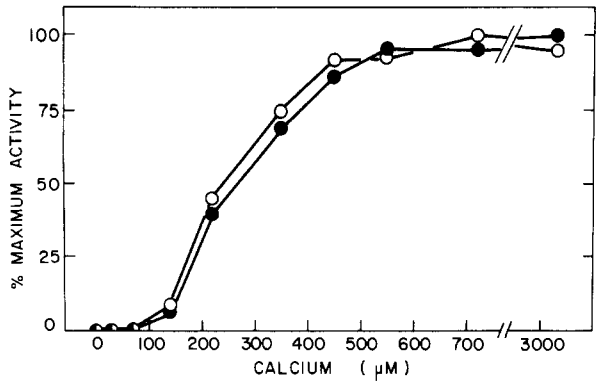

FIGURE 8: Effect of calcium concentration on the activity of calcium-dependent proteases. The calcium-dependent protease isolated either by reactive red agarose chromatography (●) or by reactive red agarose chromatography followed by casein–Sepharose affinity chromatography (○) as described in the text was assayed in the presence of the indicated concentrations of free calcium. For each protease, the activity was expressed as a percentage of the maximum activity.

transglutaminase (D. E. Croall and G. N. DeMartino, unpublished results).

Acknowledgments

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Registry No. Ca, 7440-70-2; proteinase, 9001-92-7; transglutaminase, 80146-85-6.

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Effects of Guanine Nucleotides on Cholera Toxin Catalyzed ADP-Ribosylation in Rat Adipocyte Plasma Membranes†

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ABSTRACT: ADP-ribosylation of rat adipocyte plasma membrane proteins was investigated following incubation of membranes with [α-^{32}P]NAD and cholera toxin in the presence and absence of various guanine nucleotides. In membranes incubated without guanine nucleotides, cholera toxin induced incorporation of ^{32}P into three discrete proteins of 48, 45, and 41 kDa. In membranes containing 100 μM GTP or GDP, toxin-catalyzed incorporation of ^{32}P into the 41-kDa protein was inhibited. GMP and Gpp(NH)p (100 μM) allowed moderate incorporation of ^{32}P into the 41-kDa protein. Toxin-catalyzed labeling of all proteins was rapid, reaching maximal levels between 5 and 10 min. Toxin-catalyzed ADP-ribosylation of the 48- and 45-kDa proteins was stimulated by GTP, reaching maximal levels at 10^{-5} M GTP. Inhibition of toxin-dependent labeling of the 41-kDa protein required GTP concentrations above 10^{-7} M with complete inhibition occurring between 10^{-5} and 10^{-4} M GTP. Cholera toxin catalyzed ADP-ribosylation was increased up to 2-fold in membranes supplemented with adipocyte cytosol. These results indicate that cholera toxin catalyzes ADP-ribosylation of three distinct adipocyte plasma membrane proteins, each of which is regulated by the amount and type of added guanine nucleotides.

Posttranslational covalent modification of proteins plays a pivotal role in the activation and inactivation of enzymes which act at key control points in the regulation of cellular metabolism. Although proteins undergoing reversible phosphorylation have been the most actively studied models in this area, bacterial exotoxin catalyzed modification of proteins has been the focus of a number of recent publications (Vaughan & Moss, 1981; Ueda et al., 1982). Cholera toxin, an enterotoxin of *Vibrio cholerae*, catalyzes the transfer of ADP-ribose from NAD to a variety of proteins and peptides (Gill, 1982). Most

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notably, cholera toxin catalyzes the ADP-ribosylation of guanyl nucleotide binding regulatory proteins which leads to inhibition of GTPase activity with concomitant activation of adenylate cyclase activity (Moss & Vaughan, 1979). Cholera toxin has also been observed to catalyze ADP-ribosylation of cytoskeletal elements and several unidentified proteins which do not appear to influence adenylate cyclase activity (Hawkins & Browning, 1982; Abood et al., 1982; Gill, 1979). Moreover, in addition to participation in the hormonal regulation of adenylate cyclase activity, GTP-regulatory proteins also play essential roles in a variety of biological processes. In view of the facts delineated above, it has been proposed that cholera toxin catalyzed covalent modification of GTP-regulatory proteins modulates cellular metabolism by regulating guanine nucleotide metabolism and, ultimately, by controlling the interaction of these proteins with cell membrane receptors and hormone- and neurotransmitter-sensitive enzymes (Rodbell, 1980). Thus, the ability of cholera toxin to induce the incorporation of ^{32}P into proteins present in membranes incubated with [α-^{32}P]-NAD[1] has provided a convenient means for identifying GTP-regulatory proteins and has greatly facilitated efforts designed to explore the molecular events involved in membrane signal transduction.

