Pergamon

Biochemical Pharmacology, Vol. 47, No. 10, pp. 1797–1804, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0025-G

STAUROSPORINE UP-REGULATES THE EXPRESSION OF PHORBOL DIBUTYRATE BINDING SITES IN HUMAN PLATELETS

SAMIRA RAIS,* CHRISTOPHE COMBADIERE,* JACQUES HAKIM* and AXEL PERIANIN†‡

*Laboratoire d'Hématologie, INSERM Unité 294, Hôpital Bichat, 75018 Paris and
†Departement de Pharmacologie, CNRS URA 595, Hôpital Cochin, 75014 Paris, France

(*Received* 9 *August* 1993; *accepted* 10 *January* 1994)

Abstract—Tumor-promoting phorbol esters bind to and activate protein kinase C (PKC). Staurosporine, a potent PKC inhibitor, interferes with PKC catalytic activity without altering phorbol ester binding sites in cell-free systems. We found that, unlike cell-free systems, treatment of intact platelets with staurosporine enhances the expression of phorbol 12,13-dibutyrate (PDBu) binding sites. Incubation of platelets at 37° with staurosporine (25 nM to 1 μM and 2 nM tritiated PDBu ([^{3}H]PDBu) increased the amount of [^{3}H]PDBu specifically bound to intact platelets by approximately 10 to 200% of control values. This effect was rapid and plateaued after 10 min of cell treatment. Scatchard analysis of the data showed that staurosporine (500 nM) significantly increased the total binding capacity B_{max} from $42.9 \pm 15.4 \times 10^3$ to $78 \pm 7.3 \times 10^3$ sites per platelet and reduced the apparent dissociation constant value K_d from 30.8 ± 8.6 nM to 9.4 ± 3.4 nM. Enhanced PDBu binding capacity and affinity were also observed with human mononuclear and polymorphonuclear leukocytes. Fractionation of staurosporine-treated platelets showed an increased binding capacity of the particulate fraction (102%) and decreased binding capacity of the soluble fraction (60%) compared to controls, with no change in the affinity of PDBu binding to these fractions. Chelation of internal calcium with BAPTA did not significantly attenuate the staurosporine-mediated rise in PBDu binding but prevented the platelet-activating factor-induced response, indicating that cytosolic calcium does not play an important role in these staurosporine effects. These results show that, in addition to interfering with PKC protein-phosphorylating activity, staurosporine enhances PDBu binding affinity and capacity in intact platelets. This latter effect appears to be due to translocation of soluble PDBu binding sites, presumably PKC units.

Key words: platelet; staurosporine; protein kinase C; phorbol 12,13-dibutyrate; translocation; leukocyte

PKC§ comprises a family of related enzymes which take part in cellular functions induced by various agonists [1]. Three groups of PKC have been identified: conventional PKCs which are activated by calcium, phospholipids and DAG, novel PKCs which are activated independently of calcium, and atypical PKCs which lack DAG and calcium binding sites [2, 3]. Stimulation of cells is usually associated with a redistribution of PKC in which the soluble form of the enzyme is converted to a particulate bound form [4–7]. Tumor-promoting agents such as phorbol esters substitute for DAG and activate PKC [8, 9]. Pharmacological analysis of cellular responses mediated by phorbol esters show that these agents bind to specific sites that can be measured using [^{3}H]PDBu or phorbol myristate acetate [10–13].

Staurosporine, a microbial alkaloid, is a potent PKC inhibitor [14] which interferes with the catalytic activity of PKC without altering PDBu binding [14, 15]. Staurosporine both induces tumors [16] and inhibits phorbol ester-mediated tumor promotion [17]. We recently reported that treatment of human PMN with staurosporine enhances the expression of PDBu binding sites by increasing both binding affinity and capacity [18]. In platelets, staurosporine was also found to enhance PDBu binding affinity with, however, no change in binding capacity [19], suggesting a different regulation of this latter parameter. To gain insight into the action of staurosporine, we reassessed PDBu binding to human platelets in conditions used for PMN. Staurosporine increased both PDBu binding affinity and capacity in these cells at 37°. However, in contrast to PMN, the enhancing effect of staurosporine in platelets decreased at low binding temperatures. Fractionation of platelets indicated that staurosporine may promote redistribution of PDBu binding sites.

‡ Corresponding author: A. Périanin, Laboratoire d'Hématologie, INSERM Unité 294, C.H.U.X. Bichat, 46 rue H. Huchard, 75018 Paris, France. FAX 33 (1) 40 25 88 53.

§ Abbreviations: PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; HBSS, Hanks balanced salt solution; DAG, diacylglycerol; PS, phosphatidylserine; PMN, polymorphonuclear leukocytes; Paf, platelet activating factor; BAPTA/AM, bis-(*O*-aminophenoxy-ethane-*N*,*N*,*N*′,*N*′-tetracetic acid, tetra (acetoxymethyl)-ester; B_{max}, total binding capacity.

