A protein kinase inhibitor, staurosporine, enhances the expression of phorbol dibutyrate binding sites in human polymorphonuclear leucocytes

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Staurosporine, a potent protein kinase C (PKC) inhibitor, was studied for its effects on the binding of phorbol 12,13-dibutyrate (PDBu) to human polymorphonuclear leucocytes (PMNs). Treatment of PMNs with staurosporine concentrations in the range 50 nM–2 μ M at 37 °C (but not at 4 °C) and with 1 nM [³H]PDBu at both temperatures enhanced specific PDBu binding to PMNs by approx. 10–600% relative to control values. The potentiation was rapid (detectable within 1 min) and reached a plateau after 10 min of cell treatment. Scatchard analysis of the binding showed that staurosporine increased the total number of PDBu-binding sites (B_{max}) from $(178 \pm 9) \times 10^3$ (control) to $(324 \pm 15) \times 10^3$ sites per PMN and lowered the apparent dissociation constant (K_d) from 9.6 ± 0.8 (control) to 3.3 ± 0.3 nM. In Ca^{2+} -depleted cells, staurosporine induced similar changes in K_d values, whereas the B_{max} increased by 60%. Treatment of PMNs with 500 nM staurosporine enhanced PDBu binding in the particulate fraction by $120 \pm 7\%$ and decreased PDBu binding in the soluble fraction by $69 \pm 4\%$,

whereas PKC histone-phosphorylating activity of both fractions was almost completely inhibited. Incubation of staurosporine-pretreated particulate fractions with soluble fractions enriched the particulate fraction in PDBu-binding sites at the expense of the soluble fraction, without altering the binding affinity of PDBu for either fraction. Stimulation of PMNs with chemotactic *N*-formyl peptides induced a transient increase in PDBu binding. This effect was potentiated by approx. 52% by staurosporine. These results show that, in addition to its interference with PKC protein-phosphorylating activity, staurosporine enhances both PDBu-binding capacity and affinity to PMNs, through a mechanism involving Ca^{2+} -dependent and -independent processes. Alterations of PDBu binding to soluble and particulate fractions suggest that the enhanced binding capacity in intact PMNs may be due to translocation of PDBu receptors, presumably PKC units. This phenomenon may affect PKC-dependent cellular responses mediated by physiological stimulation.

INTRODUCTION

Protein kinase C (PKC) is a family of enzymes that play a crucial role in signal transduction and other cellular processes [1,2]. PKC is activated by diacylglycerol (DAG), phospholipids and Ca^{2+} . A group of Ca^{2+} -insensitive PKC isoenzymes has also been characterized [3,4]. Tumour-promoting phorbol esters bind to and activate PKCs [5,6]. Pharmacological studies of cellular responses mediated by phorbol esters have shown that these agents operate through specific receptors that can be measured by using ³H-labelled phorbol 12,13-dibutyrate (PDBu) or phorbol myristate acetate [7–9]. One characteristic of PKC activation in stimulated cells is the subcellular redistribution of PKC, in which the soluble form of the enzyme is converted into a particulate form [10,11]. In human polymorphonuclear leucocytes (PMNs), translocation of PKC correlates with PMN stimulation by phorbol esters [11]. PKC antagonists (e.g. sphinganine and sphingosine) which interfere with DAG binding to the regulatory domain inhibit the adhesion of PKC to the particulate fraction [12] and depress PMN stimulation [13–15]. Unlike sphingosine, staurosporine, a potent PKC inhibitor that interacts with the catalytic domain of PKC [16], induces contrasting effects in many cell types. In PMN, for example, staurosporine both inhibits [17,18] and potentiates the chemoattractant-mediated respiratory burst [19,20] and promotes exocytosis [18]. Although staurosporine does not affect the binding of phospholipid and

phorbol ester to PKC in cell-free systems [21], it was recently shown to alter PDBu binding to intact platelets [22]. To gain insight into the mechanism of staurosporine action in PMNs, we analysed PDBu binding to intact PMNs and to subcellular fractions. Staurosporine was found to increase the expression of PDBu-binding sites at the surface of PMN through an enhancement of both PDBu binding affinity and total number of binding sites. Alterations in PDBu binding to subcellular fractions suggest that the increase in binding capacity may be due to a translocation of PDBu-binding sites from soluble to particulate compartments. Both Ca^{2+} -dependent and -independent processes appear to be involved in this redistribution.

