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PERTUSSIS TOXIN INHIBITS AUTOPHOSPHORYLATION AND ACTIVATION OF THE INSULIN RECEPTOR KINASE

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SUMMARY Pertussis toxin is an ADP-ribosyltransferase which alters the function of some of the GTP-binding proteins and inhibits some actions of insulin. In vivo, pertussis toxin (2µg/ml/2h) inhibited insulin-stimulated tyrosyl autophosphorylation of the insulin receptor by 50% in FaO cells, and nearly completely inhibited phosphorylation of the cellular insulin receptor substrate pp185. Similarly, insulin-stimulated autophosphorylation and kinase activity of the insulin receptor purified on wheat germ agglutinin-agarose from pertussis toxin-treated FaO cells was diminished 50%; however, treatment of cells with the catalytically inactive B-oligomer of the toxin had no effect on receptor tyrosine kinase activity in vitro. Pertussis toxin did not alter insulin binding or the cellular levels of ATP, cAMP, and cGMP. Furthermore, immunoprecipitation of the insulin receptor from intact cells with anti-insulin receptor antibodies showed that pertussis toxin did not increase the phosphorylation of serine or threonine residues in the insulin receptor. These results suggest that pertussis toxin can modulate signal transduction of insulin at the level of the insulin receptor kinase.

The insulin receptor is a tyrosyl-specific protein kinase that undergoes autophosphorylation during insulin stimulation (1,2). Autophosphorylation activates the phosphotransferase in the ß-subunit which catalyses tyrosyl phosphorylation of cellular substrates, some of which may play a role in insulin signal transmission (3,4). Guanine-nucleotide binding proteins (G-proteins) play important roles in signal transduction in a number of membrane receptor systems (5). Pertussis toxin (PT) alters the function of some receptor systems by ADP-ribosylation of the associated G-proteins (6). Recently, it has been postulated

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that the insulin receptor might interact with G-proteins as part of its signaling mechanism and it has been shown that PT can alter various insulin actions in different cell types (7-11). In this study we have found that PT treatment of FaO cells inhibits insulin-stimulated activation of the insulin receptor kinase.

MATERIALS AND METHODS

The experiments were performed with the well-differentiated and insulin-sensitive rat hepatoma cell line FaO (12). The cells were cultured in RPMI 1640 containing 10% fetal bovine serum and maintained at 37 C. Sixteen hours before each experiment the culture medium was changed to serum-free RPMI 1640.

Cell-surface insulin binding was determined by treating confluent FaO cells with or without 2 μg/ml PT (List Biologicla Laboratories, CA) for 2 h at 37 C. Then the cells were cooled to 4 C and insulin binding was measured after 16 h incubation as previously described (13).

For the phosphorylation studies in the intact cells in vivo, FaO cells were labeled for 2 h with 0.5 mCi/ml [32-P]phosphate (New England Nuclear) as previously described (14). PT (2 μg/ml) was added to the indicated dishes and the incubation was continued at 37 C for 2 h. Then, if designated, the cells were stimulated with 100 nM insulin for 1 min. Labeled proteins in the soluble cell extract were analyzed as described previously using a polyclonal anti-phosphotyrosine antibody (13).

To study insulin binding and insulin receptor kinase activity after partial receptor purification in vitro, confluent FaO cells were treated without or with PT (2 μg/ml) or an equimolar amount (1.5 μg/ml) of its enzymatic inactive derivative, the pertussis toxin-B oligomer (List Biologicl Laboratories, CA), for 2 h and then solubilized at 22 C with 3 ml of 50 mM HEPES (pH 7.4) containing 1% Triton X-100, 0.1 mg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride. Following centrifugation to remove insoluble marterial, the cell extract was passed over a WGA-agarose column, glycoproteins were eluted (14), and insulin binding to the WGA-purified insulin receptor was performed as described (14). To investigate the insulin receptor kinase in vitro, purified receptor (2.0 μg/ml; amount adjusted by insulin binding) was incubated at 22 C for 10 min in 50 μl of 50 mM HEPES containing 5 mM MnCl2 and 0.1% Triton X-100 in the absence or presence of 100 nM insulin. Autophosphorylation was measured as in (14). The reaction was initiated by adding 25 μM [g-32-P]ATP (2.5 mCi/ml, New England Nuclear) and was continued for 3 min. The tyrosine kinase activity of the WGA-purified insulin receptor was measured with Poly(Glu:Tyr)4:1 (15). In this case, artificial substrate was added to the autophosphorylation reaction at a final concentration of 2.5 mg/ml. Phosphorylation was carried out for 15 min at 20 C as described previously.

