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Staurosporine Inhibits Protein Kinase C and Prevents Phorbol Ester-Mediated Leukotriene D_4 Receptor Desensitization in RBL-1 Cells

RAJU V. K. VEGESNA, HSIAO-LING WU, SEYMOUR MONG, and STANLEY T. CROOKE

Department of Molecular Pharmacology, Smith Kline & French Laboratories, Philadelphia, Pennsylvania 19101

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SUMMARY

The aim of the present study was to investigate the effects of staurosporine on phorbol-myristate acetate (PMA)-induced activation of protein kinase C (PKC) and the desensitization of leukotriene D_4 (LTD_4)-stimulated Ca^{2+} mobilization in rat basophilic leukemia (RBL-1) cells. Staurosporine, one of the most potent PKC inhibitors known to date, markedly inhibited partially purified PKC from RBL-1 cells with an IC_{50} of 3 nM. Exposure of RBL-1 cells to PMA resulted in inhibition of LTD_4-stimulated Ca^{2+} mobilization. However, prior treatment of the cells with staurosporine completely prevented PMA-induced desensitization of LTD_4-stimulated Ca^{2+} mobilization. This reversal of Ca^{2+} desensitization by staurosporine was dose dependent with an IC_{50} of 0.1 μM. Treatment of RBL-1 cells with PMA resulted in translocation and activation of PKC from the cytosol to the membrane fraction. Pretreatment of RBL-1 cells with staurosporine inhibited the PMA-induced activation of PKC in the membrane fraction. The inhibition of PKC activity by staurosporine was time and dose dependent with an IC_{50} of 0.9 μM. These results show that PMA-induced heterologous desensitization is mediated by PKC and staurosporine prevented this process by directly inhibiting PKC in intact RBL-1 cells.

The tumor-promoting phorbol esters have been shown to activate PKC by substituting for the endogenous activator, diacylglycerol, in a variety of tissues (1). There are now numerous examples of phorbol esters altering receptor-mediated responses, presumably acting as stimulators of PKC. For example, prior treatment of cells with potent phorbol esters has been shown to inhibit hormone- and drug-induced second messenger systems such as phosphoinositide metabolism (2, 3), Ca^{2+} mobilization (4, 5), and cGMP generation (6, 7). These studies suggest that PKC plays an important role in the regulation of hormone-induced responses.

To understand and characterize the biochemical role of PKC in these responses, it is important to have a selective and potent PKC inhibitor that is active when applied to intact cells. Many compounds such as tamoxifen (8), H-7 (9–11), and concanavalin A (12) have been shown to inhibit PKC in whole cells. However, some of these studies are not conclusive because of the requirement of high concentrations of these compounds to inhibit PKC as well as its nonselective nature. Clearly, a more potent, cell-permeable inhibitor of PKC would be of value.

Recently, staurosporine, a microbial alkaloid, has been shown to be the most potent inhibitor of PKC *in vitro* with an IC_{50} value of 2.7 nM (13). We decided to use this compound as a tool to understand the role of PKC in phorbol ester-induced desensitization of LTD_4 responses. Previous studies from our laboratory have demonstrated high affinity LTD_4 receptors in RBL-1 cells (14). Our current model (15) suggests that these receptors are coupled to a guanine nucleotide-binding protein(s). Interacting via the guanine nucleotide-binding protein(s), a phosphoinositide-specific phospholipase C is activated, resulting in liberation of inositol phosphates (14), Ca^{2+} mobilization,[1] and also activation of PKC[2] in these cells. In the present report, we studied the effects of PMA and staurosporine on the activation of PKC and the desensitization of LTD_4-stimulated Ca^{2+} mobilization in RBL-1 cells.

Experimental Procedures

Materials. Reagents and materials were from the following sources. LTD_4 was prepared by total synthesis and supplied by Dr. J. G. Gleason from the Department of Medicinal Chemistry, SK&F Laboratories. Eagle's minimal essential medium, phosphate-buffered saline, and fetal

[1] S. Mong, H.-L. Wu, A. Wong, H. M. Sarau, and S. T. Crooke, unpublished observation.