MATERIALS AND METHODS

Reagents

Staurosporine was obtained from Kamiya Biochemical Co. (Thousand Oaks, CA, U.S.A.). [³²P]ATP (sp. radioactivity 30–40 Ci/mmol), [³H]PDBu (sp. radioactivity 20 Ci/mmol) and [³H]staurosporine (sp. radioactivity 160 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). Hionic Fluor was obtained from Packard (Breda, The Netherlands). Monopoly Resolving Medium was obtained from Flow Laboratories (Puteaux, France). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sphingosine was dissolved in dimethyl sulfoxide (DMSO) and diluted in an aqueous

Abbreviations used: PDBu, phorbol 12,13-dibutyrate; PMNs, polymorphonuclear leucocytes; PKC, protein kinase C; HBSS, Hanks balanced salt solution; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; PMA, phorbol 12-myristate 13-acetate.

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solution which contained an equimolar amount of fatty-acid-free BSA.

Isolation of PMNs

Heparinized human venous blood from healthy volunteers was centrifuged over a cushion of mixture of a Ficoll and Hypaque (Monopoly Resolving Medium), as described elsewhere [23,24]. The purified PMNs (97%) were subjected to hypotonic lysis, washed, resuspended in Hanks balanced salt solution (HBSS) and maintained at 4 °C until use.

Stimulation of PMNs and preparation of subcellular fractions

Neutrophils ($40 \times 10^6/20$ ml) were prewarmed for 5 min at 37 °C and treated for 15 min with staurosporine at the indicated concentrations. Cells were then diluted with 25 ml of ice-cold HBSS and collected by centrifugation at 400 *g* for 10 min at 4 °C. Pellets were resuspended in the lysis buffer containing 20 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 2 mM phenylmethanesulphonyl fluoride, 0.01 % leupeptin, 50 mM 2-mercaptoethanol and, as indicated, 2 mM EDTA and 5 mM EGTA. The cells were then disrupted by sonication at 25 W (four 10 s bursts) with a Fisher model 20/200 SV TC4C instrument. Post-nuclear supernatants were centrifuged at 100000 *g* for 60 min in a Beckman TL100 centrifuge and the soluble fraction was separated from the pellets (particulate fraction). The particulate fraction was resuspended in 250 μ l of lysis buffer containing 0.1 % Triton X-100, homogenized by sonication (two 10 s bursts) at 4 °C and then incubated in an ice bath for 30 min.

[³H]PDBu binding to PMNs

PDBu binding was performed as described previously [25]. Briefly, batches of 5×10^6 PMNs in 1 ml of HBSS containing 0.1 % BSA were pretreated at 37 °C for various periods of time in the absence (control) or presence of staurosporine at the indicated concentrations and then incubated for 5 min with 1 nM [³H]-PDBu, in the absence or presence of 1 μ M unlabelled PDBu to estimate non-specific binding. Cells were rapidly diluted with 3 ml of ice-cold 0.5 % DMSO and harvested on Whatman GF-B filters which had been soaked in fresh 0.3 % polyethylenimine solution for 1 h before use [26]. The filters were then washed with 3×3 ml of ice-cold 0.5 % DMSO and suspended in 4 ml of Hionic Fluor for radioactivity measurements. Specific binding was calculated as the difference between total and non-specific binding. PDBu-binding parameters (K_d and B_{max}) were determined at equilibrium by incubating control and staurosporine-treated PMNs with concentrations of [³H]PDBu from 0.25 to 50 nM for 20 min (37 °C) or for 2 h (4 °C). In some experiments, PMNs were depleted of Ca²⁺ as described elsewhere [27], then incubated in Ca²⁺-free HBSS in the presence of 1 μ M Fura 2 AM [24]. They were then washed and resuspended in HBSS containing 1 mM EGTA and treated with staurosporine. Binding data were analysed by using the LIGAND program developed by Munson and Rodbard [28].

