Staurosporine both activates and inhibits serine/threonine kinases in human platelets

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The effects of staurosporine on a selection of protein kinases were investigated with thrombin-stimulated and control human blood platelets. The results demonstrate that staurosporine (1 μ M) can lead to activation of certain protein kinases in intact platelets and has a general inhibitory effect on the renaturable protein kinases *in vitro*. New candidates for protein kinases involved in signal transduction are identified.

INTRODUCTION

Protein kinases are involved in signal-transduction processes in human platelets. Activation with physiological stimuli leads to increased phosphorylation of five or six major and many minor proteins [1]. Activation with thrombin leads to rapid breakdown of inositides, followed by transient elevations of intracellular Ca^{2+} and diacylglycerol (DAG) [2], which activates protein kinase C (PKC) [3]. Pleckstrin (P 47) and myosin light chain are known substrates for PKC [4,5]. The myosin light chain is also phosphorylated by the calmodulin-dependent myosin light-chain kinase that is activated by an increase in intracellular Ca^{2+} concentration [6]. Protein kinases are also involved in platelet inhibition. Membrane glycoprotein Ib and actin-binding protein are phosphorylated by cyclic AMP-dependent protein kinase when platelets are treated with prostaglandin- E_1 , thus inhibiting collagen-induced polymerization of actin [7]. Tyrosine-specific protein kinases are involved in platelet activation. Platelets contain relatively high levels of pp60^{c-src}, the normal cellular homologue of the transforming protein of Rous sarcoma virus, which is a tyrosine kinase [8]. Platelet tyrosine phosphorylation is transiently elevated after thrombin stimulation [9]. The interaction of the platelet membrane glycoprotein IIb/IIIa integrin complex with peptides that inhibit fibrinogen binding affects the phosphorylation of several tyrosine kinase substrates, suggesting their involvement in platelet-aggregation pathways [10]. Phorbol esters or DAG and exogenous Ca^{2+} ionophores act synergistically to activate platelets, thus simulating the biological effects of thrombin [3]. Staurosporine, a microbial alkaloid with antifungal properties, is widely used as a PKC inhibitor [11–15]. *In vitro* PKC is affected at nanomolar concentrations, but other protein kinases are inhibited as well at the same or higher concentrations [15–17]. However, growing evidence suggests that staurosporine not only has inhibitory effects, but may also cause stimulation *in vivo* and in intact cells [18–23]. It does not prevent PKC binding to phospholipids and phorbol esters, but inhibits substrate phosphorylation and autophosphorylation at nanomolar concentrations [15] and appears to interact directly with the catalytic moiety of the enzyme [11]. In platelets it had no effect on formation of phosphatidic acid and inositol phosphates induced by thrombin, but it inhibited the phosphorylation of pleckstrin and myosin light chain [24]. Platelet aggregation induced by collagen or ADP was inhibited by staurosporine [25]. It also had some inhibitory effects on thrombin-induced aggregation of platelets, but shape change and secretion were completely

inhibited [26]. After electrophoresis and transfer of the proteins to polyvinylidene difluoride (PVDF) membranes, kinase activity could be restored by renaturation [27]. Some novel renaturable protein kinases have increased activities *in vitro* after activation of platelets, as previously reported [28]. This increased activity was measured as increased autophosphorylation and also higher phosphorylation of exogenous substrates (e.g. BSA). Renaturation of blotted proteins was used to investigate the influence of staurosporine on the activation and activities of the renaturable and covalently modified protein kinases from human blood platelets.

MATERIALS AND METHODS

Materials and chemicals

Apyrase, BSA (fraction V, and fatty-acid-free fraction V), staurosporine, acid phosphatase (from potatoes) and the pre-stained molecular-mass markers were from Sigma, St. Louis, MO, U.S.A. Thrombin was from Merck, Zurich. Iloprost was kindly given by Schering A.G., Zurich, Switzerland. Hepes was from Calbiochem, Lucerne, Switzerland. Guanidine hydrochloride was from Fluka, Buchs, Switzerland, and Nonidet P-40 was from Grogg, Berne, Switzerland. [γ -³²P]ATP (3000 Ci/mmol) was from Amersham International, Amersham, Bucks., U.K. The PVDF membranes (Immobilon-P) were from Millipore, Bedford, MA, U.S.A. Alkaline phosphatase (from calf intestine) was from Boehringer Mannheim, Rotkreuz, Switzerland.

