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Staurosporine inhibition of intracellular free Ca^{2+} transients in mitogen-stimulated Swiss 3T3 fibroblasts

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The microbial alkaloid staurosporine is a potent inhibitor of protein kinase C [1] and a weaker inhibitor of tyrosine protein kinase activity [2]. The biological effects of staurosporine are generally ascribed to its ability to inhibit protein kinase C [3–5]. Staurosporine is a potent inhibitor of fibroblast DNA synthesis and proliferation [6], although a number of studies have shown that protein kinase C is not essential for fibroblast proliferation [6–9]. This has led to the suggestion that staurosporine has effects in addition to inhibition of protein kinase C that account for its effect on cell proliferation [6]. A common event that occurs early in the action of a number of growth factors and mitogens is a transient increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)* [10, 11]. This increase in $[Ca^{2+}]_i$ has been implicated in mediating the effects of these agents on cell proliferation [11–13]. We have examined the effects of staurosporine on the increase in $[Ca^{2+}]_i$ caused by growth factors and mitogens and on the release of Ca^{2+} from intracellular stores using Swiss 3T3 fibroblasts.

Materials and methods

Staurosporine and *myo*-inositol 1,4,5-trisphosphate [IP_3(1,4,5)] were purchased from Calbiochem (San Diego, CA). Arachidonic acid, vasopressin-^{8}Arg, bradykinin and phorbol 12-myristate 13-acetate (PMA) were purchased from the Sigma Chemical Co. (St Louis, MO). $^{45}CaCl_2$ (25 mCi/mg) was purchased from the Amersham Corp. (Arlington Heights, IL) and [^{32}P]orthophosphoric acid (9 Ci/μmol) from Dupont NEN (Boston, MA). Platelet-derived growth factor (PDGF) as the B chain homodimer was purchased from Bachem Inc. (Torrance, CA). Aequorin was provided by Dr John Blinks, Mayo Clinic. Swiss 3T3 fibroblasts were provided by Dr H. R. Herschmann, University of California, Los Angeles, CA. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and harvested at each passage with 0.5% trypsin and 0.5 mM EGTA before becoming confluent. Studies were conducted on cells between passages 37 and 58. Measurement of $[Ca^{2+}]_i$ employed Swiss 3T3 fibroblasts loaded with the Ca^{2+}-sensitive photoprotein aequorin by a low Ca^{2+} centrifugation technique as previously described [11]. The cells were plated in DMEM containing 10% fetal calf serum and allowed to attach to the surface of a culture dish for 18 hr, then they were exposed to DMEM without fetal calf serum for 2 hr. Growth factors and mitogens dissolved in 0.2 mL DMEM without fetal calf serum were added to the culture dish. Light emitted by the aequorin-loaded cells was measured as previously described [11] and converted to an estimate of $[Ca^{2+}]_i$ using the calibration method of Allen and Blinks [14]. Cells were exposed to staurosporine for 3 hr before and during the exposure to the growth factors and mitogens.

The ATP-dependent uptake and agonist-induced release of $^{45}Ca^{2+}$ by the endoplasmic reticulum of saponin-permeabilized Swiss 3T3 fibroblasts was measured by the method of Ghosh *et al.* [15]. Preliminary studies showed that $^{45}Ca^{2+}$ uptake by the permeabilized Swiss 3T3 cells had reached a plateau by 6 min. IP_3(1,4,5), 10 μM, and arachidonic acid, 50 μM, were added to the preparation at 6.25 min, and $^{45}Ca^{2+}$ remaining in the cells was measured at 7 min. Staurosporine was added to the preparation at 0 min.