Adipocytes represent excellent cells in which to investigate cholera toxin catalyzed ADP-ribosylation because of their exquisite sensitivity to hormones and a variety of exogenous stimuli. Evidence that GTP-regulatory proteins play key roles in the metabolic regulation of this system stems, in part, from the regulatory properties ascribed to its adenylate cyclase. The adipocyte adenylate cyclase is both stimulated and inhibited by GTP (Yamamura et al., 1977; Cooper et al., 1979). This dual regulation is apparently mediated by two functionally distinct guanine nucleotide binding proteins (Rodbell, 1980).

As a first step in understanding the role of ADP-ribosylation in the metabolic regulation of adipocytes, we have characterized cholera toxin catalyzed ADP-ribosylation in isolated adipocyte membranes. We show here that plasma membranes labeled with [α-^{32}P]NAD in the absence of guanine nucleotides are characterized by three radiolabeled proteins of 48, 45, and 41 kDa. On the other hand, cholera toxin induced incorporation of ^{32}P into these proteins is markedly altered in preparations containing guanine nucleotides. Most interestingly, we report that ADP-ribosylation of the 41-kDa protein is inhibited by GTP.

Experimental Procedures

Materials. Cholera toxin was purchased from Sigma, adjusted to contain 20 mM dithiothreitol and incubated for 10 min at 30 °C immediately prior to use. [α-^{32}P]NAD^+ was obtained from New England Nuclear or Amersham Corp., ATP, GTP, GDP, GMP, and thymidine were from Sigma, and Gpp(NH)p was from Boehringer Mannheim. Adipocyte plasma membranes, microsomes (enriched in endoplasmic reticulum), and cytosol were prepared from isolated rat adipocytes (Rodbell et al., 1964) by a modification (Jarett, 1974) of the procedure described by McKeel & Jarett (1970).

Assay for Cholera Toxin Catalyzed ADP-Ribosylation. Unless stated otherwise, radiolabeling of membranes with [α-^{32}P]NAD^+ was conducted in the presence of 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.4) containing 2.5 mM $MgCl_2$, 10 mM thymidine, 1 mM ATP, 300 μM EDTA, 100 μg/mL cholera toxin, and 10 μM [α-^{32}P]NAD^+ (40–80 μCi/mL). Some incubations also contained 100 μM GTP. The reaction mixture described above was incubated at 30 °C for 2 min; experiments were initiated by addition of adipocyte plasma membranes (100–125 μg of protein) and the reaction mixture was incubated an additional 5 min in a final volume of 250 μL. Experiments in which assay mixtures were preincubated with membranes in the absence of NAD and in which reactions were initiated by addition of [α-^{32}P]NAD resulted in identical patterns of radiolabeled proteins. Reactions were terminated by addition of 1.0 mL of ice-cold Tris-HCl (20 mM, pH 7.4), and membranes were collected by microcentrifugation. Membrane pellets were solubilized in 20 mM Tris-HCl (pH 7.4) containing 5% w/v SDS and 5% v/v β-mercaptoethanol. Samples were electrophoresed in 7.5% SDS–polyacrylamide gels (Laemmli, 1970). The gels were stained with Coomassie blue, destained, dried on filter paper, and exposed to Kodak XAR-5 X-ray film. The quantity of ^{32}P incorporated into membrane proteins was determined by analyzing autoradiograms in an LKB 2202 Ultroscan laser densitometer. Protein content was determined by the method of Lowry et al. (1951). Further details of these procedures are described in the figure legends.

