Regulation of glucose carrier activity by AlCl_3 and phospholipase C in fat-cells

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Recently it was speculated that activation of GTP-binding proteins and of phospholipase is involved in the transmission of a signal from the insulin-receptor kinase to effector systems in the cell. To confirm this hypothesis, we have tested the effect of AlCl_3 , which has been recently used as an experimental tool to activate GTP-binding proteins, on glucose transport in fat-cells. We found that AlCl_3 has a partial insulin-like effect on glucose transport activity (3-*O*-methylglucose uptake, expressed as % of equilibrium value per 4 s: basal 9.6 ± 2 , AlCl_3 29.6 ± 4 , insulin 74.0 ± 3). The AlCl_3 effect is totally blocked by pertussis toxin, whereas the insulin effect was not altered. The effect starts at $[\text{AlCl}_3] > 1 \text{ fM}$ and reaches its maximum at 0.1 nM . Addition of phospholipase C (PLC; 50 munits/ml) also stimulated glucose transport (maximal $53.0 \pm 5 \%$). Both substances acted faster than insulin itself (maximal values within 1 min for PLC, 2 min for AlCl_3 and 5–10 min for insulin). Using the cytochalasin-B-binding assay to determine the effects of AlCl_3 and PLC on the distribution of glucose carrier sites in subcellular fractions, we found that their glucose-transport-stimulating effect does not occur through an increase in glucose carrier sites in the plasma-membrane fraction. When PLC was combined with the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate), which increases glucose carrier sites in the plasma membrane, an additive effect on glucose transport was found [PLC (50 munits/ml), $53.0 \pm 5 \%$, TPA (1 nM), $17.3 \pm 2 \%$; PLC + TPA, $68.0 \pm 3 \%$]. In conclusion: (1) the data show that AlCl_3 , probably through activation of a pertussis-toxin-inhibitable G protein, and PLC are able to modulate the intrinsic glucose carrier activity; (2) as pertussis toxin did not modify the effect of insulin, it seems unlikely that the insulin signal on glucose transport involves activation of this specific G protein.

INTRODUCTION

Activation of the intrinsic insulin-receptor tyrosine kinase is the first post-binding event in cellular insulin action [1]. It is therefore speculated that further transmission of the insulin signal occurs then through phosphorylation of cellular protein substrates at tyrosine residues [2–6]. The nature of these substrates and the mechanisms by which they convey the signal to the metabolic effector systems of the cell is so far not understood. There are many proteins serving as substrates of the insulin-receptor kinase *in vitro* [1]. The physiological implication of most of these substrates is, however, unknown. Beside the substrates *in vitro*, several proteins have been identified in intact cells which are possible candidates for target substrates of the receptor kinase [2–6]. Two recent studies [7,8] have shown that G-proteins are among the substrates *in vitro* of the isolated insulin-receptor kinase. This finding is of particular interest, as recently other studies have suggested that activation of a glycolipid-specific PLC [9,10] is important in cellular insulin action. Like other phospholipases [11, 12] this enzyme could be regulated by G-proteins, which themselves could represent the missing link in a signal-transmitting chain between the insulin-receptor kinase and this phospholipase. To test this hypothesis we

used AlCl_3 , which has been employed as an experimental tool to activate GTP-binding proteins [13]. Recently AlCl_3 was successfully used to probe the involvement of G-proteins in the transmission of the signal of thrombin [11]. By analogy with these studies, we tested whether GTP-binding proteins might be involved in the regulation of glucose transport in fat-cells and in the transmission of an insulin signal from the insulin receptor to the glucose transport system. We found that AlCl_3 as well as exogenously added PLC can indeed mimic partially the insulin effect on glucose transport activity. These effects do not involve glucose carrier translocation, which is one accepted mechanism of insulin's stimulating effect on the glucose transport activity [14,15]. Recently, however, evidence has increased that, besides carrier translocation, a regulation of the intrinsic carrier activity also occurs after stimulation by insulin [16] and under certain other conditions [17–19], and now we show that AlCl_3 and PLC apparently modulate this intrinsic carrier activity also.

MATERIALS AND METHODS

The phorbol ester TPA and PLC (type XIV, from *Clostridium perfringens*) were purchased from Sigma, Munich, Germany. Fat-cells were prepared from male

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PLC, phospholipase C; LDM, low-density membranes; HDM, high-density membranes; CBB, cytochalasin B binding.