MATERIALS AND METHODS

Reagents. Staurosporine was obtained from the Kamiya Biochemical Co. (Thousands Oaks, CA, U.S.A.). [^{32}P]ATP (sp. act. 30–40 Ci/mmol), [^{3}H]-PDBu (sp. act. 20 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). Hionic Fluor was obtained from Packard, BV

(Breda, The Netherlands). Other reagents were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Platelet preparation. Platelets were prepared from citrated human blood as described in [20]. Briefly, blood was centrifugated at 150 *g* for 15 min at 20°. The platelet-rich plasma (10 mL) was treated with 1 mL of a citrate solution containing 38 mM citric acid and 75 mM sodium citrate and centrifuged (150 *g*, 10 min, 20°). Platelets were washed twice by centrifugation at 20° (1000 *g*, 10 min) with Tris-citrate buffer (pH 6.4) composed of 63 mM Tris, 95 mM NaCl, 5 mM KCl, 12 mM citric acid. Platelets were resuspended in the same buffer and counted with a Coulter counter. All the experiments with intact platelets were run within 4 hr following cell preparation.

Isolation of mononuclear and polymorphonuclear leukocytes. PMN and mononuclear cells were separated by one-step centrifugation of heparinized human venous blood over a cushion of a mixture of Ficoll and Hypaque (Monopoly Resolving Medium form), as described previously [18, 21]. The purified mononuclear cells were washed and stored at 4° in HBSS until use. PMN were subjected to hypotonic lysis, washed and resuspended in HBSS.

[^{3}H]PDBu binding to intact cells. PDBu binding to intact cells was performed as described previously [18]. Briefly, suspensions of 6–12 × 10^6 platelets or 2.5 × 10^6 MN or PMN in 500 μL of Tris-citrate containing 0.1% BSA were preincubated at 37° for 5 min and then treated in the absence (control) or presence of staurosporine for the indicated times and concentrations. Cells were then incubated with 2 nM [^{3}H]PDBu for 10 min in the absence or presence of 2 μM unlabelled PDBu to estimate non-specific binding. Cells were rapidly diluted with cold 0.5% DMSO, collected on Whatman GF/C filters, and counted as described previously [18]. Specific binding was calculated as the difference between total and non specific binding. B_{max} and K_d were determined at equilibrium by incubating control and staurosporine-treated cells with various [^{3}H]PDBu concentrations for 20 min at 37° or for 90 min at 4°. Binding data were analysed by using the LIGAND program developed by Munson and Rodbard [22].

Preparation of soluble and particulate platelet fractions. Platelet suspensions were incubated in the absence (control) or presence of 500 nM staurosporine for 15 min at 37° and pelleted by centrifugation (1000 *g*, 15 min) at 20°. Pellets were resuspended in 500 μL of lysis buffer containing 20 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 2 mM PMSF, 0.1% leupeptine, 50 mM 2-mercaptoethanol, 2 mM EDTA and 5 mM EGTA. The cells were then disrupted by sonication at 25 W (four 10-sec bursts) using a Fisher model 20/200 SV TC4C and centrifuged at 1000 *g* for 10 min. The supernatant was spun down at 100,000 *g* for 60 min in a Beckman TL100 and the soluble fraction was separated from the pellets (particulate fraction). The particulate fraction was resuspended in 250 μL of lysis buffer containing 0.1% of Triton X-100 and homogenized by sonication (two 10-sec bursts) at 4°; the protein content was determined by the Lowry method [23].

PDBu binding to subcellular fractions. The incubation mixture (250 μL) containing 25 μg of particulate fraction or 10 μg of soluble fraction, 100 μg PS, 1 mM $CaCl_2$, 20 mM Tris–HCl, pH 7.5 was incubated at 4° with 0.25 to 16 nM [^{3}H]PDBu in the absence or presence of a 10-fold concentration of unlabelled PDBu, as described previously [18, 24]. After 90 min of incubation, samples were filtered through Whatman GF/C paper and washed with 3 × 3 mL of ice-cold 0.5% DMSO.

Protein kinase C activity assay. Particulate fractions were treated with 0.3% Triton then centrifuged at 100,000 *g* for 30 min at 4°; the supernatant, referred to as the extractable particulate fraction, was collected and used to assess the incorporation of ^{32}P into histone, as described previously [5, 18]. The pellets were suspended in 250 μL of lysis buffer containing 0.1% Triton X-100 and homogenized by sonication. This latter fraction, referred to as the non-extractable particulate fraction, failed to show PKC activity. PKC activity was expressed in pmoles of ^{32}P incorporated per min per mg protein and was calculated by subtracting the activity measured in the absence of calcium, PS and diolein from that measured in their presence.

Statistical analysis. Results are expressed as means ± SD of at least three experiments performed in duplicate. Statistically significant differences between sets of data were determined by using Student's paired *t*-test.

RESULTS

Staurosporine effect on [^{3}H]PDBu binding in human platelets

Treatment of platelets with staurosporine concentrations from 25 nM to 1 μM for 15 min at 37° and with 2 nM [^{3}H]PDBu caused a concentration-dependent increase in the amount of PDBu bound to platelets (Fig. 1, upper panel). The maximal enhancement reached 189 ± 14% ($P < 0.001$) of control values and was obtained with 1 μM staurosporine, a concentration which had no detectable cytotoxic effect and did not induce cell aggregation (results not shown). The increase in PDBu binding was rapid, reaching 77 ± 9% ($P < 0.05$) above control values within 3 min and plateauing after 10 min (Fig. 2, lower panel). Non specific PDBu binding represented 10–15% of total binding and was not altered by staurosporine.

