Volume 291, number 2, 363–366 FEBS 10288 October 1991

ADONIS 001457939100997R

Effects of 17-hydroxywortmannin on serine/threonine-protein kinases in human blood platelets

Markus Kocher and Kenneth J. Clemetson

Theodor Kocher Institute, University of Berne, Freiestrasse 1, CH-3012 Berne, Switzerland

Received 9 August 1991

Protein kinases are involved in signal transduction in human blood platelets A number of renaturable protein kinases have increased in vitro activities compared to controls due to covalent modifications when intact platelets are activated by thrombin The effect of the platelet inhibitor 17-hydroxywortmannin (HWT) on these protein kinases was investigated in intact platelets and in vitro HWT inhibits the increase in activity of these protein kinases but it does not interact directly with their catalytic subunits We conclude that HWT blocks a step in signal transduction which is necessary to activate these protein kinases via covalent modifications

Platelet, Protein kinase, Aggregation, Signal transduction, Thrombin, Fungal metabolite

1. INTRODUCTION

Stimulation of platelets with thrombin induces the GTP-dependent activation of phospholipase C [1] which cleaves membrane-bound phosphatidylinositol bisphosphate (PIP_2) to produce the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers the release of calcium from the storage granules into the cytosol [2] while DAG activates protein kinase C [3] that phosphorylates a number of substrates [4–8]. Other pathways of platelet activation are less well characterized and may also include phosphorylation cascades.

The activity of a group of protein kinases could be restored by renaturation after electrophoresis and transfer to PVDF membranes [9] Kinase activity was monitored with [γ-^{32}P]ATP Bands on the blots were detected by autoradiography Phosphoserine and phosphothreonine were predominantly labelled and analysis of the phosphorylated protein species revealed that with most of the protein kinases, bovine serum albumin and the kinases themselves (variable amount of autophosphorylation) contained most of the ^{32}P-label [10]. Some of these protein kinases show higher in vitro activities on the blots when intact platelets were activated and therefore may somehow be involved in signal transduction pathways The changes in activity are most probably due to covalent modifications as they are conserved during the denaturation- and renaturation-treatment

17-Hydroxywortmannin (HWT) is a derivative of the sterol-like fungal metabolite wortmannin [11] It inhibited respiratory burst and secretion in neutrophils without directly affecting PKC [12]. Since HWT shows relatively specific effects on platelet activation (von Tscharner, V and Clemetson, K J., submitted), renaturation of blotted proteins was used to investigate its effects on the activation in intact platelets and in vitro activities of the renaturable and covalently modified protein kinases from human blood platelets in order to correlate these with other platelet reactions

Abbreviations HWT, 17-hydroxywortmannin, PVDF, polyvinylidene difluoride, fMLP, *N*-formyl-methionylleucylphenylalanine, PMA, phorbol myristyl acetate, IWT, inactive wortmannin derivative

Correspondence address K J Clemetson, Theodor Kocher Institute, University of Berne, Freiestrasse 1, CH-3012 Berne, Switzerland. Fax (41) (31) 65 37 99

2. MATERIALS AND METHODS

2 1 *Materials*

Apyrase, bovine serum albumin (fraction V and fatty acid free, fraction V), phorbol myristyl acetate (PMA), ionomycin and the prestained molecular mass markers were from Sigma, St Louis, MO Thrombin was from Merck, Zurich Iloprost was kindly donated by Schering AG, Zurich HEPES was from Calbiochem, Lucerne Guanidine-HCl was from Fluka, Buchs and Nonidet P-40 was from Grogg, Berne [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham International, England The PVDF membranes (Immobilon-P) were from Millipore, Bedford, MA HWT, {1,7,8,9,9a,11b-octahydro-11-acetoxy-9-hydroxy-1-(methoxymethyl)-9a,11b-dimethyl-3H,6bH-*furo*[4,3,2-de]*indeno*[4,5- h][2]*benzopyran*-3,6-dion} and the inactive wortmannin derivative IWT {6b,7,9a,10,11,11b-hexahydro-5-hydroxy-4-hydroxymethylen-1-methoxymethyl-9a,11b-dimethyl-1H,6H-*indeno*[4,5-h][2]-*benzopyran*-3,6,9(4H,8H)-trion} were supplied by Dr T G Payne, Preclinical Research, Sandoz Ltd , Basle, Switzerland

