Staurosporine clamps cytosolic free Ca^{2+} concentrations of human neutrophils

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The present studies indicate that 50 nM–10 μM -staurosporine increased cytosolic free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) of fura-2-loaded neutrophils in a non-linear manner. The rise in $[\text{Ca}^{2+}]_i$ was rapid, reaching a plateau (e.g. to 0.4 μM with 1 μM -staurosporine) within 30 s, and was maintained for more than 20 min. Pretreating cells with pertussis toxin had no effect on this reaction. The elevation of $[\text{Ca}^{2+}]_i$ was insensitive to extracellular Ca^{2+} concentrations and was due entirely to mobilization of intracellular Ca^{2+} stores. Mn^{2+} -quench studies confirmed the absence of Ca^{2+} influx. No Ca^{2+} efflux occurred in staurosporine-treated cells. In combination studies, staurosporine potentiated Ca^{2+} influx induced by *N*-formylmethionyl-leucyl-phenylalanine (FMLP) and did not block Ca^{2+} efflux associated with peptide stimulation of neutrophils. Studies with permeabilized cells showed that staurosporine did not directly release intracellular Ca^{2+} stores, nor did it affect the sequestration of Ca^{2+} by a Ca^{2+} /ATPase pump. A radioligand-binding assay failed to detect changes in the level of inositol 1,4,5-trisphosphate in neutrophils incubated with $\leq 1 \mu\text{M}$ -staurosporine, but in cells treated with 10 μM -staurosporine the assay recorded a transient increase in this second messenger similar to that induced by FMLP. Finally, lysozyme, but not β -glucuronidase, was released from staurosporine-treated cells. The present results suggest that staurosporine increased $[\text{Ca}^{2+}]_i$ by indirectly mobilizing internal Ca^{2+} stores. Staurosporine suppression of Ca^{2+} efflux and generation of a persistent signal may account for the maintained elevation of $[\text{Ca}^{2+}]_i$.

INTRODUCTION

Staurosporine is a microbial alkaloid which has been shown to be one of the most potent inhibitors of protein kinase C [concn. giving 50% inhibition (IC_{50}) = 3 nM] [1]. However, like many protein kinase inhibitors, its specificity is not great, as other kinases are inhibited at IC_{50} values close to that found for protein kinase C [2].

Its non-specificity notwithstanding, staurosporine is a useful tool for probing the role of protein kinase C in signal transduction. A good didactic example is the activation of human polymorphonuclear leucocytes (neutrophils) by chemotactic peptides such as *N*-formylmethionyl-leucyl-phenylalanine (FMLP). FMLP and other stimuli activate neutrophils by binding to specific receptors which are coupled by G-proteins to phospholipase C(s); the latter catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate to the second messengers $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol. In many cells, an initial Ca^{2+} transient arising from $\text{Ins}(1,4,5)\text{P}_3$ -dependent mobilization of intracellular Ca^{2+} stores is followed by influx of extracellular Ca^{2+} [3,4]. The latter phase in neutrophils is subject to negative feedback regulation by protein kinase C [5,6]. On a cellular level, staurosporine inhibits that part of the superoxide response driven by protein kinase C [7,8].

Recently we have observed that the gold compound auranofin elicits reactions from neutrophils independently of its inhibition of protein kinase C: with prolonged incubations auranofin causes a biphasic elevation of cytosolic free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$), an initial release of internal Ca^{2+} being followed by persistent influx of extracellular Ca^{2+} [6]. The evidence indicates that auranofin directly modulates Ca^{2+} -gating systems of at least two types of Ca^{2+} stores.

Additional studies have been done to determine whether staurosporine exerts comparative effects. Preliminary experiments using staurosporine concentrations greater than those required to inhibit protein kinase C-dependent responses in human neutrophils also show drug-induced elevation of $[\text{Ca}^{2+}]_i$. Results described in the present report demonstrate that staurosporine perturbed neutrophil Ca^{2+} homeostasis by mechanisms different from those reported for auranofin.

MATERIALS AND METHODS

Materials

FMLP, pertussis toxin, *Micrococcus lysodeikticus*, phenolphthalein glucuronidate, BSA, saponin and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Staurosporine, ionomycin, fura-2 and fura-2/AM (the acetoxymethyl ester form) were obtained from Calbiochem Biochemicals (San Diego, CA, U.S.A.). Hanks' balanced salt solution (HBSS) was purchased from GIBCO (Grand Island, NY, U.S.A.). Ficoll-Paque was supplied by Pharmacia Inc. (Dorval, P.Q., Canada). $^{45}\text{Ca}^{2+}$ (in the form of CaCl_2 dissolved in water; sp. radioactivity 30.4 mCi/mg of Ca^{2+}) was obtained from NEN–DuPont Canada (Mississauga, Ont., Canada); ACS cocktail for liquid scintillation and a radioligand assay kit for $\text{Ins}(1,4,5)\text{P}_3$ were from Amersham Canada Ltd. (Oakville, Ont., Canada). All other chemicals were of reagent grade.