To characterize further the PDBu binding alterations, binding experiments were performed with [^{3}H]PDBu concentrations from 0.6 to 45 nM at 37°. Scatchard analysis of the data shows the presence of a single population of PDBu binding in untreated cells, with an apparent K_d of 30.8 ± 8.6 nM and a B_{max} of 42.9 ± 15.4 × 10^3 sites/platelet (Fig. 2 and Table 1). Treatment of platelets with 500 nM staurosporine increased the B_{max} by approximately 85% and increased the PDBu binding affinity 3-fold. Because the high PDBu concentrations used might induce platelet activation and influence the effects of staurosporine, we analysed PDBu binding at 4° using cells pretreated at 37° with and without 500 nM staurosporine. In steady-state equilibrium conditions, the total amount of [^{3}H]PDBu bound to cells not treated with staurosporine did not differ

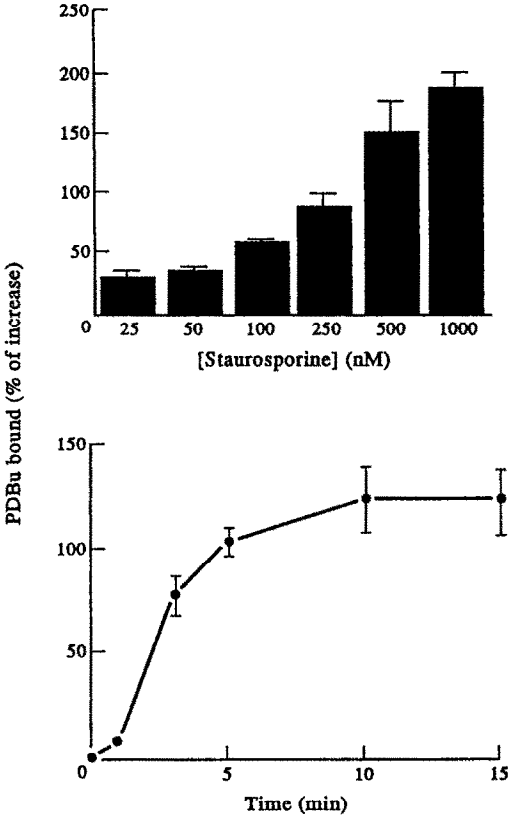

Fig. 1. Effect of staurosporine on [^{3}H]PDBu binding to platelets. Platelets were incubated for a total period of 10 min with 2 nM tritiated PDBu in the absence or presence of 2 μM unlabelled PDBu, to evaluate non-specific binding. Cells were treated in the absence (control) or presence of the indicated drug concentrations for 15 min, including a 10 min-period with PDBu (upper panel) and with 500 nM of staurosporine for the indicated periods at 37° (lower panel). Results are the means of three independent experiments and are expressed as % ±SD of control values, i.e. 1839 ± 369 dpm (upper panel) and 1445 ± 96 dpm (lower panel).

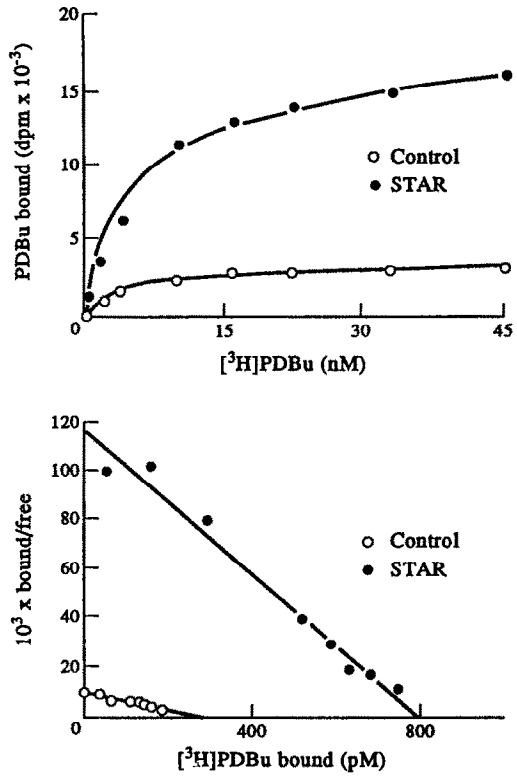

Fig. 2. Effect of staurosporine on kinetic parameters of PDBu binding to platelets. Platelets were incubated with 0.6 to 45 nM [^{3}H]PDBu concentrations for 5 min and then treated in the absence (control) or presence of 500 nM staurosporine (STAR) for 15 min at 37° (upper panel). A Scatchard analysis of the PDBu binding is shown in (lower panel). Results are derived from a single experiment performed in duplicate. Similar results were obtained in two other independent experiments.

Table 1. Kinetic parameters of PDBu binding to control and staurosporine-treated intact platelets

	Binding parameters	
Staurosporine	$10^{-3} \times B_{max}$ (sites/cell)	K_d (nM)
–	42.9 ± 15.4	30.8 ± 8.6
+	78.8 ± 7.3*	9.4 ± 3.4*

Platelets were treated as in Fig. 2. The K_d and B_{max} were calculated from the data of three separate experiments.
* Statistically significant difference between control and staurosporine-treated platelets ($P < 0.05$) ($N = 3$).

from that obtained at 37° (Fig. 3), suggesting that the bound PDBu measured at 37° was mainly present on the cell surface. Staurosporine still potentiated this binding, but less that at 37° (Fig. 3). When platelets were treated with staurosporine at 4° and PDBu binding was measured at the same temperature, staurosporine did not alter the binding parameters (data not shown), suggesting that enhanced expression of PDBu binding sites (Figs 1 and 2) may be mediated through alterations of biochemical processes induced by staurosporine. With mononuclear and PMN, staurosporine also induced a 3–4-fold enhancement of PDBu binding affinity and increased binding capacity by approximately 80% (Table 2).

