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Suppression by staurosporine of Ca^{2+}-mobilization triggered by ligation of antigen-specific receptors on T and B lymphocytes

An essential role of protein tyrosine kinase in the signal transduction

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It is known that the receptor for platelet-derived growth factor (PDGF) activates phospholipase C (PLC) by phosphorylating the γ1 isoform of PLC with the receptor protein-tyrosine kinase (PTK), whereas a guanine nucleotide-binding protein participates as a transducer in the PLC activation through the receptors for vasopressin, bombesin and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). We have shown in a rat fibroblast line that staurosporine is a potent PTK inhibitor capable of clearly discriminating the two types of receptor-stimulated Ca^{2+} mobilization and, by inference, PLC activation: the response triggered by PDGF was completely inhibited, whereas the responses triggered by vasopressin, bombesin and $PGF_{2\alpha}$ were not affected at all. The Ca^{2+} mobilization in human T and B cell lines induced by anti-CD3 and anti-immunoglobulins (Ig) was completely suppressed by staurosporine. The results indicate that the PTK activity plays an essential role in the PLC activation through the T cell receptor/CD3 complex and through membrane Ig.

T cell receptor; Membrane immunoglobulin; Staurosporine; Calcium ion mobilization; Platelet-derived growth factor; Protein-tyrosine kinase

1. INTRODUCTION

Receptor-stimulated activation of phospholipase C (PLC) is one major pathway of signal transduction through cell-surface receptors. The signaling by the PLC activation can be most easily monitored by measuring an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), the initial spike component of which is induced by inositol 1,4,5-trisphosphate [1]. Two mechanisms are known in the coupling of cell-surface receptors to PLC [2–4]. Many receptors with seven membrane-spanning segments including those for vasopressin, bombesin, and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) activate an isoform of PLC, presumably PLC-β [5], by coupling to a heterotrimeric guanine nucleotide-binding protein (G-protein), which functions as a transducer between the receptors and PLC [2,3,6]. The second mechanism has been found in the PLC activation by receptor protein-tyrosine kinases (PTK) [4]. Platelet-derived growth factor (PDGF) activates the γ1 isoform of PLC by phosphorylating the PLC with the PDGF-receptor PTK, making PLC-γ1 capable of hydrolyzing phosphatidylinositol 4,5-bisphosphate bound to profilin [7].

Cross-linking by antibodies of the antigen-specific receptors on T and B lymphocytes, T cell receptor (TCR)/CD3 complex and membrane immunoglobulins (mIg) with its associated proteins, activates PLC [8,9], resulting in an increase in $[Ca^{2+}]_i$. The mechanism of the PLC activation through the TCR/CD3 complex is poorly understood. Participation of the PTK activity has been indicated on the basis of suppression of the TCR/CD3-mediated activation of PLC by herbimycin A and genistein [10–12]. In the PLC activation through mIg, a role for a G-protein has been shown based on the effects of γ-thio and β-thio analogues of guanine nucleotides on the activation [13–15]. However, it was recently reported that herbimycin A and genistein also block the PLC activation through mIg [12,16], which indicates that a PTK is somehow involved in the PLC activation through the B cell antigen receptor.

In this paper, we assessed the susceptibility of the response with a rise in $[Ca^{2+}]_i$ (hereafter referred to as the Ca^{2+} response) in human T and B cell lines to the inhibition by staurosporine, genistein, and isoquinolinesulfonamides, H-7 and H-8. Staurosporine, a microbial alkaloid, is known to be a potent inhibitor of a variety of protein kinases including PTKs [17–20]. The susceptibility to these drugs of the Ca^{2+} responses in a rat fibroblast line, WFB, induced by PDGF, vasopressin, bombesin, and $PGF_{2\alpha}$ was also assessed

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Abbreviations: PLC, phospholipase C; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PTK, protein-tyrosine kinase; PDGF, platelet-derived growth factor; TCR, T cell receptor; mIg, membrane immunoglobulin; H-7, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide

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under the same conditions; staurosporine clearly discriminated the two mechanisms of PLC activation dependent or independent on a PTK. Our results indicate that the PTK activity plays an essential role in the PLC activation through the TCR/CD3 complex and through mIg.

2. MATERIALS AND METHODS

2.1. *Materials*

OKT3 Monoclonal antibody and PDGF were prepared as described previously [21,22]. Commercial sources of chemicals and antibodies were as follows: arginine-vasopressin (367 U/mg), bombesin, affinity-purified F(ab')$_2$ goat anti-human IgG (γ-chain-specific), and affinity-purified goat anti-human IgM (μ-chain-specific), Sigma; H-7 and H-8, Seikagaku Kogyo (Tokyo); staurosporine, Boehringer Mannheim; genistein, Funakoshi Pharmaceuticals (Osaka); rabbit anti-mouse Ig, Dakopatts.

