Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 517-523

INSULIN INDUCED PHOSPHORYLATION AND ACTIVATION OF THE cGMP-INHIBITED cAMP PHOSPHODIESTERASE IN HUMAN PLATELETS

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Received May 26, 1992

Insulin induced phosphorylation and activation of the cGMP inhibited cAMP phosphodiesterase (cGI-PDE) in human platelets were demonstrated after isolation of the enzyme with specific polyclonal cGI-PDE antibodies. The demonstration of this insulin effect required suppression of basal cGI-PDE phosphorylation, through the use of the protein kinase inhibitor H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine). The human platelet insulin receptor β-subunit, previously identified as a 97 kDa polypeptide, was detected with the use of wheat germ agglutinin chromatography and anti-phosphotyrosine antibodies. These results suggest that insulin, through phosphorylation/activation of cGI-PDE, could decrease cAMP/cAMP dependent protein kinase (cAMP-PK) activity and thereby make the platelets more sensitive towards aggregating agents.

In patients with diabetes mellitus platelets exhibit an increased sensitivity towards platelet aggregating agents and increased resistance to antiaggregatory agents (1). These observations have led to a search for possible actions of insulin on platelets. The human platelet insulin receptor has recently been partially characterized (2). Looking for cellular effects of insulin on platelets, no changes could be detected in cAMP formation or degradation, inositol phosphate formation or phosphorylation of proteins other than the receptor itself (2, 3). Insulin is reported to

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ABBREVIATIONS. $C_{13}E_{12}$, heterogeneous nonionic alkyl polyoxyethylene glycol detergent. Hepes, 4-(2-hydroxyethyl)-piperazineethanesulfonic acid. EDTA, ethylenediaminetetraacetic acid. EGTA, ethylene glycol bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

0006-291X/92 $4.00

activate a GTPase in human platelets but the physiological significance of this is not known (4). In platelets from patients with non-insulin-dependent diabetes mellitus insulin receptor number and affinity are reported to be reduced significantly (5).

Recently, we have demonstrated that in rat adipocytes insulin and isoprenaline phosphorylates and activates the cGI-PDE in a concentration- and time-dependent manner (6, 7). Synergistic phosphorylation and activation was obtained when the two hormones were added together under conditions during which insulin lowered lipolysis and cAMP-PK activity (7). These data strongly suggest the presence of two different phosphorylation sites, one for cAMP-PK and one for an unidentified insulin sensitive serine protein kinase (7).

The human platelet cGI-PDE, which represents more than 75 % of the total high affinity cAMP phosphodiesterase activity in a platelet extract, has been purified to homogeneity (8, 9). It was demonstrated that the enzyme has inhibitory, kinetic and immunological properties similar to what has been found for the rat and bovine adipose tissue, bovine cardiac and rat liver enzymes (10). In platelets, agents that increase cAMP phosphorylate and activate the cGI-PDE (11, 12) and cAMP-PK catalyses phosphorylation and activation of the isolated enzyme (13). In this report we demonstrate that also insulin induces phosphorylation and activation of the cGI-PDE. Thus, human platelets could provide an excellent model to study regulation of cGI-PDE by insulin and to examine the insulin signal chain.

METHODS

Preparation of human platelets

Outdated platelet concentrates were obtained from the University Hospital of Lund. The platelets were sedimented by centrifugation at 1100 g for 15 min, and washed twice in 20 mM Tris (pH 6.7), 135 mM NaCl, 2 mM EDTA, 15 mM trisodium citrate and 0.3 % BSA.

Purification and autophosphorylation of insulin receptors

Platelet pellets (1 x 10^{10} cells) were sonicated 15 sec in 1 ml 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 2 mM Na-O-Vanadate, 0.1 M NaF, 2 mM EGTA, 10 mM EDTA, 100 KIU/ml trasylol, 20 μM pepstatin A, 20 μM leupeptin and 0.1 mM DPF. Cellular debris was removed by centrifugation at 10.000 g for 10 min. The supernatant was added to 100 μl of wheat germ agglutinin-Sepharose 4B (Pharmacia, Uppsala,Sweden) and the glycosylated compounds were eluted with 400 μl of 50 mM Tris (pH 7.4), 0.2 % Triton X-100 (TTB) containing 0.3 M N-acetylglucosamine. Autophosphorylation was performed by incubating 200 μl of eluate with and without 10^{-5} and 10^{-7} M insulin for 10 min. Two hundred μl 400 μM ATP ([γ-^{32}P]ATP, 1 mCi) and 400 μl 15 mM $MnCl_2$ were then added and the incubation continued for an additional 30 min at room temperature, after which the samples were incubated with either anti-phosphotyrosine (generously supplied by Dr. H. Tornqvist, Department of Pediatrics, University of Lund) or anti-insulin receptor antibodies (Immunotech, Marseille, France) for 3 hours at 4 °C. The immunocomplexes were collected by adding protein A-Sepharose (Pharmacia, Uppsala, Sweden). The pellets were washed 6 times with TTB containing 0.1 mM NaCl. The samples were analyzed by SDS-PAGE (polyacrylamide gel

electrophoresis in presence of sodium dodecyl phosphate) followed by autoradiography. Parallel experiments were made with human placenta insulin receptor as a control.