2 2 *Isolation of human platelets*

Buffy coats were obtained from the Swiss Red Cross in Berne (FDA four bag system) approximately 15 h after collection Buffy coat dilution buffer (0 1 volume) was added to give an end concentration of 10 mM sodium citrate and 0 15 U/ml apyrase and the suspension was

centrifuged at 150×g for 15 min The platelet-rich plasma was acidified to pH 6 5 with 150 mM citric acid, iloprost was added to an end concentration of 1 ng/ml, and centrifugation at 500×g for 15 min pelleted the platelets The platelets were washed twice with 100 vols of 137 mM NaCl, 11 mM glucose, 11 mM sodium citrate, 0 2 U/ml apyrase, 0 25% BSA, pH 6 5, and then resuspended in 137 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0 4 mM NaH_2PO_4, 5 6 mM glucose, 5 mM HEPES, 1 U/ml apyrase pH 7 5 to an end concentration of 10^9 platelets per ml Experiments were performed at 37°C HWT was added from stock solutions in Me_2SO to yield a final Me_2SO concentration of 0 1%, which did not change the pattern of phosphorylation in the renatured kinases

2 3 *Kinase assays*

Samples were solubilized immediately in SDS-PAGE sample buffer at a concentration of 2 3% SDS, 0 1% dithiothreitol, 0 1 mM EDTA, 62 5 mM Tris, 10% glycerol, pH 6 8, vortexed and boiled for 2 min Separation of platelet proteins in 7 5% polyacrylamide gels [13] and the transfer to PVDF membranes were as described [14] Renaturation and kinase reactions were performed according to Ferrell and Martin [10] The X-ray films were analysed on a DD2 densitometer from Kipp & Zonen connected to a chart recorder

2 4 *In vitro treatment*

After renaturation and blocking, sections of the blots were treated with HWT in 30 mM Tris, 10 mM $MgCl_2$, 2 mM $MnCl_2$, pH 7 5 for 10 min at room temperature They were washed once with the same buffer without HWT and then incubated with the kinase reaction buffer [10]

3. RESULTS

Washed human platelets were preincubated with different concentrations of HWT for 10 min and then stimulated with thrombin (Fig 1). Blots from untreated control or thrombin-stimulated platelets showed patterns of labelled bands similar to those reported earlier [10] A combination of the phorbol ester PMA and ionomycin induced the activities of the protein kinases similar to that induced by thrombin (not shown). Preincubation of unstimulated control platelets with HWT had no effect on the activities of the renaturated protein kinases (Fig 1A) HWT preincubation inhibited the increase of activity of PK 48, PK 52, PK