Staurosporine promotes redistribution of PDBu binding sites in platelets

Potential mechanisms for this enhanced binding capacity include a redistribution of PDBu sites binding from the cytosol to the plasma membrane and/or an unmasking effect on cryptic-membrane PDBu binding sites. To examine the possibility that staurosporine induces PDBu binding site translocation binding assays were performed with particulate and soluble fractions derived from control

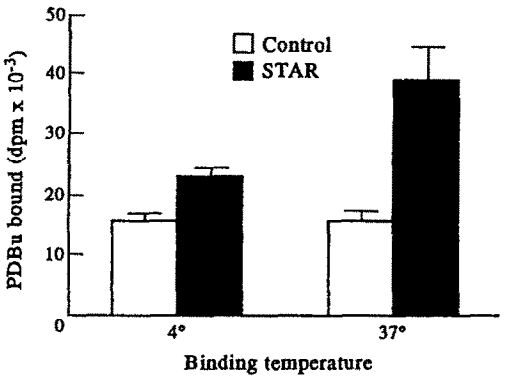

Fig. 3. Effect of temperature on PDBu binding to control and staurosporine-treated platelets. Cells were first treated in the absence (control) or presence of 500 nM staurosporine (STAR) for 15 min at 37°. Some cells were then incubated at 4° for 10 min before being treated with 35 nM tritiated PDBu for 90 min. The other cells were assayed for PDBu at 37° for 10 min. Each column represent the mean ± SD of the amount of PDBu specifically bound to platelets.

Table 2. Effect of staurosporine on PDBu binding to polymorphonuclear and mononuclear cells

		Binding parameters	
	Staurosporine	$10^{-3} \times B_{max}$ (sites/cell)	K_d (nM)
PMN	−	181 ± 21	9.6 ± 1.8
	+	294 ± 45*	3.5 ± 0.4*
MN	−	287 ± 57	16.6 ± 2.2
	+	510 ± 54*	4.2 ± 0.2*

Cells were incubated with 1–45 nM tritiated PDBu for 5 min at 37° and then in the absence (control) or presence of 500 nM staurosporine (STAR) for 15 min. Results represent the means ± SD of three separate experiments.
* A statistical significance between control and staurosporine-treated platelets.

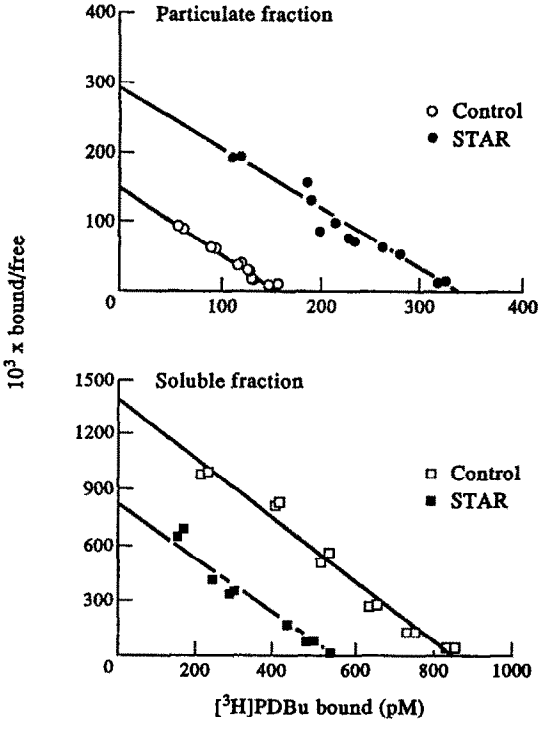

Fig. 4. Kinetic parameters of PDBu binding to subcellular fractions from control and staurosporine-treated platelets. Cells were incubated in the absence or presence of 500 nM staurosporine (STAR) for 15 min at 37° and pelleted by centrifugation. They were then fractionated into particulate and cytosolic fractions. The binding assays were carried out by incubating 25 μg of particulate fraction (upper panel), or 10 μg of soluble fraction (lower panel), with 0.25–20 nM tritiated PDBu for 90 min at 4°. The results are derived a single experiment run in duplicate. Similar results were obtained in two other experiments.

and staurosporine-treated platelets. Scatchard analysis of the data showed the presence of a single population of PDBu binding sites in both fractions from untreated platelets (Fig. 4). However, these fractions exhibited higher PDBu binding affinity than in intact platelets. Treatment of cells with 500 nM staurosporine failed to alter PDBu affinity to either fraction (Table 3). By contrast, staurosporine increased the binding capacity of the particulate fraction by 102 ± 6% ($P < 0.01$) and decreased that of the soluble fraction by 60 ± 11% ($P < 0.01$).

