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SELECTIVE REGULATION BY PERTUSSIS TOXIN OF INSULIN-INDUCED ACTIVATION OF PARTICULATE cAMP PHOSPHODIESTERASE ACTIVITY IN 3T3-L1 ADIPOCYTES

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Incubation of 3T3-L1 adipocytes with insulin or isoproterenol for 10 min increased particulate "low K_m" cAMP phosphodiesterase activity by 42% and 50%, respectively. Pertussis toxin catalyzed the [^{32}P]-ADP ribosylation of a 41,000 dalton protein in adipocyte particulate fractions; prior incubation of adipocytes with toxin markedly reduced incorporation of radiolabel. Exposure of adipocytes to pertussis toxin (0.3 µg, 18 hr) increased glycerol production and inhibited activation of cAMP phosphodiesterase by insulin, but not by isoproterenol. These results suggest that pertussis toxin can interfere with receptor-mediated processes that stimulate cAMP hydrolysis as well as those that inhibit cAMP formation.

Both insulin and lipolytic hormones cause a rapid increase in particulate "low K_m" cAMP phosphodiesterase activity of rat (1,2,3) and 3T3-L1 adipocytes (4). A recent report suggests a role for guanyl nucleotides in regulating the activity of a rat liver plasma membrane phosphodiesterase that is activated by insulin (5). In retinal rod outer segments, light-induced activation of a cGMP phosphodiesterase is mediated by transducin, a GTP-binding protein that is similar to the stimulatory and inhibitory guanyl nucleotide-binding proteins of the adenylate cyclase system (6). By catalyzing the transfer of ADP-ribose from NAD to the 41,000 dalton subunit of the inhibitory GTP-binding protein (7,8,9), pertussis toxin (PT) apparently alters the affinity of inhibitory receptors (10), inhibits GTP hydrolysis (11), and decreases GTP-dependent inhibition of adenylate cyclase (7). In this way, PT enhances cellular responses to agents that increase cAMP

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formation and reduces responses to inhibitory effectors (12). In fat cells, the PT-induced loss of inhibitory responses results in activation of lipolysis (13). We have now found that in 3T3-L1 adipocytes, PT selectively inhibits the insulin-induced increase in particulate "low K_m" cAMP phosphodiesterase activity.

METHODS

3T3-L1 fibroblasts were grown and induced to differentiate as described (4). Differentiated adipocytes in 6-well plates (Costar, each well 35 mm) were incubated for 16 hr with 2 ml of medium containing 0.3 µg pertussis toxin or vehicle (1 µl of 50% glycerol or normal saline). One hr before termination of the incubation, medium was replaced with 2 ml of Hanks' medium containing bovine serum albumin, 10 mg/ml. For the final 10 min, diluent (H_2O), insulin, or isoproterenol was added. At the end of the incubation, medium was removed and frozen until glycerol content was assayed (14). Cells were washed with cold phosphate-buffered saline, scraped, and homogenized. Supernatant and particulate fractions were separated and assayed for protein content and phosphodiesterase activity as described (4). Hormone-sensitive particulate phosphodiesterase was assayed with 0.5 µM (^{3}H)cAMP as substrate (4).

For experiments like that in Fig. 1, adipocytes (in 10 cm tissue culture dishes) were incubated with PT (0.575 µg/dish) or vehicle for 16 hr, then collected in phosphate-buffered saline. Cells, pelleted by centrifugation, were homogenized in 1 ml of 20 mM Tris HCl, pH 8.0. Homogenates were centrifuged (20,000 g, 20 min) and particulate fractions suspended in 0.5 ml of Tris buffer (10.3 mg protein/ml). Pertussis toxin was activated by incubation (10 min, 30°) in 50 mM glycine (pH 8.0), with 20 mM dithiothreitol and ovalbumin, 1 mg/ml. Samples of particulate fractions (25 µl) were incubated for 60 min at 30° with PT (0.575 µg) or vehicle in a total volume of 100 µl containing 50 mM potassium phosphate buffer (pH 7.5), 20 mM thymidine, 3 mM GTP, 0.5 mM ATP, and 20 mM [^{32}P]-NAD (~ 1 µCi/assay). After addition of 1.5 ml of cold 10% trichloroacetic acid and centrifugation, precipitated proteins were solubilized in 1% sodium dodecylsulfate/50 mM dithiothreitol. Electrophoresis (in 10% polyacrylamide gels) and autoradiography were performed as described (15).

