Biochem. J. (1989) 264, 389–396 (Printed in Great Britain)

Role of guanine nucleotide regulatory proteins in insulin stimulation of glucose transport in rat adipocytes

Influence of bacterial toxins

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The potential role of guanine nucleotide regulatory proteins (G-proteins) in acute insulin regulation of glucose transport was investigated by using bacterial toxins which are known to modify these proteins. Cholera-toxin treatment of isolated rat adipocytes had no effect on either 2-deoxyglucose transport or insulin binding. Pertussis-toxin treatment resulted in an inhibition of both insulin binding and glucose transport. Insulin binding was decreased in pertussis-toxin-treated cells by up to 40%, owing to a lowering of the affinity of the receptor for hormone, with no change in hormone internalization. The dose–response curve for insulin stimulation of glucose transport was strongly shifted to the right by pertussis-toxin treatment [EC_{50} (half-maximally effective insulin concn.) = 0.31 ± 0.04 ng/ml in control cells; 2.29 ± 1.0 in treated cells), whereas cholera toxin had only a small effect ($EC_{50} = 0.47 \pm 0.02$ ng/ml). Correcting for the change in hormone binding, pertussis toxin was found to decrease the coupling efficiency of occupied receptors (50% of maximal insulin effect with 928 molecules bound/cell in control and 3418 in treated cells). Pertussis-toxin inhibition of insulin sensitivity was slow in onset, requiring 2–3 h for completion. Under conditions where pertussis-toxin inhibition of insulin sensitivity was maximal, a 41000 Da protein similar to the α subunit of G_i (the inhibitory G-protein) was found to be fully ribosylated. These results are consistent with the concept that pertussis-toxin-sensitive G-protein(s) can modify the insulin-receptor/glucose-transport coupling system.

INTRODUCTION

The family of guanine nucleotide regulatory (G) proteins has received considerable attention as elements in the stimulus–response sequence coupling binding to action for many hormones [1]. Various G proteins are coupled to such key signalling events as adenylate cyclase, guanylate cyclase, K^+ influx and phosphoinositide turnover [1,2], mediating the actions of hormones and agents as dissimilar as glucagon, catecholamines, adenosine and acetylcholine [1,2]. The mechanism by which insulin binding to its receptor is coupled to its subsequent actions is not as fully understood as for many G-protein-linked hormones. However, recent work has proposed a possible role for G-proteins in insulin action as well [3–5].

Much of the evidence for G-protein involvement in insulin action is derived from the use of bacterial toxins which modify the proteins by ADP-ribosylation [6]. The toxins most commonly used are cholera toxin, which acts mainly on the protein mediating stimulation of adenylate cyclase, designated G_s [1,2], and pertussis toxin, which primarily influences the protein inhibiting adenylate cyclase, G_i [1,2]. At least one other pertussis-toxin substrate (G 'other' = G_o) has been identified, but the effectors to which it is linked have yet to be established. Pertussis-toxin treatment, either *in vivo* or of isolated cells, has been shown to influence lipolysis [7–10], glucose transport [11,12], glucose oxidation [7], lipogenesis [10] and cyclic AMP phosphodiesterase [13], either acting directly on the effector system or by altering insulin sensitivity. Decreasing G-protein content, either by induction of diabetes [5,14] or by incubation of cultured rat adipocytes with an adenosine-receptor agonist [15], correlates with defects in insulin action. Thus G-proteins appear to be important in both insulin action and control of effector systems. The current studies were designed to explore further the potential role of G-proteins in the insulin-response signalling system.

MATERIALS AND METHODS

Materials

Pig insulin was given by Dr. R. E. Chance of Eli Lilly Co. (Indianapolis, IN, U.S.A.). [^{125}I-Tyr^{A14}]insulin was given by Mr. Edwin Shermer, also of Eli Lilly Co. Bovine serum albumin (fraction V) and N^6-phenylisopropyladenosine were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Collagenase was from Cooper Biochemicals (Freehold, NJ, U.S.A.). 2-Deoxy-D-[1,2-^{3}H]glucose and L-[1-^{3}H(n)]glucose were purchased from New England Nuclear (Boston, MA, U.S.A.). The silicone oil for deoxyglucose-transport and binding assays was from A. H. Thomas Co. (Philadelphia, PA, U.S.A.). Adenosine deaminase, 2-deoxyglucose, L-glucose and

Abbreviations used: G-protein, guanine nucleotide regulatory protein; EC_{50}, half-maximally effective insulin concn.

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cholera toxin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and pertussis toxin was from List Laboratories (Campbell, CA, U.S.A.). Both toxins were used without further activation.

Preparation of isolated adipocytes

Fat-cells were isolated from the epididymal fat-pads of Sprague–Dawley rats (180–220 g) by a modification [16] of the method of Rodbell [17]. Minced tissue was incubated for 60 min at 37 °C in Krebs–Ringer phosphate buffer [16] containing 3 mM-glucose/4% (w/v) albumin and collagenase (2 mg/ml), pH 7.4. The cell suspension was filtered through a 500 μm nylon mesh and washed twice in reaction buffer. The cells were then filtered a second time through 250 μm mesh and washed twice more. Washes were performed with a buffer containing 150 mM-NaCl, 5 mM-KCl, 1.2 mM-$MgSO_4$, 1.2 mM-$CaCl_2$, 2.5 mM-NaH_2PO_4, 10 mM-Hepes, 2 mM-pyruvate and 4% albumin, pH 7.4, and this buffer was used in all subsequent studies unless otherwise noted. Cell counting was performed by a modification [17] of Method III of Hirsch & Gallian [18] and a model ZM Coulter Counter with a 400 μm-aperture tube.