Stock solutions of staurosporine and ionomycin were made up in dimethyl sulfoxide (Me_2SO), and stored at -80°C ; when added to aqueous reaction mixtures, the final concentration of the carrier solvent did not exceed 0.5% (e.g. 2 μl of stock solution/ml of cell suspension).

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic or cytoplasmic free Ca^{2+} concentration; FMLP, *N*-formylmethionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; Me_2SO , dimethyl sulfoxide; HBSS, Hanks' balanced salt solution.

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Isolation of neutrophils

Neutrophils were isolated and purified from peripheral venous blood of healthy donors by following procedures outlined previously [6,9] and involving cell separation in a Ficoll-Paque density gradient.

Purified neutrophils containing > 95 % viable cells were normally resuspended in HBSS, pH 7.4. When experiments called for the stimulation of cells in Ca^{2+} -free medium, neutrophils were washed and resuspended in HBSS made up without CaCl_2 ($[\text{Ca}^{2+}] < 1 \mu\text{M}$, estimated by a Ca^{2+} -sensitive electrode).

Fluorimetric measurement of $[\text{Ca}^{2+}]_i$

Purified neutrophils (1×10^7 cells/ml of HBSS) were incubated with $1 \mu\text{M}$ -fura-2/AM for 30 min at 37°C . After washing twice with HBSS, cells were resuspended at 1×10^6 cells/ml of HBSS at room temperature. Samples (2 ml) were prewarmed for 5 min at 37°C in disposable fluorimetric cuvettes before assays. Fura-2 fluorescence was continuously monitored in a Perkin-Elmer (MKF-4) fluorescence spectrophotometer equipped with a thermostatically controlled cuvette compartment. Monochromator settings, unless stated otherwise, were 339 nm (excitation) and 505 nm (emission). $[\text{Ca}^{2+}]_i$ levels were calculated from the formula

$$[\text{Ca}^{2+}]_i = K_d(F - F_{\min.}) / (F_{\max.} - F)$$

where F is arbitrary fluorescence units and K_d (224 nM) is the dissociation constant for Ca^{2+} binding to fura-2 in an intracellular milieu [10]. For cells suspended in buffer containing 1 mM - Ca^{2+} , $F_{\max.}$ was determined by lysing the cells with $50 \mu\text{M}$ -digitonin or alternatively with 0.1 % (v/v) Triton X-100; $F_{\min.}$ was determined by adding 20 mM-Tris base and 2–3 mM-EGTA to lysed cells.

Quantification of Ca^{2+} efflux

Neutrophils (2×10^7 cells/ml, in Mg^{2+} -free HBSS) were incubated with $5 \mu\text{Ci}$ of $^{45}\text{Ca}^{2+}$ /ml for 50 min at 37°C , then washed twice and resuspended to a similar density in the same medium without the radioisotope. Upon exposure to reagents, 0.5 ml samples were removed and layered on top of 0.3 ml of Harwick (SF-1250) silicone oil in conical Microfuge tubes. Intact cells were separated from the supernatant by centrifugation for 1 min in an Eppendorf Microfuge, the cells forming a pellet at the bottom of the oil layer. Radioactivity released was assayed by combining 0.45 ml of the supernatant with 8 ml of ACS cocktail (Amersham) and counting in a Beckman (6800) liquid-scintillation counter.

Measurement of Ca^{2+} release in permeabilized neutrophils

Saponin permeabilizes the neutrophil plasma membrane without affecting the membranes of intracellular organelles [6]. Neutrophils (3×10^7 cells/ml) were incubated with $75 \mu\text{g}$ of saponin/ml at 37°C for approx. 30 min, and the extent of permeabilization was gauged by uptake of Trypan Blue by cells. Permeabilization was judged adequate when > 90 % of cells took up the dye. Such cells were washed and then resuspended at 1×10^7 cells/ml in a low- Ca^{2+} buffer with the following constituents: NaCl, 20 mM; KCl, 100 mM; MgSO_4 , 5 mM; NaHCO_3 , 25 mM; NaH_2PO_4 , 0.96 mM; BSA, 0.2 %. Samples of cells (2 ml, transferred to two-sided polystyrene cuvettes) were incubated with $1 \mu\text{M}$ -fura-2 at 37°C while being stirred by a magnetic flea. Reagents were added in microlitre volumes. Solvent vehicles, normally Me_2SO , were kept below 0.5 % (v/v). Before addition of ATP, cells were further incubated for 5 min in $10 \mu\text{M}$ each of antimycin A and oligomycin, inhibitors of mitochondrial ATP synthesis and Ca^{2+} transport. Ca^{2+} uptake into permeabilized neutrophils was activated by addition of 1.5 mM-ATP, 10 units of creatine kinase/ml and 5 mM-phosphocreatine. The ambient

Ca^{2+} levels were monitored in the spectrofluorimeter by using excitation and emission wavelengths of 339 and 505 nm respectively.