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Sprague-Dawley rats fed *ad libitum* (180–220 g body wt.). Krebs-Ringer-Hepes buffer (pH 7.4, 37 °C) containing 21.5 g of crystalline bovine serum albumin/dl and 5 mM-D-glucose was used for all incubations.

3-O-Methylglucose transport in adipocytes

This was measured as previously described [20]. Samples (100 μ l) of the concentrated cell suspension (5×10^6 cells/ml) were drawn together with 200 μ l of 3-O-methylglucose (final concn. 0.5 mM) and a tracer of 0.1 μ Ci of 3-O-methyl-D-[14 C]glucose (The Radiochemical Centre, Amersham, Bucks., U.K.) into a mixing pipette (Gilson Medical Electronics, Villier-le-Bel, France). After 4 s, the uptake was stopped by diluting the cells in 5 ml of NaCl (0.9 g/dl) which contained phloretin (1 mM). Cells and medium were separated by centrifugation through silicone oil (1000 *g* for 60 s). The cell layer was removed by a pipette and added to scintillation fluid, and the radioactivity was measured. The amount of 3-O-methylglucose in extracellular fluid or taken up by diffusion was determined in samples which contained 1 mM-phloretin. All other uptake data were corrected by this value. 3-O-Methylglucose accumulation measured during 4 s is expressed as a percentage of the 3-O-methylglucose accumulated at equilibrium in fat-cells in a 30 min incubation under saturating insulin concentrations.

Subcellular fractionation of adipose cells

Plasma- and microsomal-membrane fractions were prepared from incubated cells by a differential-centrifugation method described by McKeel & Jarret [21], with modifications described by Karnieli *et al.* [22].

The reproducibility of the fractionation procedure was assessed by measuring the specific 5'-nucleotidase activity (enriched in plasma-membrane fraction) [23], rotenone-insensitive NADH-cytochrome *c* reductase activity (enriched in endoplasmic-reticulum fraction) [24] and UDP-galactosyltransferase activity (enriched in Golgi apparatus) [25] of each fraction and the original homogenates.

Membrane protein concentration was determined by the Coomassie Brilliant Blue method (Bio-Rad assay).

Lipocrit was determined by measuring the impedance in a haematocrit-electrolyte-combination apparatus.

Cytochalasin-B-binding assay

The concentration of D-glucose transporters in the membrane fractions was assessed by using a specific D-glucose-inhibitable CBB assay developed by Cushman & Wardzala [14] and modified by Karnieli *et al.* [22]. In each assay we used 11 different cytochalasin B concentrations in the test. [14 C]urea was used to determine trapped unbound [3 H]cytochalasin B in the pelleted membranes, obtained during the binding assay. Therefore it was added so that the final concentrations of 14 C and 3 H were the same. From the resulting 11-point CBB curves the number of glucose-inhibitable binding sites (R_0) and the affinity constants (K_d) were determined by Scatchard analysis.

The CBB assay was carried out as outlined recently [26]. Incubation was carried out at 22 °C for 60 min. All other working steps were performed at 24 °C. After incubation, 60 μ l of 1.17% (v/v) acetic acid was added to each sample for neutralization, and the tubes were vortex-mixed and transferred bottom to top to a

scintillation vial for measuring bound [3 H]cytochalasin B.

RESULTS

3-O-Methylglucose transport

AlCl_3 was shown to activate a PLC in fibroblasts through activation of G-proteins [11]. Simultaneous addition of NaF increased this effect [11], and it is believed that the active compound which interacts with GTP-binding proteins is AlF_3 [11]. By analogy with that study, AlCl_3 alone or in combination with NaF was added in a wide concentration range between 1 fM and 1 μ M to isolated fat-cells for 20 min. The effect of insulin was also measured in parallel incubation. Fig. 1 shows the dose/response curve for the effect of AlCl_3 on glucose transport activity. AlCl_3 stimulates the glucose transport activity in a dose-dependent way. The combination with NaF had no additive effect (results not shown) suggesting that fluoride is already present in sufficient amounts in the fat-cell to form AlF_3 complexes. We therefore used in all further experiments AlCl_3 alone. The effect of AlCl_3 starts at concentrations greater than 1 fM and reaches the maximum at 0.1 nM. The stimulation at the maximally effective concentrations of AlCl_3 is 3-fold (3.1 ± 0.3 , $n = 4$), whereas insulin stimulates the glucose transport

Fig. 1. Effect of AlCl_3 on glucose transport activity in isolated rat adipocytes

3-O-Methylglucose transport activity in isolate rat adipocytes is expressed as percentage of equilibrium uptake reached in 4 s. (a) Dose-response curve for AlCl_3 (means $\pm$ S.E.M. from 14 experiments are shown). (b) Time course of the effect of 1 nM- AlCl_3 (●) and 2 nM-insulin (■) (means $\pm$ S.E.M. from three parallel experiments are shown).