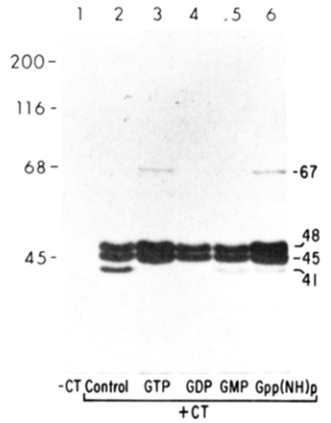

FIGURE 1: Effect of guanine nucleotides on cholera toxin catalyzed ADP-ribosylation of adipocyte plasma membrane proteins. Representative autoradiographic profiles of ^{32}P-labeled proteins present in membranes incubated with [α-^{32}P]NAD according to the conditions described under Experimental Procedures. Lane 1 shows membranes labeled with [α-^{32}P]NAD in the absence of cholera toxin. Lanes 2–6 represent membranes incubated with cholera toxin (CT) in the absence of guanine nucleotides (lane 2) and presence of GTP (lane 3), GDP (lane 4), GMP (lane 5), and Gpp(NH)p (lane 6). Final concentrations of added guanine nucleotides were 100 μM. Migration of molecular mass standards expressed in kilodaltons is designated.

Results

Effect of Guanine Nucleotides on Cholera Toxin Catalyzed ADP-Ribosylation in Rat Adipocyte Plasma Membranes. Cholera toxin catalyzes the transfer of ^{32}P from [α-^{32}P]NAD to several discrete proteins present in isolated rat adipocyte plasma membranes. Autoradiographic profiles of radiolabeled membrane proteins following incubation of rat adipocyte plasma membranes with [α-^{32}P]NAD in the presence of cholera toxin and various guanine nucleotides are shown in Figure 1. Membranes labeled in the absence of cholera toxin revealed negligible incorporation of ^{32}P into proteins (lane 1). In the absence of guanine nucleotides, cholera toxin catalyzed ADP-ribosylation of three discrete proteins possessing molecular mass values of 48, 45, and 41 kDa (lane 2). Following incubation of membranes with GTP, cholera toxin treatment

[1] Abbreviations: NAD, β-nicotinamide adenine dinucleotide; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GMP, guanosine 5′-monophosphate; Gpp(NH)p, guanosine 5′-(β,γ-iminotriphosphate); kDa, kilodaltons; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; SDS, sodium dodecyl sulfate.

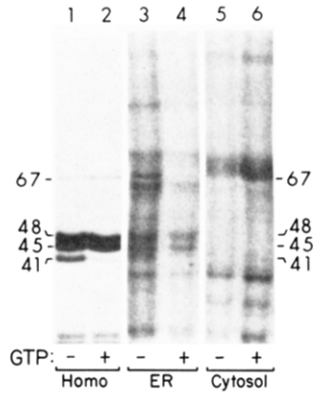

FIGURE 2: Cholera toxin catalyzed ADP-ribosylation in adipocyte homogenates, endoplasmic reticulum, and cytosol. Representative autoradiographic profiles of ^{32}P-labeled proteins present in adipocyte subcellular preparations following incubation with cholera toxin and [α-^{32}P]NAD in the absence (–) and presence (+) of GTP (100 μM). Assays were conducted in accordance with the procedure described for plasma membranes under Experimental Procedures. Lanes 1 and 2 represent homogenates (Homo), lanes 3 and 4 represent endoplasmic reticulum (ER), and lanes 5 and 6 represent cytosol. Final protein concentrations were 0.5 mg/mL. Migration of the 67-, 48-, 45-, and 41-kDa proteins which undergo cholera toxin dependent radiolabeling in plasma membranes (Figure 1) is designated.