[2] R. V. K. Vegesna, S. Mong, and S. T. Crooke, unpublished observation.

ABBREVIATIONS: PKC, protein kinase C; LTD_4, leukotriene D_4, 5(*S*)-hydroxy-6(*R*)-*S*-1-cysteinylglycyl-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid; PMA, 4β-phorbol 12β-myristate 13α-acetate; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; EDTA, ethylenediaminetetraacetate, disodium salt; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; DDT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; Fura 2,1-[2-(5-carboxy-oxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentasodium salt; KRH buffer, Krebs-Ringer-Henseleit buffer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PDBU, phorbol 12,13-dibutyrate; DMSO, dimethyl sulfoxide.

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calf serum were from Grand Island Biological Co.; [γ-^{32}P]ATP was from New England Nuclear. PMA, leupeptin, Triton X-100, Histone 111-S, EDTA, EGTA, PIPES, DTT, sodium pyrophosphate, trichloroacetic acid, Tris·HCl, $MgCl_2$, $CaCl_2$, and PMSF were from Sigma Chemical Co. Phosphatidylserine was obtained from Avanti Polar Lipids; DEAE-cellulose (DE-52) and Whatman 3MM filters were obtained from Thomas Scientific Co. Staurosporine was a gift from Drs. R. Johnson and J. Westley, SK&F Laboratories, obtained from Dr. S. Omura, The Kitasato Institute, Japan.

Cell Culture. RBL-1 cells were obtained from The American Type Tissue Collection (CRL-1378) and were grown at 37° in Eagle's medium supplemented with 10% (v/v) fetal calf serum and 0.4% (v/v) streptomycin in an atmosphere of 5% CO_2/95% humidified air. Cells were subcultured weekly and culture medium was changed daily. Cells were grown to a density of 1–1.5 × 10^6 cells/ml and used for the experiments.

Fura-2 loading and Ca^{2+} measurement. RBL-1 cell suspension (2 × 10^6/ml) was incubated with 2 μM of Fura-2/AM in KRH buffer, pH 7.4 (118 mM NaCl, 4.6 mM KCl, 1.1 mM $MgSO_4$ $7H_2O$, 24.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 11.1 mM glucose, and 1.8 mM $CaCl_2$) to a final concentration containing 5 mM HEPES (pH 7.4), 0.1% bovine serum albumin, and 1 mM $CaCl_2$ at 37° for 30 min. The cells were then centrifuged (500 × g for 5 min), resuspended in the same buffer, and further incubated at 37° for 15 min to hydrolyze the entrapped Fura-2 ester. The cells were centrifuged as described above, resuspended in the same buffer at a concentration of 4 × 10^6/ml, and stored at 4° until used for the measurement of fluorescence within 4 hr.

Fluorescence was monitored using a fluorimeter designed by the Johnson Biomedical Instrumentation Group (Philadelphia, PA), equipped with a 75-W xenon lamp for illumination. An interference filter (339 nm, 3–4 μm half-bandwidth) and another filter (499 nm, 5 nm half-bandwidth) were used to select the respective excitation and emission wavelengths. The quartz cuvette (1 cm path length) was secured in a holder equipped for constant temperature control and magnetic stirring. The concentration of cell was adjusted to 1 × 10^6/ml with the HEPES-buffered KRH buffer (containing 0.1% bovine serum albumin and 1 mM $CaCl_2$). Two ml of the cell suspension were transferred to the cuvette and equilibrated to 37° for 2 min before addition of agonist stimuli or other reagents. All the experiments for measuring calcium-induced Fura-2 fluorescence were performed using this fluorimeter at 37° with constant mixing.

Cell incubations for PKC estimation. RBL-1 cells were harvested by centrifuging at 1000 × g for 5 min. The pellet was washed twice with PBS and suspended in KRH buffer, pH 7.4, to a final concentration of 2–3 × 10^7 cells/ml. Drugs were added in 15-μl aliquots to a total incubation volume of 1.5 ml and incubated at 37° for the indicated times. Incubations were terminated by adding 10 ml of ice-cold KRH buffer (without $CaCl_2$ and $MgCl_2$), and the cells were washed twice and recovered by centrifuging at 1000 × g for 5 min at 4°.