Radioligand-binding assay for $\text{Ins}(1,4,5)\text{P}_3$

Neutrophils preincubated for 10 min with 10 mM-LiCl at 37°C before treatment with various agents were fixed by addition of 5 % trichloroacetic acid as outlined previously [6,11]. Ether-washed extracts were processed and assayed for $\text{Ins}(1,4,5)\text{P}_3$ by using a radioligand-binding kit purchased from Amersham Canada Ltd. This assay is based on the competition between unlabelled $\text{Ins}(1,4,5)\text{P}_3$ and a fixed quantity of ^3H -labelled $\text{Ins}(1,4,5)\text{P}_3$ and for binding sites on bovine adrenal binding protein. Maximal cross-reactivity as reported by Amersham for $\text{Ins}(1,3,4,5)\text{P}_4$ was 6.4 %, for $\text{Ins}(1,3,4)\text{P}_3$ 0.22 %. We found that standard curves generated with or without the inclusion of extracts from resting cells were similar, indicating that non-specific interference with $\text{Ins}(1,4,5)\text{P}_3$ binding was negligible.

Degranulation assays

Neutrophil suspensions treated with staurosporine or solvent vehicle for defined periods were centrifuged for 1 min in an Eppendorf 5412 Microfuge. Collected supernatants were assayed for lysozyme (EC 3.2.1.17) and β -glucuronidase (EC 3.2.1.31) activity, with *Micrococcus lysodeikticus* and phenolphthalein glucuronidate (Sigma) as respective substrates [9,12].

Cytotoxicity assays

Cell viability was assessed by use of the Trypan Blue and lactate dehydrogenase activity assays outlined previously [6,11].

RESULTS

Potential of Ca^{2+} transients in neutrophils

As shown previously, staurosporine potentiates Ca^{2+} transients in neutrophils activated with the chemotactic peptide FMLP [5,6]. Published results obtained from studies on fura-2-loaded neutrophils are reproduced in Fig. 1 (traces a–c) for comparison. In summary, FMLP induces a biphasic elevation of $[\text{Ca}^{2+}]_i$ (trace

Fig. 1. $[\text{Ca}^{2+}]_i$ transients in neutrophils treated with a combination of FMLP and staurosporine

Neutrophils (1×10^6 /ml HBSS) loaded with fura-2 were activated with $1 \mu\text{M}$ -FMLP throughout at points indicated by arrows. Traces: (a), cells treated with FMLP only; (b), staurosporine (STA; 50 nM) added before FMLP; (c), 10 nM-PMA added about 2 min before FMLP; (d), staurosporine ($1 \mu\text{M}$) added about 2 min before FMLP. Traces (a)–(c) were published previously [6] and are reproduced here by permission of the Journal of Biological Chemistry.

a). A rapid transient rise of $[Ca^{2+}]_i$ to $\geq 1 \mu M$ resulting from mobilization of intracellular Ca^{2+} stores is followed by influx of extracellular Ca^{2+} , which maintains $[Ca^{2+}]_i$ above basal levels for several minutes (trace a). As reported above, the secondary influx of Ca^{2+} is negatively regulated by protein kinase C, one evidence being the suppression of this phase by prior incubation of neutrophils with phorbol myristate acetate (PMA) (trace c). Added to cells before FMLP, 50 nM-staurosporine potentiates the extent of the secondary influx of Ca^{2+} (trace b). Here, the drug is inhibiting the feedback effects of the physiological kinase C activator, diacylglycerol.

Staurosporine increased $[Ca^{2+}]_i$

It was observed that staurosporine alone elevated $[Ca^{2+}]_i$, a response more evident at staurosporine concentrations ≥ 50 nM. Traces shown in Fig. 2(a) demonstrate the dose/response effect of staurosporine on $[Ca^{2+}]_i$ of neutrophils. The traces show that the elevation of $[Ca^{2+}]_i$ was rapid and attained a plateau in about 1 min. Traces followed for over 20 min showed that enhanced levels of $[Ca^{2+}]_i$ were maintained indefinitely. Fig. 2(b) correlates staurosporine concentration versus $[Ca^{2+}]_i$ measured after incubation for 14 min. Up to $1 \mu M$, staurosporine increased $[Ca^{2+}]_i$ from basal levels of approx. $0.1 \mu M$ to about $0.4 \mu M$. Greater concentrations of staurosporine increased $[Ca^{2+}]_i$ to micromolar levels, although the accuracy of measurements at this range was limited by the dissociation constant of fura-2 for Ca^{2+} of 224 nM. The elevation of $[Ca^{2+}]_i$ was due entirely to the mobilization of internal Ca^{2+} stores, since decreasing extracellular Ca^{2+} concentrations to about $0.04 \mu M$ from 1.26 mM by addition of 3 mM-EGTA (dotted trace, Fig. 2a) did not affect the extent of the rise in $[Ca^{2+}]_i$. {Calculations for $[Ca^{2+}]_i$ in a multiple-equilibria condition were made with the EQCAL program purchased from Biosoft (Cambridge, U.K.).} Depending on reagents and water quality, Ca^{2+} is present at micromolar or sub-micromolar levels in nominally Ca^{2+} -free HBSS. Further addition of 2 mM-EGTA effectively decreased $[Ca^{2+}]_i$ to < 1 nM. The isolated trace in Fig. 2(a) shows that when cells under such conditions were treated with staurosporine, $[Ca^{2+}]_i$ rose as shown in preceding results; the difference was that $[Ca^{2+}]_i$ gradually declined under the influence of the much steeper Ca^{2+} gradient. Although not shown, responses induced by other concentrations of staurosporine were similarly insensitive to EGTA. These results also indicate that fura-2 leakage was not a factor.