Isolation of human platelets

Buffy coats were obtained from the Swiss Red Cross in Berne (FDA four-bag system) approx. 15 h after collection. Buffy-coat dilution buffer (0.1 vol.) was added to give final concentrations of 10 mM-sodium citrate and 0.15 unit of apyrase/ml, and the suspension was centrifuged at 150 *g* for 15 min. The platelet-rich plasma was removed and acidified to pH 6.5 with 150 mM-citric acid, iloprost was added to final concentration of 1 ng/ml, and centrifugation at 500 *g* for 15 min pelleted the platelets. They were washed with 2 $\times$ 100 vol. of 137 mM-NaCl, containing 11 mM-glucose, 11 mM-sodium citrate, 0.2 unit of apyrase/ml and 0.25 % BSA (fatty-acid-free), pH 6.5, and then resuspended in 137 mM-NaCl/2 mM-KCl/1 mM-MgCl₂/1 mM-CaCl₂/0.4 mM-NaH₂PO₄/5.6 mM-glucose/5 mM-Hepes, containing 1 unit of apyrase/ml, pH 7.5, to a final concentration of 10⁹ platelets/ml. Experiments were performed in 1.5 ml Eppendorf tubes gently

Abbreviations used: PKC, protein kinase C; DAG, diacylglycerol; PVDF, polyvinylidene difluoride.

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Fig. 1. Effect of staurosporine treatment and thrombin stimulation of intact platelets on the activities *in vitro* of the renaturable protein kinases from human blood platelets

(a) Effect of staurosporine. Portions of washed platelets from the same buffy coat were treated with different concentrations of staurosporine (StSp) as indicated. Samples were taken after fixed times given in min ($\frac{1}{3}$ min = 20 s), solubilized and treated as described in the Materials and methods section. The positions of the molecular-mass markers on the blots are indicated in kDa. (b) Effect of thrombin with staurosporine. Platelets from the same buffy coat were treated with 1 unit of thrombin/ml added together with different concentrations (none, 1 μ M, 0.01 μ M) of staurosporine (StSp). Samples were taken after times as indicated. The 0 min samples contain proteins from platelets taken just before staurosporine or thrombin was added. Molecular-mass markers are indicated in kDa. PK indicates protein kinase with molecular mass in kDa, according to nomenclature used in [28].

shaken in a water bath at 37 °C. Staurosporine was added from stock solutions in dimethyl sulphoxide to yield a final dimethyl sulphoxide concentration of 0.1 %, which did not change the pattern of phosphorylation in the renatured kinases.

PAGE

Samples were taken at fixed times and solubilized immediately in SDS/PAGE sample buffer, which contained 2.3 % SDS, 0.1 % dithiothreitol, 0.1 mM-EDTA, 62.5 mM-Tris, 10 % glycerol, pH 6.8, then vortex-mixed and boiled for 2 min. The samples were separated by electrophoresis in 7.5 %-polyacrylamide gels [29] in a mini-gel system with 30 μ g of platelet protein loaded per lane.

Renaturation procedure

Proteins were transferred to PVDF membranes by semi-dry blotting using 0.3 M-Tris/20 % ethanol, pH 10.4, as anode buffer I, 25 mM-Tris/20 % ethanol, pH 10.4, as anode buffer II, and 40 mM- ϵ -aminohexanoic acid, pH 9.4, as cathode buffer with 160 mA constant current for 1 h. The blots were then immersed for 1 h at room temperature in 7 M-guanidine hydrochloride/50 mM-Tris/50 mM-dithiothreitol/2 mM-EDTA, pH 8.3. The renaturation was performed as described by Ferrell & Martin [28] in 100 mM-NaCl/50 mM-Tris/2 mM-dithiothreitol/