Preparation of cytosol and membrane fractions. The cell pellet recovered after incubation was resuspended in 1.5 ml of buffer A (20 mM Tris·HCl, pH 7.5, 5 mM DTT, 2 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 1 mM PMSF, and 0.01% leupeptin), and the cells were disrupted at 4° by sonication for 10 sec at a power setting of 6 using a Branson sonicator fitted with a microprobe. The homogenate was centrifuged at 100,000 × g for 60 min at 4°. The supernatant representing the cytosolic fraction was applied to a DE-52 column (0.8 × 2 cm) which was previously equilibrated with buffer B (20 mM Tris·HCl, pH 7.5, 5 mM DTT, 2 mM EDTA, 2 mM EDTA, and 1 mM PMSF). The column was washed with 10 ml of buffer B and the enzyme was eluted with 2 ml of 0.1 M NaCl in buffer B at 4°. The pellet (particulate fraction) was resuspended by homogenizing in 1.5 ml of buffer A and stirred with 1% Triton X-100 at 4° for 30 min. The resulting detergent-solubilized membrane fraction was applied to a DE-52 column and eluted as described for the cytosolic fraction. The eluates obtained from the supernatant and pellet fractions were employed to determine PKC activity. In previous experiments, we have characterized the elution profile of PKC enzyme activities in RBL-1 cell samples. Greater than 90% of the cytosolic and/or the detergent-extracted particulate form of PKC activities from RBL-1 cells were collected in the 0.1 M NaCl eluted fraction under all conditions employed.

PKC Assay. PKC activity was assayed by the incorporation of ^{32}P into histone (16). The reaction mixture contained 50 mM PIPES buffer (pH 7.0), 10 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM DTT, 50 μg of Histone III-S, 12.5 μM [γ-^{32}P]ATP (~2 × 10^6 cpm), 1 mM $CaCl_2$, 40 μg/ml phosphatidylserine, 0.16 μM PMA, and 15 μl of the enzyme eluate in a total volume of 100 μl. The samples were incubated for 5 min at 30°. The reactions were terminated by spotting 70 μl of the reaction mixture onto Whatman 3MM filter paper discs (2.3-cm diameter). The filters were then washed in 10% trichloroacetic acid containing 2.5% pyrophosphate (17). Filters were dried and the bound ^{32}P was determined by liquid scintillation spectrophotometry. To determine Ca^{2+} and phospholipid-dependent enzyme activity, assays were performed with and without Ca^{2+}, phosphatidylserine, and PMA, and the difference is expressed as PKC activity.

[^{3}H]PDBU binding assay to PKC. Binding of [^{3}H]PDBU to DEAE-cellulose-eluted cytosolic PKC was determined by the method of Tanaka *et al.* (18). Briefly, the reaction mixture of 200 μl contained 20 mM Tris-maleate at pH 6.8, 100 mM KCl, 0.15 mM $CaCl_2$, 0.05 mM EGTA, 50 μg/ml phosphatidylserine, 30 nM [^{3}H]PDBU (15.8 Ci/mmol), 0.5% dimethyl sulfoxide (DMSO), and 100 μl of the enzyme eluate (~8–10 μg protein). After incubating for 20 min at 30°, the reaction was stopped by adding 4 ml of ice-cold 0.5% DMSO. The mixture was subjected to vacuum filtration using glass-fiber (Whatman GF/C) filters presoaked in polyethylenemine, and filters were washed four times with 4 ml of ice-cold 0.5% DMSO. Filters were dried and bound radioactivity was determined. Nonspecific binding was measured in the presence of 10 μM nonradioactive PDBU. The difference between total and nonspecific binding represents specific binding.

Protein assays. The concentration of protein in the eluate was determined in triplicate by the Bradford microassay method (19) using the Bio-Rad reagent and BSA as a standard.