[³H]PDBu binding to particulate and soluble fractions

The incubation mixture (250 μ l), containing 20 μ g of soluble fraction or 100 μ g of particulate fraction plus 100 μ g of phosphatidylserine/ml, 50 mM Tris/HCl, pH 7.5, and 0.5 mM CaCl₂,

was treated for 30 min at 30 °C with 40 nM [³H]PDBu in the absence or presence of 40 μ M unlabelled PDBu. The mixture was diluted with 3 ml of ice-cold 0.5 % DMSO, filtered through Whatman GF-C filters and washed three times as described above [26]. Maximal non-specific binding represented approx. 25 % of total binding.

Assay of PKC activity

The particulate fraction was centrifuged at 100000 *g* for 30 min at 4 °C and the supernatant, referred to below as the particulate elutable fraction, was collected. The pellets were suspended in 250 μ l of lysis buffer containing 0.1 % Triton X-100 and homogenized by sonication. This latter fraction is referred to as the non-elutable particulate fraction. PKC activity was assayed by measuring the incorporation of ³²P into histone [11]. Briefly, the reaction mixture contained 10 mM MgCl₂, 1 mM CaCl₂, 2 μ g of phosphatidylserine, 0.1 μ g of 1,2-*sn*-diolein, 20 μ g of histone, 10 μ M ATP (containing 0.5 μ Ci of [³²P]ATP) and 2 μ g of soluble or elutable protein in 100 μ l of 20 mM Tris/HCl, pH 7.5. Reactions were started with ATP, allowed to run for 10 min at 30 °C and stopped by addition of 400 μ l of ice-cold 20 % trichloroacetic acid followed by 100 μ l of BSA (2.5 mg/ml) as carrier. Precipitates were collected by centrifugation at 10000 *g* for 10 min and washed twice after dissolution of the pellet in 0.5 M NaOH. The pellets were dissolved in 0.5 M NaOH and mixed with 4 ml of Hionic fluor for radioactivity measurement. PKC activity was expressed in pmol of ³²P incorporated/min per mg of protein and was calculated by subtracting the activity measured in the absence of Ca²⁺, phosphatidylserine and diolein from that measured in their presence. No significant PKC activity could be measured in the non-elutable particulate fraction, and the PKC activity of the particulate fraction is therefore that found in the elutable fraction.

[³H]Staurosporine binding to PMNs

Batches of PMNs (2×10^6) were prewarmed for 5 min at 37 °C in 200 μ l of HBSS and treated with 25 nM [³H]staurosporine for the indicated time. Cells were centrifuged at 10000 *g* for 15 s on a mixture of silicone oil and paraffin (13 %) placed over a cushion of 0.5 M sucrose/HBSS in Eppendorf tubes. After discarding the supernatants and oil mixtures, the bottom of each tube was cut off. The pellets were lysed in 250 μ l of Soluene 350 and mixed with 4 ml of Hionic fluor for radioactivity measurement. For analysis of staurosporine distribution in PMNs, pellets were resuspended in HBSS and sonicated at 25 W (2×10 s). The sonicated preparations were centrifuged for 30 min at 100000 *g* at 4 °C and the supernatants were drawn off. The pellets were finally resuspended in HBSS containing 0.1 % Triton X-100 and homogenized by sonication.

Statistical analysis

Values are given as means $\pm$ S.E.M. of at least three experiments run in duplicate. Statistically significant differences between experiments were identified by Student's paired *t* test.

RESULTS

Staurosporine increased PDBu binding to PMNs

Treatment of PMNs with staurosporine concentrations from 50 nM to 2 μ M for 15 min at 37 °C caused a concentration-dependent enhancement of specific PDBu binding (Figure 1a).

Figure 1 Effect of staurosporine on [^3H]PDBu binding to intact PMNs

PMNs ($5 \times 10^6/\text{ml}$) were incubated in the absence (control) or presence of various staurosporine concentrations for 15 min (a) and with 500 nM staurosporine for various times (b). They were then incubated at 37°C for 5 min with 1 nM [^3H]PDBu in the absence or presence of excess unlabelled PDBu. Results are expressed as percentages ($\pm$ S.E.M.) of the control value (10450 ± 673 sites/PMN).

Figure 2 Effect of staurosporine on the kinetic parameters of PDBu binding to PMNs

PMNs were incubated in the absence (control: $\circ$) or presence ($\bullet$) of 500 nM staurosporine for 15 min at 37°C . They were then incubated with [^3H]PDBu concentrations from 0.25 to 50 nM for 30 min at 37°C (a) or for 2 h at 4°C (b). The values shown are from a representative experiment run in duplicate. Similar results were obtained in three other separate experiments.