2 mM-EDTA/0.1 % Nonidet P-40/1 % BSA, pH 7.5, at 4 °C for 14–16 h with gentle rocking. The membranes were blocked with 30 mM-Tris/3 % BSA, pH 7.5, at room temperature for 1 h. Kinase reactions were performed by immersing the blocked blots in 30 mM-Tris/10 mM-MgCl₂/2 mM-MnCl₂, pH 7.5, containing 50 μ Ci of [γ -³²P]ATP/ml for 30 min at room temperature. The reaction was stopped by transferring the blots to 30 mM-Tris, pH 7.5. The blots were then washed three times with 30 mM-Tris, pH 7.5, and twice with 30 mM-Tris/0.05 % Nonidet P-40, pH 7.5, followed by treatment with 1 M-KOH for 10 min at room temperature, rinsing several times with water and 10 % acetic acid, and drying. Autoradiograms were made with the dried blots from the same experiment together on the same film. The film was photographed, and Fig. 1 was prepared by aligning the appropriate lanes by cutting out empty lanes and lanes with molecular-mass markers.

Stimulation procedure *in vitro*

After renaturation and blocking, sections of the blots were treated with staurosporine in 30 mM-Tris/10 mM-MgCl₂/2 mM-MnCl₂, pH 7.5, for 10 min at room temperature. They were washed once with the same buffer without staurosporine and then incubated with the kinase reaction buffer as described above. The blots were treated accordingly with the phosphatases.

Fig. 2. Time courses of protein kinase activities

The labelled bands of the blots from which the autoradiogram in Fig. 1 was made were cut out and counted for radioactivity in a scintillation counter. Each point represents the amount of ^{32}P incorporated in one cut band in this typical experiment. The symbols represent: ○, control; ●, thrombin (1 unit/ml); △, staurosporine (1 μM); ▲, thrombin plus staurosporine. The values marked with * are corrected (-80 c.p.m.) for an elevated background in this lane on the blots.

After blocking with BSA, the sections were equilibrated with 30 mM-Tris/10 mM-MgCl₂/2 mM-MnCl₂, pH 5.0, or pH 7.5 for the control. The pH 5.0 sections were then incubated for 10 min with 10 units of acid phosphatase/ml. The reaction was stopped by rinsing several times with the pH 7.5 buffer. The sections were then incubated with the [γ - ^{32}P]ATP solution as above.

RESULTS AND DISCUSSION

In order to investigate the effect of staurosporine on re-naturable protein kinases probably involved in signal transduction, washed human platelets were activated with thrombin and treated with different amounts of the inhibitor. After blocking with BSA, the renatured proteins on the blots were overlaid with [γ - ^{32}P]ATP. Labelling of the bands agreed well with that previously reported, and the same nomenclature is used [28]. The effects of staurosporine on the activities of the renatured protein kinases were investigated (Fig. 1). To our surprise, although the activities *in vitro* of the renatured protein kinases from platelets treated with staurosporine were expected to decrease, some increased. Treatment of platelets with staurosporine (1 μM) increased the activity of three protein kinases (Fig. 1a). Staurosporine increased PK 60 (protein kinase of 60 kDa) activity strongly, whereas the increase in PK 170 activity was not

as intense as that obtained with thrombin. The activity of PK 52 reached a peak very quickly at 20 s, and then decreased slowly. In some experiments staurosporine depressed the activity of PK 56 below the control level. These effects were still detectable but weaker with 0.1 μM -staurosporine and disappeared at concentrations of 0.01 μM and less. When staurosporine (1 μM) was added together with thrombin (1 unit/ml), the kinase activities of PK 52, PK 60, PK 150 and PK 170 increased (Fig. 1b). The increases in these activities no longer correlated with the time course of the thrombin-induced increases, especially for PK 60, where the maximum was reached only at 5 min after the addition (Fig. 2). The increased activities of PK 56 and also of PK 64 after thrombin stimulation were inhibited by staurosporine, although a direct measurement of the radioactivity (c.p.m.) incorporated into PK 64 on the blots was not possible.