RESULTS AND DISCUSSION

To determine the effect of PT on insulin receptor tyrosine kinase activity (Fig. 1), intact FaO cells were labeled with [32-P]orthophosphate for 2h, and then treated with or without 2μg/ml PT for 2h. The control or toxin-treated cells were either

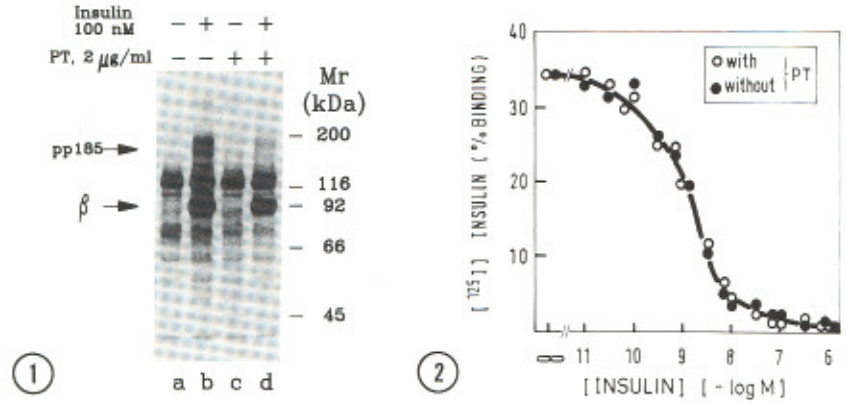


Figure 1. The effect of PT on tyrosyl phosphorylation in vivo. Confluent FaO cells were labeled with [32-P]phosphate and treated without (lanes a and b) or with 2 µg/ml PT (lanes c and d) at 37 C for 2 h. Then, cells were incubated for 1 min. without (lanes a and c) or with 100 nM insulin (lanes b and d). The phosphotyrosine-containing proteins were immunoprecipitated from whole cell extract with αPY and separated by SDS-PAGE. A representative autoradiogram of five separate experiments is shown.

Figure 2. The effect of PT on insulin binding. Confluent FaO cells were treated without (●) or with (○) 2 µg/ml PT for 2h at 37C. Insulin binding to intact cells was measured at 4 C as described under "Materials and Methods".

directly solubilized with Triton X-100 or stimulated with 100 nM insulin for 1 min. before solubilization. Phosphotyrosine-containing proteins were analysed using antiphosphotyrosine antibodies (α-PY). Insulin stimulated tyrosyl phosphorylation of a 95 kDa and 185 kDa protein. The 95 kDa protein corresponds to the ß-subunit of the insulin receptor and the 185 kDa protein (pp185) has been previously suggested to be an endogenous substrate of the insulin receptor in these cells (4,13,16-18). Pretreatment of cells with 2 µg/ml PT inhibited the insulin-stimulated autophosphorylation of the insulin receptor by 50% and also inhibited tyrosyl phosphorylation of pp185. Maximum inhibition of receptor autophosphorylation was reached at 2 µg/ml toxin, and the half-maximal effect occured at 1 µg/ml; inhibition was observed after 10 min. of incubation with PT (data not shown).

The inhibition of the insulin receptor tyrosine kinase activity by PT was not mediated by alterations in receptor number or affinity (Fig.2) and the toxin had no significant effect on the basal concentrations of ATP, cAMP, and cGMP (data not shown). Increased serine and threonine phosphorylation of the insulin receptor in the basal state inhibits insulin-stimulated tyrosyl

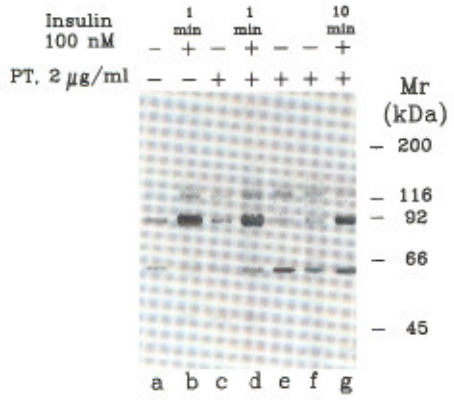
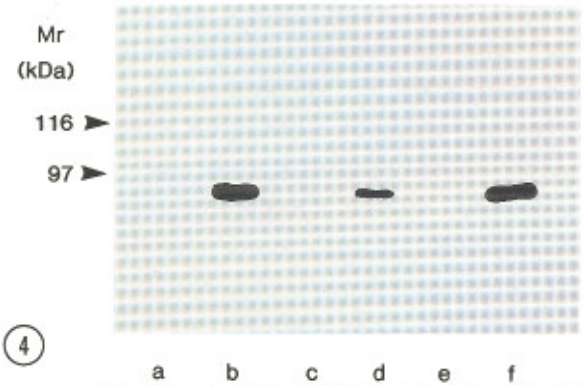