This increase was statistically significant ($P < 0.05$) for staurosporine concentrations above 50 nM. The maximal enhancement was $596 \pm 30\%$ of control values and was obtained with $2 \mu\text{M}$ staurosporine, a concentration which had no detectable cytotoxic effect, as assessed by the Trypan Blue exclusion method. Non-specific PDBu binding represented approx. 10% of total binding and was not modified by staurosporine. The staurosporine-induced potentiation of PDBu binding was rapid, reaching $150 \pm 3\%$ of control values within 1 min with 500 nM staurosporine, and reaching a plateau after 10 min of PMN treatment (Figure 1b). To determine whether these staurosporine effects were due to alterations of the dissociation constant (K_d) or the total number of binding sites (B_{max}), binding experiments were performed at equilibrium and at 37°C . Scatchard analysis of the binding confirmed the presence of a single class of binding sites on control cells, with an apparent K_d of 9.6 ± 0.8 nM and a maximal binding capacity (B_{max}) of $(178 \pm 9) \times 10^3$ sites per cell (Figure 2 and Table 1). Treatment of PMNs with 500 nM staurosporine increased B_{max} to $(324 \pm 15) \times 10^3$ sites and lowered K_d to 3.3 ± 0.3 nM. Because the high phorbol ester concentrations used for binding assay also induce cell activation at 37°C [29] and could thus have influenced the effects of staurosporine, we measured PDBu binding at 4°C , with PMNs pretreated with staurosporine at 37°C . Under these conditions, B_{max} for control PMNs was $(166 \pm 4) \times 10^3$ sites, a value similar to that observed at 37°C , and K_d was 13.0 ± 2 nM. Staurosporine treatment of PMNs increased B_{max} to $(276 \pm 15) \times 10^3$ sites but had no significant effect on K_d (8.7 ± 1.7 nM). No alteration of B_{max} or the PDBu-binding affinity was observed when PMN were treated with staurosporine at 4°C (results not shown).

Staurosporine-induced redistribution of PDBu-binding sites in PMNs

The enhancement of the number of PDBu-binding sites at the surface of staurosporine-treated PMNs can be interpreted as being due to translocation of PKC molecules from the cytosol to the plasma membrane or to an unmasking of cryptic membrane PDBu-binding sites. To gain insight into the mechanism of staurosporine action, binding studies were performed with particulate and soluble fractions derived from control and staurosporine-treated PMNs. The total number of PDBu-binding sites measured in the soluble and particulate fractions derived from control PMNs were $(492 \pm 26) \times 10^3$ and $(78 \pm 4) \times 10^3$ per cell

Table 1 Staurosporine effect on [^3H]PDBu binding parameters

Dissociation constant (K_d) and total number of PDBu-binding sites (B_{max}) were calculated from a Scatchard analysis of binding data in Figure 2. Means values $\pm$ S.E.M. were from four experiments; *statistically significant difference between control and staurosporine-treated PMNs.

Temperature	Staurosporine (500 nM)	Binding parameter	
		$10^{-3} \times B_{\text{max}}$ (sites/PMN)	K_d (nM)
37°C	—	178 ± 9	9.6 ± 0.8
	+	$324 \pm 15^*$	3.3 ± 0.3
4°C	—	166 ± 4	13.0 ± 2.0
	+	$276 \pm 15^*$	$8.7 \pm 1.7^*$

Table 2 Staurosporine effect on PDBu-binding-site distribution and PKC activity in PMNs

PMNs were treated in the absence or presence of 100 or 500 nM staurosporine and fractionated into soluble and particulate compartments. PKC activities of both fractions were measured by histone [32 P] phosphorylation and [3 H]PDBu binding. Results are expressed as numbers of PDBu sites per PMN for PDBu binding and as pmol of 32 P incorporated/min per mg of protein for PKC activity. Means values $\pm$ S.E.M. were from four experiments; * statistically significant difference between control and staurosporine-treated PMNs.