To examine a possible unmasking effect of staurosporine on cryptic-membrane PDBu binding sites, the particulate fraction of untreated platelets (control) was incubated in the absence or presence of staurosporine concentrations from 50 to 500 nM and assayed for PDBu binding. As shown in Fig. 5, staurosporine had no effect on PDBu binding to the particulate fraction, in agreement with reported

Table 3. Effect of staurosporine on PDBu binding to subcellular fractions

		Binding parameters	
	Staurosporine	$10^{-3} \times B_{max}$ (sites/cell)	K_d (nM)
Particulate	−	10.6 ± 3.9	1.3 ± 0.4
	+	20.7 ± 3.3*	1.4 ± 0.3
Soluble	−	53.7 ± 5.9	0.6 ± 0.06
fraction	+	32.2 ± 3.2*	0.7 ± 0.05

Platelets were incubated in the absence or presence of 500 nM staurosporine for 90 min at 4° and pelleted by centrifugation. Cells were fractionated as described in Fig. 5. The K_d and B_{max} are expressed per cell-equivalent. Means ± SD were from three separate experiments.
* Significant difference between control and staurosporine-treated platelets ($P < 0.05$) ($N = 3$).

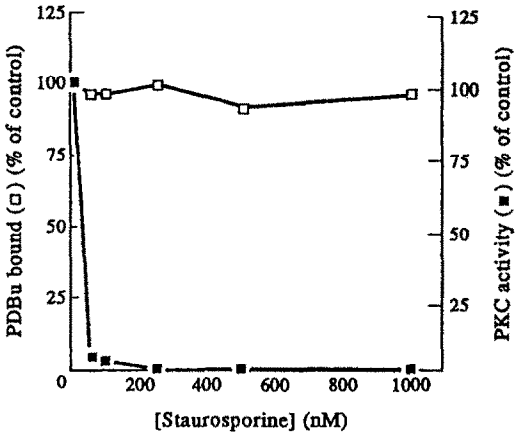

Fig. 5. Effect of staurosporine on PDBu binding to the particulate fraction and on histone phosphorylation. Particulate fractions were incubated in the absence (control) or presence of the indicated staurosporine concentrations. PKC activity was measured in terms of histone phosphorylation (■) and [^{3}H]PDBu binding (□) and is expressed as the percentage of control values (326 ± 35 pmol/min/mg protein and 12.787 ± 781 dpm/50 μg protein, respectively.

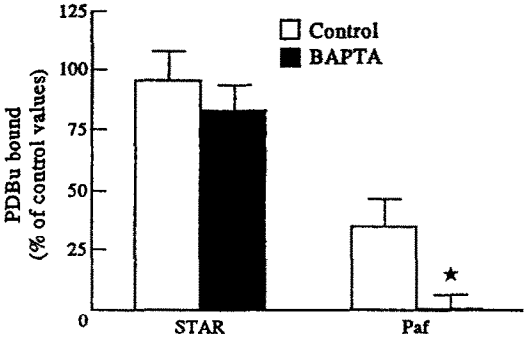

Fig. 6. Effect of BAPTA on PDBu binding to staurosporine- and Paf-treated platelets. Cells were first treated in the absence (control) or presence of 30 μM BAPTA/AM for 30 min at 37°, then washed and treated at 37° for 10 min with 35 nM tritiated PDBu in the absence of presence of 35 μM cold PDBu. Before the end of this latter treatment, cells were incubated in the absence or presence of either 500 nM staurosporine for 10 min or 1 μM Paf for one min. Results represent the net increase in PDBu binding induced by staurosporine and Paf, and are expressed as % of the values for resting cells [respectively, 20.004 ± 2.785 (control) and 19.806 ± 2.854 (BAPTA) dpm per 12 × 10^6 cells].

findings [15]. However, the PKC histone-phosphorylating activity of particulate fractions was strongly inhibited, indicating that staurosporine acts on membrane PKC. The phosphorylation of histone in the absence of calcium and PS was also completely inhibited (result not shown). These results suggest that the staurosporine-mediated increase in the expression of PDBu binding sites may not be due to unmasking of cryptic-membrane PDBu sites.

Effect of BAPTA on PDBu binding to staurosporine- and Paf-treated platelets

Cytosolic calcium has been shown to prime the expression of PDBu binding by cells stimulated with thrombin [19] and Paf [6, 25]. To determine a possible role of cytosolic calcium in the staurosporine-mediated enhancement of PDBu binding capacity, cells were treated with a permeant calcium chelator, BAPTA and assayed for PDBu binding at steady state in the presence of staurosporine or Paf. The results in Fig. 6 show that BAPTA did not significantly alter the rise in PDBu binding mediated by staurosporine but completely inhibited the Paf-mediated rise in PDBu binding. BAPTA had no effect on PDBu binding resting cells. These data suggest that intracellular calcium does not play an important part in the mechanism of PDBu binding site redistribution by staurosporine.

DISCUSSION

Our results show that staurosporine enhances the specific binding of PDBu to platelets, which is assumed to bind to PKC [10–13]. This phenomenon, also observed with PMN [18] and mononuclear cells (Table 1), comprises a rise in both K_d and B_{max} values (Fig. 2, Table 1). The order of increase in PDBu binding expression at 37° was similar with platelets, PMN and MN, i.e. approximately 80% in B_{max} and a 3-fold enhancement of K_d (Table 2), although differences in the two parameters were observed according to the cell type. Unlike PMN [18], platelets showed a markedly smaller increase in B_{max} at 4° than at 37° (Fig. 3). This may explain why binding experiments performed at 0° failed to show changes in the PDBu binding capacity of staurosporine-treated platelets, although modifications in binding affinity were preserved [19]. A possible interpretation of these temperature effects is that the rise in binding capacity of platelets at 37° may result from a combined effect of staurosporine and PDBu; Phorbol esters have been reported to stimulate both platelet activation and aggregation [25]. In our conditions of PDBu binding measurement (absence of divalent cations), staurosporine and PDBu failed to induce platelet aggregation (results not shown). It is therefore likely that the increased expression of PDBu binding resulted from a synergistic effect of both agents but not to platelet aggregation.