Figure 3. The effect of PT on the basal and insulin-stimulated phosphorylation of the insulin receptor. Confluent FaO cells were labeled with [32-P]phosphate and treated without (lanes a and b) or with 2 µg/ml PT (lanes c to g) at 37 C for 2 h. Then, cells were incubated for 1 min. without (lanes a,c,e and f) or with 100 nM insulin (lanes b and d) or 10 min. (lane g). The insulin receptor was immunoprecipitated from whole cell extract with anti-insulin receptor antibodies and analysed by SDS-PAGE. An autoradiogram is shown.

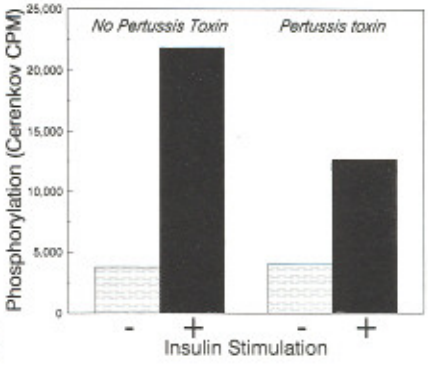
phosphorylation in vivo and in vitro (19-23). To determine whether PT stimulated basal phosphorylation of the insulin receptor ß-subunit, FaO cells were labeled with [32-P]orthophosphate and the receptor was immunoprecipitated with anti-insulin receptor antibody (B2). PT inhibited the insulin-induced autophosphorylation of the ß-subunit by 50%, but did not increase the phosphorylation of the receptor in the basal state (Fig. 3).

To examine whether the inhibitory effect of PT was due to a modification tightly associated with the receptor, intact cells were treated with or without PT and then, after incubation of the intact cells with PT, the insulin receptor was purified on immobilized wheat germ agglutinin to test the recpetor kinase activity in vitro. Insulin stimulated autophosphorylation of the partially purified insulin receptor from control and PT-treated cells in vitro. However, insulin-induced autophosphorylation of the ß-subunit was reduced at least 50% when cells were pretreated with PT (Fig. 4, lane d). Consistent with these results, receptors purified from PT-treated cells showed a 50% reduction in insulin-stimulated tyrosyl phopshorylation of the synthetic substrate poly(Glu,Tyr)4:1 (Fig. 5). These results show that the inhibitory effect of PT was retained by the insulin receptor after partial purification.

To determine whether the inhibitory action of PT on insulin receptor kinase activity is related to PT-induced ADP-ribosylation



(4)



(5)

Figure 4. The effect of PT and the PT-B oligomer on in vitro autophosphorylation of the insulin receptor. Cells were treated without (lanes a and b), with 2 μg/ml PT (lanes c and d), or with an equimolar concentration (1.5 μg/ml) PT-B oligomer (lanes e and f) for 2 h at 37 C. The insulin receptor was purified by WGA-chromatography. In vitro, WGA-purified insulin receptor (adjusted to equal amounts of insulin binding in the solubilized receptor preparations) was incubated without (lanes a,c and e) or with 100 nM insulin (lanes b,d and f) for 30 min. and phosphorylated in presence of [g-32P]ATP for 3 min. The tyrosyl phosphorylated insulin receptor was immunoprecipitated by αPY and separated by SDS-PAGE. A representative autoradiogram of three separate experiments is shown.

Figure 5. The effect of PT on substrate phosphorylation of the insulin receptor in vitro. Cells were treated without or with 2 μg/ml PT for 2 h at 37 C. The insulin receptor was purified by WGA-chromatography. In vitro, WGA-purified insulin receptor (adjusted to equal amounts of insulin binding in the solubilized receptor preparations) was incubated without or with 100 nM insulin for 30 min., and then the phosphorylation of Poly(Glu:Tyr)4:1 (2.5 mg/ml) during 15 min. in presence of 25 μM [g-32P]ATP was determined as described under "Materials and Methods". Values are the mean of triplicate determinations.

