Contrasting Actions of Staurosporine, a Protein Kinase C Inhibitor, on Human Neutrophils and Primary Mouse Epidermal Cells

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ABSTRACT

Staurosporine, a recently described microbial alkaloid, is uniquely potent as an inhibitor of protein kinase C in vitro, being active at nM concentrations rather than the μM concentrations typical of other inhibitor classes. Like these other inhibitors, however, staurosporine exhibits only limited selectivity among different protein kinases. We report here that, in intact human neutrophils, nM concentrations of staurosporine blocked the action of the phorbol ester tumor promoters. In mouse primary epidermal cells, on the other hand, staurosporine failed to block the effects of phorbol 12,13-dibutyrate on epidermal growth factor binding sites and on induction of ornithine decarboxylase and epidermal transglutaminase. Unexpectedly, staurosporine induced morphological changes in keratinocytes to a dendritic shape resembling that induced by the phorbol esters. It also induced epidermal transglutaminase and cornified envelope production, markers of the differentiative pathway in the epidermal cells. We conclude that the effectiveness of staurosporine as a protein kinase C inhibitor in intact cells may depend markedly on the cell system. Other actions of staurosporine may predominate, and, in keratinocytes, its activity is suggestive of a tumor promoter rather than of an inhibitor of tumor promotion.

INTRODUCTION

Protein kinase C has attracted great research interest (1, 2). It is the major receptor of the phorbol ester tumor promoters (3, 4) and mediates one arm of the phosphatidylinositol second messenger system. This system is involved in signal transduction for a broad array of hormones and cellular effectors, including both growth factors and several oncogenes (5, 6).

A number of compounds inhibit protein kinase C enzymatic activity. These include H-7 (7), sphingosine (8), palmitoyl carnitine (9), and chlorpromazine (10). These inhibitors unfortunately have proven to be of limited usefulness in biological systems because of a combination of low potency—typically μM—and limited selectivity. H-7, for example, shows similar KiS for protein kinase C and for the cAMP-dependent and cGMP-dependent protein kinases, although its Ki for myosin light chain kinase is 15- to 20-fold greater (7). H-7 is, moreover, competitive with ATP, which has a Ki of 7 μM for protein kinase C (11), so effective biological concentrations of H-7 would need to be in appreciable excess over those for ATP in biological systems, which may be nm.

Recently, a new class of protein kinase C inhibitors has been reported which are unique in terms of their high potencies. Staurosporine and the related compounds K252a,b inhibit protein kinase C enzymatic activity in vitro with KiS of 2.7 nM (12) and 20 to 25 nM (13), respectively. Although the details of their inhibitory mechanisms remain to be established, we have shown that staurosporine potently inhibits the proteolytically generated catalytic domain of protein kinase C (14), whereas it has no effect on phorbol ester binding activity (12, 14). Likewise, inhibition by K252a was reported to be competitive with ATP but not with the phosphate-accepting substrate or with Ca2+ and phospholipid, modulators of the regulatory domain. Like H-7, however, staurosporine and K252a,b show limited selectivity among different protein kinases. Staurosporine inhibited the p60V-src tyrosine kinase with an IC50 of 6.4 nM (15), and K252a inhibited the cAMP-dependent and cGMP-dependent protein kinases with KiS of 18 and 20 nM, respectively (13).

In intact platelets, staurosporine blocked the phorbol ester-induced rise in intracellular calcium (IC50 = 27 nM) and phorbol ester-induced alkalization (at a staurosporine concentration of 100 nM) (16). Effects of staurosporine on the response to phorbol esters in other systems have not as yet been reported. Other biological effects of staurosporine include induction of differentiation in human NB-1 neuroblastoma cells at 20 nM (17) and inhibition of platelet aggregation in response to collagen or ADP at μM concentrations (18). Cytotoxic concentrations ranged from 4 x 10⁻¹² M for HeLa S3 cells upon a 72-h incubation (12) to 2.1 x 10⁻⁷ M for the NB-1 cells (17). The related rebeccamycin displayed an IC50 of 6 μg/ml for a 1-h exposure of human lung adenocarcinoma cells (19). The stability of staurosporine under culture conditions has not been determined.

We analyze here the effect of staurosporine on phorbol ester action in two biological systems—human neutrophils and mouse primary epidermal cells. Neutrophils have been extensively studied as a model system for stimulus-response coupling (20). The NADPH-oxidase of neutrophils can be activated both through protein kinase C-dependent and -independent pathways. Mouse primary epidermal cells are of great relevance for exploring the tumor-promoting activity of the phorbol esters. Subpopulations of the epidermal cells display both proliferative and differentiative responses; the induction of the differentiative pathway is thought to be essential for the promotion response (21, 22).