It was noted that staurosporine emitted considerable autofluorescence, especially at concentrations greater than $1 \mu M$. Traces were corrected for this. Alternate excitation of staurosporine-treated suspensions at 339 and 380 nm generated emission signals (corrected) which increased and decreased respectively; this meets another criterion for Ca^{2+} -fura-2 interactions [10,13]. Other control studies show that the dose range of staurosporine used under applied conditions was not cytotoxic. Neutrophils treated with staurosporine for 20 min excluded Trypan Blue and released little or no cytosolic lactate dehydrogenase compared with control cells.

Trace (d) in Fig. 1 illustrates the effect of adding FMLP to neutrophil suspensions after $[Ca^{2+}]_i$ was clearly increased by $1 \mu M$ -staurosporine. The ensuing response resembled trace (b) in Fig. 1. A biphasic elevation of $[Ca^{2+}]_i$ was immediately triggered; the enhancement of the secondary influx of Ca^{2+} was commensurate with the concentration of staurosporine applied. Over the next 8–10 min, $[Ca^{2+}]_i$ declined slowly, but did not fall below the plateau value induced by staurosporine alone.

Insensitivity to pertussis toxin

Further studies asked the question whether the staurosporine-mediated increase in $[Ca^{2+}]_i$ was sensitive to pertussis toxin.

Fig. 2. Effect of staurosporine on $[Ca^{2+}]_i$ of human neutrophils

(a) Fura-2 traces. Neutrophils (1×10^6 cells/ml HBSS) were loaded with fura-2 as outlined in the Materials and methods section. Cells were incubated with 0.1 – $10 \mu M$ -staurosporine, and changes in $[Ca^{2+}]_i$ were monitored continuously. The dotted line represents the reproduction of a trace in which 3 mM-EGTA was added to the neutrophil suspension immediately before $1 \mu M$ -staurosporine. The isolated trace was generated from cells resuspended in Ca^{2+} -free HBSS, then treated in turn with EGTA and staurosporine (STA). Traces have been corrected for staurosporine autofluorescence and superimposed over each other to facilitate comparison. (b) Dose-response effect of staurosporine on $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ at 14 min incubation was calculated from fura-2 traces obtained for separate concentrations of staurosporine. Results are means ($\pm$ S.E.M.) of four separate experiments.

Experiments were carried out in which neutrophils were incubated for approx. 60 min at $37^\circ C$ with pertussis toxin ($1 \mu g/ml$), washed, and then loaded with fura-2 in the normal manner. Results consistently show that pertussis-toxin pretreatment totally suppressed FMLP ($1 \mu M$)-induced Ca^{2+} transients while having no effect on the mobilization of intracellular Ca^{2+} by 0.1 – $1 \mu M$ -staurosporine (results not shown).

Mn²⁺ quench of fluorescence studies

The observation that nominal influx of extracellular Ca^{2+} occurred in staurosporine-treated cells raised the possibility that Ca^{2+} entry might be blocked. Mn^{2+} entry into neutrophils and

Fig. 3. Cation entry into neutrophils monitored by Mn^{2+} -mediated quenching of cytosolic fura-2

Neutrophils loaded with fura-2 were washed and resuspended in Ca^{2+} -free medium as described in the Materials and methods section. Staurosporine (STA; $1 \mu M$), FMLP ($1 \mu M$) and Mn^{2+} ($0.1 mM$) were added to cell suspensions at points indicated by arrows. Traces (a), (b), (c) and (d) were generated in one experiment using cells from a single donor. Excitation wavelength was 339 nm. Additions made in traces (a') and (b') were similar to (a) and (b) respectively, except that the excitation wavelength was 360 nm. Results are representative of three experiments.

subsequent quenching of fura-2 fluorescence were used to assess cation influx directly [6,14,15].

