothetical sites at the transporter, which would be modified by forskolin.

Acknowledgments

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