Fig. 2. Comparison of glucose transport activity in isolated rat adipocytes

Cells were preincubated under following conditions: basal controls (Ba; $n = 6$), insulin (Ins) 10 nM ($n = 6$), AlCl₃ 0.1 nM ($n = 6$), AlCl₃ 0.1 nM + pertussis toxin (P.T.) 0.01 μ g/ml ($n = 4$), insulin 10 nM + pertussis toxin 0.01 μ g/ml ($n = 4$), PLC 50 munits/ml ($n = 6$).

activity more than 7-fold (7.7 ± 0.2 , at 10 nM-insulin). The effect of AlCl₃ on glucose transport activity occurs even more rapidly than does the effect of insulin. Fig. 1(b) shows a time course at the maximally effective AlCl₃ concentration.

To confirm that the effect of AlCl₃ is attributable to an action on G-proteins, we tested if pertussis toxin blocks the effect of AlCl₃. Pertussis toxin is known to inhibit activation of G_i [27–29], transducin [30] and G-proteins linked to PLC [12]. Fig. 2 shows that the AlCl₃ effect at the maximal stimulatory concentration of 0.1 nM is indeed fully abolished by pertussis toxin (maximally inhibitory concn. 0.01 μ g/ml). Furthermore, we investigated the effect of AlCl₃ on catecholamine-stimulated cyclic AMP concentrations in isolated rat fat-cells as described in [20], using the maximal effective concentrations for both substances. We found a 30% decrease under these conditions (results not shown). These data so far show that AlCl₃ has a partial insulin-like effect on the glucose transport activity of fat-cells. This effect apparently occurs through interaction with G-proteins. One possible mechanism by which AlCl₃, through G-protein action, might convey an insulin-like signal is the activation of an intrinsic PLC [12]. To test this hypothesis, we studied whether the effect of AlCl₃ can also be mimicked by addition of PLC. We used PLC in the concentration range which was previously shown to activate pyruvate dehydrogenase in fat-cells [31]. PLC indeed mimics the AlCl₃ effect; however, it is even

more stimulatory. At the maximally effective PLC concentrations a 5.5-fold ($n = 5$) stimulation was found (Fig. 2). We next attempted to analyse the mechanism through which AlCl₃ and PLC increase the glucose transport activity. It is widely accepted that insulin action on glucose transport activity of isolated fat-cells involves the translocation of glucose carrier sites from intracellular storage sites to the plasma membrane [14,15]. In addition, recently evidence is increasing that a modulation of the intrinsic glucose carrier activity occurs also after insulin binding [26] and under certain other conditions [17–19]. The translocation process can be measured by determination of CBB sites in subcellular fractions of isolated fat-cells. To test whether the regulatory influence of G-proteins on the glucose transport activity of fat-cells also involves glucose carrier translocation, we determined the effect of AlCl₃ and PLC on the distribution of CBB sites in isolated fat-cells.

Cytochalasin B-binding assay in subcellular fractions of fat-cells treated with AlCl₃, phospholipase C or insulin