For measurement of ligand-dependent PDGF receptor phosphorylation, Swiss mouse 3T3 cells were grown to confluency, in 35 mm tissue culture dishes, in DMEM containing 10% fetal calf serum. Forty-eight hours after the last medium change, the confluent cultures were washed in 37° serum-free labeling medium (phosphate-free DMEM containing 2% bovine serum albumin, 2 mM L-glutamine, buffered to pH 7.2 with 10 mM HEPES). The washed cultures were then incubated for 4 hr in serum-free labeling medium (4 mL/dish) containing 2 mCi [^{32}P]orthophosphate

* Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PMA, phorbol 12-myristate 13-acetate; IP_3(1,4,5), *myo*-inositol 1,4,5-trisphosphate; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; and HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

per dish. Staurosporine, 1 μM, was added 1 hr after labeling was initiated and treatment continued for the remainder of the labeling period. Subsequently, selected samples were stimulated with PDGF at a final concentration of 3×10^{-9} M. After 10 min at 37°, the dishes were placed in an ice bath, the medium was rapidly aspirated, and the cell monolayers were washed with ice-cold phosphate-buffered saline containing 0.5 mM EDTA. The cells were then solubilized with 1 mL/dish of 20 mM Tris–HCl, pH 7.6, 30 mM $Na_2P_2O_7$, 50 mM NaF, 40 mM NaCl, 5 mM EDTA, 100 μM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer) containing 1 mg/mL bovine serum albumin and 1% (w/v) Triton X-100. The lysates were incubated for 10 min at 0° with intermittent vortexing and centrifuged at 7000 *g* for 5 min to remove nuclei and other insoluble material. Post-nuclear supernatant fractions were cleared with 100 μL Sepharose 4B beads coupled to bovine serum albumin at a density of 10 mg bovine serum albumin/mL. After 1 hr at 4°, the cleared supernatant fractions were immunoprecipitated with 20 μL of Sepharose 4B coupled with the monoclonal antiphosphotyrosine antibody, 1G2 [16], at a density of 15 mg antibody/mL of beads. After 3 hr of rotation at 4°, the immunoprecipitates were washed three times in lysis buffer containing 1 mg/mL bovine serum albumin and 1% Triton-X-100, followed by two washes with lysis buffer plus 1% Triton X-100 (no bovine serum albumin). Antibody-bound phosphoproteins were specifically eluted from the beads with the competitive ligand, phenylphosphate (10 mM), in lysis buffer plus 100 μg/mL ovalbumin and 1% Triton X-100. Proteins were precipitated with −20° acetone, and solubilized by boiling in sodium dodecyl sulfate (SDS)-containing sample buffer prior to discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The proteins were electrophoresed through 10% polyacrylamide gels along with protein molecular weight standards (Bio-Rad, Richmond, CA). Radiolabeled phosphoproteins were visualized by autoradiography at −70°.

Groups of data were analyzed using Student's *t*-test [17], and $P < 0.05$ was considered statistically significant.

Results and discussion

Staurosporine inhibited the growth of Swiss 3T3 fibroblasts in serum-supplemented medium with an IC_{50} for continuous exposure of 3.5×10^{-8} M (results not shown).

Bradykinin, vasopressin and PDGF are all known to induce the proliferation of Swiss 3T3 fibroblasts [11, 18, 19] and to cause an increase in $[Ca^{2+}]_i$ [11, 20, 21]. The concentrations of the agents chosen for the study gave the maximum increases in $[Ca^{2+}]_i$ (Fig. 1). Staurosporine pretreatment had no effect on resting $[Ca^{2+}]_i$ in Swiss 3T3 fibroblasts at 3 hr but reduced the increase in $[Ca^{2+}]_i$ caused by PDGF but not by bradykinin and only marginally reduced the increase in $[Ca^{2+}]_i$ caused by vasopressin (Fig. 2). For example, the inhibition of the $[Ca^{2+}]_i$ increases seen with 10^{-6} M staurosporine was for PDGF 80 ± 3% ($P < 0.01$ compared to PDGF alone), for vasopressin 20 ± 6% ($P > 0.05$ compared to vasopressin alone) and