Trace (b) in Fig. 3 is a control experiment in which fura-2-loaded neutrophils were treated with Mn^{2+} in a Ca^{2+} -free medium. The ensuing decline in the fluorescence signal is attributed to passive Mn^{2+} diffusion into the resting cells. The simultaneous addition of staurosporine and Mn^{2+} produced trace (a), where the initial rise in $[Ca^{2+}]_i$ is due to release of internally stored Ca^{2+} . The following slow rate of quenching was not significantly different from the control (trace b); thus drug treatment did not increase the permeability of cells to Mn^{2+} and by extension to Ca^{2+} . This corroborates results in Fig. 2(a), which show that Ca^{2+} influx did not accompany staurosporine treatment. Traces (a') and (b') were similar to conditions applied in traces (a) and (b) respectively, except that the excitation wavelength used was 360 nm, the isosbestic point where fura-2 is insensitive to changing $[Ca^{2+}]_i$. Trace (a') differs from (a) in that an initial rise in the signal due to Ca^{2+} release was not recorded.

In trace (d), neutrophils were treated with FMLP and then Mn^{2+} . The response confirms those reported previously [6,14]; that is, in the absence of a significant Ca^{2+} gradient, FMLP induced a monophasic elevation of $[Ca^{2+}]_i$ resulting from mobilization of internal stores. The delayed addition of Mn^{2+} after $[Ca^{2+}]_i$ declined to basal levels resulted in accelerated quenching of fluorescence. The conclusion advanced previously is that cation channels remain open during the indicated period and that the empty state of internal Ca^{2+} stores may regulate their

Fig. 4. Effect of staurosporine on Ca^{2+} sequestration in permeabilized neutrophils

Neutrophils permeabilized with saponin as described in the Materials and methods section were resuspended in the KCl buffer containing $1 \mu M$ fura-2. Relative changes in the ambient Ca^{2+} concentration was monitored in the spectrofluorimeter. Ca^{2+} sequestration into internal stores was activated by addition of ATP (together with phosphocreatine and creatine kinase) at points indicated by arrows. Sequestration of Ca^{2+} lowered the ambient Ca^{2+} concentration. At other points in the traces shown, final concentrations of reagents added were: staurosporine (STA), $1 \mu M$ (except in the bottom trace, in which $5 \mu M$ was added); ionomycin (ION), $1 \mu M$; $Ins(1,4,5)P_3$ (IP3), $20 \mu M$. Similar results were obtained with cells from three different donors.

opening. Trace (c) monitors changes in fluorescence in neutrophils treated with a combination of staurosporine and FMLP. Staurosporine ($1 \mu M$) generated an expected rapid rise in $[Ca^{2+}]_i$, which levelled off as previously. FMLP added 4 min after staurosporine caused a transient elevation of $[Ca^{2+}]_i$, arising from more release of internal Ca^{2+} . Again, the absence of a significant Ca^{2+} chemical gradient precluded Ca^{2+} influx. Ca^{2+} efflux or reuptake into stores rapidly lowered $[Ca^{2+}]_i$ but only to the raised level of $[Ca^{2+}]_i$ maintained by staurosporine alone (cf. Fig. 1, trace d). Addition of Mn^{2+} at this point in the trace resulted in a rapid rate of quench similar to that observed in trace (d). These results suggest that pretreating neutrophils with staurosporine did not inhibit cation entry mediated by FMLP or block processes associated with opening of cation channels.

Effect of staurosporine on Ca^{2+} sequestration and release in permeabilized neutrophils

Recent evidence reported by our group indicates that agents such as auranofin and the diltiazem analogue TA-3090 directly release Ca^{2+} from intact stores in permeabilized neutrophils [6,11]. This mechanism may account for the ability of those drugs to elevate $[Ca^{2+}]_i$ in intact cells. Similar experiments were carried out to assess whether staurosporine affects Ca^{2+} fluxes associated with intracellular compartments. The traces reproduced in Fig. 4

Fig. 5. $Ins(1,4,5)P_3$ formation in neutrophils treated with staurosporine

$Ins(1,4,5)P_3$ levels in neutrophils were quantified by radioligand assay as outlined in the Materials and methods section. Neutrophils were incubated with the following: (a) Me_2SO control (○, ●); 1 μM -FMLP (△, ▲); 1 μM -staurosporine (□, ■); 0.1 μM -staurosporine (▽). (b) Me_2SO control (○, ●); 1 μM -FMLP (△, ▲); 10 μM -staurosporine (□, ■); 10 μM -staurosporine and 1 μM -FMLP (▽, ▼). The white and black symbols represent results from two different experiments.

Fig. 6. Ca^{2+} efflux from neutrophils treated with staurosporine

Neutrophils loaded with ${}^{45}Ca^{2+}$ as outlined in the Materials and methods section were incubated with FMLP or staurosporine; portions of cell suspensions were removed at various times and rapidly centrifuged. The supernatants were assayed for radioactivity, and the results are expressed as means ($\pm$ S.E.M.) of three separate experiments. Neutrophils were treated with: ○, Me_2SO vehicle (control); ●, 1 μM -FMLP; ▲, 5 μM -staurosporine; or △, with both agents (staurosporine was added to suspensions 30 s before FMLP; a zero-time sample was removed just before FMLP was added).

clearly point to two negative findings. Compared with the control (top trace), the second trace shows that staurosporine, added to permeabilized cells 2 min before ATP, did not inhibit sequestration of Ca^{2+} mediated by a Mg^{2+}/Ca^{2+} -ATPase pump. The bottom two traces show that staurosporine when added to suspensions after Ca^{2+} sequestration had reached a plateau did not release Ca^{2+} from stores. Both the Ca^{2+} ionophore ionomycin and $Ins(1,4,5)P_3$ effected release of Ca^{2+} .