RESULTS

In particulate fractions from 3T3-L1 adipocytes, PT catalyzed the transfer of [^{32}P]ADP-ribose from [^{32}P]NAD to a protein of 41,000 daltons. Labelling of this protein was markedly reduced in fractions from adipocytes previously incubated with PT (Fig. 1) presumably because it had been already ADP-ribosylated during exposure of the cells to toxin.

Glycerol production was increased 60 to 200% after incubation of adipocytes for 18 hr with PT (Table 1). Similar effects on glycerol production by rat fat cells have been reported (16). Equivalent amounts of glycerol were produced in 1 hr by PT-treated cells and by control cells

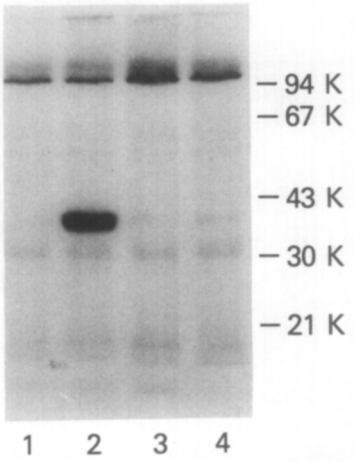

Figure 1. Analysis by autoradiography of in vitro ADP-ribosylation of membrane proteins from 3T3-L1 adipocytes incubated with and without pertussis toxin.
Adipocytes were incubated for 16 hr without (lanes 1 and 2) or with PT (lanes 3 and 4). Membrane fractions were prepared, incubated with [^{32}P]NAD without (lanes 1 and 3) or with PT (lanes 2 and 4), and subjected to electrophoresis in SDS and autoradiography as described in Methods.

incubated with a maximally effective concentration of isoproterenol (1 μM) (data not shown).

Incubation of adipocytes for 10 min with 1 nM insulin or 1 μM isoproterenol increased particulate cAMP phosphodiesterase activity by 42 ±

TABLE 1
Effect of Pertussis Toxin on Glycerol Production and Hormone-sensitive Particulate cAMP Phosphodiesterase Activity

Exp No.	Additions Conc.	Glycerol Production (nmol/well/hr) No PT	Plus PT	Phosphodiesterase activity (% of control) No PT	Plus PT
1	None	156 ± 26	261 ± 52	100 ± 9	115 ± 7
	Insulin, 1 nM	-------	------	154 ± 9	127 ± 3
	Isoproterenol, 1 μM	-------	------	161 ± 10	170 ± 10
2	None	88 ± 7	300 ± 41	100 ± 3	91 ± 9
	Insulin, 1 nM	------	------	139 ± 13	99 ± 4
	Isoproterenol, 1 μM	------	------	145 ± 9	131 ± 9
3	None	120 ± 30	218 ± 26	100 ± 4	97 ± 2
	Insulin, 1 nM	------	------	134 ± 1	110 ± 4
	Isoproterenol, 1 μM	------	------	145 ± 1	137 ± 7

Nine (Exp. 1) or ten (Exp. 2 and 3) days after initiation of differentiation, 3T3-L1 adipocytes were incubated with or without 0.3 μg of PT for 16 hours. Glycerol production and cAMP phosphodiesterase activity were assayed as described in Methods. Data are means ± S.E.M. (n = 4). Phosphodiesterase activities of control cells (not exposed to PT, insulin, or isoproterenol) were 59 ± 5.9, 64 ± 2.4, and 71 ± 5.9 pmol/min/mg protein in Exp. 1, 2, and 3, respectively.

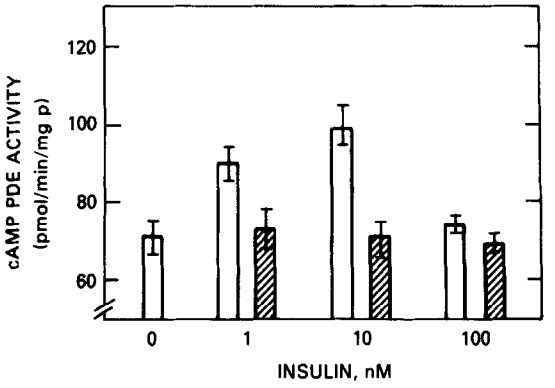

Figure 2. Effect of insulin on particulate phosphodiesterase activity in 3T3-L1 adipocytes incubated with or without pertussis toxin. Adipocytes were incubated without (open bars) or with (hatched bars) PT (0.15 µg) for 18 hr and then without or with the indicated concentrations of insulin for 10 min before preparation and assay of particulate fractions. Data are means ± S.E.M., n = 3.