The rise in PDBu binding capacity of staurosporine-treated platelets was associated with a 2-fold increase in B_{max} of the particulate fraction and a decrease in binding capacity of the cytosol (Table 3). Although a loss of soluble binding sites was observed in staurosporine-treated cells, likely due to proteolysis, these data suggest that staurosporine induces association of PDBu binding sites to the particulate fraction. A translocation of PKC by other agents that are not structurally related to phorbol esters has been described, particularly in rat kidney cells treated with okadaic acid or calyculin A [26], both of which are protein phosphatase inhibitors.

Thapsigargin, a calcium ATPase inhibitor [27] that does not appear to inhibit PKC [28], also induces redistribution of PKC ε and δ GH4Cl cells [29], whereas okadaic acid failed to alter the distribution of these isoforms. These results suggest that alterations of PDBu binding expression induced by staurosporine may occur through indirect rather than direct interaction of staurosporine with PKC. Moreover, the observation that PKC redistribution occurs with high concentrations of staurosporine which blocks PKC activation raises the possibility that phosphorylation may not be required for enzyme redistribution. Further support for this assumption is that, in cell-free systems in the absence of ATP, staurosporine promotes association of PDBu binding sites to the cell membrane [30, 18]. Alternatively, the mechanism whereby staurosporine induces PKC translocation may occur through inhibition of PKC dissociation from the particulate fraction or to proteolysis-induced PKC down-regulation. Further studies are required to clarify these points.

PKC association to the cell membrane has been shown to be regulated by calcium in a different manner. In platelets, for example, thrombin induces a translocation of PKC which is either sustained or transient depending on the presence or absence of external calcium [7]. The calcium mobilizing agonist fMLP induces transient PKC translocation through both cytosolic calcium-independent and calcium-dependent mechanisms [6]. Phorbol esters stimulate sustained and tight PKC binding to cell membranes, which becomes resistant to extraction in calcium chelator-containing buffers [5, 25, 31]. The increase in PBDu binding induced by staurosporine was persistent (Fig. 1). Furthermore, with cells depleted of calcium with BAPTA (Fig. 6) or with Fura 2 in the presence of EGTA [18], staurosporine markedly enhanced PDBu binding capacity. This suggests that calcium may not play an important role in the staurosporine-mediated rise in PDBu binding capacity of platelet, unlike the rise in PDBu affinity [18, 19].

The use of antibodies against PKC isoforms has shown that human platelets contain predominantly PKC α, βI, βII and δ with minor immunoreactivity for PKC ε, ζ and η [7, 32, 33]. Thrombin and PMA induce translocation of all these isoforms [7] whereas oleic acid induces a selective translocation of PKC α, βII and δ, with little effect on βI [33]. It is not known which PKC isoform is redistributed in staurosporine-treated platelets. In GH4Cl cells, in which PKC α, β, γ, δ and ε have been identified [34], staurosporine only induced translocation of PKC ε and, to a lesser extent, PKC δ, indicating that redistribution of PKC δ and ε may be regulated by a process that does not influence other isoforms [29]. A translocation of PKC ε by staurosporine also occurs in SH-SY5Y neuroblastoma cells [35]. PKC ε does not associate with the particulate fraction in the presence of an excess of calcium *in vitro* [36] or *in vivo* [37]. It has therefore been suggested that calcium mobilization may be involved indirectly in PKC ε redistribution [29]. We have recently found that staurosporine, by itself, induces stimulation of phospholipase D in human PMN [38]. A similar effect observed with rabbit peritoneal PMN appears to follow the activation of a pertussis toxin-sensitive G protein, likely of the G_i type [39]. Activation of these proteins induces stimulation of various phospholipases, suggesting a potential role of lipid second messengers in the mechanism of PKC redistribution. It remains to be determined whether the early transduction events contribute to the numerous activating and priming effects of staurosporine, including activation of protein kinases in platelets [40], exocytosis [41], priming of the respiratory burst of PMN [42–45] and production of interleukin 2 and 4 [46], changes in cell morphology and differentiation [47], induction of neuropeptides [48] and interleukin 8 gene expression [49].

In conclusion, staurosporine enhances the expression of PDBu binding site in human platelets by increasing both PDBu binding affinity and capacity. The rise in this latter parameter appears to be due to a redistribution of PDBu binding sites between the cytosol and the particulate fraction. These effects occurred with staurosporine concentrations which inhibit PKC activation, suggesting they may be mediated by indirect rather than direct interaction of staurosporine with PKC. Staurosporine may thus be of use in clarifying the mechanism of PDBu binding site redistribution.