Fraction	Staurosporine (nM)	$10^{-3} \times$ PDBu binding (sites/PMN)	Histone phosphorylation (pmol of P/min per mg)
Soluble	0	492 ± 26	2353 ± 312
	100	$197 \pm 10^*$	$282 \pm 141^*$
	500	$149 \pm 12^*$	$0 \pm 0^*$
Particulate	0	78 ± 4	151 ± 29
	100	$118 \pm 3^*$	$23 \pm 15^*$
	500	$172 \pm 5^*$	$0 \pm 0^*$

Figure 3 Effect of staurosporine on [3 H]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 (●). Results are expressed as percentages of control values (see Table 2).

equivalent respectively (Table 2). PMN treatment with 100 nM and 500 nM staurosporine enhanced the number of particle-associated PDBu-binding sites to 151 ± 4 and $220 \pm 7\%$ of control values respectively, whereas the number of PDBu-binding sites in the cytosol was decreased to 40 ± 2.5 and $30 \pm 2\%$ of control values respectively. However, the PKC activity of the soluble and particulate fractions, as evaluated by histone phosphorylation, was completely inhibited. These results are compatible with a translocation of soluble PDBu-binding sites to the particulate fraction. To investigate a possible unmasking effect of staurosporine on cryptic membrane PDBu-binding sites, the particulate fraction from control PMNs was incubated in the absence and presence of staurosporine concentrations from 25 nM to $1 \mu\text{M}$ and then assayed for PDBu binding. As shown in Figure 3, staurosporine had no effect on PDBu binding, but inhibited PKC activation, as evaluated by Ca^{2+} and phospholipid-dependent histone phosphorylation. Similar effects were observed with the

Table 3 Effect of staurosporine on [3 H]PDBu binding to subcellular fractions

Particulate fractions obtained from resting PMNs were treated in the absence (—) or presence of $1 \mu\text{M}$ staurosporine (+) for 15 min and then incubated with soluble fraction for 15 min. Soluble and particulate fractions were separated and tested for their ability to bind PDBu. Mean values $\pm$ S.E.M. were from three experiments; * statistically significant difference between control and staurosporine-treated PMNs.

Fraction	Staurosporine	Binding parameter	
		$10^{-3} \times B_{\text{max}}$ (sites/PMN)	K_d (nM)
Soluble	—	312 ± 8	1.9 ± 0.2
	+	$237 \pm 7^*$	2.3 ± 0.2
Particulate	—	115 ± 3	5.6 ± 0.1
	+	$148 \pm 5^*$	5.8 ± 0.1

cytosolic fraction (results not shown). The concentration inhibiting 50% of control activity (IC_{50}) was approx. 50 nM for both fractions. These results confirmed that staurosporine is a potent inhibitor of membrane PKC and suggested that its ability to enhance PDBu-binding capacity in PMNs is not due to an unmasking of cryptic membrane PDBu-binding sites.

Model *in vitro* for translocation of PDBu-binding sites by staurosporine

To examine further the possibility of staurosporine inducing translocation of PDBu-binding sites from the soluble to the particulate compartment, we set up a model in which particulate fractions from control PMNs were first treated without (control) or with $1 \mu\text{M}$ staurosporine for 15 min at 37°C and then incubated with the soluble fraction from control PMNs. The two fractions were then separated by ultracentrifugation and tested for their PDBu-binding capacity. Scatchard analysis of the binding (Table 3) showed the presence of a single class of binding sites in both the soluble and particulate fractions, with K_d values of 1.9 ± 0.2 and 5.6 ± 0.1 nM respectively, and B_{max} values of $(312 \pm 8) \times 10^3$ and $(115 \pm 3) \times 10^3$ sites per cell equivalent. Staurosporine treatment of the particulate fraction did not affect the PDBu-binding affinity of either fraction. In contrast, the binding capacity of the soluble fraction was significantly decreased by 24% ($P < 0.05$), whereas that of the particulate fraction was increased by 29% ($P < 0.05$). These results show that staurosporine induced a translocation of PDBu-binding sites from the soluble to the particulate fraction. However, in this reconstitution model *in vitro*, staurosporine was active only at high concentrations. This may be due to the large amount of particulate protein used. This result also suggests that staurosporine may reach high concentrations in intact PMN. We next examined this possibility. Incubation of PMNs for various times with 25 nM [3 H]staurosporine enhanced the amount of [3 H]staurosporine associated with PMNs (Figure 4), with a maximum at less than 3 min and a plateau which lasted for at least 20 min. At 4°C staurosporine uptake by PMNs was also rapid, but the maximum value was only approx. 60% of that observed at 37°C . Given that 5×10^6 PMNs have a volume of approx. $1.2 \mu\text{l}$ at 37°C [30], the above results indicate that the cellular staurosporine concentration was approx. 375 nM, i.e. about 15 times that in the extracellular medium. Fractionation of staurosporine-treated PMNs showed that $57 \pm 3\%$ of the staurosporine was present in the particulate fraction and $43 \pm 3\%$ in the soluble fraction.