To investigate whether staurosporine acts directly on the protein kinases, PVDF membranes were treated with staurosporine *in vitro* after renaturation and before the incubation with labelled ATP. The activity of almost all the kinases was completely blocked by staurosporine at 10 μM , except for a very weak remnant activity of PK 52, PK 60 and PK 170 (Fig. 3). With lower concentrations (0.01 μM) the inhibition of these kinases was weaker, and the increase in activity of PK 52, PK 56, PK 60 and PK 170 with the thrombin-treated platelets compared with the control re-appeared. Staurosporine may affect the kinases

Fig. 3. Comparison of staurosporine treatment in intact platelets and *in vitro*

Platelets were preincubated with staurosporine (StSp) at different concentrations as indicated in the Figure for 10 min. They were then treated with (T) or without (C) thrombin at a concentration of 1 unit/ml for 20 s. The activities of the protein kinases that increase after thrombin stimulation had not yet reached a peak, in contrast with those which increased after preincubation with staurosporine (see the text). After renaturation and blocking, the three sections of the blot were treated with different concentrations of staurosporine *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 overlaid with 50 μ Ci of [γ - 32 P]ATP/ml for 30 min. Autoradiograms were prepared from the washed and dried blots. Molecular-mass markers are indicated in kDa.

from control and thrombin-treated platelets differently, since the relatively higher intensities found with the latter disappear with increasing staurosporine concentrations. The group of protein kinases between 80 and 100 kDa (PK 82, PK 86, PK 94) was still completely inhibited at 0.01 μ M-staurosporine, although after renaturation they seemed to have been unaffected by staurosporine treatment of intact platelets (Fig. 1). These three kinases have masses similar to those of PKC (80 kDa) [30]. However, the way in which PKC is activated suggests that it is not detected in this assay system, as it requires at least phosphatidylserine and DAG for activation [31]. The inconsistent weak activity changes after thrombin stimulation also argue against the involvement of the kinases detected in this position in signal transduction. In addition, purified PKC from platelets could not be detected in the kinase assay after SDS/PAGE, Western blotting and renaturation (results not shown).

Phosphorylations of proteins such as pleckstrin that increase during platelet activation are inhibited by staurosporine [24]. In addition, staurosporine also depresses the level of phosphorylation of several phosphoproteins in resting platelets compared with control values (V. von Tscharner & K. J. Clemetson, unpublished work). Interaction with the catalytic subunit of the protein kinases, probably by blocking the ATP-binding site, are responsible for the inhibition [11]. The direct effect of staurosporine is due to non-covalent interaction with the enzymes, and activity is restored after renaturation (Fig. 3). As staurosporine inhibits all the detected protein kinases *in vitro*, it probably has the same effect in intact platelets.

Treatment of the renaturated proteins on the blocked blots with acidic phosphatase to create newly vacant phosphorylation sites before treatment with [γ - 32 P]ATP did not result in increased labelling of bands (Fig. 4). The same was found with alkaline phosphatase (results not shown). The slight overall decrease in activity might be due to hydrolysis of ATP by a small remnant of phosphatase that bound to the membrane. A specific and

marked decrease in the activity of PK 170, PK 150 and PK 60 was found that must be due to the direct action of the acid phosphatase on the proteins. This suggests an inactivation of these protein kinases by dephosphorylation *in vitro*. With alkaline phosphatase the specific decreases in the activities of PK 170 and PK 150 were not found, and the decrease in PK 60 was not so pronounced. These results show that non-specific dephosphorylation of the renaturated proteins on the blots does not increase the apparent activity of the protein kinases by providing free sites for phosphorylation. This suggests an increase in the specific activity of the renaturated protein kinases. The activity towards exogenous substrates (e.g. BSA) is increased by staurosporine in the same way as by thrombin. The pattern (SDS/PAGE) of phosphoproteins in the bands from renaturated blots treated with [γ - 32 P]ATP from staurosporine- or thrombin-treated platelets was conserved (result not shown). Changes in the activity of the protein kinases are most probably due to covalent modifications, as they are conserved after denaturation and renaturation. Possible covalent modifications involved in activation of protein kinases in intact platelets are phosphorylation and dephosphorylation where protein phosphatases are also involved. Other mechanisms could be proteolysis or modification with other functional groups where the regulation might be more complex. Without directly purifying the protein kinases, the method used here provides the possibility of predicting their most probable regulation. From the results with staurosporine presented here, it is possible to divide the renaturable protein kinases from platelets into two groups. First, the protein kinases that are activated by phosphorylation increase in activity if they are involved in signal transduction. If they are controlled by a feedback inhibition the increase might only be transient. When staurosporine blocks all the protein kinases in platelets, this thrombin-induced increase should disappear. This is the case for PK 56 and PK 64. Secondly, for protein kinases that increase in activity after staurosporine treatment, such as PK 52, PK 60,