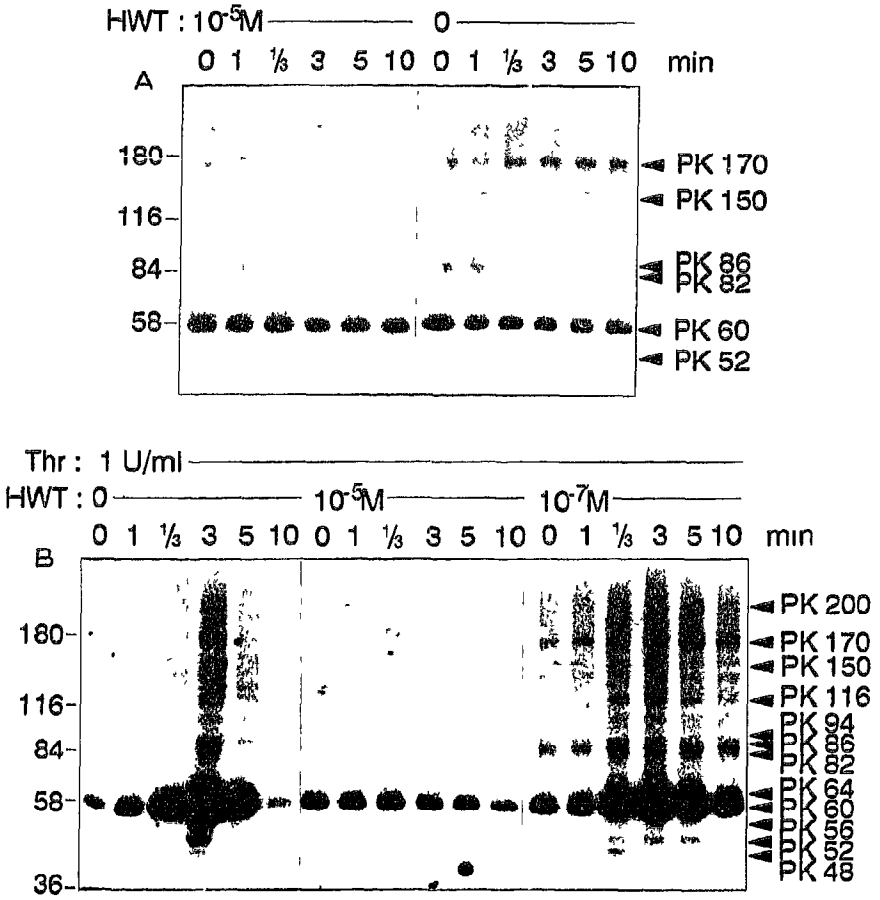

Fig 1 Effect of HWT treatment and thrombin stimulation in intact platelets on the in vitro activities of the renaturable protein kinases from human platelets Autoradiogram of PVDF-membranes with renaturated proteins labelled in vitro with [γ-^{32}P]ATP from a typical experiment where platelets were preincubated for 10 min with different concentrations of HWT (A) Shows unstimulated platelets preincubated with and without HWT (B) Shows platelets activated with 1 U/ml thrombin The 0 time samples were taken just before the addition of thrombin, then the other samples were taken after times as indicated and all were treated as described in Materials and Methods The stimulations were performed with washed platelets from the same buffy coat All the blots were treated the same way and autoradiographed together on the same film Molecular mass markers are indicated in kilodaltons

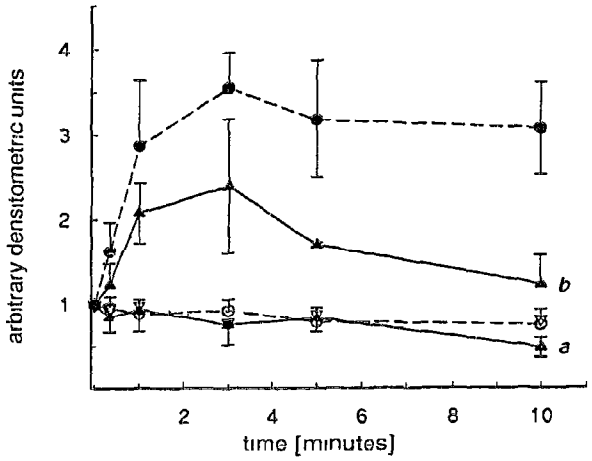

Fig 2 Kinetics of PK 60 activation Densitometric analysis of the autoradiograms of several similar experiments of the activity of PK 60 The amount of processed ^{32}P was measured as peak surfaces relative to the control values at 0 time that were set to 100 The symbols are ○ control, n=4, ▽ HWT 10^{-5} M alone, n=2, ▲ HWT 10^{-5} M, n=3, *b* HWT 10^{-7} M, n=2, both stimulated with thrombin 1 U/ml ● thrombin 1 U/ml, n=4