Results

Effect of staurosporine on PMA-mediated inhibition of LTD_4-induced Ca^{2+} mobilization in RBL-1 cells. As shown in Fig. 1A, treatment of RBL-1 cells with LTD_4 resulted in mobilization of Ca^{2+}. When these cells were pretreated with PMA (0.01 μM) for 5 min, LTD_4-induced mobilization of Ca^{2+} was completely inhibited (Fig. 1B). Staurosporine (0.5 μM) alone had no effect on the basal or LTD_4-induced $[Ca^{2+}]_i$ levels (data not shown). However, as shown in Fig. 1C, prior treatment of the cells with staurosporine resulted in reversal of the PMA-induced inhibition of Ca^{2+} mobilization by LTD_4. This reversal of PMA effects by staurosporine was concentration dependent (Fig. 2), with an IC_{50} value of 0.1 μM. When RBL-1 cells were treated with staurosporine at concentrations greater than 2.5 μM, the rise in Ca^{2+} following the addition of LTD_4 was reduced (data not shown). This may be due to the cytotoxic effect of this drug or, in addition to PKC inhibition, other mechanisms that may be operative at higher concentrations of this drug (13).

Effect of staurosporine on enzyme activity and [^{3}H]PDBU binding of partially purified PKC from RBL-1 cells. To understand the molecular mechanism of staurosporine in RBL-1 cells, we studied the direct effect of staurosporine on enzymatic activity and [^{3}H]PDBU binding of partially purified PKC (see Experimental Procedures). As shown in Fig. 3, staurosporine inhibited PKC activity in a concentration-dependent manner and, at the concentrations employed, had no significant effect on basal activation. The IC_{50} value was 3 nM under the conditions described under Experimental Procedures.

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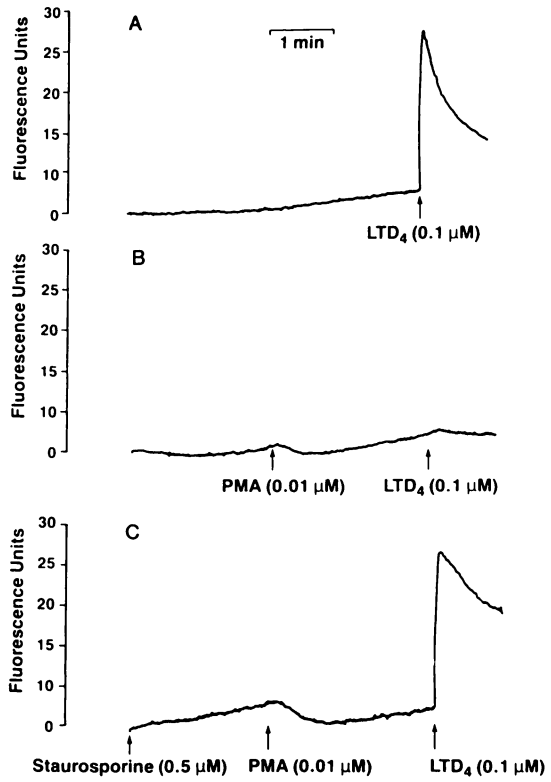

Fig. 1. The effect of PMA and staurosporine on LTD_4-induced Fura-2/Ca^{2+} fluorescence in RBL-1 cells. RBL-1 cells were labeled with Fura-2 and resuspended in Krebs buffer containing 1.0 mM $CaCl_2$ as described under Experimental Procedures. Representative tracings for each experimental protocol are shown. Cells were exposed to PMA for 2 min and staurosporine for 4 min. The basal intracellular Ca^{2+} concentration in RBL-1 cells was 118 ± 15 nM.