10% or 50 ± 9%, (mean ± S.E.M., n = 3) respectively (Table 1). In PT-treated adipocytes, activation by insulin was almost abolished, whereas activation by isoproterenol was decreased little if at all (Table 1). Higher concentrations of insulin (100 nM) did not overcome the effect of PT (Fig. 2). As previously reported (4), 100 nM insulin did not increase phosphodiesterase activity in control cells (Fig. 2). In this experiment, particulate cAMP phosphodiesterase activity after incubation with 1 µM isoproterenol was 124 ± 7.4 and 114 ± 9.2 pmol/min/mg protein (n = 3) in control and PT-treated cells, respectively. PT did not alter supernatant cAMP, particulate cGMP or supernatant calmodulin-sensitive or -insensitive phosphodiesterase activities (Table 2). Assays of combinations of fractions from control and PT-treated cells indicated that PT inhibition of insulin-stimulated activity was not related to the presence of a freely diffusible inhibitor (data not shown).

DISCUSSION

Both insulin and lipolytic hormones increase particulate cAMP phosphodiesterase activity in adipocytes; it is not certain, however, that the same enzyme is affected (4). With isoproterenol, the transient rise and fall in phosphodiesterase activity parallels and may be secondary to changes in cAMP content. A cAMP-induced increase in phosphodiesterase activity could serve to modulate the cAMP signal produced by activation of adenylate cyclase.

TABLE 2
Effect of Pertussis Toxin on Supernatant and Particulate cAMP and cGMP Posphodiesterase Activities

Cell Fraction	Substrate	Phosphodiesterase activity (pmol/min/mg protein) No PT	Plus PT
Supernatant	[^{3}H]cAMP	14 ± 1.3 (10.2)†	13 ± 3.6 (8.8)
	[^{3}H]cGMP	27 ± 3.9 (19.6)	30 ± 3.9 (20.1)
	[^{3}H]cGMP*	84 ± 15.1 (60.9)	84 ± 5.7 (56.3)
Particulate	[^{3}H)cGMP	15 ± 2.6 (7.5)	14 ± 1.6 (6.7)
	[^{3}H]cAMP	59 ± 5.3 (29.5)	68 ± 6.4 (32.9)

Nine days after initiation of differentiation, 3T3-L1 adipocytes were incubated with or without 0.3 µg of PT for 16 hours. Phosphodiesterase activity was assayed with 0.5 µM [^{3}H]cAMP or 0.5 µM [^{3}H]cGMP. Data are means ± S.E.M. (n = 3).
* Assay contained 2 mM $CaCl_2$ and 0.16 µg of calmodulin.
† In parentheses, total phosphodiesterase activity in pmol/min/well.

In the presence of insulin, phosphodiesterase activation persists for at least 30 min; activity falls when insulin is removed. Activation of phosphodiesterase may represent one mechanism whereby insulin reduces cAMP content and glycerol production elevated by lipolytic hormones. Incubation of adipocytes with PT decreased insulin-induced activation of particulate cAMP phosphodiesterase, without altering isoproterenol-induced activation or other adipocyte phosphodiesterase activities. This is the first evidence of selective regulation of hormone-sensitive particulate phosphodiesterase activity in adipocytes and strongly supports the notion that the mechanisms of activation by insulin and the lipolytic hormones are different.

PT reverses GTP-dependent inhibition of adenylate cyclase by catalyzing the ADP-ribosylation of the 41,000 dalton subunit of the inhibitory GTP-binding protein (7-12). A GTP-binding protein, analagous to the inhibitory GTP-binding protein of the adenylate cyclase system (17) mediates the photoactivation of a cGMP phosphodiesterase in rod outer segments (6,18). An analagous protein, that can serve as a substrate for PT-catalyzed ADP-ribosylation, may play a role in the activation of adipocyte particulate phosphodiesterase as well as in the effects of guanyl nucleotides on a liver plasma membrane cAMP phosphodiesterase (5). Until the mechanism of phosphodiesterase activation by insulin is defined, however, it remains

possible that the effect of PT on this process is unrelated to modification of a GTP-binding protein; e.g., it could be secondary to lectin-like effects of the β-oligomer (binding subunits) of the toxin (19). In any case, it appears that PT in addition to preventing inhibition of adenylate cyclase by ligands acting through specific receptors, can interfere with enhancement of cAMP degradation by insulin. Perhaps the toxin will be as useful in understanding the mechanism of insulin activation of the phosphodiesterase as it has been in probing the structure and function of the inhibitory GTP-binding protein of the cyclase system.

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