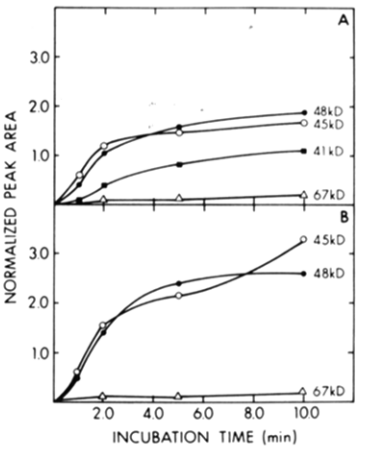

FIGURE 3: Effect of incubation time on cholera toxin catalyzed ADP-ribosylation in adipocyte plasma membranes. Autoradiographs of ^{32}P-labeled proteins present in membranes incubated with cholera toxin and [α-^{32}P]NAD for varying time intervals were analyzed by laser densitometry. Absorbance signals corresponding to radiolabeled proteins on autoradiographic film were converted to arbitrary units (normalized peak area) through the use of chart recording and automatic digital printout of integrated values (LKB 2220 Recording Integrator). Panel A represents membranes labeled in the absence of GTP. Membranes incubated with GTP (100 μM) are depicted in panel B.

was characterized by the presence of the ^{32}P-labeled 48- and 45-kDa proteins as well as a minor species of 67 kDa. Under these conditions, however, incorporation of radioactivity into the 41-kDa protein was barely detectable.

The pattern of ^{32}P-labeled proteins is clearly altered by the type of guanine nucleotide added. Addition of GDP virtually abolished the ability of cholera toxin to induce ^{32}P incorporation into the 41-kDa protein (lane 4). ADP-ribosylation of the 41-kDa protein was, however, permitted in preparations incubated with GMP (lane 5). Radiolabeling of the 48- and 45-kDa proteins in membranes containing Gpp(NH)p was strikingly similar to that observed in GTP-supplemented preparations (compare lanes 3 and 6). On the other hand, cholera toxin catalyzed incorporation of ^{32}P into the 41-kDa protein in the presence of Gpp(NH)p was highly comparable to that noted in GMP-containing preparations (compare lanes 5 and 6).

The autoradiographic profiles of ^{32}P-labeled proteins illustrated in Figure 1 are highly reproducible. It is interesting to note that these proteins probably represent an extremely small percentage of the total plasma membrane protein pool since none of the labeled species were observed to comigrate with readily discernible Coomassie-stained membrane proteins (results not shown).

Evidence that the radiolabeling of these proteins is attributable to ADP-ribosylation was provided by (i) the ability of snake venome phosphodiesterase to quantitatively remove ^{32}P from all the proteins (results not shown) and (ii) the absence of radiolabeled proteins corresponding to those labeled in membranes incubated with [α-^{32}P]NAD following cholera toxin treatment of preparations incubated with unlabeled NAD and [γ-^{32}P]- or [α-^{32}P]ATP (results not shown). Nonenzymatic transfer of ADP-ribose to the proteins seems unlikely since membranes incubated with *N. crassa* NADase in lieu of cholera toxin did not result in incorporation of ^{32}P into any of the proteins depicted in Figure 1 (results no shown).

The radiolabeled proteins illustrated in Figure 1 are located in the plasma membrane. This was established by comparing cholera toxin catalyzed radiolabeling of proteins in adipocyte homogenates, microsomes (enriched in endoplasmic reticulum), and cytosol under identical conditions (Figure 2). Cholera toxin did not induce appreciable incorporation of ^{32}P into the proteins present in microsomes (lanes 3 and 4) and cytosol (lanes 5 and 6) corresponding to those illustrated in Figure 1. The pattern of ADP-ribosylated proteins in homogenates was, however, virtually identical with that observed in plasma membranes.

Further Characterization of Cholera Toxin Catalyzed ADP-Ribosylation in Rat Adipocyte Plasma Membranes. Cholera toxin catalyzed ADP-ribosylation of adipocyte plasma membrane is rapid and reaches steady-state levels in a short time frame (i.e., 5–10 min). Membranes were incubated with cholera toxin and [α-^{32}P]NAD in the absence (Figure 3A) and presence (Figure 3B) of GTP for varying periods of time. The quantity of ^{32}P incorporated into the 48-, 45-, and 41-kDa proteins was determined by densitometric analysis of autoradiographic profiles. As shown in Figure 3, cholera toxin catalyzed a time-dependent incorporation of ^{32}P into the 48-, 45-, and 41-kDa proteins in the absence of GTP reaching plateau levels between 5 and 10 min. Labeling of the 67-kDa protein remained fairly constant throughout the 10-min time frame but represented only a small percentage (1.5–3.5%) of the total radioactivity incorporated at each time point. Cholera toxin dependent incorporation of ^{32}P into the 41-kDa protein only occurred in membranes labeled in the absence of GTP and was approximately 30% less than that of the ^{32}P incorporated into either the 48- or 45-kDa proteins at 10 min.