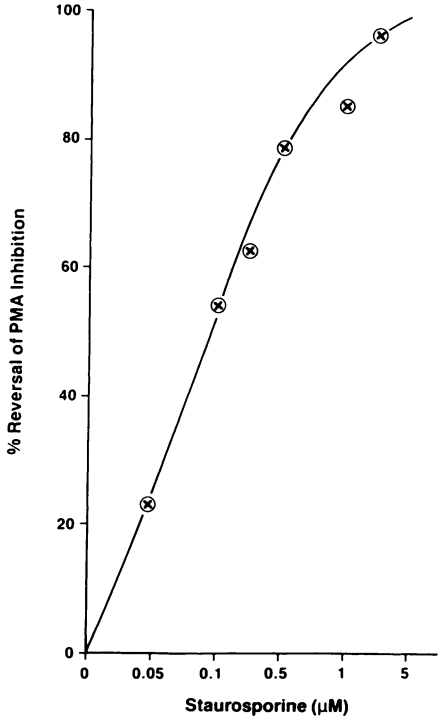

Fig. 2. Staurosporine reversal of PMA-mediated inhibition of LTD_4-stimulated Ca^{2+} mobilization. The experimental protocol was similar to that of Fig. 1. The concentrations of LTD_4 and PMA were 0.1 μM and 0.01 μM, respectively. Cells were exposed to staurosporine for 4 min and to PMA for the last 2 min of the pretreatment. Values are expressed as per cent maximum of 0.1 μM LTD_4 response alone. Values are the average of two separate experiments which differed by <10%.

This value was similar to the value previously reported for inhibition of PKC partially purified from rat brain (IC_{50} of 2.7 nM, Ref. 13). As shown in Fig. 3, staurosporine did not interfere with [^{3}H]PDBU binding at concentrations which completely inhibited PKC activity. These results further confirm the earlier observation (13) that staurosporine inhibits PKC via a mechanism other than inhibition of the binding of phorbol esters to PKC.

Effect of staurosporine pretreatment on the PMA-induced redistribution of PKC in RBL-1 cells. Table 1 shows the effect of staurosporine on PMA-induced translocation of PKC in RBL-1 cells. The majority of PKC activity (~90%) in RBL-1 cells was localized in the cytosol. Prior treatment of the cells with staurosporine (1 μM) alone had no significant effect on the distribution of PKC or on the total PKC activity (Table 1). When RBL-1 cells were treated with PMA (1 μM) for 5 min, PKC was translocated from the cytosol to the particulate fraction. In these experiments, we used 1 μM PMA instead of 0.01 μM (as was the case with Ca^{2+} mobilization experiments) because this concentration was maximally effective with respect to translocation of PKC in RBL-1 cells (data not shown). Prior treatment of the cells with staurosporine (1 μM) inhibited (by ~50%) the PMA-induced increase of PKC in the particulate fraction of RBL-1 cells. However, it did not reverse the PMA-induced decrease of PKC activity in the cytosol. Similar results were obtained when we calculated the total activity of PKC in these experiments (Table 1). These results suggest that the inhibition of phorbol ester-mediated effects by staurosporine may be due to direct inhibition of activated PKC. When staurosporine-treated, partially purified PKC was dialyzed against 500 volumes of 10 mM Tris·HCl for 7 hr at 4°, greater than 65% of PKC activity was regenerated (results not shown). This result suggests that staurosporine did not produce the inhibitory effect by a covalent and irreversible inhibition of the enzyme.

Concentration-dependent and time-dependent effect of staurosporine on PMA-induced activation of PKC in the intact RBL-1 cells. Fig. 4 shows the effects of pretreating RBL-1 cells with increasing concentrations of staurosporine on PMA (1 μM)-induced activation of PKC in the particulate fraction. Treatment of RBL-1 cells with different concentrations of staurosporine alone did not significantly change the particulate PKC activity. However, pretreatment of the cells with staurosporine resulted in inhibition of the PMA-induced activation of PKC. This inhibition was dose dependent, with an IC_{50} of 0.9 μM.

Similarly, the effect of 10 μM staurosporine pretreatment on the time course of PMA-induced translocation of PKC in the particulate fraction is shown in the Fig. 5. This concentration of staurosporine alone did not change the particulate PKC activity compared to the control and was maximally effective with respect to inhibition of PMA-induced PKC (see also Fig. 4). PMA alone, in a time-dependent manner, increased particulate PKC activity which was sustained for at least 10 min. As shown in Fig. 5, staurosporine significantly inhibited (by ~90%) PMA-induced activation of PKC. These results suggest that