Cell treatment

The exact conditions of incubation and treatment are described in the legends of the Figures. In general, adipocytes [(3–4) $\times 10^5$ cells/ml] were incubated with or without toxin for 2–3 h at 37 °C. At the end of the pretreatment period, cells were distributed to tubes containing the noted additions, and the incubations were continued before assay of insulin binding and glucose transport.

Insulin binding

This was measured as described previously [16], in a 500 μl volume. Adipocytes (2×10^5 cells/ml) were incubated with [^{125}I-Tyr^{A14}]insulin (0.1 ng/ml) and various concentrations of unlabelled insulin. After 60 min at 37 °C, duplicate 200 μl samples were removed, layered over silicone oil in 400 μl Microfuge tubes and then centrifuged at 11000 g for 30 s in a Beckman Microfuge. The tubes were sliced through the oil layer and the cell plugs placed into tubes for direct measurement of radioactivity. Non-specific binding was determined in the presence of 200 μg of unlabelled insulin/ml, and all results are corrected for this value.

The amount of internalized cell-associated radioactivity was determined by a modification [19] of the protocol of Haigler *et al.* [20]. This procedure is based on the principle that essentially all cell-surface ligand can be removed by incubation of cells at low temperature and pH. After binding for 60 min at 37 °C, samples were removed for measurement of total cell-associated radioactivity and the remaining cells were concentrated and then resuspended in reaction buffer adjusted to pH 4.0 at 10 °C. After a 5 min incubation at 10 °C, the cells were allowed to float and the buffer was removed. The cells were then washed twice with equal volumes of 10 °C reaction buffer (pH 7.4), resuspended to the original volume, and 200 μl samples were placed over silicone oil for determination of internalized radioactivity.

Insulin degradation was measured in the cell-free buffer under the oil layer by precipitation with trichloroacetic acid (final concn. 7.5%, w/v), as described previously [16].

Glucose transport

2-Deoxyglucose transport was measured by a modification of a previously reported technique [21] in a 500 μl volume. These conditions are the same as for the measurement of binding. After preincubation of cells [(2–4) $\times 10^5$/ml] with insulin and agents, the reaction was initiated by adding a small volume of mixed [1,2-^{3}H]-deoxyglucose and unlabelled sugar (0.1 mM, 0.2 μCi/ml) to the cells. The reaction was continued at 37 °C, and then duplicate 200 μl samples were layered over silicone oil as in the binding studies, and the reactions were terminated after 3 min by centrifugation. The contributions of trapping and diffusion were corrected for by parallel incubations with L-[^{3}H]glucose. This value was subtracted from total uptake to determine carrier-mediated transport.

Assay for cyclic AMP

Adipocytes [(3–5) $\times 10^5$ cells/ml] were incubated for 3 h at 37 °C, with no additions, cholera toxin (1 μg/ml), or pertussis toxin (0.5 μg/ml). At that time samples (250 μl) of the cell suspension were centrifuged at 50 g for 1 min, and the infranatant was removed. A stopping buffer consisting of 50 mM-sodium acetate (pH 4.0) and 0.2 mM-isobutylmethylxanthine was added to the cells, and the tubes were placed in a boiling-water bath for 5 min [22]. The clarified cellular extract was assayed for cyclic AMP by using a competitive binding protein assay [23]. Duplicate determinations were made on duplicate extracts from each of two cell preparations.

Preparation of adipocyte membranes

The ability of pertussis-toxin treatment of intact cells to modify G-proteins was monitored by subsequent ADP-ribosylation of membranes. Cells were treated with or without toxin as described in Fig. 4 legend, and a sample was removed for assay of glucose transport and insulin-sensitivity. The remaining cells were washed once in chilled (4 °C) 255 mM-sucrose/1 mM-EDTA/20 mM-Tris, pH 7.4 (TES), and resuspended in the same buffer. Cells were homogenized with a Teflon–glass homogenizer, and the homogenate was centrifuged for 10 min at 1000 $g_{max.}$ The post-nuclear supernatant was then centrifuged for 100 min at 55000 $g_{max.}$ and the crude total membrane pellet was resuspended in TES buffer for subsequent analysis. Protein concentrations were determined by the method of Bradford [24]. The generation of cyclic AMP was measured in membranes (prepared from 10^7 cells) by the method of Salomon *et al.* [25]. The assay period was 10 min.

ADP-ribosylation of G-proteins

This was performed by the method of Owens *et al.* [26]. Pertussis toxin was activated by treatment with 20 mM-dithiothreitol for 10 min at 37 °C before use. The reaction mixture contained 25 mM-Hepes (pH 7.4), 1 mg of bovine serum albumin/ml, 2.5 mM-$MgCl_2$, 0.3 mM-EDTA, 10 mM-thymidine, 1 mM-ATP, 0.1 mM-GTP, 5 μM-[^{32}P]NAD^+ and ~ 1 mg of membrane protein/ml. The reaction was for 15 min at 30 °C, and was stopped by washing the membrane twice in 10 mM-Tris (pH 7.4)/5 mM-EDTA. The pellets were solubilized with 30 μl of 1.3% (v/v) SDS/3 mM-Tris/HCl (pH 8)/1.3 mM-EDTA/50 mM-dithiothreitol/0.2 M-sucrose before electrophoresis.

SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli [27] in homogeneous 10% acrylamide gels in the presence of 0.1% SDS. The gels were then fixed and dried, and autoradiography was performed by established methods. After autoradiography, bands (1.5–2 mm) were excised from the dried gel, heated at 55 °C in 30% (v/v) H_2O_2 for 16 h, and then the radioactivity was counted.

Statistical analysis

Results are presented as means ± S.E.M. (n = number of observations). Statistical significance was evaluated by Student's t test for paired observations, and two-tailed P values were calculated. The EC_{50} values for transport stimulation were obtained from log–logit transformations of individual dose–response curves.

RESULTS

A major result of G-protein modification is an increase in adenylate cyclase activity and cyclic AMP levels [1,2]. This result was confirmed for treatment for 3 h of intact adipocytes with cholera toxin (1 μg/ml) or pertussis toxin (0.5 μg/ml). The level of cyclic AMP in cholera-toxin-treated cells (5.8 ± 0.4 pmol/2 × 10^5 cells; n = 4) was increased 4–5-fold over that in control cells (1.4 ± 0.1; n = 4), and petussis-toxin treatment caused a 7-fold increase (8.7 ± 1.2; n = 4). Cellular metabolic integrity was not significantly altered by toxin treatment, as treated cells maintained normal maximal responsiveness of glucose transport to insulin. The modification by cholera toxin was not transient. When membranes prepared from control cells were challenged with forskolin (0.1 μM), there was a large increase in cyclic AMP accumulation (from 5 to 51 pmol/min per 10^7 cells). Cholera treatment of intact cells elevated the basal adenylate cyclase activity of membranes to that attained after forskolin exposure.

The effects of toxin treatment on the major steps in the insulin action sequence in isolated rat adipocytes were studied next. Cells were treated with a range of toxin concentrations for 2 h at 37 °C before measurement of insulin binding and glucose transport. Cholera toxin was found not to influence specific insulin binding to any significant extent over the entire concentration range tested (0–10 μg/ml). Pertussis toxin, however, caused a dose-dependent decrease in binding, to 40% of control values (60% inhibition) at the highest dose tested (4 μg/ml). Insulin degradation was the same in control and both toxin-treated groups (5.5–7.3 %/h per 2 × 10^5 cells). Cholera toxin was also without significant effect on deoxyglucose transport, in either the absence or the presence of insulin (Fig. 1). Meanwhile, pertussis toxin inhibited basal transport by up to 80%, with lesser effects on maximally insulin-stimulated transport (Fig. 1). From these data, the concentrations of toxins chosen for further study were 1 μg/ml for cholera toxin, which has been shown to ribosylate G_s extensively [6], and 0.5 μg/ml for pertussis toxin, where transport rates were not significantly altered. The lack of effect of treatment with these concentrations of toxins on glucose transport was confirmed with 3-*O*-methylglucose, a glucose analogue that is transported but not phosphorylated.

Several earlier reports noted that when ^{125}I-insulin is incubated with pertussis-toxin-treated cells, the toxin treatment led to a decrease in cell-associated radioactivity

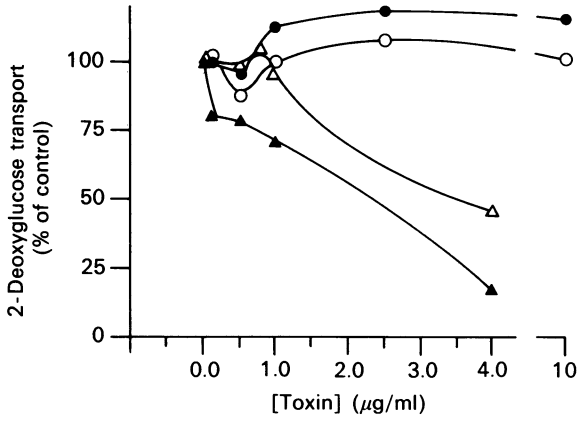

Fig. 1. Concentration-dependence of toxin effect on adipocyte glucose transport

Cells were treated for 3 h at 37 °C with various concentrations of cholera toxin (○, ●) or pertussis toxin (△, ▲). Cells were incubated for an additional 60 min in the absence (●, ▲) or presence (○, △) of a maximally stimulating insulin concentration (50 ng/ml) before assay of 2-deoxyglucose transport. Results are normalized to control (untreated) values in either the absence or presence of insulin for each experiment, and are the averages of four experiments. The control values for transport are 0.15 ± 0.06 nmol/3 min per 2 × 10^5 cells for basal cells and 1.11 ± 0.15 for insulin-treated cells.