REFERENCES

1. Nishizuka Y, The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* **308**: 693–698, 1984.
2. Nishizuka Y, The molecular heterogeneity of protein kinase C and its implication for cellular regulation. *Nature* **334**: 661–665, 1988.
3. Nishizuka Y, Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**: 607–613, 1992.
4. Kraft AS and Anderson WB, Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* **301**: 621–623, 1983.
5. Wolfson M, McPhail LC, Nasrallah VN and Snyderman R, Phorbol myristate acetate mediates redistribution of protein kinase C in human neutrophils: potential role in the activation of the respiratory burst. *J Immunol* **135**: 2057–2062, 1989.
6. O'Flaherty JT, Jacobson DP, Redman JF and Rossi AG, Translocation of protein kinase C in human polymorphonuclear neutrophils: Regulation by Ca^{2+}-independent and Ca^{2+}-dependent mechanisms. *J Biol Chem* **265**: 9146–9152, 1990.
7. Baldassare JJ, Henderson P, Burns D, Loomis C and Fischer GJ, Translocation of protein kinase C isoenzymes in thrombin-stimulated human platelets-correlation with 1,2 diaglycerol levels. *J Biol Chem* **267**: 15585–15590, 1992.
8. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U and Nishizuka Y, Direct activation of calcium-activated, phospholipid-dependent protein kinase C by tumor-promoting phorbol esters. *J Biol Chem* **257**: 7847–7851, 1982.
9. Niedel J, Kuhn L and Vandenbark GR, Phorbol esters copurifies with protein kinase C. *Proc Natl Acad Sci USA* **80**: 36–40, 1983.
10. Kikkawa U, Takai Y, Tanaka RM and Nishizuka Y, Protein kinase C, as a possible receptor protein of tumor-promoting phorbol esters. *J Biol Chem* **258**: 11442–11445, 1983.
11. Lehrer RL and Cohen L, Receptor-mediated regulation

of superoxide production in human neutrophils stimulated by phorbol myristate acetate. *J Clin Invest* **68**: 1314–1320, 1981.
12. Tauber AI, Brettler DB, Kennington EA and Blumberg PM, Relation of human neutrophil phorbol ester receptor occupancy and NADPH oxidase. *Blood* **60**: 333–339, 1982.
13. Nishihira J and O'Flaherty JT, Phorbol myristate acetate receptors in human polymorphonuclear neutrophils. *J Immunol* **135**: 3439–3447, 1985.
14. Tamaoki T, Nomoto H, Takahashi I, Kato U, Morimoto M and Tomita F, Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase C. *Biochem Biophys Res Commun* **135**: 397–402, 1986.
15. Nakadate T, Jeng AY and Blumberg PM, Comparison of protein kinase C functional assays to clarify mechanisms of inhibitory action. *Biochem Pharmacol* **37**: 1541–1545, 1988.
16. Yoshizawa S, Fujiki H, Suguri H, Suganuma M, Nakayasu M, Matsushima R and Sugimura R, Tumor-promoting activity of staurosporine, a protein kinase inhibitor on mouse skin. *Cancer Res* **50**: 4974–4978, 1990.
17. Yamada S, Hirota K, Chida K and Kuroki T, Inhibition of phorbol ester-caused induction of orithine decarboxylase and tumor promotion in mouse skin by staurosporine. *Biochem Biophys Res Commun* **157**: 9–15, 1988.
18. Combadière C, Pedruzzi E, Hakim J and Périanin A, A protein kinase inhibitor, staurosporine, enhances the expression or phorbol dibutyrate binding sites. *Biochem J* **289**: 695–701, 1993.
19. Takaya J, Kimura M, Lasker N and Aviv A. Phorbol 12, 13 dibutyrate binding to intact human platelets—the role of cytosolic free calcium. *Biochem J* **278**: 411–415, 1991.
20. Lorenzet R, Niemets J, Marcus AJ and Broekman MJ, Enhancement of mononuclear procoagulant activity by platelet 12-hydroxyeicosatetraenoic acid. *J Clin Invest* **78**: 418–423, 1986.
21. Ferrante A and Thong YH, Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leucocytes from human peripheral blood by the Hypaque-Ficoll method. *J Immunol Methods* **36**: 109–117, 1980.
22. Munson PJ and Rodbard D. Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107**: 220–224, 1980.
23. Lowry OH, Rosenbrough NH, Farr AL and Randall JR, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
24. Tanaka Y, Miyake R, Kikkawa U and Nishizuka YJ, Rapid assay of binding of tumor-promoting phorbol esters to protein kinase C. *Biochemistry (Tokyo)* **99**: 257–261, 1986.
25. Siess W and Lapetina EG, Ca^{2+} mobilization primes protein kinase C in human platelets. Ca^{2+} and phorbol esters stimulate platelet aggregation and secretion synergistically through protein kinase C. *Biochem J* **255**: 309–318, 1988.
26. Gopalakrishna R, Chen ZH and Gundimed U, Non phorbol tumor promotors okadaic acid and calyculin A induce membrane translocation of protein kinase C. *Biochem Biophys Res Commun* **189**: 950–957, 1992.
27. Thastrup O, Cullen JP, Dobrack BK, Hanley MR and Dawson AP, Thaspsigargin, a tumor promoter, discharges intracellular Ca^{2+} store by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc Natl Acad Sci USA* **87**: 2464–2470, 1990.
28. Haakii H, Fujikii H, Sugunuma M, Nakayashu M, Tahira T, Sugumira T, Scheuer PJ and Christiensen SB, Thapsigargin, a histamine secretagogue, is a non 12-*O*-tetradecanoylphorbol-13-acetate (TPA) type tumor promoter in two-stage mouse skin carcinogenesis. *J Cancer Res Clin Oncol* **111**: 177–181, 1986.
29. Kiley SC, Parker PJ, Fabbro D and Jaken S, Selective redistribution of protein kinase C isozymes by Thapsigargin and staurosporine. *Carcinogenesis* **13**: 1997–2001, 1992.
30. Wolf M and Baggiolini M, The protein kinase inhibitor staurosporine, like phorbol esters induces the association of protein kinase C with membranes. *Biochem Biophys Res Commun* **154**: 1273–1279, 1988.
31. Gopalakrishna R, Barsky SH, Thomas P and Anderson WB, Factors influencing chelator-stable, detergent-extractable, ophorbol ester-induced membrane association of protein kinase C. *J Biol Chem* **261**: 16438–16445, 1986.
32. Crabos M, Imber R, Woodtli T, Fabbro D and Erne P, Different translocation of three distinct PKC isoforms with tumor-promoting phorbol ester in human platelets. *Biochem Biophys Res Commun* **178**: 878–883, 1991.
33. Wasiuddin AK, Blobe G, Halpern A, Taylor W, Wetsel WC, Burns D, Loomis C and Hannun YA, Selective regulation of protein kinase C isoenzymes by oleic acid in human platelets. *J Biol Chem* **268**: 5063–5068.
34. Kiley SC, Parker PJ, Fabbro D and Jaken S, Differential regulation of protein kinase C isoform by thyrotropin-releasing hormone in GH4Cl cells. *J Biol Chem* **266**: 23761–23768, 1991.
35. Jalava A, Akerman K and Heikkila J, Protein kinase inhibitor, staurosporine, induces a mature neuropnal phenotype in SH-SY5Y human neuroblastoma cells through an alpha protein, neta-protein and zeta-protein kinase C-independent pathway. *J Cell Physiol* **155**: 301–312, 1993.
36. Kiley S, Schaap D, Parker P, Hsieh LL and Jaken S, Protein kinase C heterogeneity in GH4Cl rat pituitary cells-characterization of a Ca^{2+}-independent phorbol ester receptor. *J Biol Chem* **265**: 15704–15712, 1990.
37. Kiley SC, Parker PJ, Fabbro D and Jaken S, Hormone and phorbol ester-activated protein kinase C isoenzymes mediate a reorganization of the cytoskeleton associated with prolatin secretion in GH4Cl cells. *Mol Endocrinol* **6**: 120–131, 1991.
38. Périanin A, Combadière C, Pedruzzi E, Djerdjouri B and Hakim J, Staurosporine, a protein kinase inhibitor, induces phospholipase D activation in human polymorphonuclear leukocytes. *FEBS Lett* **315**: 33–37, 1993.
39. Kanaho Y, Nishida A and Nozawa Y, Calcium rather than protein kinase C is the major factor to activate phospholipase D in FMLP-stimulated rabbit peritoneal neutrophils. *J Immunol* **149**: 622–628, 1992.
40. Kocher M and Clemetson KJ, Staurosporine both activates and inhibits serine/threonine kinases in human platelets. *Biochem J* **275**: 301–306, 1991.
41. Dewald B, Thelen M, Wymann MP and Baggiolini M, Staurosporine inhibits the respiratory burst and induces exocytosis in human neutrophils. *Biochem J* **264**: 879–884, 1989.
42. Combadière C, Hakim J, Giroud JP and Périanin A, Staurosporine, a protein kinase inhibitor, up-regulates the stimulation of human neutrophil respiratory burst by N-formyl peptides and platelet activating factor. *Biochem Biophys Res Commun* **168**: 65–70, 1990.
43. Combadière C, El Benna J, Pedruzzi E, Hakim J and Périanin A, Stimulation of the human neutrophil respiratory burst by formyl peptides is primed by a protein kinase inhibitor, staurosporine. *Blood* **82**: 2890–2898, 1993.
44. Twomey B, Muid RE and Dale MM, The effect of putative protein kinase C inhibitors, K 252a and staurosporine, on the human neutrophil respiratory burst activated by both receptor stimulation and