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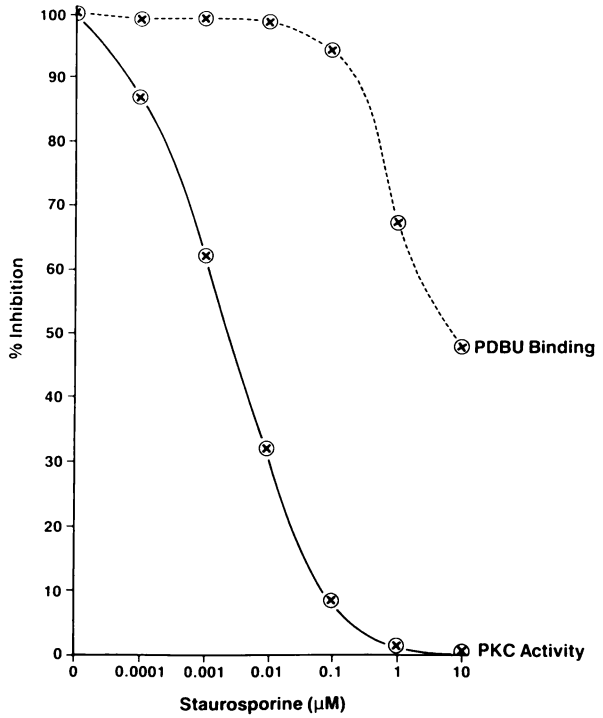

Fig. 3. Effect of staurosporine on enzymatic activity and [^{3}H]PDBU binding to PKC. Protein kinase C was partially purified from the cytosolic fraction of RBL-1 cells and assayed for PKC activity and [^{3}H]PDBU binding as described under Experimental Procedures in the presence and absence of various concentrations of staurosporine. Each value represents the average of two separate experiments assayed in triplicate which differed by <8%. Values are expressed as percentage inhibition of respective controls. Values for control cells are as follows: 658 pmol/mg/min for PKC activity and 7444 fmol/mg for [^{3}H]PDBU binding.

staurosporine is also an effective inhibitor of activated PKC in the intact RBL-1 cells.

Discussion

Consistent with previous findings in other systems (4, 5), treatment of RBL-1 cells with PMA resulted in suppression of LTD_4 receptor-mediated Ca^{2+} mobilization (Fig. 1). In the present experiments, we also found that PMA caused a translocation of PKC from the cytosol to the particulate fraction in RBL-1 cells (Table 1). A similar effect of PMA on PKC has been reported in many cell systems (for example, see Ref. 20), indicating that tight association of the cytosolic enzyme with the particulate fraction is a general biochemical mechanism of action for phorbol ester activation of PKC. Our results suggest that the effect of PMA on LTD_4 receptor responsiveness may be due to its ability to activate PKC in RBL-1 cells.

The PMA-induced translocation of PKC to the particulate fraction and subsequent phosphorylation of proteins may trigger the uncoupling of the signal transduction process in RBL-1 cells. In other systems, several possible candidates as target substrates for PKC have been identified, including the receptor itself (2), the guanine nucleotide-binding protein (21), or phospholipase C (22). In RBL-1 cells, the site of phosphorylation appears to occur at a step preceding the LTD_4-induced phosphoinositide hydrolysis.[3] It was previously demonstrated that a phosphoinositide-specific phospholipase C was phosphorylated upon treatment of RBL-1 cells with PMA (22); thus, PKC may phosphorylate phospholipase C to attenuate agonist-induced phosphoinositide hydrolysis in this cell line.

If PKC activation by PMA is involved in its ability to block LTD_4 receptor-mediated Ca^{2+} mobilization, one would expect that the presence of a PKC inhibitor might prevent such desensitization. We have employed staurosporine as a tool to understand the role of PKC in RBL-1 cells. Staurosporine is the most potent inhibitor of partially purified PKC from rat brain (13). In RBL-1 cells also, staurosporine inhibited partially purified PKC from the cytosolic fraction (Fig. 3). Staurosporine appears to act on PKC at a site different from the PDBU-binding site (Fig. 3), and its effects are probably due to a direct interaction with the catalytic site of PKC (13).