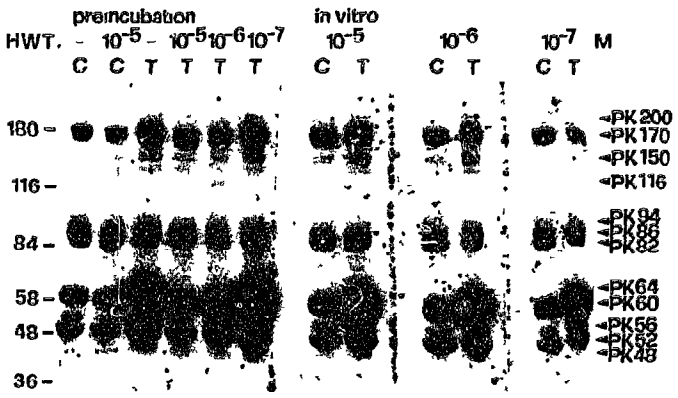

Fig 3 Comparison of HWT-treatment in intact platelets and in vitro Platelets were preincubated with HWT at different concentrations as indicated above for 10 min They were then treated with (T) or without (C) thrombin at a concentration of 1 U/ml for 20 s After renaturation and blocking the four sections of the blot were treated with different concentrations of HWT in vitro for 10 min as indicated on the 'in vitro' side of the top line The left part of the blot was treated with buffer alone All four pieces were then treated with 50 μCi/ml [γ-^{32}P]ATP for 30 min Autoradiograms were prepared from the washed and dried blots Molecular mass markers are indicated in kilodaltons

56, PK 60, PK 64 and PK 170 after thrombin treatment (Fig 1B) The inactive wortmannin derivative (IWT) had no effect on the activities of the renaturated protein kinases from either unstimulated control platelets or from thrombin-stimulated platelets (not shown) The activities of PK 82, PK 86 and PK 94 were little affected when platelets were activated with thrombin HWT did not change this The activity of PK 150 after thrombin stimulation was increased although the difference was very small This was not inhibited by HWT. PK 150 activity was also increased by PMA alone and not affected by HWT (not shown)

PK 60 was the strongest labelled band detected in this kinase assay method in platelets and densitometric analysis of the relative intensities of these bands (Fig 2) revealed that the IC_{50} of HWT was approximately 10^{-7} M At 10^{-5} M HWT the increase in activity was completely inhibited At HWT concentrations of 10^{-8} M and lower, the time course of the kinase activity followed roughly the time course of thrombin activation (data not shown, n=1).

To investigate whether HWT directly affected the protein kinases, the renaturated proteins on the membranes were subjected to in vitro HWT treatment with different concentrations after renaturation and blocking and before incubation with the radioactive ATP (Fig 3). HWT had no effect on any renaturable protein kinase in vitro. The differences in the activities of the renaturated protein kinases from control and from thrombin treated platelets were still conserved.

4 DISCUSSION

From two previous series of experiments the renaturable protein kinases from platelets could already be divided into different groups [10] according to the changes in their renaturated in vitro activity with different platelet activators in intact platelets PK 52, PK 56, PK 60, PK 64 and PK 170 were activated when platelets were treated with a combination of phorbol ester and ionophore indicating their possible involvement in signal transduction downstream of PIP_2-breakdown. PK 52 and PK 64 are most probably activated by phosphorylation as their activation is inhibited by the protein kinase inhibitor staurosporine and PK 52, PK 60, PK 150 and PK 170 are activated by dephosphorylation or another mechanism since their activation was enhanced by staurosporine [14].

In neutrophils HWT inhibited the fMLP-induced respiratory burst at a concentration of 10^{-6} M but the PMA-induced burst was not affected suggesting that HWT does not inhibit PKC directly and it does not interfere with the phospholipase C mediated rise in intracellular calcium concentration (via IP_3) and therefore a novel signal transduction pathway was proposed [12]. There is evidence that DAG production and the resulting PKC activation is not only phospholipase C dependent and the activation of phospholipase D to produce DAG via another pathway to fully activate PKC was proposed [15] This leads to the speculation that PKC activation is blocked indirectly by HWT by reducing DAG production via the other pathway Since in platelets HWT inhibits aggregation and pleckstrin phosphorylation (von Tscharner, V and Clemetson, K J , submitted) and the increase in activity of several Ser/Thr protein kinases (this paper) at a comparable concentration (10^{-5} M) to that effective in neutrophils, a similar signal transduction pathway might be affected that leads to the activation of protein kinases DAG is

not enough to activate the renaturable protein kinases since PMA alone only stimulates one of them (PK 150) and they are therefore not activated via PKC alone. The way in which PKC itself is activated suggests that it is not detected in this assay system, as it requires at least phosphatidylserine and DAG for activation [16]. As all the protein kinases inhibited by HWT (PK 48, PK 52, PK 56, PK 60, PK 64 and PK 170) are affected to roughly the same extent (Fig 1) they may all be involved in a later phase of platelet activation HWT blocks a step in signal transduction which is necessary to activate these protein kinases via covalent modifications. It was suggested that this may be a G-protein dependent step leading to phospholipase D activation [17]