Insulin induced activation and phosphorylation of the cGI-PDE

For phosphorylation experiments the washed platelets were resuspended at a concentration of 2 x 10^8 cells per ml in 10 ml of 10 mM Hepes (pH 6.6), 1 mM $MgCl_2$, 145 mM NaCl, 5 mM KCl, 20 mM glucose, 10 μM leupeptin and 0.3 % BSA. Carrier-free ^{32}Pi was added (1 mCi/ml) and the suspension incubated at 37 ºC for 90 min. The platelet suspension was centrifuged at 1100 g for 20 min, and the pellet was resuspended at the same cell concentration in 50 mM Tris (pH 7.5), 20 mM $MgCl_2$, 150 mM NaCl, 10 mM benzamidine, 2 mM EGTA, 5 μM pepstatin A and 40 μM leupeptin. Five hundred μl alicuotes were treated with insulin and/or H-7 (Sigma, St. Louis, USA). An equal volume of buffer without $MgCl_2$ and containing 10 mM NaF and 20 mM sodium pyrophosphate was added to the samples to stop the reaction and the cells pelleted (10000 g, 1 min). Five hundred μl of ice cold homogenization buffer (50 mM Tes, 250 mM sucrose, 1 mM EDTA, 0.1 mM EGTA, 3 mM benzamidine, 10 mM sodium pyrophosphate, 50 mM NaF, 10 μM pepstatin A, 10 μM leupeptin and 1% $C_{13}E_{12}$) was added to the platelet pellet before sonication for 15 sec and centrifugation. The supernatants were incubated for 12 h at 4 ºC with the cGI-PDE antibodies raised against the human platelet cGI-PDE (9) followed by immunoprecipitation with *S. aureus* protein A (Sigma, St. Louis, USA) and collection of the immunoprecipitates by centrifugation. The pellets were washed five times in 0.1 M sodium phosphate buffer (pH 7.5) containing 1 % N-lauryl-sarcosine. The samples were analyzed by SDS-PAGE followed by autoradiography. Phosphoamino acid analysis of the electroeluted phosphorylated cGI-PDE from SDS-PAGE gels was performed as described in (6).

Parallel experiments were conducted in the absence of $^{32}P_i$ to determine the effect of phosphorylation on cGI-PDE activity. In these experiments, after washing the immunoprecipitate with 50 mM Tris (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 0.03% $C_{13}E_{12}$ and 10% glycerol twice, phosphodiesterase activity was assayed using a modification of the Manganiello and Vaughan method (14).

RESULTS AND DISCUSSION

Insulin induced autophosphorylation of the platelet insulin receptor

Platelet insulin receptor was recently identified using wheat germ agglutinin chromatography and anti-insulin receptor antibodies (2). Two subunits were detected. One had a molecular weight of 97 kDa, and could be phosphorylated in a dose dependent manner in response to insulin stimulation of cells, and the other had a molecular weight of 69 kDa and was not phosphorylated. The classical 135 kDa α-subunit was not detected (2). As shown in Fig. 1, incubation of a wheat germ agglutinin eluate with $Mn^{2+}[\gamma\text{-}^{32}P]ATP$ and different concentrations of insulin, followed by immunoprecipitation with anti-phosphotyrosine antibodies, resulted in the isolation of a 97 kDa phosphorylated polypeptide presumably representing the insulin receptor β-subunit. In addition two other polypeptides were tyrosine phosphorylated in response to insulin with molecular weights of 120 and 60 kDa respectively (Fig. 1), which were not demonstrated after insulin stimulation of intact cells (2). The same phosphoprotein pattern was obtained after immunoprecipitation with anti-insulin