Fig. 4. Effect of phosphatase treatment on the activities *in vitro* of the renaturated protein kinases

Platelets were treated with 1 unit of thrombin/ml (T) for 3 min, with staurosporine at $1 \mu\text{M}$ (S) for 5 min, or with both (ST) for 5 min. Control lanes are indicated with C. After SDS/PAGE, blotting and blocking with 3% BSA, the right section of the blot was treated with acid phosphatase (P'ase) before the [γ - ^{32}P]ATP treatment as described in the Materials and methods section. Autoradiograms were prepared from the washed and dried blots. Molecular-mass markers are indicated in kDa.

PK 150 and PK 170 (Figs. 1 and 2), activation other than via phosphorylation can be postulated. This includes the possibility that the kinase is activated by a dephosphorylation step. Again, the increase of activity after thrombin stimulation of the platelets becomes transient when the kinase is under feedback-inhibition control. This increase cannot be inhibited by staurosporine. In addition, staurosporine stimulates their activation. This could be explained by a higher activity of the protein phosphatases (or other enzymes), owing to the activation of platelets with thrombin leading to the larger increase observed with PK 60. Protein kinases that show no change in activity after thrombin treatment with or without staurosporine, or with staurosporine alone, are probably not involved in signal transduction. Such kinases have a constant activity, and no phosphorylation or dephosphorylation is responsible for their activation, as staurosporine would have affected that process. A special case might be protein kinases that are activated by phosphorylation but remain unchanged with thrombin stimulation. That constant activity would decrease with staurosporine. Such protein kinases were not observed. PK 150 is activated in platelets treated with phorbol 12-myristate 13-acetate alone [28], suggesting activation in response to DAG [3]. As this kinase is not activated by staurosporine alone, and the transient increase of activity that reaches a peak after 1 min of thrombin treatment (Fig. 2) is not affected, phosphorylation by PKC or modification by an enzyme downstream from PKC in the phosphorylation cascade can be excluded. A direct involvement of DAG or even another pathway for its activation must be proposed.

These results clearly indicate that staurosporine has not only inhibitory effects in intact platelets. Other biological responses point also in this direction. Recently, staurosporine has been shown to stimulate various responses concurrent with inhibition of phosphorylation in several cell types [18–23]. *In vitro*, staurosporine induced the association of purified PKC with inside-out vesicles from erythrocyte membranes in a concentration-dependent manner [32], suggesting that phorbol 12-myristate 13-acetate and staurosporine act similarly, but, in

addition, phosphorylation was inhibited. In the present study we give evidence that staurosporine affects a set of renaturable protein kinases in intact human platelets, leading to activation of three of them (PK 52, PK 60 and PK 170) and inhibiting the activation of one (PK 56). In contrast, it inhibited all the detectable serine/threonine kinases in platelets *in vitro* in a concentration-dependent manner. This shows that staurosporine, as well as inhibiting kinases directly, can also cause increased platelet responses. As long as no protein kinase is found which is unaffected or even stimulated directly *in vitro* by staurosporine, this method distinguishes between two general ways of protein kinase control. PK 52 and PK 64 are probably activated by phosphorylation. PK 52 may be activated by another mechanism, including dephosphorylation. As phosphatase treatment *in vitro* decreased the activities of PK 60, PK 150 and PK 170 (Fig. 4), activation by another mechanism that is not dephosphorylation can be proposed.