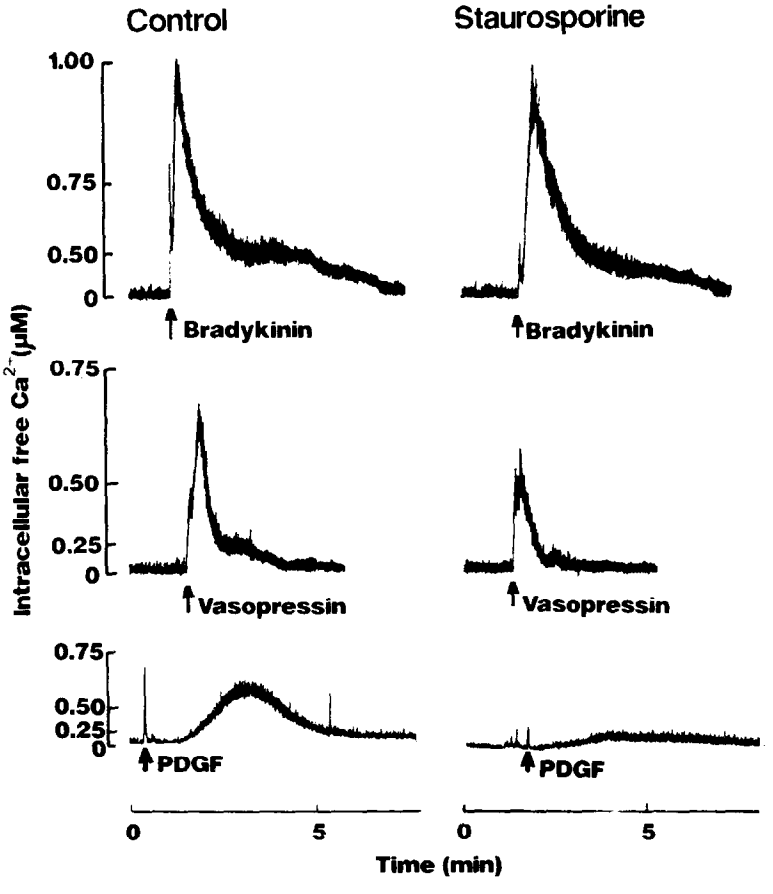

Fig. 1. Effect of staurosporine on $[Ca^{2+}]_i$ changes caused by growth factor and mitogens in Swiss 3T3 fibroblasts. The agents used were: upper panel, bradykinin, 2×10^{-7} M; middle panel, vasopressin, 10^{-7} M; and, lower panel, PDGF, 3×10^{-9} M. Additions were made at the arrows. Left panels, control cells; right panels, cells exposed to 10^{-7} M staurosporine for 3 hr prior to addition of the agent.

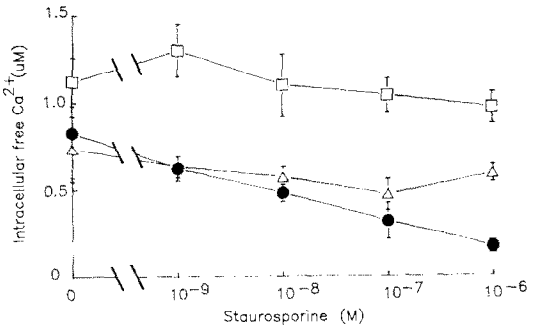

Fig. 2. Staurosporine concentration–response curve for the inhibition of the $[Ca^{2+}]_i$ increase caused by 3.3×10^{-9} M PDGF, (●), 10^{-7} M vasopressin (△) and 2×10^{-7} M bradykinin (□) in Swiss 3T3 fibroblasts. Each point is the mean of at least five experiments and bars are SD.