Fig. 7. Lysosomal enzyme release from neutrophils treated with staurosporine

Neutrophils were incubated for 5 min at 37 $^{\circ}C$ with various concentrations of staurosporine (or, for the control, Me_2SO); recovered supernatants were assayed for lysozyme or β -glucuronidase activity as detailed in the Materials and methods section. Enzyme activity assayed was calculated as percentage of the total activity released from the same cells lysed with digitonin. Results are means ($\pm$ S.E.M.) of three separate experiments. In the lysozyme results, all points except for incubations carried out in the presence of 20 nM-staurosporine were significantly different from control (0 staurosporine) ($P < 0.01$, by Student's paired t test). ○, Lysozyme released from neutrophils suspended in HBSS; ●, lysozyme released from cells suspended in Ca^{2+} -free HBSS; △, β -glucuronidase released from cells suspended in HBSS.

Status of $Ins(1,4,5)P_3$ in staurosporine-treated neutrophils

The failure of staurosporine to release Ca^{2+} directly from intracellular stores raised the possibility that staurosporine might do so by inducing $Ins(1,4,5)P_3$ formation in intact cells. This hypothesis was investigated by measuring $Ins(1,4,5)P_3$ levels in neutrophils incubated with a combination of staurosporine and FMLP (Fig. 5). Results show that FMLP induced a transient elevation of $Ins(1,4,5)P_3$ as expected (Figs. 5a and 5b); $Ins(1,4,5)P_3$ concentrations increased to maximal levels within 5–10 s, and then declined to basal levels within 2 min, thus confirming previous results [6,11]. Staurosporine at 0.1 and 1 μM did not influence $Ins(1,4,5)P_3$ levels significantly (Fig. 5a). However, staurosporine at 10 μM did induce a transient elevation of $Ins(1,4,5)P_3$ with a time course similar to that found for FMLP-activated neutrophils (Fig. 5b). When FMLP (1 μM) and staurosporine (10 μM) were added simultaneously to cells, the effect on $Ins(1,4,5)P_3$ levels was approximately additive (Fig. 5b).

Effect of staurosporine on Ca^{2+} efflux

The observation that $[Ca^{2+}]_i$ remained elevated in staurosporine-treated neutrophils suggests that mechanisms responsible for decreasing $[Ca^{2+}]_i$ might be blocked. As Ca^{2+} uptake by internal stores was not affected (Fig. 4), the possibility that Ca^{2+} transport in the plasma membrane might be inhibited was studied by measuring Ca^{2+} efflux in cells incubated with combinations of staurosporine and FMLP (Fig. 6). Experiments carried out with ${}^{45}Ca^{2+}$ -loaded cells show that rates of Ca^{2+} efflux increased after exposure of cells to FMLP. Transport systems were presumably activated in response to peptide-induced elevation of $[Ca^{2+}]_i$. Ca^{2+} efflux subsided to basal rates about 2 min after FMLP was added. In contrast, no significant Ca^{2+} efflux

occurred in cells treated with 5 μM -staurosporine, despite an increase of $[\text{Ca}^{2+}]_i$ to $\geq 1 \mu\text{M}$. Additional experiments show that this concentration of staurosporine did not modulate the accelerated Ca^{2+} efflux associated with FMLP in neutrophils incubated with both reagents.

Lysosomal enzyme release from neutrophils treated with staurosporine

The degranulation response of neutrophils was assessed by assaying supernatants for lysozyme and β -glucuronidase (Fig. 7). Results show that insignificant β -glucuronidase activity was released into the supernatant in cell suspensions treated with up to 1 μM -staurosporine. The same supernatants expressed increasing lysozyme activity with increasing staurosporine concentration in incubation mixtures. Time-course studies show that maximal release of lysozyme was reached after 5 min for all staurosporine concentrations (results not shown). In separate experiments, lysozyme release was measured in cells suspended in Ca^{2+} -free medium. Results indicate a similar dose-dependent effect of staurosporine on lysozyme release. Since β -glucuronidase is found only in azurophilic granules, whereas lysozyme is present in both azurophilic and specific granules [9], these results suggest that staurosporine caused degranulation of the latter. Dewald and co-workers, who assayed a more comprehensive list of enzyme markers, reached the same conclusion [7].