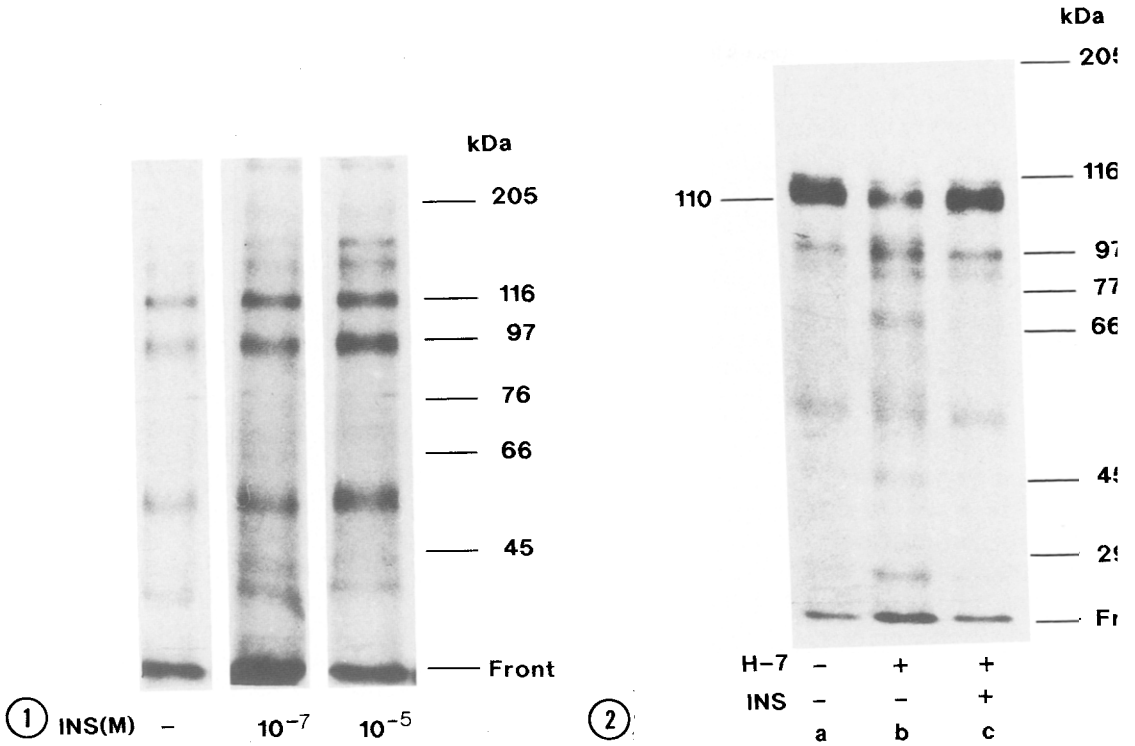

FIGURE 1. Insulin induced autophosphorylation of platelet insulin receptor.

Partially purified platelet insulin receptor was incubated without or with 10^{-5} and 10^{-7} M insulin for 10 min at room temperature. The phosphorylation reaction was initiated by adding $MnCl_2$ and [γ-^{32}P]ATP and the incubation was continued for 30 min. The insulin receptor was immunoadsorbed using anti-phosphotyrosine antibodies and the complexes were analyzed by SDS-PAGE and autoradiography as described in Methods.

FIGURE 2. Insulin induced 32P-incorporation into platelet cGI-PDE.

Platelets were prelabeled with ^{32}Pi for 90 min at 37 °C and then preincubated with vehicle (a) or with 100 µM H-7 (b, c) for 15 min. Thereafter insulin (c) or vehicle (a, b) was added and the incubations continued for 15 min. The phosphorylated cGI-PDE was immunoadsorbed using cGI-PDE antibodies and the immunocomplexes analyzed by SDS-PAGE and visualized by autoradiography as described in Methods.

receptor antibodies (not shown). The identity of these phosphoproteins in platelets is unknown but insulin-dependent phosphorylation of 115-120 kDa and 60 kDa polypeptides has been described in different cells (15-18). The 60 kDa polypeptide has been suggested to function as a serine protein kinase (17, 18).

In order to verify the methodology for insulin receptor isolation/phosphorylation a human placenta insulin receptor preparation was used in parallel experiments. Insulin dependent phosphorylation of the 95 kDa placenta insulin receptor ß-subunit was demonstrated (not shown).

Insulin induced serine-phosphorylation of cGI-PDE

In a search for insulin induced cellular effects in platelets we used specific cGI-PDE antibodies (9) to immunoadsorb the cGI-PDE from control and insulin stimulated

cells prelabeled with $^{32}P_i$. Approximately 90 % of the cGI-PDE activity in the cell sonicates was immunoprecipitated with the cGI-PDE antibodies and could be recovered in an active form in the immunopellet. No measurable differences with regard to the efficiency in immunoprecipitation was found in extracts from insulin stimulated and control cells.