Figure 4 Staurosporine uptake by PMNs

PMNs (2×10^6 /ml) were incubated with 25 nM [3 H]staurosporine for the indicated time and centrifuged on a cushion of oil/paraffin. The radioactivity associated with the cell pellet was measured and expressed as fmol of [3 H]staurosporine/ 2×10^6 PMNs.

Table 4 Effect of Ca^{2+} depletion on [3 H]PDBu binding parameters

PMNs loaded in Fura 2 were treated in the absence or presence of 500 mM staurosporine. Dissociation constant (K_d) and total number of PDBu-binding sites (B_{max}) were calculated from a Scatchard analysis of binding data. Mean values $\pm$ S.E.M. were from four experiments; * statistically significant differences between control and staurosporine-treated PMNs. ^a and ^b compared PMNs incubated in presence of Ca^{2+} or EGTA ($P = 0.037$).

	Staurosporine	Binding parameter	
		$10^{-3} \times B_{\text{max}}$ (sites/PMN)	K_d (nM)
Calcium (1.2 mM)	—	126 ± 12	9.7 ± 0.6
	+	$370 \pm 26^*$	$2.8 \pm 0.4^*$
EGTA (200 μ M)	—	132 ± 15	14.1 ± 1.1^b
	+	$282 \pm 46^{a*}$	$2.9 \pm 0.4^*$

Effect of staurosporine on PDBu binding to Ca^{2+} -depleted and *N*-formylmethionyl-leucyl-phenylalanine (fMLP)-stimulated PMNs

Translocation of PKC in PMNs stimulated by the Ca^{2+} ionophore A23187 and chemoattractants is influenced by cellular Ca^{2+} levels [29]. To investigate the potential role of Ca^{2+} in the effects of staurosporine, PMNs were Ca^{2+} -depleted as described elsewhere [29] by incubating them with the Ca^{2+} chelator Fura 2 and then in the presence of 1 mM EGTA. In these conditions, staurosporine still enhanced both the PDBu-binding affinity and the total binding capacity of PMN, but to a lesser extent than in the presence of Ca^{2+} (Table 4), suggesting that both Ca^{2+} -dependent and -independent mechanisms are involved in the PDBu-binding alterations. The cytosolic Ca^{2+} level, which is approx. 100 nM in resting PMNs [24], was not markedly altered by staurosporine treatment (results not shown), which indicates that the alterations of PDBu binding (Table 4) may be influenced by basal Ca^{2+} levels. In contrast with staurosporine, chemoattractants, such as *N*-formyl peptides, induce a translocation of PDBu-binding sites which is transient [29]. To examine whether staurosporine alters the physiological stimulation of the PDBu-binding redistribution, PMNs were treated with 100 nM staurosporine and challenged with 100 nM fMLP. Results in Figure 5 confirm that fMLP induced a transient increase in PDBu binding, which reached a peak at 30 s and returned to baseline by 4 min. Treatment of PMNs with staurosporine significantly ($P < 0.05$)

Figure 5 Effect of staurosporine on [3 H]PDBu binding to fMLP-stimulated PMNs

PMNs (5×10^6 /ml) were treated in the absence (control; $\circ$) or presence ($\bullet$) of 100 nM staurosporine for 15 min and then incubated at 37 °C for 5 min with 1 nM [3 H]PDBu in the presence or absence of excess unlabelled PDBu. During the incubation of PMNs with [3 H]PDBu, cells were challenged with 100 nM fMLP for the indicated time. Results represent the increase in the amount of specifically bound to PMN upon stimulation by fMLP and are expressed as PDBu-binding sites per PMN. The basal amount of PDBu bound to resting PMNs represented 10547 ± 321 and 11869 ± 467 sites per PMN treated in the absence (control) and presence of staurosporine respectively. Results are means $\pm$ S.E.M. of three different experiments; statistically significant differences between control and staurosporine-treated PMNs are designated by * ($P < 0.05$).

enhanced the maximal fMLP-induced response by approx. 52 % but inhibited the return to basal level.