2.2. *Cell culture and determination of $[Ca^{2+}]_i$*

A human T cell line, Jurkat (clone E6-1), and human B cell lines, RPMI 8075 [23] and Daudi, were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum as described previously [21]. The cells harvested at late log phase of growth were loaded with fluo-3 by incubating the cells (3×10^7 cells/ml in serum-free medium) with 10 μM fluo-3/acetoxymethyl ester (fluo-3 AM) at 23°C for 1 h. Fluo-3 AM was added from a dimethyl sulfoxide solution containing 2 mM fluo-3 AM and Pluronic F-127 (a non-ionic detergent) at 50 mg/ml. After loading, the cells were washed twice with HEPES-buffered saline, which contained 1 mM Ca^{2+}, and then suspended in the saline at 2×10^6 cells/ml.

WFB cells were cultured as described previously [2]. The cells kept for 3 days after growth to confluence were loaded with fluo-3 by incubating the cells in monolayer with 20 μM fluo-3 AM, added from the solution described above, in serum-free medium at 23°C for 2 h. After loading, the cells were washed twice with HEPES-buffered saline, harvested by scraping with a rubber policeman, washed again, and then suspended in the saline at 1.2×10^6 cells/ml.

Fluo-3 fluorescence from 1 ml of the cell suspension was recorded at 20°C with a Hitachi 650-10S fluorescence spectrophotometer. Excitation and emission wavelengths were 500 and 530 nm. Ligands used in stimulating cells were added in a volume of 1–5 μl to 1 ml of fluo-3-loaded cells at the following doses: 108 ng of the PDGF preparation described previously [2,22], 1.2 mU of vasopressin, 40 pmol of bombesin, 1 nmol of $PGF_{2\alpha}$, 1 μg of OKT3, 50 μg of anti-mouse Ig, 2 μg of anti-human IgG F(ab')$_2$, and 1 μg of anti-human IgM. Staurosporine (1 mM) in dimethyl sulfoxide was added 1 min before the ligand addition at 1 μl per ml of cell suspension. Genistein (10 mg/ml) in dimethyl sulfoxide was added 15 min before the ligand addition at 1.1 μl per ml of cell suspension. H-7 (10 mM) and H-8 (10 mM) in water were added 6 min befored the ligand addition at 3 μl per ml of cell suspension.

3. RESULTS

3.1. *Effects of protein kinase inhibitors on the Ca^{2+} responses of a rat fibloblast line, WFB*

WFB cells respond to the stimulation by PDGF, vasopressin, bombesin, and $PGF_{2\alpha}$ with the PLC activation and an increase in $[Ca^{2+}]_i$ [2]. The susceptibility of the Ca^{2+} responses in WFB cells to the inhibition by staurosporine, genistein, H-7, and H-8 was determined by the use of a fluorescent Ca^{2+} indicator, fluo-3 [24]. Fluo-3 was chosen in place of fura-2 for its longer excitation and emission wavelengths to avoid an interference by the fluorescence of staurosporine. All Ca^{2+} responses reported in this paper were measured at 20°C to avoid and excessive leak of fluo-3 from the cells at higher temperature. The Ca^{2+} response to PDGF was completely inhibited by 1 μM staurosporine added 1 min before the PDGF addition and by 40 μM genistein (Fig. 1). H-7 and H-8, inhibitors of protein kinase C and cAMP-dependent protein kinase [25], had no marked effects on the Ca^{2+} response to PDGF at 30 μM (Fig. 1): a concentration high enough for the inhibition by these drugs [25]. The Ca^{2+} response in WFB cells to vasopressin was not inhibited by staurosporine, H-7, or H-8 under the same conditions as described above (Fig. 2) Genstein had a small inhibitory effect on the Ca^{2+} response to vasopressin (Fig. 2). The Ca^{2+} responses in WFB cells to bombesin and $PGF_{2\alpha}$ were not affected at all by staurosporine, H-7, or H-8 and suppressed to a limited extent by genistein as found in the response to vasopressin (data not shown). These results indicate that staurosporine is an inhibitor capable of clearly discriminating two types of the receptor-stimulated PLC activation either dependent or independent on a PTK.

3.2. *Effects of protein kinase inhibitors on the Ca^{2+} responses of human T and B cell lines*

The susceptibility of Ca^{2+} responses in human T and B cell lines to the inhibition by staurosporine was determined under the same conditions as used in WFB cells. The Ca^{2+} response of a human T cell leukemia line, Jurkat, to anti-CD3, OKT3, was completely inhibited by 1 μM staurosporine (Fig. 3). Genistein at 40 μM inhibited the response to some extent but the inhibition was not complete (Fig. 3). H-7 and H-8 did not have any obvious effect on the Ca^{2+} response of Jurkat cells (Fig. 3).