Isolated fat-cells were pretreated with insulin (10 nM), AlCl₃ (0.1 nM) or PLC (50 munits/ml) for 20 min. Glucose transport activity was then determined in one sample. Another sample was used for fractionation of fat-cell membranes as described in the Materials and methods section. The characteristics of the marker enzymes in the different membrane fractions were monitored as described previously [26]. The purity of the membrane fractions matched our earlier findings [26], which were within the range reported by other groups [16]. Fig. 3(a) shows the CBB data in the plasma-membrane fraction. The data are presented as the derived Scatchard plot of CBB. Under basal conditions a glucose-transporter number of 5.4 pmol/mg of membrane protein is found. Insulin increased in these experiments the number of glucose carrier sites to approx. 15 pmol/mg of protein. In contrast, AlCl₃ and PLC had no significant increasing effect on the number of glucose carrier sites in the plasma membrane fraction (6.3 pmol/mg of protein). There was also no statistically significant effect on the dissociation constant of the glucose carrier sites. These data suggest that the increased glucose-transport activity of the fat-cells observed after addition of AlCl₃ or PLC is not explained by an increased number of glucose carriers in the plasma membrane. Fig. 3(b) shows the Scatchard plot of CBB to LDM. Under basal conditions a glucose carrier concentration of 10 pmol/mg was found. Insulin treatment decreased the number of glucose carrier sites in the LDM fraction to approx. 5 pmol/mg. Interestingly, AlCl₃ also induced a decrease in the number of glucose carrier sites in the LDM fraction to 5.7 pmol/mg, whereas PLC had no effect. Fig. 3(c) shows the derived Scatchard plot for CBB to HDM. Under basal conditions 9 pmol/mg of protein are found. Insulin increases the number of CBB sites in the HDM fraction to 18 pmol/mg. AlCl₃ and PLC do not increase the number of glucose carrier sites in HDM. The data can be summarized as follows: as shown above, insulin causes a decrease in the intracellular pool of CBB sites and a concomitant increase in the plasma-membrane and HDM pool of glucose transporters. This phenomenon is interpreted as translocation of glucose carriers. AlCl₃ also causes a decrease in the number of intracellular glucose transporters in LDM, but no increase in plasma-membrane glucose transporters. PLC neither decreases

Fig. 3. Derived Scatchard analysis of [^3H]cytochalasin-B binding to rat adipocyte membranes isolated from basal (●), insulin- (▲), AlCl_3 - (■) and PLC- (△) stimulated cells

Results are the means of six experiments. Concentrations are the same as described in the legend of Fig. 2. Dissociation constants are given in nM.

glucose transporters in the LDM fraction nor increases glucose transporters in the plasma membrane. Apparently no translocation occurs after addition of AlCl_3 or PLC, suggesting that the stimulating effect of AlCl_3 and PLC on glucose transport activity is exclusively due to a modulation of the intrinsic glucose carrier activity.

Effect of pertussis toxin on insulin action

The insulin effect on glucose transport apparently consists of two additive steps, carrier translocation and also carrier activation [26]. To test if the effect of insulin on carrier activity is transmitted through the AlCl_3 -activated pertussis-toxin-inhibited G-protein, we tested the effect of pertussis toxin on insulin's effect. Fig. 4

Fig. 4. Comparison of dose-response curves of 3-O-methylglucose transport activation by insulin (●) and insulin + pertussis toxin (0.01 $\mu\text{g/ml}$) (□)

Mean values $\pm$ S.E.M. are shown for three experiments.

shows that pertussis toxin has no inhibitory effect on insulin's action on 3-O-methylglucose uptake, suggesting that this specific G-protein is not used as a signal transmitter by insulin.

Fig. 5. Comparison of glucose transport activity in isolated rat adipocytes, stimulated by maximally effective concentrations of: insulin (Ins; 10 nM), TPA (1 nM), AlCl_3 (0.1 nM), PLC (50 munits/ml)

Substances were combined for AlCl_3 and TPA as well as PLC and TPA in concentrations given above ($n = 6$ for basal, insulin, AlCl_3 and PLC; $n = 4$ for TPA and the combinations with TPA).

Fig. 6. 3-O-Methylglucose transport in rat adipocytes: time course of the effect of 10 nM-insulin (●), 50 munits of PLC/ml (▲) and the combination of 50 munits of PLC/ml with 1 nM-TPA (□)

Results are means $\pm$ S.E.M. ($n = 3$).