DISCUSSION

The conversion of soluble PKC into its particulate form (translocation) in stimulated cells is considered as a prerequisite for PKC activation by intravesicular DAG. Physiological stimuli promote transient and reversible PKC redistribution, whereas non-physiological stimuli such as phorbol esters induce tight PKC association to the membrane [11,29,31,32]. PKC binds to the plasma membrane via the regulatory PKC domain and is inhibited by competitive inhibitors of DAG binding such as sphinganine [12] and dioctanoylglycerol [33]. In the present study, we have analysed the effect of PMN treatment by staurosporine on the kinetic parameters of PDBu-binding sites, which, on the basis of the known specificity of phorbol esters, are assumed to be PKC [5,6,14,25,29]. Results show that staurosporine enhanced both binding capacity and PDBu affinity for PMNs. This effect appears to be due to translocation of PDBu-binding sites. This was first suggested by the rapid and marked increase in specific PDBu binding to the PMN surface after treatment at 37 °C, but not at 4 °C (Figure 1). Analysis of this event at binding equilibrium (Table 1) revealed that staurosporine both decreased K_d by 34 % and increased the total number of available PDBu-binding sites (B_{max}) by 82 %. Fractionation of staurosporine-treated PMNs confirmed that the number of PDBu-binding sites in the particulate fraction rose, whereas that in the soluble fraction fell (Table 2). The enhancement of PDBu binding to the particulate fraction did not appear to be due to an unmasking of cryptic PDBu-binding sites, since treatment of the particulate fractions with staurosporine did not augment PDBu-binding capacity but strongly inhibited PKC catalytic activity (Table 2). These results are consistent with the observation that staurosporine does not interact with DAG-binding sites in cell-free systems [16]. They further suggest that the increase in the affinity of PDBu binding to intact staurosporine-treated PMNs

may be due to alterations of the PDBu binding-site environment, likely by staurosporine-mediated metabolic events rather than an effect of staurosporine itself. Indeed, treatment of PMN with staurosporine at 4 °C did not alter PDBu binding (results not shown), although staurosporine did bind to PMNs (Figure 4). PDBu binding-site redistribution was further confirmed *in vitro* by using a subcellular model in which the particulate fraction from resting PMNs was treated with staurosporine and then with the soluble PMN fraction. The binding capacity of the particulate fraction increased significantly by approx. 29 % at the expense of the soluble fraction, whereas PDBu binding affinity was unchanged. This model shows that staurosporine has effects *in vitro* that may contribute to the observed changes *in vivo*. This ability of staurosporine to induce translocation *in vitro* of PDBu-binding sites is consistent with previous data showing that staurosporine promotes the association of purified PKC with inside-out vesicles from erythrocytes [34].

PDBu-binding capacity to particulate fractions of PMN treated with 100 nM and 500 nM staurosporine was increased by approx. 40 000 and 94 000 sites respectively, whereas there was a loss of 295 000 and 343 000 sites respectively from the soluble fraction (Table 2). In the subcellular model of translocation of PDBu-binding sites approx. 33 000 sites were gained by the particulate fraction, whereas about 75 000 sites disappeared from the cytosol. One possible explanation for this overall loss of PDBu-binding sites is that some translocated PKC molecules may bind to the particulate fraction via the DAG-binding site and thus become inaccessible to PDBu, as indicated by the inhibitory effect of sphinganine on PKC adhesion to vesicles [12]. Alternatively, it may have been due to proteolysis [11,35].