We studied the effect of staurosporine on the PMA-induced desensitization response in intact RBL-1 cells. As shown in Fig. 1, preincubation of the cells with staurosporine resulted in complete reversal of the PMA-mediated inhibition of LTD_4-induced Ca^{2+} mobilization. The antagonism was concentration dependent (Fig. 2). These results illustrate that the action of PMA on the LTD_4-induced Ca^{2+} mobilization is through the activation of PKC.

To further understand the site of action of staurosporine in the intact cells, we studied the effects of PMA and staurosporine on the distributiuon of PKC in the RBL-1 cells. Staurosporine effectively reduced the PMA-induced increase of PKC in the particulate fraction of RBL-1 cells (Table 1). However, it did not reverse the PMA-induced decrease of PKC activity in the cytosolic fraction. Instead, we observed a further decrease of the cytosolic PKC activity. This could be due to the greater inhibitory effect of accumulated staurosporine on the small

[3] S. Mong, H.-L. Wu, A. Wong, H. M. Sarau, and S. T. Crooke, unpublished observation.

TABLE 1

Effect of staurosporine pretreatment on PMA-induced translocation of PKC in RBL-1 cells

RBL-1 cells were incubated with and without staurosporine or PMA and assayed for PKC activity in the cytosol and particulate fractions as described under Experimental Procedures. Values are the mean ± standard error of triplicate determinations from a representative experiment. Similar results were obtained in three separate experiments.

Treatment	Cytosolic PKC		Particulate PKC	
	Specific activity	Total activity	Specific activity	Total activity
	pmol/mg/min	*pmol/min*	*pmol/mol/min*	*pmol/min*
Control	1160 ± 22	601 ± 11	298 ± 47	92 ± 15
PMA (1 μM), 5 min	201 ± 27	107 ± 14	2309 ± 31	661 ± 9
Staurosporine (1 μM), 10 min	1050 ± 55	582 ± 8	315 ± 24	102 ± 8
Staurosporine (1 μM), 10 min + PMA (1 μM) last 5 min	102 ± 7	54 ± 4	962 ± 17	349 ± 6

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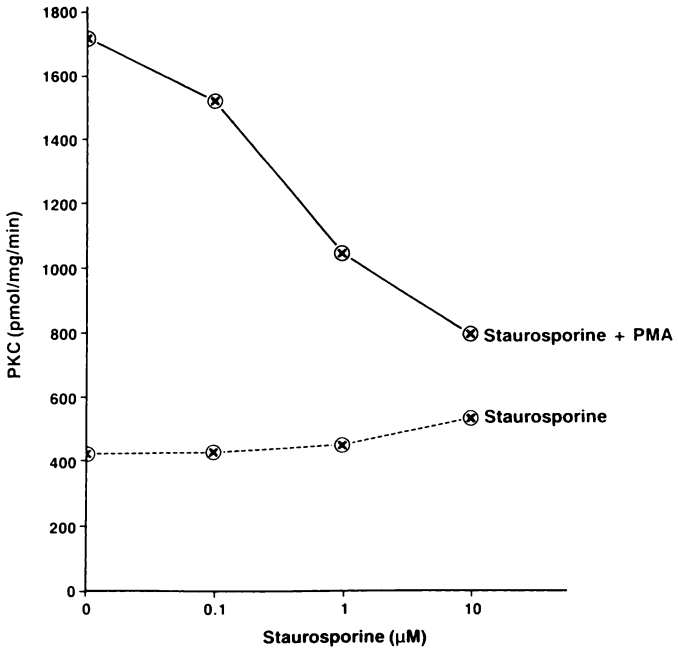

Fig. 4. Concentration response study of staurosporine pretreatment on PMA-induced activation of PKC in RBL-1 cells. Cells were incubated with and without staurosporine for 2 min and then treated with PMA (1 μM) for 2 min, as described in Fig. 1. Particulate fractions were prepared and assayed for PKC activity as described under Experimental Procedures. Each value represents the average of two separate experiments assayed in triplicate which differed by <5%. Specific activity is expressed as pmol/mg/min.