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REFERENCES

- Haslam, R. J., Salama, S. E., Fox, J. E. B., Lynham, J. A. & Davidson, M. M. L. (1980) in *Platelets: Cellular Response Mechanisms and their Biological Significance* (Rotman, A., Meyer, F. A., Gitler, C. & Silberberg, A., eds.), pp. 213–231, John Wiley and Sons, Chichester
- Berridge, M. J. & Irvine, R. F. (1989) *Nature* (London) **341**, 197–205
- Nishizuka, Y. (1984) *Nature* (London) **308**, 693–698
- Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 6701–6704
- Naka, M., Nishikawa, M., Adelstein, R. S. & Hikada, H. (1983) *Nature* (London) **306**, 490–492
- Hathaway, D. R. & Adelstein, R. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1653–1657
- Fox, J. E. B. & Berndt, M. C. (1989) *J. Biol. Chem.* **264**, 9520–9526
- Golden, A., Nemeth, S. P. & Brugge, J. S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 852–856
- Ferrell, J. E. & Martin, G. S. (1988) *Mol. Cell. Biol.* **8**, 3603–3610
- Ferrell, J. E. & Martin, G. S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2234–2238
- Nakadate, T., Jeng, A. Y. & Blumberg, P. M. (1988) *Biochem. Pharmacol.* **37**, 1541–1545
- Linden, D. L. & Routtenberg, A. (1989) *J. Physiol. (London)* **419**, 95–119
- Wong, R. C. K., Remold-O'Donnell, E., Vercelli, D., Sancho, J., Terhorst, C., Rosen, F., Geha, R. & Chatila, T. (1990) *J. Immunol.* **144**, 1455–1460
- Friedman, B., Fujiki, H. & Rosner, M. R. (1990) *Cancer Res.* **50**, 533–538
- Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402
- Staats, J., Marquardt, D. & Center, M. S. (1990) *J. Biol. Chem.* **265**, 4084–4090
- Nakano, H., Kobayashi, I., Takahashi, I., Tamaoki, T., Kuzuu, Y. & Iba, H. (1987) *J. Antibiotics* **40**, 706–708
- Sako, T., Tauber, A. I., Jeng, A. Y., Yuspa, S. H. & Blumberg, P. M. (1988) *Cancer Res.* **48**, 4646–4650
- Hashimoto, S. & Hagino, A. (1989) *Exp. Cell Res.* **184**, 351–359
- Morioka, H., Ishihara, M., Shibai, H. & Suzuki, T. (1985) *Agric. Biol. Chem.* **49**, 1959–1964
- Dewald, B., Thelen, M., Wymann, M. P. & Baggiolini, M. (1989) *Biochem. J.* **264**, 879–884
- Reinhold, S. L., Prescott, S. M., Zimmerman, G. A. & McIntyre, T. M. (1990) *FASEB J.* **4**, 208–214
- Hedberg, K. K., Birrell, G. B., Habliston, D. L. & Griffith, O. H. (1990) *Exp. Cell Res.* **188**, 199–208
- Watson, S. P., McNally, J., Shipman, L. J. & Godfrey, P. P. (1988) *Biochem. J.* **249**, 345–350

25. Oka, S., Kodama, M., Takeda, H., Tomizuka, T. & Suzuki, T. (1986) *Agric. Biol. Chem.* **50**, 2723–2727
 26. Watson, S. P. & Hambleton, S. (1989) *Biochem. J.* **258**, 479–485
 27. Celenza, J. L. & Carlson, M. (1986) *Science* **233**, 1175–1180
 28. Ferrell, J. E. & Martin, G. S. (1989) *J. Biol. Chem.* **264**, 20723–20729
 29. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
 30. Watanabe, M., Hagiwara, M., Onoda, K. & Hikada, H. (1988) *Biochem. Biophys. Res. Commun.* **152**, 642–648
 31. Nishizuka, Y. (1986) *Science* **233**, 305–311
 32. Wolf, M. & Baggiolini, M. (1988) *Biochem. Biophys. Res. Commun.* **154**, 1273–1279
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