MATERIALS AND METHODS

PDBU (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide at a concentration of 50 μg/ml, stored at −20°C, and diluted with medium containing serum for treatment of cells. The final concentration of dimethyl sulfoxide did not exceed 0.1%. Staurosporine, the generous gift of Kyowa Hakko Kogyo Co., Ltd., was dissolved in dimethyl sulfoxide at a concentration of 2 mM, stored at −20°C in small aliquots, and diluted with medium containing serum to the desired concentration immediately before treatment of cells.

Neutrophils were isolated from normal human donors as described (23). Neutrophil respiratory burst activation was assessed by measuring O2− generation by a modification of the method of Cohen and Chov-
anemic (24). Briefly, neutrophils (10⁶ cells) were incubated for 3 min at 37°C in 140 mM NaCl-3 mM KCl-1 mM K₂HPO₄-1 mM CaCl₂-1 mM MgCl₂-5 mM glucose, pH 7.4, 0.9-ml final volume, in the presence or absence of the indicated concentrations of staurosporine. An additional 0.10 ml of the above solution containing cytochrome c (final concentration in 1 ml, 30 μM) PMA (final concentration in 1 ml, 100 ng/ml), and, for the reference cuvette, superoxide dismutase (30 μg) was added to yield a final volume of 1.0 ml. The sample was incubated for 5 min at 37°C with continuous monitoring of absorbance at 550 nm. For measurement of staurosporine inhibition of opsonized zymosan or arachidonate-induced respiratory burst activity, PMA was replaced by 2-mg/ml particles or 82 μM (final concentration) arachidonic acid, respectively; for stimulation by FMLP, 5 μM FMLP was used in place of PMA, and 5 μg/ml of cytochalasin B were included in the 3-min preincubation period.

Cell Culture. Epidermal cells were prepared from newborn BALB/c mice by the trypsin floatation technique and cultured in low calcium (0.05 mM) Eagle's minimal essential medium (MA Bioproducts, Walkersville, MD) prepared with reduced calcium and supplemented with 8% Chelated-treated fetal calf serum (Reheis Chemical Co., Kankakee, IL) as described previously (25). All media contained 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY). Cells were plated at an initial density of 0.24 x 10⁶/cm² in Costar 6-well tissue culture dishes for determination of ODC and TGase induction and cornified envelope formation. Media were changed daily for the first 3 days and every 2 to 3 days thereafter. All experiments were done on confluent cells at 3 to 7 days after plating. Cultures were routinely incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Enzyme Assays. For assay of ODC, TGase, and protein, cells were treated as described in the figure legends. They were then washed 3 times with Dulbecco’s phosphate-buffered saline (0.2 g of KCl-0.2 g of KH₂PO₄-8 g of NaCl-2.16 g of NaHPO₄-7H₂O/liter), frozen on dry ice, and stored at −20°C until assay. The cells were then scraped from the culture dish with 300 μl of lysis buffer containing 0.02 M sodium phosphate (pH 7.5), 0.5 mM EDTA, 10 mM dithiothreitol, and 50 μg/ml of phenylmethanesulfonyl fluoride. Aliquots were analyzed for ODC or for TGase and for protein.

ODC was measured by the release of ¹⁴CO₂ from [¹⁴C]-ornithine (57 mCi/mmol; Amersham, Arlington Heights, IL) as described (26). TGase activity was assayed by transfer of [2,3-³H]putrescine (New England Nuclear, Boston, MA) to casein (27). Protein was determined by the method of Lowry et al. (28) after destruction of dithiorthiol with chloramine T (29).

EGF binding was assayed with ¹²⁵I-EGF (150 to 200 μCi/μg; New England Nuclear, Boston, MA), with or without unlabelled EGF, 1 μg/ml (receptor grade; Collaborative Research, Lexington, MA), as previously described (30, 31). Treatment of cells with staurosporine and/or PDBu was carried out in freshly changed low calcium medium containing 8% chelated-treated serum, 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY). Cells were plated at an initial density of 0.24 x 10⁶/cm² in Costar 6-well tissue culture plates for assay of EGF binding and in 60-mm Falcon tissue culture dishes for determination of ODC and TGase induction and cornified envelope formation. Media were changed daily for the first 3 days and every 2 to 3 days thereafter. All experiments were done on confluent cells at 3 to 7 days after plating. Cultures were routinely incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

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Inhibition of NADPH-Oxidase Activation. Staurosporine was a potent inhibitor of superoxide generation by human neutrophils stimulated by PMA at 100 ng/ml (Fig. 1). The IC₅₀, derived from the averaged data from 5 experiments, was 3.8 nm. In contrast to its activity as an inhibitor of superoxide production upon phorbol ester stimulation, staurosporine at up to 40 nm caused little (<10%) inhibition of stimulation by FMLP (Fig. 1). Likewise, no inhibition of stimulation by arachidonic acid or opsonized zymosan was observed at staurosporine concentrations of up to 100 nm (2 to 3 experiments each, data not shown); for these latter experiments, a positive control was included confirming the inhibition by staurosporine of phorbol ester stimulation in each case. These results are consistent with our previous findings utilizing these agonists, which also indicated, based on different criteria, that these agonists activated the respiratory burst by a protein kinase C-independent pathway (33, 34).