DISCUSSION

Studies with fura-2-loaded neutrophils (Fig. 2) have clearly shown that staurosporine raised $[\text{Ca}^{2+}]_i$ by mobilizing intracellularly stored Ca^{2+} . Mn^{2+} -quenching experiments corroborated the absence of Ca^{2+} influx in such cell suspensions (Fig. 3). In contrast with present findings, Dewald *et al.* [7] reported that staurosporine had no effect on neutrophil $[\text{Ca}^{2+}]_i$. The difference may be attributed to their use of quin-2 rather than fura-2 in their fluorescence assays; the former dye is not as sensitive to changes in Ca^{2+} and may have buffered increases in $[\text{Ca}^{2+}]_i$ [10]. Staurosporine clamping of $[\text{Ca}^{2+}]_i$ at elevated values stems from multiple effects on regulatory systems (discussed below). It should be noted that significant mobilization of Ca^{2+} (and effects contributing to it) occurred at staurosporine concentration $\geq 0.1 \mu\text{M}$. At concentrations $< 0.1 \mu\text{M}$, the inhibition of protein kinase C is the dominant effect. This is evident in the enhancement of Ca^{2+} influx in cells treated with FMLP and nanomolar levels of staurosporine (Fig. 1) [5,6]. Here the drug has dampened the feedback effects of protein kinase C.

Elevated $[\text{Ca}^{2+}]_i$ may account for some of the phenomena reported for staurosporine-treated neutrophils. As shown by others [7,16] and confirmed here (Fig. 7), staurosporine alone acts as an incomplete secretagogue by inducing the degranulation of specific granules. This may arise as a result of activation of Ca^{2+} -dependent processes. Previously Wolf & Baggiolini [17] have shown that staurosporine translocates protein kinase C to the plasma membrane in a dose- and Ca^{2+} -dependent manner while maintaining its inhibition of the enzyme. Concomitant elevation of $[\text{Ca}^{2+}]_i$ by staurosporine probably enhanced this translocation. Protein kinase C translocation may be the clue to the paradoxical observation that PMA, a protein kinase C activator, and staurosporine, an inhibitor, both induce degranulation of specific granules [7,16]. As Dewald and co-workers have suggested [7], phorbol esters and staurosporine may share a common activation step, namely that exocytosis from specific granules may be caused by a non-catalytic part of protein kinase C.

It is not known at present whether staurosporine increases

$[\text{Ca}^{2+}]_i$ of other cells in the manner shown here for neutrophils. We suggest that this potential effect should be measured in studies involving staurosporine, as it may influence the interpretation of results. As examples, changes in $[\text{Ca}^{2+}]_i$ may play a role in (a) staurosporine-induced dissolution of microfilament bundles by a protein kinase C-independent pathway in a variety of cultured cells [18] and (b) staurosporine mimicry of nerve-growth-factor effects on PC12 cells [19].

The mechanism by which staurosporine mobilized internal Ca^{2+} appears to be indirect, since it did not release sequestered Ca^{2+} from semi-permeabilized neutrophils (Fig. 4). A logical second messenger might be $\text{Ins}(1,4,5)\text{P}_3$ generated by drug stimulation of phospholipase C. If true, the staurosporine and FMLP pathways differed in that the former was insensitive to pertussis toxin, whereas responses elicited by the chemo-attractant are inhibited [3,4]. As results show (Fig. 5), staurosporine up to 1 μM did not significantly raise $\text{Ins}(1,4,5)\text{P}_3$ levels, in contrast with increases in $[\text{Ca}^{2+}]_i$. On the other hand, 10 μM -staurosporine, which increased $[\text{Ca}^{2+}]_i$ to values matching peak levels attained in FMLP-treated cells, induced a transient increase in $\text{Ins}(1,4,5)\text{P}_3$ similar to that produced by the peptide. Based on present evidence, it is not likely that sub-micromolar levels of staurosporine mobilized Ca^{2+} via $\text{Ins}(1,4,5)\text{P}_3$ generation. At 10 μM , staurosporine may have activated phospholipase C directly or mobilized sufficient Ca^{2+} to activate this enzyme. Previous studies with semi-permeabilized neutrophils or neutrophil membranes have shown that 1 mM- Ca^{2+} alone was sufficient to stimulate $\text{Ins}(1,4,5)\text{P}_3$ formation [20,21]; in the presence of guanine nucleotides, the Ca^{2+} dose-response curve was shifted to sub-micromolar concentrations [20]. A similar chemistry may pertain in intact neutrophils treated with relatively high concentrations of staurosporine.

The persistent elevation of $[\text{Ca}^{2+}]_i$ found in staurosporine-treated neutrophils may arise from a combination of effects. However, a cogent interpretation of results and modelling must await additional studies. Ca^{2+} homeostasis is based on complex interactions between a host of parameters, among them Ca^{2+} -ATPases of the plasma and intracellular membranes, Ca^{2+} channels and exchangers, Ca^{2+} -gating components [the $\text{Ins}(1,4,5)\text{P}_3$ receptor may be one of several] and leakage through plasma and internal membranes. It is clear that the balance of Ca^{2+} -transport processes is shifted in the presence of this agent. The present results suggest that passive Ca^{2+} efflux and influx were unchanged in neutrophils incubated with staurosporine alone (Figs. 3 and 6) and that Ca^{2+} -ATPase pumps of intracellular stores were not inhibited (Fig. 4). As discussed above, we have no knowledge of the kinetics of Ca^{2+} release mediated by staurosporine, one important question being 'is the signal induced by staurosporine persistent or transient?'.