[7,11]. However, these studies did not determine whether this was due to decreased receptor number, lowered binding affinity, or decreased hormone internalization. Such questions were explored by constructing full competition curves for insulin binding at 37 °C (Fig. 2). The control and cholera-toxin curves are essentially superimposable, whereas in pertussis-toxin-treated cells there is a 40% decrease in binding at tracer insulin concentrations. This curve converges with that of the control cells at higher insulin levels, suggesting a change in the affinity of insulin for its receptor. The

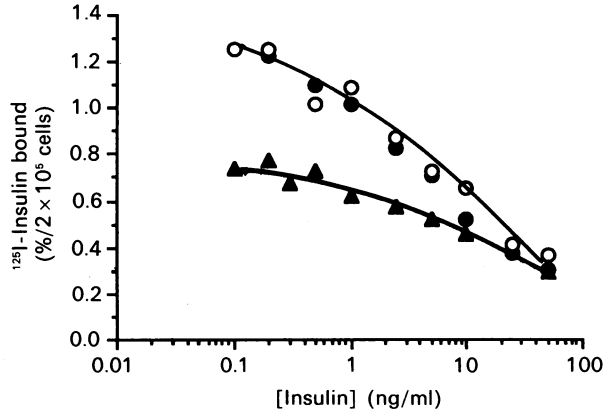

Fig. 2. Competition displacement curves for insulin binding to rat adipocytes after toxin treatment

Cells were incubated for 3 h at 37 °C with no addition (●), 0.5 μg of pertussis toxin/ml (▲) or 1 μg of cholera toxin/ml (○) before assay of tracer ^{125}I-insulin binding. Results are averages of 3–8 studies.

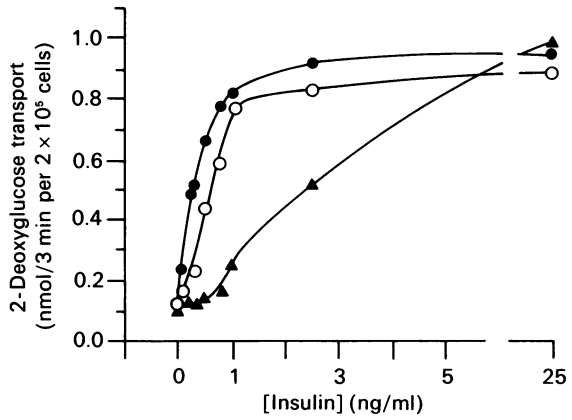

Fig. 3. Dose-dependency of insulin stimulation of glucose transport in the absence and presence of toxin treatment

Treatment conditions and symbols are as described in Fig. 2. Results are averages of 3–7 studies.

concentrations of insulin which cause 50% inhibition of tracer ^{125}I-insulin binding, an approximate measure of receptor affinity, were 8.1 ± 0.1 ($n = 11$), 10.3 ± 1.5 ($n = 4$) and 27.3 ± 6.4 ($n = 7$) ng/ml for control, cholera-toxin- and pertussis-toxin-treated cells respectively. Only the value for pertussis-toxin-treated cells differed significantly from control ($P < 0.01$).

The cell-associated radioactivity measured in Fig. 2 represents, at 37 °C, both insulin bound to the cell surface and internalized hormone. It is possible that pertussis-toxin treatment (0.5 ng of toxin/ml for 2 h) could alter the balance between intracellular and surface-bound hormone. This was studied by directly measuring intracellular insulin after acid dissociation of cell-surface-bound insulin. In this series of studies specific binding of tracer (0.1 ng/ml) insulin in pertussis-toxin-treated cells (0.80 ± 0.11 %/2×10^5 cells; $n = 5$) was decreased to 50% of the value in control cells (1.62 ± 0.21, $n = 5$; $P < 0.05$). The proportion of specific cell-associated radioactivity that is internalized after 60 min was the same in control (20.9 ± 1.1 %, $n = 5$) and treated (20.8 ± 1.1 %) cells, showing that the pertussis-toxin-induced decrease in insulin binding involves a decrease in insulin binding to the cell-surface receptor.

The influence of toxin treatment on insulin sensitivity was investigated by constructing dose–response curves for insulin stimulation of glucose transport after pretreatment with cholera toxin (1 μg/ml) and pertussis toxin (0.5 μg/ml) (Fig. 3). Neither basal nor maximally stimulated deoxyglucose transport rates differed significantly between treatment groups at these toxin concentrations. Thus final hormone responsiveness was the same in all groups. The results show that toxin-untreated cells are highly sensitive to insulin ($EC_{50} = 0.31 \pm 0.04$ ng/ml; $n = 7$). Cholera-toxin-treated cells behave similarly, but with slightly decreased sensitivity ($EC_{50} = 0.47 \pm 0.02$ ng/ml; $P < 0.05$; $n = 4$). Higher cholera-toxin concentrations had no further influence on sensitivity. The greatest difference is in pertussis-toxin-treated cells, with a large rightward shift in the dose–response curve ($EC_{50} = 2.29 \pm 1.0$ ng/ml; $P < 0.01$; $n = 7$), yet with normal maximal responsiveness.