for bradykinin 13 ± 8% ($P > 0.05$ compared to bradykinin alone). Staurosporine did not affect the ability of aequorin to respond to an increase in $[Ca^{2+}]_i$. This was shown in two ways. Staurosporine, 10^{-7} M, had no effect on the light emitted when aequorin was mixed with EGTA-buffered Ca^{2+} at various concentrations. Staurosporine also did not affect the transient increase in $[Ca^{2+}]_i$ when Swiss 3T3 fibroblasts in Ca^{2+}-free DMEM were exposed to DMEM containing 1.8 mM Ca^{2+}. Without staurosporine the external Ca^{2+}-induced increase in $[Ca^{2+}]_i$ was (± SD, N = 17) 1.25 ± 0.25 μM and with 10^{-7} M staurosporine the increase in $[Ca^{2+}]_i$ was 1.22 ± 0.39 μM ($N = 8, P > 0.05$). This observation also shows that staurosporine was not altering Ca^{2+} fluxes across the plasma membrane. Voltage-dependent Ca^{2+} channels are known to be present in the plasma membrane of Swiss 3T3 fibroblasts [22] and to contribute, in part, to the increase in $[Ca^{2+}]_i$ caused by PDGF [21]. Staurosporine was also unlikely to be stimulating the efflux of Ca^{2+} from the cell either by activating the plasma membrane Ca^{2+} pump [23] or increasing Na^+/Ca^{2+} exchange [24].

Staurosporine at concentrations greater than about 10^{-8} M has been reported previously to block the increase in $[Ca^{2+}]_i$ caused by thrombin in platelets [25] and by concanavalin A, phytohemagglutinin and monoclonal antibody directed against the antigen receptor/CD3 complex in human T lymphoblasts [26]. Inhibition of a positive feedback control exerted by protein kinase C on changes in $[Ca^{2+}]_i$ was suggested to explain the action of staurosporine in human T lymphoblasts [26]. Such an action, however, cannot explain the effects of staurosproine in Swiss 3T3 fibroblasts where protein kinase C exerts a negative feedback control of growth factor-induced changes in $[Ca^{2+}]_i$ [27–29]. This is shown in the present study by the ability of 10^{-7} M PMA, an activator of protein kinase C [30], to inhibit the increase in $[Ca^{2+}]_i$ in Swiss 3T3 fibroblasts caused by PDGF and vasopressin (Table 1).

The increase in $[Ca^{2+}]_i$ in Swiss 3T3 fibroblasts caused by bradykinin and vasopressin, and part of the increase in $[Ca^{2+}]_i$ caused by PDGF are produced by the release of Ca^{2+} from endoplasmic reticulum stores [21]. We examined the possibility that staurosporine might be producing its effect on $[Ca^{2+}]_i$ by blocking the release of Ca^{2+} from the endoplasmic reticulum. To do this we measured the ability of staurosporine to block the release of $^{45}Ca^{2+}$ from saponin-permeabilized Swiss 3T3 fibroblasts using the putative intracellular second messengers inositol 1,4,5-trisphosphate [31] and arachidonic acid [32]. Staurosporine, 10^{-7} M, had no effect on $^{45}Ca^{2+}$ release by either agent (Table 2). Even at 10^{-6} M, staurosporine did not affect $^{45}Ca^{2+}$ release by inositol 1,4,5-trisphosphate significantly (results not shown). The possibility remains, however, that staurosporine inhibits the release of $^{45}Ca^{2+}$ from endoplasmic reticulum stores that is mediated by some other mechanism. Recent evidence has suggested that the increase in $[Ca^{2+}]_i$ caused by PDGF is not mediated by an increase in inositol 1,4,5-trisphosphate [33, 34] but may involve a different class of inositol phosphates [33]. Another mechanism that may account for a decreased $[Ca^{2+}]_i$ response caused by staurosporine is a decreased accumulation of Ca^{2+} by the endoplasmic reticulum stores. However, staurosporine had no effect on the uptake of $^{45}Ca^{2+}$ by saponin-permeabilized Swiss 3T3 fibroblasts (Table 2).