The mechanism by which staurosporine enhances the redistribution of PDBu-binding sites is not known. Both direct and indirect effects may be involved. Indirect effects may occur through factors known to facilitate PKC translocation, such as DAG and acidic phospholipids [12,36] or Ca^{2+} [27,29]. In human PMNs, the breakdown of phosphatidylcholine through the phospholipase D pathway provides a major source of phosphatidic acid and DAGs [37,38], and treatment of PMNs with staurosporine enhances fMLP-mediated activation [39]. We confirmed this finding (results not shown) and found that treatment of PMNs for 15 min with 0.1, 0.5 or 1 μM staurosporine itself increased the basal level of phosphatidic acid by respectively 114, 200 and 212 % relative to control values, suggesting that phosphatidic acid or its metabolites (i.e. lyso-phosphatidic acid or DG) may be potential candidates for the PDBu-binding-site translocation. Alteration in cytosolic Ca^{2+} has also been involved in PKC translocation mediated by chemoattractants such as fMLP [27,29]. Unlike fMLP, staurosporine failed to induce marked alterations in Ca^{2+} -levels under our conditions [24], whereas an increase in cytosolic Ca^{2+} concentration has been reported [40]. A role of Ca^{2+} is further supported by our observation that the Ca^{2+} depletion of PMNs inhibited approx. 40 % of the increase in B_{max} values (Table 4). These data suggest that both Ca^{2+} -dependent and -independent processes may be involved in PDBu-binding-site redistribution. By contrast, Ca^{2+} depletion failed to alter the enhancement of PDBu binding affinity induced by staurosporine (Table 4). In platelets, staurosporine was found to increase PDBu-binding affinity through a Ca^{2+} -dependent process [22], but failed to alter PDBu-binding capacity. These differences between platelets and PMNs may be due to cell selectivity, but could also be explained by differences in the experimental conditions. Alternatively, staurosporine may enhance PDBu-binding capacity through a direct effect. This is suggested by its ability to interact with ATP-binding sites on PKC [21]. Such an interaction may occur

between membrane-associated staurosporine and soluble PKC and thus facilitate the adhesion of the latter to the particulate fraction.

The redistribution of PDBu-binding sites mediated by staurosporine was associated with a strong decrease in the PKC histone-phosphorylating activity of the particulate fraction (Figure 3). Staurosporine has also been reported to inhibit the phosphorylation of some proteins *in vivo* [18,41], including the 47 kDa species, implicated in the PMN respiratory-burst response to phorbol 12-myristate 13-acetate (PMA) and chemoattractants. Although inhibition of protein phosphorylation is generally associated with a depressed respiratory burst in PMA-stimulated PMNs, the use of staurosporine under certain conditions has dissociated the inhibition of the phosphorylation of the 47 kDa species from the fMLP-induced PMN respiratory burst [41]. It was suggested that PMNs may use a simulatory pathway involving a kinase distinct from that which is activated by phorbol ester [41]. We previously showed that staurosporine enhanced the chemoattractant-induced respiratory burst under certain conditions in which that induced by PMA was depressed [19]. This finding is compatible with the above hypothesis and confirms the utility of staurosporine is dissociating signal-transduction pathways [41]. We show here that staurosporine also potentiated the transient redistribution of PDBu binding in PMNs stimulated by fMLP, resulting in the persistence of PDBu-binding sites at the surface of PMNs (Figure 5). Whether this effect is related to enhancement of the fMLP-stimulated respiratory burst [19] remains to be examined. Staurosporine has also been reported to have other several activating effects. In PMNs, it promotes actin polymerization [42] and induces a prolonged release of components of secretory vesicles and specific granules, with a time course and pattern of activity similar to those induced by PMA [18]. Staurosporine-mediated exocytosis, like PKC translocation (Table 4), occurred in Ca^{2+} -depleted PMNs. These similarities suggest the existence of a common pathway leading to exocytosis and PKC translocation. In platelets, staurosporine potentiates PDBu-binding-site translocation induced by thrombin [22] and promotes marked thrombin-mediated accumulation of inositol phosphates [43]. In other cell types, staurosporine promotes changes in morphology and differentiation similar to those induced by phorbol ester [44] and mimics nerve growth factor for the induction of neuropeptide gene expression [45]. Whether or not these stimulating and priming properties of staurosporine are related to the metabolic consequences of PKC redistribution remains to be determined. In conclusion, staurosporine promotes the expression of PDBu-binding sites, through the enhancement of both PDBu affinity (K_d) and total binding capacity (B_{max}). The latter effect is associated with redistribution of PDBu-binding sites between the soluble and the particulate compartment; this occurs with no apparent change in cytosolic Ca^{2+} levels, but appears to be mediated by both Ca^{2+} -dependent and -independent processes. Staurosporine may thus provide an additional tool for investigating the mechanism of PKC translocation. However, the fact that it increases the affinity of ligands to PKC regulatory units in intact cells, but not in cell-free systems, will complicate its use as a pharmacological probe for PKC.

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