As seen in Fig. 2a, incubation of the cGI-PDE antibodies with sonicates from control cells resulted in the isolation of a phosphoprotein with a molecular weight of 110 kDa, previously established to be the intact subunit molecular weight of human and bovine platelet cGI-PDE (8, 11-13). Addition of 10^{-8} M insulin resulted in increased phosphorylation of the cGI-PDE but the increase in phosphorylation varied between cell batches. Since it has been demonstrated that agents that increase cAMP result in phosphorylation and activation of the platelet cGI-PDE (11, 12) and that cAMP-PK phosphorylates and activates the platelet cGI-PDE (13), it was likely that cAMP-PK phosphorylation was responsible for some of the phosphorylation of the cGI-PDE observed in control cells. Addition of 100 μM H-7, a cGMP-dependent protein kinase, cAMP-PK and protein kinase C inhibitor (19), to the cell incubations reduced phosphorylation of the cGI-PDE in control cells by 70-80% (Fig. 2b). The stimulation of platelets with 10^{-8} M insulin in the presence of H-7 resulted in a marked (300-400 %, n= 6) increase in phosphorylation of the enzyme (Fig. 2c).

Partial acid hydrolysis of the phosphorylated cGI-PDE, immunoisolated from cells stimulated with insulin in the presence of H-7, followed by two-dimensional thin-layer electrophoresis revealed the presence of [^{32}P]phosphoserine but no detectable [^{32}P]phosphotyrosine or [^{32}P]phosphothreonine (not shown).

Insulin induced activation of cGI-PDE

To test whether the phosphorylation of the cGI-PDE in response to insulin was accompanied by activation of the enzyme, cGI-PDE activity was determined in immunoprecipitates obtained from control cells, cells incubated with H-7 and cells stimulated with insulin in the presence of H-7. As shown in Fig. 3, 100 μM H-7 in the incubation medium produced a 20 % decrease in the cAMP phosphodiesterase activity probably due to inhibition of cAMP-PK phosphorylation/activation of the cGI-PDE, as previously reported by others (11, 12). Incubation of platelets with three different insulin concentrations from 10^{-9} to 10^{-7} M in the presence of H-7 for 15 min at 37 ºC resulted in a small (15-25 %) but reproducible ($p < 0.001$, n= 6) increase in cGI-PDE activity.

The results are consistent with the hypothesis that in human platelets insulin, by interaction with its specific cell surface receptor and activation of the receptor tyrosine kinase, increases cGI-PDE activity by activating a serine protein kinase responsible for the phosphorylation of the cGI-PDE. In platelets cAMP is an inhibitory second messenger and an insulin induced activation of the cGI-PDE could therefore render the platelets more sensitive towards aggregating agents (1) by lowering cellular cAMP/cAMP-PK. A failure to demonstrate insulin induced decrease of cAMP in

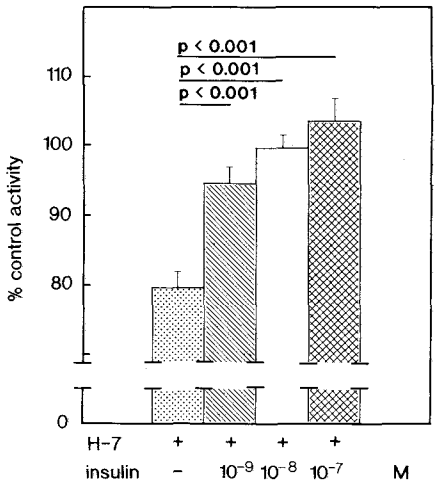

FIGURE 3. Insulin mediated phosphorylation-activation of platelet cGI-PDE.
Platelets were preincubated for 15 min with 100 μM H-7. Thereafter vehicle or insulin at different concentrations was added as indicated in the figure. cGI-PDE was immunoadsorbed using cGI-PDE antibodies and the cGI-PDE activity was assayed. PDE activity in absence of H-7 and agonists varied from 70 to 189 pmol/min/ml and was assigned to 100 %. The data represent mean ± S. E. M. from 6 separate experiments. Statistical analysis was formed using the t' Student test for paired data.

platelets was recently reported (2). Measurements of changes in cAMP-PK ratios in platelets in response to insulin could be a more sensitive method to detect small but physiological changes in cAMP. It has been demonstrated that insulin lowers cAMP-PK ratios in fat cells under conditions where the hormone exerts its antilipolytic effect (20) whereas the changes in cAMP have been more difficult to detect (21, 22).

Several serine protein kinases that are activated by insulin have been described in different tissues (23-25). Recently, evidence for the presence in rat adipocytes of an insulin stimulated protein kinase which activates cGI-PDE has been provided (26). Our next step will be to attempt to isolate and purify the insulin sensitive serine protein kinase from human platelets that is responsible for the phosphorylation and activation of the cGI-PDE.