It is not likely that the > 6-fold decrease in insulin sensitivity seen after pertussis-toxin (0.5 μg/ml) treatment is the result solely of the 50% decrease in insulin binding (Fig. 2). To test this idea directly, however, we measured binding and transport in the same experiments and plotted transport stimulation as a function of receptor occupancy (Fig. 4). For the studies summarized in Fig. 4, neither basal [control 0.18 ± 0.03 (nmol/3 min per 2×10^5 cells), toxin-treated 0.16 ± 0.02; $n = 5$] nor maximally stimulated (control 1.24 ± 0.16, toxin-treated 1.19 ± 0.16) 2-deoxyglucose transport rates were different after treatment. However, the curve for pertussis-toxin-treated cells was markedly shifted to the right ($EC_{50} = 1.57 \pm 0.17$ ng/ml; $n = 5$) of the control curve ($EC_{50} = 0.33 \pm 0.11$; $P < 0.01$; $n = 5$). In control cells insulin stimulation was maximal at a low receptor occupancy, ~ 10 pg/2×10^5 cells, compared with 70 pg/2×10^5 cells for toxin-treated cells. The receptor occupancy at 50% of maximal effect (defined as EB_{50}) was calculated as a measure of coupling efficiency. This value was 928 ± 101 ($n = 5$) molecules of insulin bound/cell in controls, compared with 3418 ± 530 ($n = 5$) in pertussis-toxin-treated cells ($P < 0.01$). Thus, at the concentration tested, pertussis-toxin treatment decreases insulin binding, owing to an affinity change (Fig. 2). Additionally, it further decreases by 3-fold the coupling efficiency of receptors that are occupied.

We have recently reported that treatment of adipocytes with adenosine deaminase to remove adenosine causes a 3-fold decrease in coupling efficiency [28], similar to that reported above. The possibility that pertussis toxin decreases sensitivity by perturbing A_1 adenosine receptors linked to G_i was tested by measuring dose–response curves in cells treated first with adenosine deaminase. In this series of paired experiments (Table 1), adenosine deaminase treatment resulted in a 2–3-fold shift in sensitivity, whereas pertussis toxin caused an 11-fold increase in the EC_{50}. There was a 4-fold decrease in receptor affinity after toxin treatment in these studies. The combination of treatments resulted in no further decrease in sensitivity (Table 1). Insulin binding was not

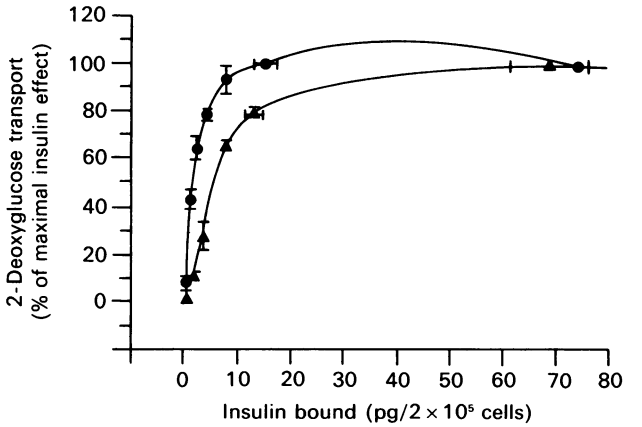

Fig. 4. Influence of pertussis-toxin (0.5 μg/ml) treatment (▲) on coupling efficiency of glucose transport stimulation

Treatment conditions are as described in Fig. 2 legend. (●) control cells. Results are expressed as the fraction of maximal insulin effect, for each group, obtained at each level of receptor occupancy. Binding and function were measured in the same study each time. Results are averages ±S.E.M. ($n = 5$).

Table 1. Effect of pertussis toxin (PT) and adenosine deaminase (ADA) treatment on insulin-sensitivity of glucose transport

Cells were treated as described before further incubation with various concentrations of insulin (60 min) and assay of 2-deoxyglucose transport. Results are means ± S.E.M. of three paired experiments. Differences between all values are statistically significant ($P < 0.05$), except for the PT versus ADA/PT comparison.

1st treatment	2nd treatment	EC_{50} (ng/ml)
None	None	0.38 ± 0.04
PT (0.5 μg/ml, 2 h)	None	4.64 ± 0.27
ADA (2 μg/ml, 2 h)	None	0.86 ± 0.08
ADA (10 min)	PT (2 h)	3.91 ± 0.41

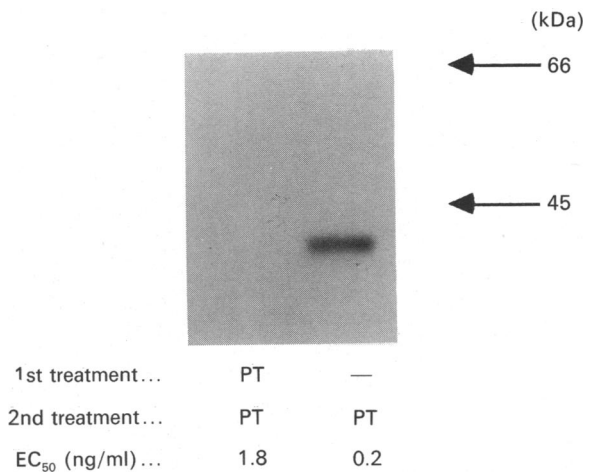

Fig. 5. Effect of toxin treatment of intact adipocytes on labelling of G-proteins in membranes

Cells were treated for 2 h ('1st treatment') with or without pertussis toxin (PT; 0.5 μg/ml). Plasma membranes were prepared and G-proteins labelled with [^{32}P]NAD$^+$ by using activated toxin ('2nd treatment'). Insulin-sensitivity of glucose transport was determined from full dose–response curves by using the same treated cells as for membrane preparation.

influenced by the combined treatment beyond the effect seen with toxin treatment alone.