Radiolabeling of adipocyte plasma membrane proteins was dependent upon increasing concentrations of cholera toxin. (results not shown). Cholera toxin catalyzed labeling of the 48-, 45-, and 41-kDa proteins was maximal at and above 100 μg/mL concentrations of the toxin under GTP-deficient conditions. In GTP-supplemented membranes, labeling of the 48- and 45-kDa proteins also increased as a function of cholera toxin concentration, reaching apparent plateau levels between 100 and 200 μg/mL concentrations. Radiolabeling of the 67-kDa protein was virtually identical in both GTP-deficient and -supplemented membranes, showing a small yet discernible dependence on toxin concentration.

Role of GTP in Cholera Toxin Catalyzed ADP-Ribosylation in Adipocyte Plasma Membranes. As illustrated

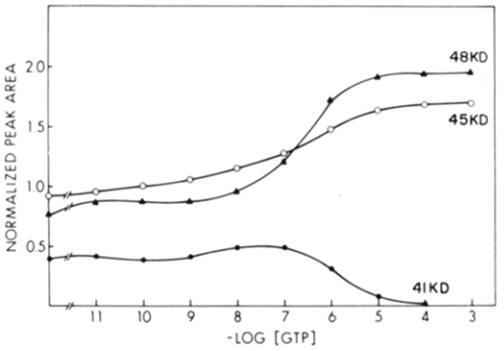

FIGURE 4: Effect of GTP concentration on cholera toxin catalyzed ADP-ribosylation in adipocyte plasma membranes. Plasma membranes were incubated with cholera toxin and [α-^{32}P]NAD in the presence of the GTP concentrations indicated in the abscissa. The quantity of ^{32}P incorporated into the 48- (▲), 45- (○), and 41-kDa (●) proteins were determined by densitometry as described in Figure 3.

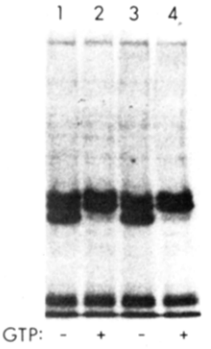

FIGURE 5: Effect of assay conditions on cholera toxin catalyzed ADP-ribosylation in adipocyte plasma membranes. Plasma membranes were incubated with [α-^{32}P]NAD and cholera toxin for 15 min as described under Experimental Procedures (lanes 1 and 2) and by Cooper et al. (1981) (lanes 3 and 4) in the absence (−) and presence (+) of 70 μM GTP. The assay method of Cooper et al. (1981) was conducted in a final volume of 250 μL containing cholera toxin (100 μg/mL), [α-^{32}P]NAD (25 μM), dithiothreitol (5 mM), thymidine (1 mM), ADP-ribose (1 mM), and arginine (1 mM) in 10 mM phosphate buffer (pH 7.4). All reactions were initiated by addition of membranes prepared according to Jarett (1974). Assays were terminated and samples processed as described under Experimental Procedures.