A contributing factor for maintained elevation of $[\text{Ca}^{2+}]_i$ may come from staurosporine inhibition of Ca^{2+} pumps located in the plasma membrane. As evidence, Ca^{2+} efflux was not significantly different in staurosporine-treated cells and controls (Fig. 6). An ATP-dependent Ca^{2+} pump regulated by both calmodulin and protein kinase C is present in the plasma membrane of neutrophils (see [4] for review and additional references cited therein). Of relevance to the present discussion is the report that staurosporine interacted directly with Ca^{2+} -calmodulin complexes and expressed anti-calmodulin activity [22]. Therefore it is possible that staurosporine inhibited the plasma-membrane Ca^{2+} -ATPase by inhibiting both protein kinase C and calmodulin.

The failure of staurosporine to block Ca^{2+} efflux in FMLP-treated cells may be rationalized by the presence of multiple Ca^{2+} transporters in membranes. In this vein, Perianin & Snyderman [23] reported evidence for two distinct mechanisms for lowering $[\text{Ca}^{2+}]_i$ in human neutrophils: one activated by protein kinase C,

the other activated by FMLP and independent of protein kinase C. It is possible that the latter is insensitive to staurosporine.

The preceding hypothesis, if true, raises the question why, in combination studies (Fig. 1, trace d; Fig. 3, trace c), the FMLP-activated pump did not lower $[Ca^{2+}]_i$ to values closer to that found in resting cells. A combination of factors may account for this observation. It is clear that the FMLP-induced pump operated transiently and that an unaltered turnover number might have moved similar amounts of Ca^{2+} to the extracellular medium. Furthermore, in the intact cell (Fig. 1) the permeability of the plasma membrane to extracellular Ca^{2+} is prolonged by staurosporine suppression of negative-feedback effects of protein kinase C [6].

The absence of Ca^{2+} entry from the medium in staurosporine-treated neutrophils was of some interest. Recent results suggest that Ca^{2+} influx in neutrophils can be interpreted in terms of the capacitative Ca^{2+} -entry model of Putney [24,25]. This model proposes that the status of internal Ca^{2+} stores regulates Ca^{2+} entry, and that emptying of such stores triggers a signal(s) which opens plasma-membrane channels or activates carrier proteins. This model predicts that agents which directly release Ca^{2+} from internal stores will also induce Ca^{2+} influx. This was observed in thapsigargin-treated parotid and lacrimal acinar cells, and neutrophils [15,26,27]. We in turn have shown that Ca^{2+} entry accompanies mobilization by auranofin or TA-3090 (an 8-chloro derivative of diltiazem) of intracellular Ca^{2+} [6,11]. All three agents act directly on intracellular sites to release Ca^{2+} from stores. Even though staurosporine did not act in the same manner to mobilize intracellular Ca^{2+} , we expect that Ca^{2+} entry might be a significant component of its action. Negative findings raised the conjecture that staurosporine simultaneously generated a signal for Ca^{2+} entry and inhibited the conduits of Ca^{2+} entry. The latter could be putative second-messenger-operated channels or a Ca^{2+}/Na^+ exchanger [28,29]. However, this argument is not supported by the inability of staurosporine to block Ca^{2+} entry induced by FMLP (Fig. 3). Taken at face value, the staurosporine results suggest that Ca^{2+} mobilization and Ca^{2+} influx are dissociable events and that the capacitative Ca^{2+} -entry model may have to be modified for neutrophils.

It could be argued that Ca^{2+} entry in neutrophils is triggered only by emptying of $Ins(1,4,5)P_3$ -sensitive Ca^{2+} stores and that staurosporine released Ca^{2+} from different storage compartments. In order to resolve this question, the additivity or non-additivity of staurosporine and $Ins(1,4,5)P_3$ actions would have to be measured. One approach is to determine the effect of staurosporine on Ca^{2+} release under conditions where Ca^{2+} stores sensitive to $Ins(1,4,5)P_3$ are completely emptied. At present this experiment is not feasible with semi-permeabilized neutrophils, since we have yet to find conditions under which the indirect mechanism of staurosporine operates.

In summary, the present experiments indicate that staurosporine mobilized intracellular Ca^{2+} stores of neutrophils in a dose-dependent manner. This response was insensitive to pertussis-toxin pretreatment and was not accompanied by Ca^{2+} influx or efflux. Maintenance of $[Ca^{2+}]_i$ at elevated levels is

probably due to (a) inhibition of plasma-membrane Ca^{2+} pumps and (b) new equilibrium conditions for Ca^{2+} fluxes between the cytosol and internal storage compartments.

This study was supported by research grants from MRC Canada and the Arthritis Society of Canada.

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