of cellular proteins, the PT-B oligomer was used in control experiments. The B oligomer consists of the fragment of PT that is responsible for cell surface binding, but lacks the catalytically active A-protomer containing the ADP-ribosyltransferase activity (6). Cells were incubated with the B oligomer (1.5 μg/ml, equimolar to a concentration of 2 μg/ml of PT) for 2h at 37 C, and the insulin receptor was partially purified by WGA-chromatography. Insulin-stimulated autophosphorylation was not significantly affected by the b oligomer alone (Fig. 4, compare lanes b and f). These results suggest that the effects of PT are not mediated by the extracellular binding of the B oligomer, but are a consequence of the action of the catalytic subunit. In a series of experiments we have studied in vitro, using [32-P]NAD, as well as in vivo, labeling intact cells with [3-H]ADP-ribose, whether a protein in the WGA eluate or the insulin receptor itself is ADP-ribosylated by PT.

We could not identify a potential substrate of PT in the WGA eluate using the antibodies B2, αPY or none. In accordance to that, Davis and McDonald (24) could also not detect a G-protein in WGA receptor preparations using an antibody raised to a peptide sequence common to the α-subunits of various G-proteins.

Taken together, this study shows that PT inhibits insulin-stimulated autophosphorylation, activation of the insulin receptor kinase, and insulin-stimulated tyrosyl phosphorylation of pp185. Therefore PT may affect signal transduction of insulin by interrupting the insulin-induced tyrosyl phosphorylation cascade.

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REFERENCES

1. Kahn, C.R., and White, M.F. (1988) J. Clin. Invest. 82, 1151-1156.
2. Rosen, O. (1987) Science 237, 1452-1458.
3. Kasuga, M., Izumi, T., Tobe, K., Shiba, T., Momomura, K., Tashiro-Hashimoto, Y., and Kadowaki, T. (1990) Diabetes Care 13, 317-326.
4. White, M.F., Livingston, J.N., Backer, J.M., Lauris, V., Dull, T.J., Ullrich, A., and Kahn, C.R. (1988) Cell 54, 641-649.
5. Gilman, A.G. (1990) Ann. Rev. Biochem. 56, 615-649.
6. Moss, J., and Vaughan, M. (1988) Adv. Enzymol. 61, 303-379.
7. Lutrell, L.M., Hewlett, E.L., Romero, G., and Rogol, A.D. (1988) J. Biol. Chem. 263, 6134-6141.
8. Heyworth, C.M., Grey, A.M., Wilson, S.R., Hansli, E., and Houslay, M.D. (1986) Biochem. J. 235, 145-149.
9. Heyworth, C.M., Whetton, A.D., Wong, S., Martin, B.R., and Houslay, M.D. (1985) Biochem. J. 228, 593-603.
10. Lutrell, L., Kilgour, E., Larner, J., and Romero, G. (1990) J. Biol. Chem. 265, 16873-16879.
11. Goren, H.J., Northup, J.K., and Hollenberg, M.D. (1985) Can. J. Physiol. Pharmacol. 63, 1017-1022.
12. Crettaz, M., and Kahn, C.R. (1983) Endocrinology 113, 1201-1209.
13. White, M.F., Stegmann, E.W., Dull, T.J., Ullrich, A., and Kahn, C.R. (1987) J. Biol. Chem. 262, 9769-9777.
14. Takayama, S., White, M.F., and Kahn, C.R. (1988) J. Biol. Chem. 263, 3440-3447.
15. Grigorescu, F., Flier, J.S., and Kahn, C.R. (1984) J. Biol. Chem. 259, 15003-15006.
16. White, M.F., Maron, R., and Kahn, C.R. (1985) Nature 318, 183-186.
17. Sun, X.J., Rothenberg, P., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J., and White, M.F. (1991) Nature 352, 73-77.
18. White, M.F., and Kahn, C.R. (1986) The Enzymes XVII, 247-310.

19. Stadtmauer, L., and Rosen, O.M. (1986) J. Biol. Chem. 261, 3402-3407.
20. Lewis, R.E., Wu, G.P., MacDonald, R.G., and Czech, M.P. (1990) J. Biol. Chem. 265, 947-955.
21. Koshio, O., Akanuma, Y., and Kasuga, M. (1989) FEBS Lett. 254, 22-24.
22. Lewis, R.E., Cao, L., Perregaux, D., and Czech, M.P. (1990) Biochemistry 29, 1807-1813.
23. Roth, R.A., and Beaudoin, J. (1987) Diabetes 36, 123-126.
24. Davis, W., and Mc.Donald, J.M. (1990) Biochem. J. 270, 401-407.