in Figure 3, cholera toxin induced incorporation of ^{32}P into proteins was markedly altered in membranes supplemented with GTP (100 μM): (i) radiolabeling of the 48- and 45-kDa proteins was enhanced, and (ii) radiolabeling of the 41-kDa protein was inhibited. In an effort to further characterize these properties, cholera toxin catalyzed ADP-ribosylation was investigated in membranes incubated in the presence of a wide range of GTP concentrations. As shown in Figure 4, the quantity of ^{32}P incorporated into the 41-kDa protein in membranes incubated without GTP was similar to that observed in membranes supplemented with GTP at concentrations between 10^{-11} and 10^{-7} M. At GTP concentrations above 10^{-7} M, toxin-dependent labeling of the 41-kDa protein declined markedly and was totally inhibited at concentrations exceeding 10^{-4} M GTP. Toxin-catalyzed labeling of the 48- and 45-kDa proteins exhibited a similar dependence on, but an opposing response to, increasing concentrations of GTP. Incorporation of ^{32}P into these proteins remained fairly constant at GTP concentrations below 10^{-8} M. Radiolabeling of the 45- and 48-kDa proteins increased steadily above 10^{-8} M GTP, reaching plateau levels between 10^{-4} and 10^{-5} M GTP. Toxin-catalyzed ADP-ribosylation of the 48- and 45-kDa proteins at 10^{-4} M GTP was increased 2.5- and 1.8-fold, respectively, over that observed in membranes incubated without GTP. These results indicate that the stimulation of ^{32}P incorporation into the 48- and 45-kDa proteins and inhibition of radiolabeling of the 41-kDa protein by GTP are concentration dependent. With respect to the 41-kDa protein in particular, these data and the results described in the previous section demonstrate that the inhibitory effects of GTP are overcome by decreasing added concentrations of GTP but not by increasing incubation time and cholera toxin concentration.

There are several criteria which suggest that the 45- and 48-kDa proteins represent guanine regulatory subunits of the hormone-sensitive adenylate cyclase of adipocyte tissue: (i) they are membrane proteins possessing molecular weights consistent with those reported for guanyl nucleotide binding regulatory proteins in other systems (Gill, 1982; Cooper et al., 1979), (ii) ADP-ribosylation of these polypeptides is cholera toxin dependent, and (iii) cholera toxin catalyzed radiolabeling of the proteins is stimulated by GTP. Although the 41-kDa protein exhibits the properties described in (i) and (ii), our results indicate that GTP inhibits toxin-dependent ADP-ribosylation of this polypeptide in adipocyte plasma membranes. While the basis of this apparent anomaly and the relationship of these proteins to adenylate cyclase are presently unknown, it is noteworthy that this observation opposes the findings reported by other investigators. The results of Malbon & Gill (1979) and Cooper et al. (1981) have indicated that a 42-kDa protein represents the major substrate for cholera toxin catalyzed ADP-ribosylation in fat cell membranes containing GTP. In an effort to further explore this apparent discrepancy, we conducted assays in membranes according to the procedure described by Cooper et al. (1981). In contrast to our protocol, the method of Cooper et al. (1981) included ADP-ribose and arginine in phosphate-buffered solution in which ATP, $MgCl_2$, and EDTA were absent. As shown in Figure 5, resultant patterns of radiolabeled proteins were virtually identical in both assay systems. Thus, the ability of GTP to inhibit toxin-induced incorporation of ^{32}P into the 41-kDa protein was not altered when assays were conducted in the reaction mixture described by Cooper et al. (1981).

It is, however, important to point out that the results of Cooper et al. (1981) were obtained by using membranes prepared according to the procedure outlined by Avruch et al. (1971). The experiments depicted in Figure 5 were conducted with membranes prepared by the protocol described by Jarett (1974). Furthermore, numerous studies have demonstrated that cholera toxin catalyzed ADP-ribosylation in membranes containing optimal quantities of NAD and GTP is enhanced by a cytosolic factor (Gill, 1982; Enomoto & Gill, 1979, 1980). Although this cytosolic factor has not been definitively characterized, it appears that the factor is ubiquitous in vertebrate cells and significantly reduces the concentration of toxin required to produce a given effect. These collective properties raised questions concerning the potential role of cytosolic components in the mechanism by which GTP alters toxin-dependent labeling of proteins in isolated adipocyte plasma membranes. Cholera toxin catalyzed ADP-ribosylation was therefore investigated in membranes supplemented with dialyzed and undialyzed preparations of adipocyte cytosol. As shown in Figure 6, incubation with cytosol did not result in labeling of the 41-kDa protein in membranes containing GTP. Thus, the inhibitory effects of GTP observed in our studies do not appear to be attributable to the absence of a regulatory element of cytosolic origin.