Pertussis-toxin treatment has other effects on adipocytes, including elevation of cyclic AMP levels and increased lipolysis [10–13]. The possibility that the decrease in insulin sensitivity after pertussis-toxin treatment is the result of some factor released into the media, such as non-esterified fatty acids [29], and not to any direct actions of the toxin, was tested. Cells were treated for 3 h with pertussis toxin (0.5 μg/ml), and half the cells were washed three times before incubation with insulin. Absolute glucose transport rates were not different in treated and treated/washed cells (results not shown), and the EC_{50} values were similar (1.75 ± 0.15 ng/ml and 1.43 ± 0.12, treated and treated/washed respectively; not significant; $n = 4$), suggesting that the important change is cellular. In fact, sensitivity remained decreased in treated cells for at least 4 h after washing, even though the cells were still fully responsive to insulin.

Pertussis toxin is known to catalyse ADP-ribosylation of the α-subunit of susceptible G-proteins [6,30]. For G_i, this results in dissociation of the holoprotein and prevents coupling to adenosine and α_2-adrenergic receptors [1]. The functional result of such covalent modification is a loss of inhibitory control of adenylate cyclase [1,2]. That the pertussis-toxin treatment of intact adipocytes employed in these studies results in modification of a G-protein is supported by the data in Fig. 5, where a G-protein is identified by toxin-catalysed ADP-ribosylation of membranes prepared from the cells shown. Pertussis-toxin treatment of membranes causes ^{32}P-labelling of a 41 000 Da protein similar in size to the α-subunit of G_i. This protein can be fully labelled in control cells (no first treatment), which display normal insulin-sensitivity. Pertussis-toxin pretreatment of cells prevents further ribosylation of the 41 000 Da protein *in vitro*; this occurs along with a large decrease in insulin sensitivity. The identification of the 41 000 Da protein as the α-subunit of G_i is not absolute, as pertussis toxin can also label a 40 000 Da protein [31]. Whatever the identity of the protein(s) involved, modification of these proteins occurs together with a large increase in EC_{50} (Fig. 5).

In intact adipocytes, complete ribosylation of G_i requires several hours [7]. If a G-protein is involved in insulin signal transmission, then any effect on sensitivity might be expected to follow a similar time course. Such a slow onset of inhibition is seen in Fig. 6, where the time of pretreatment with pertussis toxin, before an additional incubation with insulin (60 min), is shown. Also shown is the transport rate after stimulation by a sub-maximal (0.8 ng/ml) insulin level. The inset displays the change in insulin sensitivity seen at 0.8 ng of insulin/ml. Co-incubation ($t = 0$) of cells with insulin and toxin had no effect on sensitivity. Pretreatment for 2–3 h is required for optimal inhibition of sensitivity by the toxin, stimulation by 0.8 ng of insulin/ml being decreased from 60 % of maximal effect to 8 %.

Although pertussis-toxin action is slow in onset and cannot alter insulin-sensitivity when added together with the hormone (Fig. 6), an interesting result was observed when the toxin treatment followed incubation with insulin. If the toxin was added after 60 min exposure to insulin and just before transport assay, then sensitivity was normal (Fig. 6). However, if toxin treatment continued for 2 h after insulin, allowing the toxin to complete its action, then sensitivity was greatly decreased ($EC_{50} = 2.9 \pm 0.4$ ng/ml; $n = 3$). This shift in the dose/response curve was as great as that seen in paired cells where toxin treatment preceded incubation with insulin ($EC_{50} = 2.3 \pm 0.3$ ng/ml; $n = 3$).

DISCUSSION

The mechanisms by which transmembrane signal transduction occur after insulin binding to its receptor are still open to conjecture. The receptor itself has been identified as a tyrosine-specific protein kinase [32–34], and much evidence has accumulated suggesting that activation of this kinase activity is the initial step in insulin receptor–effector coupling [32,33]. Attention has focused recently on the possibility that elements of the family of guanine nucleotide regulatory (G) proteins might also be involved in insulin action [3–5]. There are

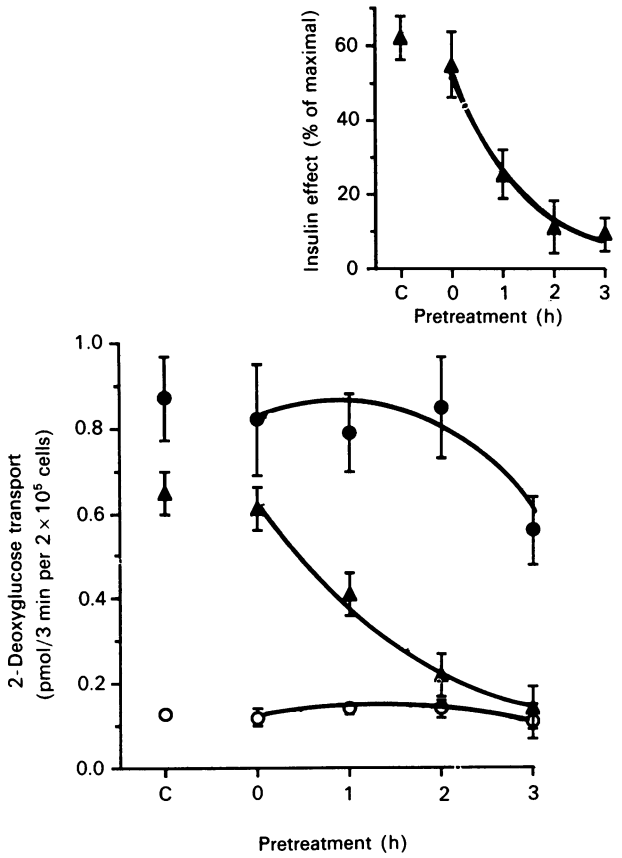

Fig. 6. Time course of onset of pertussis-toxin action on glucose transport and insulin-sensitivity