Combination of AlCl₃ or phospholipase C with the phorbol ester TPA

The data so far show that AlCl₃ and PLC stimulate the glucose transport of fat-cells through a modulation of the intrinsic glucose carrier activity. Both substances do not stimulate glucose carrier translocation, and their effect on glucose uptake reaches only 25–50% of the insulin effect. To answer the question whether additional stimulation of carrier translocation by phorbol ester would mimic the full insulin effect, we combined AlCl₃ or PLC with TPA. Fig. 5 shows the effect of TPA (1 nM), AlCl₃ (0.1 nM) alone or in combination with TPA (1 nM) and of PLC (50 munits/ml) alone or in combination with TPA (1 nM). The effect of AlCl₃ is only minimally increased by the combination with TPA. At higher AlCl₃ concentrations or after preincubation with TPA, both substances apparently inhibit each other's effect (results not shown). For PLC, however, a clearly increased effect in combination with TPA was found, even though the insulin effect was not fully mimicked. We also investigated the time course of the PLC effect, as well as the effect of PLC plus TPA. Fig. 6 shows the kinetics of PLC (50 munits/ml) alone, PLC (50 munits/ml) plus TPA (1 nM) and, for comparison, insulin (10 nM). Insulin shows the known time course. After a short initial lag phase, maximal values are reached within 10 min. In contrast, PLC acts apparently without a lag phase, reaching maximal values already within 1 min. Simultaneous addition of TPA does not significantly alter the initial time course of PLC action; however, at time points later than 2 min, significantly higher values are found.

DISCUSSION

The aim of our study was a further characterization of the mechanisms regulating glucose transport of fat-cells, and more specifically to answer two questions. (1) Are G-proteins involved in the regulation of the glucose

transport activity of fat-cells? (2) Are G-proteins part of the signal-transmitting chain between the insulin receptor and the glucose transport system? The data allow the following conclusions and speculations.

Glucose carrier translocation is not a necessary prerequisite of glucose transport activation

It has long been recognized that insulin acts on glucose transport activity through stimulation of glucose carrier translocation from intracellular storage sites to the plasma membrane [14–16]. Recently we have proposed that insulin affects glucose transport by two different mechanisms, involving firstly glucose carrier translocation and in addition regulation of glucose carrier activity [26]. One aspect of this two-step model [26] is now confirmed. The data clearly show that the glucose transport activity of fat-cells can be increased by a rapid modulation of intrinsic glucose carrier activity independently of glucose carrier translocation.

Activation of a G-protein-dependent phospholipase C is the underlying mechanism of the AlCl₃ effect

AlCl₃ in combination with NaF was shown to activate G-proteins and a G-protein-dependent phospholipase [11,12]. Here AlCl₃ alone is able to activate the intrinsic glucose carrier activity. Even though there is no definite proof that this effect occurs through activation of G-proteins and G-protein-dependent phospholipases, the observations that the AlCl₃ effect is abolished by pertussis toxin, and that AlCl₃ decreases catecholamine-elevated cyclic AMP concentrations in fat-cells significantly, strongly suggest a G-protein mechanism. Exogenously added PLC can mimic the AlCl₃ effect, and this also suggests that activation of an intrinsic G-protein-dependent PLC is a likely possibility to explain the AlCl₃ effect.

Evidence for a two-step model of insulin action on glucose transport

From the two-step model [26] discussed above, we expected that AlCl₃ or PLC, in combination with the carrier-stimulating phorbol ester TPA, would be able to mimic fully the insulin effect on glucose transport, or at least would act in an additive way with TPA. However, TPA and AlCl₃ showed only a small additive effect. When the AlCl₃ concentration was increased or the cells were preincubated with AlCl₃, even an inhibitory interaction with TPA was observed. It is unclear if AlCl₃ at higher concentrations interferes somehow with the TPA–protein kinase C mechanism, and this complicates the interpretation of the data. In contrast, the combination of TPA and PLC is clearly additive. Even though the combination is still somewhat less effective than insulin itself, it appears possible that the insulin effect on glucose transport occurs through a parallel activation of protein kinase C- and of intrinsic PLC-dependent steps.

No evidence for a G-protein as transmitter of insulin's effect on glucose carrier activity

Even though AlCl₃ (presumably through a G-protein) and PLC have insulin-like effects on glucose carrier activity, it appears impossible that the insulin signal occurs through activation of this particular G-protein, as the insulin effect is not decreased by pertussis toxin. This does not exclude the possibility that the insulin effect in

the intact cell occurs through activation of an intrinsic phospholipase by another G-protein which is not pertussis-toxin-dependent. For other insulin effects a role of an insulin-dependent G-protein (G_{ins}) was demonstrated [32]. If a role of an insulin-dependent G-protein which can substitute for the $AlCl_3$ -dependent G-protein is assumed, a G-protein-dependent phospholipase as signal-transmitting system would be an attractive model to explain insulin's effect on intrinsic carrier activity. Further studies will be needed to investigate this possibility.

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