Incubation of membranes with cytosolic fractions did, however, enhance the ability of cholera toxin to induce the incorporation of ^{32}P into the 48-, 45-, and 41-kDa proteins.

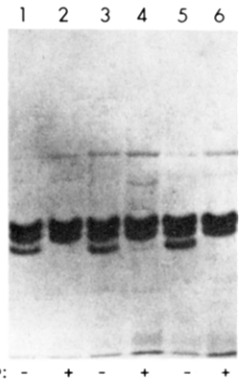

FIGURE 6: Effect of cytosol on cholera toxin catalyzed ADP-ribosylation in adipocyte plasma membranes. Cholera toxin dependent incorporation of ^{32}P into adipocyte plasma membrane proteins was investigated in preparations supplemented with adipocyte cytosol in the absence (−) and presence (+) of 100 μM GTP. Cytosol was adjusted to contain protein concentrations of 1 mg/mL. Radiolabeling of membrane proteins and gel autoradiography were conducted as described under Experimental Procedures. Lanes 1 and 2 represent membranes labeled with ^{32}P in the absence of cytosol. Lanes 3 and 4 represent membranes labeled in the presence of 50 μL of cytosol. Lanes 5 and 6 represent membranes labeled in the presence of 50 μL of cytosol previously dialyzed against 150 mM NaCl and 10 mM phosphate, pH 7.4, in dialysis tubing with a 12000–14000 molecular weight cutoff.

Table I: Effect of Cytosol on Cholera Toxin Dependent Incorporation of ^{32}P into Adipocyte Plasma Membrane Proteins

components added[b]	^{32}P-labeled protein[a]		
	41 kDa	45 kDa	48 kDa
none	0.29	0.39	0.67
GTP	ND[c]	0.61 (1.56)[d]	1.15 (1.72)
cytosol (50 μL)	0.33 (1.14)	0.53 (1.36)	1.02 (1.52)
cytosol (50 μL) + GTP	ND	0.72 (1.85)	1.47 (2.19)
dialyzed cytosol (50 μL)	0.39 (1.34)	0.51 (1.31)	0.99 (1.47)
dialyzed cytosol (50 μL) + GTP	ND	0.83 (2.13)	1.59 (2.37)
cytosol (100 μL)	0.35 (1.21)	0.65 (1.67)	1.08 (1.61)
cytosol (100 μL) + GTP	ND	0.81 (2.07)	1.62 (2.41)
dialyzed cytosol (100 μL)	0.59 (2.03)	0.66 (1.69)	1.27 (1.89)
dialyzed cytosol (100 μL) + GTP	ND	0.72 (1.85)	1.47 (2.19)

[a] ^{32}P incorporation was determined following densitometric analysis of autoradiographs as described in the legend to Figure 3. [b] Experiments were conducted as described in the legend to Figure 6. [c] ND indicates that radioactivity was not detectable. [d] Fold increases over control (no additions) are shown in parentheses.

As shown in Table I, addition of cytosol resulted in up to 2-fold increases in the quantity of ^{32}P incorporated into the three proteins in membranes incubated without GTP. The stimulatory properties of cytosol was most prominent in preparations containing GTP as toxin-dependent labeling of the 48- and 45-kDa proteins was increased between 1.8- and 2.4-fold. The ability of cytosol to increase toxin-catalyzed ADP-ribosylation was not appreciably altered following dialysis. These data indicate that a nondialyzable factor in adipocyte cytosol accelerates toxin-induced ^{32}P incorporation into adipocyte plasma membranes. Although the putative factor enhances GTP-mediated increases in radiolabeling of the 48- and 45-kDa proteins, it does not appear to participate in the regulatory mechanism by which GTP inhibits ADP-ribosylation of the 41-kDa protein.