Cells [(2–3) × 10^5/ml] were incubated in the presence of pertussis toxin (0.5 ng/ml) for the indicated time before addition of insulin for an additional 60 min and subsequent assay of deoxyglucose transport. Control cells were incubated for 2 h before insulin exposure. Insulin concentrations used are 0 (○), 0.8 (▲) and 50 (●) ng/ml. Results are averages ±S.E.M. of 4–5 studies. C, Control untreated cells. Inset: Insulin-sensitivity. Results presented as percentage of maximal insulin effect, for each study, obtained in the presence of 0.8 ng of insulin/ml, added at the indicated times after toxin treatment.

several lines of evidence supporting this conjecture. For example, agents coupled to G_i are reported to increase glucose-transporter intrinsic activity [35] and insulin-sensitivity [30,36], whereas agents acting on G_s often inhibit insulin action on glucose transport and metabolism [16,35]. Bacterial toxins which covalently modify G-proteins can also alter insulin action. Cholera toxin can prevent insulin stimulation of the low-K_m cyclic AMP phosphodiesterase in liver [4]. Pertussis toxin stimulates lipolysis in adipocytes [7–10,37] and, in some hands, blunts the anti-lipolytic effects of insulin [7]. In other cases this insulin action is unimpaired [9,35]. In addition, changes in G-protein content often coincide with changes in insulin action. Decreasing G_i levels in rat adipocytes by culture with an adenosine agonist appears together with decreased insulin-sensitivity and, under certain conditions, a decrease in responsiveness of glucose transport [15]. Induction of insulin deficiency and insulin-resistance by streptozotocin injection also results in an impaired ability of G_i to mediate inhibition of adenylate cyclase in liver plasma membranes, as well as a lower G_i content [5]. Also, it has recently been reported that streptozotocin-induced diabetes results in a decrease in G_i in rat adipocytes [14]. This formulation is not absolute, however, as fasting [38] and hypothyroidism [39], both insulin-resistant states, have either normal or increased G_i levels.

The current findings extend and clarify our understanding of the possible involvement of G-proteins in insulin action. One conclusion to be drawn is that coupling mechanisms may vary from tissue to tissue, for, unlike the situation in liver [4], cholera-toxin treatment of adipocytes had little effect on the components of the stimulus–response system studied (Figs. 1–3). Further evidence of tissue differences in toxin effects was recently reported in cultured BC3H-1 muscle cells, where cholera toxin actually stimulated glucose transport [40].

Pertussis-toxin treatment, however, can influence all of the elements of this stimulus–response system. The decrease in insulin binding after toxin treatment has been established here not to be due to differences in hormone degradation, internalization or apparent receptor number, but rather to a large decrease in the overall affinity. In many proven G-protein-linked systems, G-protein modification by guanine-nucleotide binding or toxin treatment alters agonist binding to receptors through an affinity change [2,41]. A similar change in insulin-receptor affinity after pertussis-toxin treatment (Fig. 2) is suggestive of a relationship between the receptor and some species of G-protein. While glucose transport rates are altered by pertussis toxin (Fig. 1), treatment conditions can be selected so that transport remains unaltered (Fig. 3), even while insulin binding and sensitivity are decreased. One novel observation in this report is that the 6–7-fold change in sensitivity (measured as EC_{50}, Fig. 3) has two components. One is the decrease in binding affinity, and the other is the approx. 3-fold decrease in the coupling efficiency of occupied receptors (Fig. 4). The change in coupling efficiency is a common feature of adenosine deaminase and pertussis-toxin treatments ([26]; Table 1) and may be the result of action via a shared mechanism. However, the receptor affinity change appears to be a unique result of pertussis-toxin treatment.

Several factors suggest that pertussis-toxin action on insulin-sensitivity may be a result of covalent modification of a G-protein. The inhibition of sensitivity is slow in onset (Fig. 6) and occurs when further ribosylation of G_i *in vitro* is prevented by ribosylation *in vivo* (Fig. 5). A novel finding in the present paper is that if time is permitted for full toxin action and complete G_i modification, then the decrease in sensitivity is observed, even if insulin had been allowed to act first. This result suggests that insulin signalling is a continuous process, and possible modification of G_i can cause reversion of the transport system to a new steady-state activity.

Pertussis toxin is an oligomeric protein, and the B-oligomer moiety, which binds to the cell surface, can generate some biological effects independent of G-protein ribosylation [42]. There are several reasons why we consider that the B-oligomer action is not responsible for the reported effects on insulin binding and coupling efficiency. The effects reported here are maximal at a toxin concentration of 0.5 μg/ml, whereas B-oligomer levels of 20 μg/ml are needed to block toxin-stimulated glycerol release [42]. The B-oligomer also has insulin-

like effects to stimulate adipocyte glucose oxidation [42], which are maximal at concentrations 100-fold greater (40 μg/ml) than the levels of pertussis toxin which decrease the action of sub-maximal insulin concentrations. In our hands higher toxin concentrations decrease glucose transport (Fig. 1), the opposite of the B-oligomer effect.