Table 1. Effect of modulation of protein kinase C activity on growth factor induced changes in $[Ca^{2+}]_i$ in Swiss 3T3 fibroblasts

Treatment	Vasopressin	PDGF
None	0.73 ± 0.19	0.81 ± 0.28
Staurosporine	0.47 ± 0.09*	0.32 ± 0.11*
PMA	0.36 ± 0.05*	0.58 ± 0.06*

Peak $[Ca^{2+}]_i$ responses were recorded after exposure of aequorin-loaded Swiss 3T3 fibroblasts to either vasopressin, 10^{-7} M, or PDGF, 3.3×10^{-9} M. Treatments modulating protein kinase C activity were exposure to 10^{-7} M staurosporine for 3 hr and 10^{-7} M PMA for 5 min. Values are means ± SD of up to 12 experiments.

* $P < 0.05$ compared to value with no treatment.

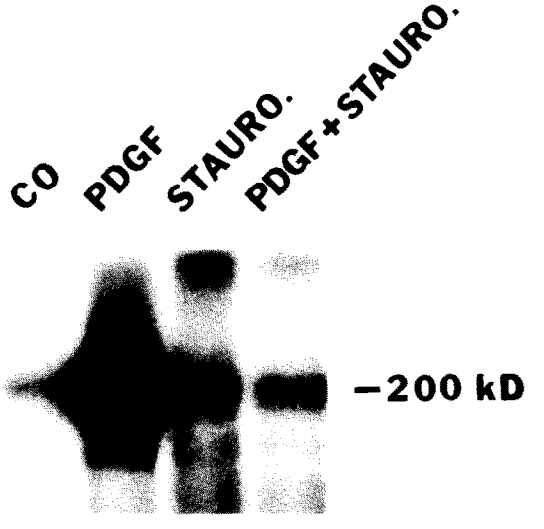

Fig. 3. Block of PDGF autophosphorylation by staurosporine. Confluent 3T3 cells were labeled with [^{32}P]orthophosphate for 4 hr in serum-free medium. Staurosporine, 1 μM (Stauro), was added to the indicated samples 1 hr after labeling was initiated (3-hr pretreatment). Labeled cells were subsequently stimulated with 3×10^{-9} M PDGF or buffer only (Co), and the incubations were continued for 10 min. Detergent-soluble proteins were immunoprecipitated with anti-phosphotyrosine antibodies and analyzed by SDS–PAGE in 10% polyacrylamide gels. Radiolabeled phosphoproteins were detected by autoradiography for 20 hr at −70°. Gels were calibrated with non-radioactive molecular weight standards. The autoradiogram depicts the region of the gel predicted to contain the phosphorylated PDGF receptor (185–195 kD).

Table 2. Effect of staurosporine on $^{45}Ca^{2+}$ uptake and release by permeabilized Swiss 3T3 fibroblasts

Treatment at 6 min		Cell $^{45}Ca^{2+}$ (pmol/10^6 cells)	
		Without staurosporine	Staurosporine
None	6 min	204 ± 5	210 ± 5
	7 min	182 ± 7 (89)	183 ± 6 (87)
$IP_3(1,4,5)$	6 min	202 ± 7	199 ± 4
	7 min	117 ± 5* (56)	123 ± 8* (62)
$C_{20:4}$	6 min	215 ± 7	206 ± 9
	7 min	116 ± 5* (54)	101 ± 5* (49)

Uptake of $^{45}Ca^{2+}$ by saponin-permeabilized Swiss 3T3 fibroblasts was measured between 0 and 6 min. *myo*-Inositol 1,4,5-trisphosphate [$IP_3(1,4,5)$], 10 μM, and arachidonic acid ($C_{20:4}$), 50 μM, were added at 6.25 min, and Ca^{2+} release was measured at 7 min. Staurosporine, 10^{-7} M, was added to the incubation at 0 min. Values are means ± SD of 5 experiments. Numbers in parentheses are the percent of the appropriate 6-min value.