Discussion

Numerous studies have demonstrated that cholera toxin catalyzed ADP-ribosylation results in inhibition of the GTPase activity which is associated with hormonal activation of adenylate cyclase (Gill, 1982; Rodbell, 1980). Inhibition of GTP hydrolysis prevents the cyclase from being converted to an inactive form, thereby maintaining the enzyme in an activated state (Gill & Meren, 1978; Cassel & Pfeuffer, 1978; Cassel & Selinger, 1977). Cholera toxin dependent ADP-ribosylation of the cyclase guanyl nucleotide regulatory protein (G/F) is stimulated by GTP (Watkins et al., 1981; Enomoto & Gill, 1980). The results of the studies presented here demonstrate that cholera toxin induces the incorporation of ^{32}P into several discrete proteins present in rat adipocyte plasma membranes incubated with [α-^{32}P]NAD. While the molecular weights of these proteins closely approximate those ascribed to G/F subunit proteins in other systems, their toxin-catalyzed ADP-ribosylation in response to guanine nucleotides was different than that reported for the G/F protein in the adenylate cyclase system.

Our findings indicate the GTP inhibits toxin-dependent radiolabeling of a 41-kDa protein in fat cell membranes. This inhibition appears to be partially attributable to hydrolysis of GTP to GDP since studies conducted with the nonhydrolyzable GTP analogue, Gpp(NH)p, resulted in an attenuation of this effect. Additional experiments revealed that GDP also prevented toxin-catalyzed incorporation of ^{32}P into the 41-kDa protein, while in those membranes supplemented with GMP, labeling of the 41-kDa protein was permitted. It has been proposed that the catecholamine-dependent stimulation of adenylate cyclase is promoted by dissociation of bound GDP which allows subsequent binding of GTP and GTP-like nucleotides (Cassel & Selinger, 1978; Abramowitz et al., 1980). In view of the ability of GMP to induce the dissociation of protein-bound GDP and Gpp(NH)p to inhibit endogenous production of GDP, it seems plausible that the 41-kDa protein may contain bound GDP and that removal of GDP is limiting in the toxin-dependent ADP-ribosylation of the protein. Although the mechanism by which guanine nucleotides alter toxin-catalyzed labeling of the 41-kDa protein remains unknown, it appears likely that the effects are mediated at the substrate rather than the enzyme level since a mechanism imposed upon the latter would be of a general nature and not one limited to the 41-kDa protein.

Although GTP has been observed to enhance toxin-catalyzed ADP-ribosylation of the G/F protein in the adenylate cyclase system, the inhibitory effect of the nucleotide described in our investigation is not without precedence. Abood et al. (1982) observed that cholera toxin catalyzed ADP-ribosylation of transducin, a 41-kDa guanine nucleotide binding regulatory protein of the rod outer segment (ROS), is inhibited when ROS membranes are labeled in the presence of GTP. Watkins et al. (1980) demonstrated that choleragen induces incorporation of ^{32}P into a wide variety of proteins and that the effects of GTP on the process varied widely depending on the nature of the substrate and its milieu (i.e., soluble, particulate). Together these findings indicate that cholera toxin catalyzes the covalent modification of numerous GTP regulatory proteins and suggest that ADP-ribosylation appears to be regulated by the interaction of these proteins with guanine nucleotides.

In conclusion, these studies demonstrate that the proteins which serve as substrates for cholera toxin catalyzed ADP-ribosylation in fat cell membranes are significantly influenced by the type of exogenously added guanine nucleotide. Radiolabeling of a 41-kDa protein was inhibited in membranes

supplemented with GTP and GDP but permitted in guanine nucleotide deficient and Gpp(NH)p and GMP containing preparations. Toxin-induced incorporation of ^{32}P into proteins of 48 and 45 kDa was enhanced in the presence of GTP. The relationship of these proteins with the G/F component of adenylate cyclase is presently unknown. The adipocyte cyclase system is subjected to a dual regulatory mechanism in which GTP evokes both inhibitory and activatory responses via interaction with distinct components. It is significant to note that systems subject to dual regulation are characterized by multiple toxin-dependent ADP-ribosylated proteins while cholergen treatment of plasma membranes from systems in which GTP evokes stimulation alone results in labeling of a 42-kDa protein (Cooper et al., 1981). It will be of interest to determine if the effect of GTP and the multiple ADP-ribosylated proteins observed in these studies are associated with inhibitory actions of cholera toxin and GTP on adenylate cyclase activity.

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