While staurosporine directly blocks the protein kinases in a general way but cannot prevent their activation and even induces the activation of PK 52, PK 60 and PK 170 directly [14], HWT probably inhibits the initiation of the pathway leading to their activation The above results indicate that HWT is a useful tool for differentiating between early and late signal transduction events. Protein kinases may be involved in the regulation of other enzymes or receptors by phosphorylation and together with phosphatases may be responsible for amplification or fine tuning of signals. Since aggregation of platelets is not completely inhibited by staurosporine [18] the activation of PK 52, PK 56, PK 60, PK 64 and PK 170 is not sufficient to lead to platelet aggregation. Their inhibition by HWT correlates well with the inhibition of the late phase of platelet activation including aggregation. Other similarities are found when the kinetics of activation of these kinases are compared to the increases in Tyr-phosphorylation of platelets. Tyr-phosphorylation increases also in a group of proteins in platelets 3 minutes after thrombin stimulation [19]. Although it remains unresolved whether the renaturable protein kinases are involved in the signal transduction leading to aggregation and secretion the results obtained here suggest that activation of these protein kinases goes in parallel with these processes.

Acknowledgements We thank Prof E F Lüscher for carefully reading and criticizing the manuscript We also thank the Swiss Red Cross Central Laboratory for providing buffy coats This work was supported by the Swiss National Foundation Grant 31 25633 88

REFERENCES

[1] Wilson, B B , Neufeld, E J and Majerus, P W (1985) J Biol Chem 260, 1046–1051
[2] Berridge, M J and Irvine, R F (1989) Nature 341, 197–205
[3] Nishizuka, Y (1984) Nature 308, 693–698
[4] Kaibuchi, K , Takai, Y , Sawamura, M , Hoshijima, M , Fujikura, T and Nishizuka, Y (1983) J Biol Chem 258, 6701–6704
[5] Naka, M , Nishikawa, M , Adelstein, R S and Hikada, H (1983) Nature 306, 490–492
[6] Werth, D K , Niedel, J E and Pastan, I (1983) J Biol Chem 258, 11426–11432
[7] Connolly, T M , Lawing, W J Jr and Majerus, P W (1986) Cell 46, 951–958
[8] Gould, K L , Woodgett, J R , Cooper, J A , Buff, J E , Shalloway, D and Hunter, T (1985) Cell 42, 849–856
[9] Celenza, J L and Carlson, M (1986) Science 233, 1175–1180
[10] Ferrell, J E and Martin, G S (1989) J Biol Chem 264, 20723–20729
[11] Baggiolini, M , Dewald, B , Schnyder, J , Ruch, W , Cooper, P H and Payne, T G (1987) Exp Cell Res 169, 408–418
[12] Dewald, B , Thelen, M and Baggiolini, M (1988) J Biol Chem 263, 16179–16184
[13] Laemmli, U K (1970) Nature 227, 680–685
[14] Kocher, M and Clemetson, K J (1991) Biochem J 275, 301–306.
[15] Reinhold, S L Prescott, S M , Zimmerman, G A and McIntyre, T N (1990) FASEB J 4, 208–214
[16] Nishizuka, Y (1986) Science 233, 305–311
[17] Bonser, R W , Thompson, R W , Randall, R W , Tateson, J E , Spacey G D , Hodson, H F and Garland, L G (1991) Br J Pharmacol 103, 1237–1241
[18] Watson, S P and Hambleton, S (1989) Biochem J 258, 479–485
[19] Ferrell, J E and Martin G S (1989) Proc Natl Acad Sci USA 86, 2234–2238