ACKNOWLEDGMENTS. We are grateful to Dr. Hans Tornqvist for advice regarding the detection and phosphorylation of the insulin receptor. This work was supported by grants from the following foundations: A. Påhlsson, Malmö; Nordic Insulin, Copenhagen; Swedish Diabetes Association; Hoechst's Diabetes, Stockholm; Medical Faculty, University of Lund; The Swedish Medical Research Council (Project Nos. 3362 and 8689) and the Spanish Ministry of Education and Science.

REFERENCES

1. Davis, J. M. E., Laurie, D. and Colwell, J. E. (1985) Diabetologia, 28, 274-277.
2. Falcon, C., Pfliegler, G., Deckmyn, H. and Vermylen, J. (1988) Biochem. Biophys. Res. Commun., 157, 1190-1196.

3. Kahn, N. N. and Sinha, A. K. J. (1990) J. Biol. Chem., 265, 4976-4981.
4. Gawler, D. and Houslay, M. D. (1987) FEBS Letters, 216, 94-98.
5. Udvardy, G. Pfliegler, G. and Rak., K. (1985) Experientia, 41, 422-423.
6. Degerman, E., Smith, C. J., Tornqvist, H., Vasta, V., Belfrage, P. and Manganiello, V. C. (1990) Proc. Natl. Acad. Sci., USA, 87, 533-537.
7. Smith, C. J., Vasta, V., Degerman, E., Belfrage, P. and Manganiello, V. C. (1991) J. Biol. Chem., 266, 13385-13390.
8. Grant, P. G. and Colman, R. W. (1984) Biochemistry, 23, 1801-1807.
9. Degerman, E., Moos, M., Rascón, A., Vasta, V., Meacci, E., Smith, C. J., Lindgren, S., Andersson, K.-E., Belfrage, P. and Manganiello, V. C. (1992). Unpublished.
10. Manganiello, V. C., Smith, C. J., Degerman, E. and Belfrage, P. (1990) In Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and drug Action (Beavo, J. and Houslay M. D. Eds.), pp. 87-116. John Wiley and Sons, Ltd., England.
11. Macphee, C. H., Reifsnyder, D. H., Moore, T. A. and Beavo, J. A. (1987) J. Cycl. Nucl. Prot. Phosph. Res., 11, 487-496.
12. Grant, P. G., Mannarino, A. F. and Colman, R. W. (1988) Proc. Natl. Acad. Sci., USA, 85, 9071-9075.
13. Macphee, C. H., Reifsnyder, D. H., Moore, T. A., Lerea, K. M. and Beavo, J. A. (1988) J. Biol. Chem., 263, 10353-10358.
14. Manganiello, V. C. and Vaughan, M. (1973) J. Biol. Chem., 248, 7164-7170.
15. Machicao, F., Häring, H., White, M. F., Carrascosa, J. M., Obermaier, B. and Wieland, O. H. (1987) Biochem. J., 243, 797-801.
16. Sadoul, J. L., Peyron, J. F., Ballotti, R., Debant, A., Fehlmann, M. and Van Obberghen, E. (1985) Biochem. J., 227, 887-892.
17. Häring, H. U., White, M. F., Machicao, F., Ermel, B., Schleicher, E. and Obermaier, B. (1987) Proc. Natl. Acad. Sci., USA, 84, 113-117.
18. Obermaier-Kusser, B., Ermel, B., Mühlbacher, C., and Häring H. U. (1988) Diabetes Res. Clin. Pract., 60 (Suppl. 1), 67-93.
19. Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Biochemistry, 23, 5036-5041.
20. Londos, C., Honnor, R. C. and Dhillon, G. S. (1985) J. Biol. Chem., 260, 15139-15145.
21. Siddle, K. and Hales, C. N. (1974) Biochem. J., 142, 97-103.
22. Wong, E. H. A. and Loten, E. G. (1981) Eur. J. Biochem., 115, 17-22.
23. Czech, M. P., Klarlund, J. K., Yagalott, K. A., Bradford, A. P. and Lewis, R. E. (1988). J. Biol. Chem., 263,11017-11019.
24. Sturgill, T. W., Ray, L. B., Eriksson, E. and Maller, J. L. (1985) Nature (London), 334, 715-718.
25. Sommercorn, J., Mulligan, J. A., Fred, J. L. and Krebs, E. G. (1987) Proc. Natl. Acad. Sci., USA, 84, 8834-8838.
26. Shibata, H. and Kono, T. (1990) Biochem. Biophys. Res. Commun., 167, 614-620.S