EGF Binding. Phorbol ester treatment leads to rapid inhibition of binding of ¹²⁵I-EGF to EGF receptors on primary mouse epidermal cells (30, 35, 36), as is the case for many other cell types. Incubation of the epidermal cells for 1 h with staurosporine paradoxically reduced ¹²⁵I-EGF binding in a dose-dependent fashion (Fig. 2). The magnitude of inhibition of ¹²⁵I-EGF binding was less than that observed for PDBu treatment within the same experiment. It was not determined whether the effect of staurosporine was on the number of binding sites or
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on binding affinity. The inhibition of EGF binding by PDBu was a little decreased by staurosporine at high concentrations, although complete blockage was not observed.

ODC Activity. The induction of ODC (EC 4.1.1.17), the enzyme that converts ornithine to putrescine and CO₂, has been associated with an increased rate of proliferation in epidermal cells and is an early response to phorbol ester exposure in keratinocytes (37). Treatment for 3 h with staurosporine did not stimulate ODC activity at all. Staurosporine did not inhibit ODC induction by PDBu at concentrations of 20 nM (Fig. 3). Reduction of PDBu-induced activity at the concentration of 200 nM staurosporine may be due to cytotoxicity, as suggested by morphology under phase-contrast microscopy.

Cell Morphology. Mouse epidermal cells grown in low Ca²⁺ medium (0.05 mM Ca²⁺) maintain a continuously proliferating population, although at all times a fraction of the cells undergoes terminal differentiation and becomes detached from the monolayer. Under these conditions monolayers were confluent by 3 days. These cells had a polygonal shape with distinct intercellular space giving a paving stone appearance. Many round cells were also observed floating in the low Ca²⁺ medium.

Primary mouse epidermal cells plated and maintained at low calcium levels (0.05 mM) were treated with staurosporine or PDBu. After 1-h incubation with each compound, cells were observed by phase-contrast microscopy (Fig. 4). Treatment with PDBu (100 nM) led to marked morphological change characterized by contraction to give a dendritic shape and by cell rounding, followed by eventual release from the monolayer and terminal differentiation of a subpopulation of cells (Fig. 4B). Treatment with staurosporine (60 nM) induced similar morphological change to that by PDBu upon 1-h incubation (Fig. 4C).

Transglutaminase Activity. TGase (EC 2.3.2.13) is the enzyme that is presumed to catalyze the formation of the glutamyl-lysyl cross-links abundant in the cornified envelopes of keratinocytes. TGase is thus considered to be a good marker for differentiation induced by the phorbol esters in epidermal cell cultures (38, 39).

Epidermal cells grown in low Ca²⁺ medium were treated with PDBu (200 nM) and/or with staurosporine at varying concentrations for 9 h. This incubation time is optimal for PDBu induction of TGase (39). Staurosporine at concentrations of 6 to 20 nM stimulated TGase induction to the same degree as did PDBu. There was no additional effect of staurosporine on PDBu-induced TGase activity when cells were exposed to both compounds simultaneously. The decrease of TGase induction at high concentrations of staurosporine may be due to cytotoxicity or additional sites of action (Fig. 5).

Cornified Cell Production. The formation of an insoluble cell envelope is a second prominent characteristic of the maturation and terminal differentiation process in epidermal cells. Treatment with phorbol ester for 24 h in the presence of high Ca²⁺ medium has been known to enhance formation of cornified cells (38). As was the case for transglutaminase induction, staurosporine by itself in the presence of high Ca²⁺ medium...
epidermal cells indicate that staurosporine did not effectively block phorbol ester responses in this system. Intriguingly, staurosporine by itself induced phorbol ester-like effects on morphology and differentiation. Other protein kinase C inhibitors, H-7 or palmitylcarnitine, failed to induce markers of terminal differentiation in the epidermal cells (data not shown), suggesting that these effects were not an unexpected consequence of protein kinase C inhibition. We have postulated that induction of differentiation in epidermal cells represents a critical feature of the cellular response to tumor promoters (21). The differentiative action of staurosporine suggests that this in vitro inhibitor of protein kinase C may function in vivo as a tumor promoter. Consistent with our results in tissue culture, recent studies by Fujiki and coworkers indeed indicate moderate tumor-promoting activity of staurosporine in mouse skin (43).

Our findings in mouse epidermal cells, together with those reported earlier for the NB-1 neuroblastoma cells, demonstrate potent differentiating activity for staurosporine. Exploration of the mechanism for this response and understanding of its relation to kinase inhibitory activity remain important priorities. Our results indicate that staurosporine may be a useful tool for blocking phorbol ester responses. Its in vitro activity on other kinases and the biological evidence for other effects, however, dictate marked caution in the interpretation of results with this agent.

REFERENCES


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