postreceptor mechanisms. *Br J Pharmacol* **100**: 819–825, 1990.
45. Goodman EB and Tenner AJ, Signal transduction mechanism of C1q-mediated superoxide production—Evidence for the involvement of temporally distinct staurosporine-insensitive and sensitive pathways. *J Immunol* **148**: 3920–3928, 1992.
46. Dornand J, Bouaboula M, Dupuy d'Angeac A, Favero J, Shire D and Casselas P, Contrasting effects of the protein kinase inhibitor staurosporine on the interleukine-1 and phorbol ester activation pathways in the EL4-6.1 thymona cell line. *J Cell Physiol* **151**: 71–80, 1992.
47. Sako T, Tauber AI, Jeng AY, Yuspa SH and Blumberg PM, Contrasting effects of staurosporine, a potent kinase C on human neutrophils and primary mouse epidermal cells. *Cancer Res* **48**: 4646–4650, 1988.
48. Tischer AS, Ruzicka LA and Dobner PR, A protein kinase inhibitor, staurosporine, mimics nerve growth factor induction of neurotensin/neuromedin N gene expression. *J Biol Chem* **266**: 1141–1146, 1991.
49. Cassatella MA, Aste M, Calzetti F, Constantantin G, Guasparri I, Cesta M and Rossi F, Studies on the regulatory mechanism of interleukin-8 gene expression in resting and IFN-γ treated neutrophils. Evidence on the capability of staurosporine of inducing the production of interleukin 8 by human neutrophils. *Biochem Biophys Res Commun* **190**: 660–667, 1993.