* $P < 0.01$ compared to 6-min value.

A final possibility to be considered is that the decreased $[Ca^{2+}]_i$ response caused by staurosporine is due to inhibition of receptor-dependent protein kinase activities. Staurosporine has been reported to be a weak inhibitor of some tyrosine protein kinase activities [2]. Recent data indicate that growth factors may regulate phosphoinositide hydrolysis in fibroblasts by stimulating the phosphorylation of phospholipase C at tyrosine residues [35]. The PDGF receptor contains an intrinsic tyrosine kinase domain that phosphorylates both the receptor itself and other cellular proteins as a consequence of ligand binding [35]. PDGF receptor autophosphorylation, therefore, serves as a convenient marker for the activation of the receptor tyrosine kinase subsequent to ligand binding. To determine whether staurosporine blocked PDGF receptor tyrosine kinase activity, PDGF receptors were immunoprecipitated from [^{32}P]orthophosphate-labeled 3T3 cells with a monoclonal antiphosphotyrosine antibody. As shown in Fig. 3, PDGF stimulated a profound increase in the labeling of a protein in the 170–200 kD region of the gel, which corresponds to the previously described electrophoretic mobility of the PDGF receptor [36]. Although pretreatment with 1 μM staurosporine for 3 hr had no effect on PDGF receptor phosphorylation (Fig. 3, lane 3), PDGF-dependent PDGF receptor autophosphorylation was inhibited almost completely in staurosporine-pretreated Swiss 3T3 cells (Fig. 3, lane 4). Staurosporine also blocked the tyrosine phosphorylation of 116 kD and 74 kD (broad triplet), and 60 kD and 42 kD proteins induced by a 10-min stimulation with PDGF (data not shown). These results suggest that inhibition of the ligand-activated PDGF receptor tyrosine kinase may contribute to the inhibitory effects of staurosporine on PDGF-dependent $[Ca^{2+}]_i$ increases in 3T3 fibroblasts. It should be noted, however, that while qualitatively, PDGF-receptor autophosphorylation was inhibited completely by 10^{-6} M staurosporine, the increase in $[Ca^{2+}]_i$ caused by PDGF was inhibited only 80%. It is possible that the remaining increase in $[Ca^{2+}]_i$ caused by PDGF utilizes a mechanism that does not involve PDGF receptor tyrosine kinase activation, although what this mechanism might be is not clear.

Binding of vasopressin to the V_1 vascular-type vasopressin receptor responsible for Ca^+ mobilization by vasopressin also leads to phosphorylation of cellular proteins [37]. However, the pattern of protein phosphorylation resembles that seen with phorbol esters, suggesting that it is due to activation of protein kinase C [37]. It is possible that other protein kinases may also be activated by vasopressin leading to an increase in $[Ca^{2+}]_i$, and these could be inhibited by staurosporine leading to a partial block in the increase in $[Ca^{2+}]_i$.

In summary, we have shown that staurosporine inhibited the increase in $[Ca^{2+}]_i$ caused by PDGF and to a lesser degree that caused by vasopressin but not that caused by bradykinin in Swiss 3T3 fibroblasts. The effect is unlikely to be mediated by the inhibition of protein kinase C by staurosporine since activation of protein kinase C by PMA resulted in feedback inhibition of the increases in $[Ca^{2+}]_i$ in the same cells. Staurosporine did not block an increase in $[Ca^{2+}]_i$ caused by the influx of external Ca^{2+}, nor did it block the release of Ca^{2+} from the endoplasmic reticulum of permeabilized Swiss 3T3 fibroblasts by putative intracellular second messengers. The most likely explanation for the effect of staurosporine is inhibition of protein tyrosine kinase activity involved in signalling cascades for the release of internal Ca^{2+}. In support of this mechanism staurosporine was found to be a potent inhibitor of PDGF-dependent PDGF receptor autophosphorylation.

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