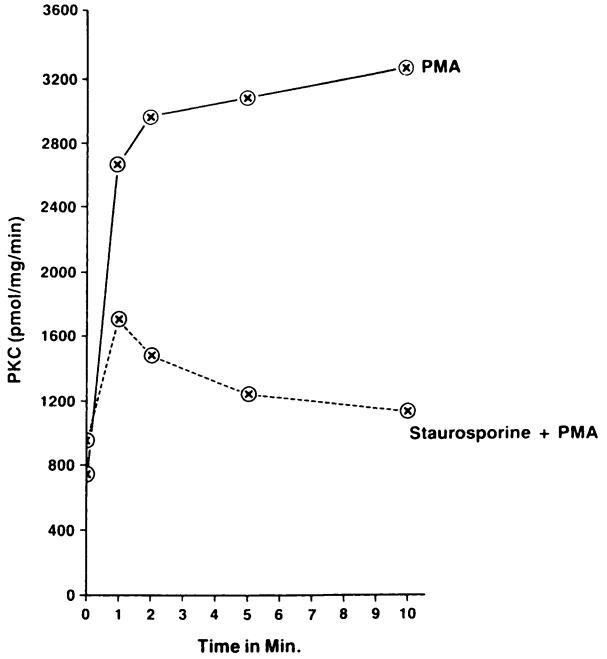

Fig. 5. Pretreatment of staurosporine on time response study of PMA on PKC in intact RBL-1 cells. RBL-1 cells were incubated with and without staurosporine (10 μM) for 10 min and then treated with 1 μM PMA for 10 sec to 10 min. The particulate fractions were prepared and assayed for PKC activity as described under Experimental Procedures. Each value represents the average of two separate experiments, each assayed in triplicate, which differed by <5%. Specific activity is expressed as pmol/mg/min.

pool of PKC available in the cytosol after PMA treatment. These results suggest that the inhibition of the phorbol ester-mediated effects by staurosporine may be due to direct inhibition of activated enzyme rather than blockade of the PKC translocation. The results showing the dose-dependent (Fig. 4) and time-dependent (Fig. 5) inhibition of the PMA activated PKC in the particulate fraction of RBL-1 cells by staurosporine further support this hypothesis.

It has been indicated that staurosporine inhibits cAMP- and cGMP-dependent protein kinases with the same potency as it inhibits PKC (13). The interference of these kinases in our experimental results appears to be less likely because, in our experiments, the cells were stimulated by PMA which is known to be a specific activator of PKC. However, we also believe that caution should be exercised in interpreting the data using staurosporine under other experimental conditions. The relatively higher concentration of staurosporine required to inhibit the PMA-induced activation of PKC (Figs. 4 and 5) as compared to that required to inhibit partially purified PKC (Fig. 3) might reflect the permeability of RBL-1 cells for staurosporine or the possibility that it interacts with other cellular components. We also observed a slight difference in the IC_{50} values for staurosporine reversal of LTD_4-induced Ca^{2+} mobilization (0.1 μM, Fig. 2) and inhibition of PMA-induced translocation of PKC to membranes (0.9 μM, Fig. 4). This could be due to the higher concentration of PMA (1 μM) we used to maximally activate PKC in these experiments. Still, staurosporine appears to be the most potent inhibitor of PKC *in vitro* and in intact cells compared to the other available inhibitors.

In summary, our results demonstrate that PKC is the mediator of PMA-induced heterologous desensitization in RBL-1 cells. The evidence to support this hypothesis includes the following.

1. Staurosporine is a potent inhibitor of partially purified PKC in RBL-1 cells.
2. Staurosporine prevented the PMA-induced inhibition of the LTD_4-stimulated Ca^{2+} mobilization in RBL-1 cells.
3. Staurosporine effectively inhibited PMA-induced activation of PKC in the intact RBL-1 cells.

Our results also further suggest that staurosporine can be used as a valuable tool to understand the role of PKC in intact cells.

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Send reprint requests to: Dr. Stanley T. Crooke, Department of Molecular Pharmacology, Smith Kline & French Laboratories, P. O. Box 1539, King of Prussia, PA 19406-0939.

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