Sensitivity of a biological response to pertussis or cholera toxin does not serve as direct proof that a G-protein is involved in the signal-transmission sequence for that response, and this caution must be kept in mind while drawing conclusions. However, this finding can be suggestive, and there are a number of situations where G-protein involvement initially indicated by pertussis-toxin sensitivity in intact cells has been confirmed by other criteria in more purified systems [1,6,43–50]. Reference has also been made in this report to G_i as the possible pertussis-toxin substrate involved in insulin stimulation of transport. This identification cannot be taken as absolute, as there are multiple G-proteins which are ribosylated by this toxin [29,49,50]. The appearance of a single 41000 Da band upon labelling by [^{32}P]NAD$^+$ ribosylation (Fig. 5) suggests that we are dealing with the α-subunit of G_i, but G-protein distribution varies from tissue to tissue [1,2,50], and other, novel, G-proteins cannot be excluded.

Although both cholera toxin and pertussis toxin cause large increases in cellular cyclic AMP, the inability of cholera toxin to influence insulin-sensitivity (Fig. 3) suggests that control of insulin coupling efficiency in adipocytes is largely independent of cyclic AMP. The influence of cholera toxin on adenylate cyclase and lipolysis has been extensively studied in adipocytes [44,51,52], but little is known about cholera-toxin effects on insulin action in these cells to compare with the work in liver [5] and muscle cells [40]. Adrenergic agents can regulate insulin sensitivity [12,36], and Lonnroth *et al.* [11] have shown that adenosine can influence this regulation in both cyclic-AMP-dependent and -independent ways. The current findings serve to support that conclusion. We and others [29,53] have shown that dibutyryl cyclic AMP and lipolytic agents decrease both insulin-sensitivity and receptor binding. However, these may be distinct events [29]. The comparison of results from cholera-toxin and pertussis-toxin treatment suggest that both the change in binding affinity and in coupling efficiency seen here are independent of cyclic AMP.

Keeping in mind the indirect nature of information obtained by using bacterial toxins [50], the current findings suggest several possible mechanisms for linkage between adenosine receptors, a purported G-protein and insulin stimulation of glucose transport. In one scheme, A_1 adenosine-receptor occupancy serves to maintain a high efficiency of coupling, and pertussis-toxin treatment could decrease coupling efficiency by modifying the G-protein which links the A_1 receptor to the insulin coupling mechanism. G-protein modification would have the additional effect of decreasing receptor affinity, which would also serve to lower insulin-sensitivity. We have also reported that the decrease in insulin-sensitivity seen after adenosine deaminase treatment involves the insulin receptor kinase and decreases in autophosphorylation in response to insulin [54]. It is possible that pertussis-toxin treatment could also be altering receptor kinase activity. In support of this supposition, it has been recently reported that pertussis-toxin treatment of rat hepatoma (Fao) cells results in a large decrease in insulin-stimulated receptor kinase activity toward both the receptor itself and exogenous substrates [55]. Yet in these cells toxin treatment did not alter insulin binding [55], another example of tissue variability of toxin effects. It has also been shown that, in phospholipid vesicles, G-proteins can increase insulin-induced autophosphorylation [56]. The insulin receptor kinase can also phosphorylate the α-subunits of G_i and G_o [57] in a soluble system. More recently, however, phosphorylation of G proteins in isolated hepatocytes was shown to be insulin-independent [58].

Attention in the present paper has focused on the relationship between bacterial toxins, G-proteins and insulin-sensitivity. However, as mentioned previously, pertussis toxin also has major effects on the adipocyte glucose transport system (Fig. 1). Several reports have suggested that lipolytic and anti-lipolytic compounds express a portion of their glucose-transport stimulatory action through changes in the intrinsic activity of glucose transporters present on the cell surface [35,59]. More recently, Obermaier-Kusser *et al.* [60] studied a number of agents, some of which stimulated transport by an increase in the activity of transporters pre-existing in the plasma membrane. The stimulatory effect of these compounds could be blocked in part with pertussis-toxin treatment, implicating a G-protein in control of glucose-transporter intrinsic activity. In the present studies, conditions were manipulated to dissociate toxin effects on transport and coupling (Figs. 3 and 4), and this could suggest the existence of multiple pertussis-toxin-sensitive G-proteins involved in both regulation of coupling efficiency and control of glucose transport activity.

In summary, there is a pertussis-toxin-sensitive protein(s) present in rat adipocytes which can regulate all the elements of the insulin stimulus–response sequence for glucose transport. The toxin acts to decrease both receptor binding affinity and the efficiency of the signalling process, but need not impair final responsiveness. The effects occur together with ribosylation of a 41000 kDa membrane protein, which may be involved in these effects. The results suggest that insulin might belong to the family of hormones whose signal transduction occurs in part via G-proteins.

We thank David Brady for technical assistance, Dr. Jerrold Olefsky for helpful review of the manuscript, and Elizabeth Martinez and Cleon Tate for their expert assistance in preparing the manuscript.

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Received 21 April 1989/24 July 1989; accepted 3 August 1989