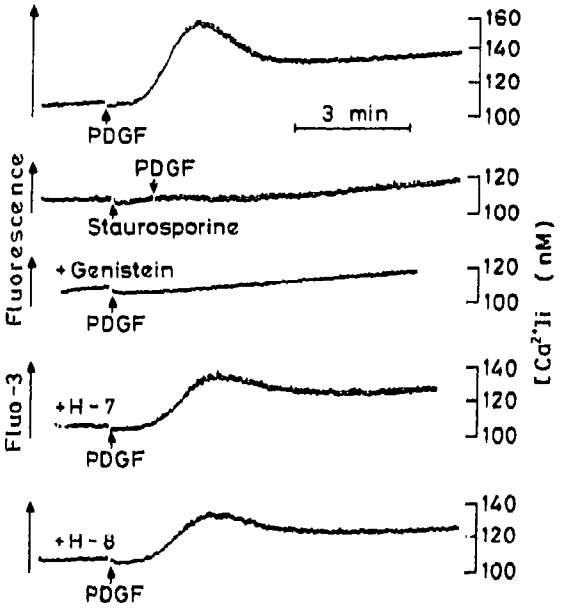

Fig. 1. Effects of staurosporine, genistein, H-7 and H-8 on the PDGF-induced Ca^{2+} response in WFB cells.

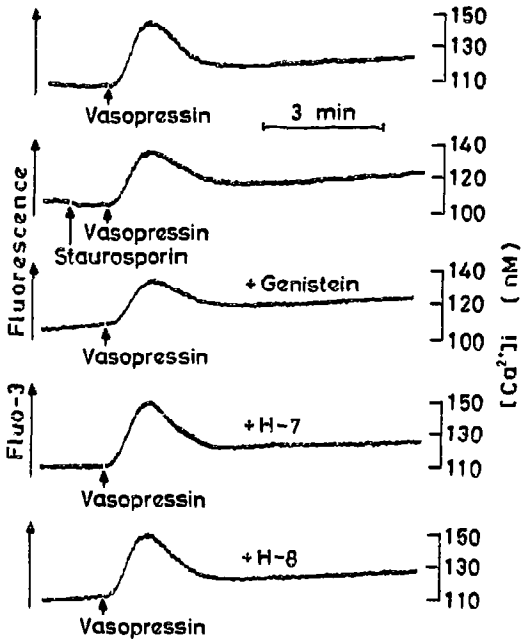

Fig. 2. Effects of staurosporine, genistein, H-7 and H-8 on the vasopressin-induced Ca^{2+} response in WFB cells.

The Ca^{2+} response of an Epstein-Barr virus-transformed human B cell line, RPMI 8075, to anti-human IgG was completely inhibited by 1 μM staurosporine (Fig. 4). Genistein at 40 μM inhibited the

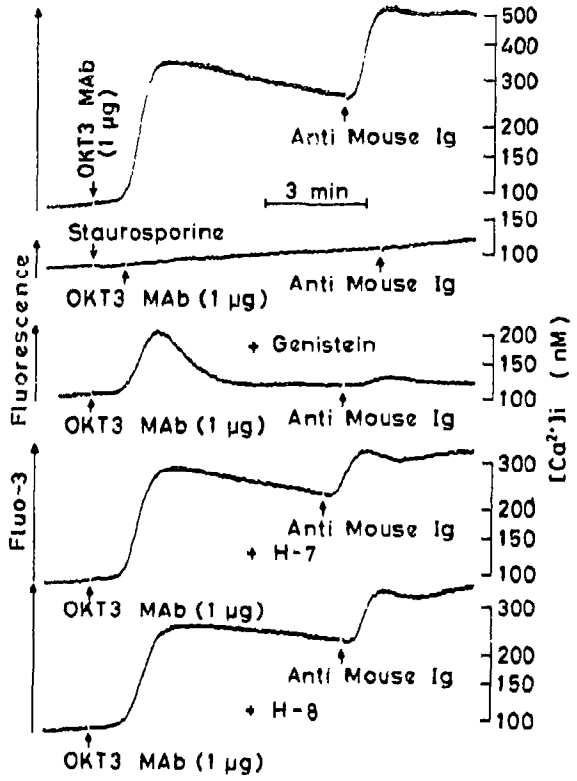

Fig. 3. Effects of staurosporine, genistein, H-7 and H-8 on the OKT3-induced Ca^{2+} response in Jurkat cells. Anti-mouse Ig was added to cross-link OKT3 in order to augment the response to OKT3.

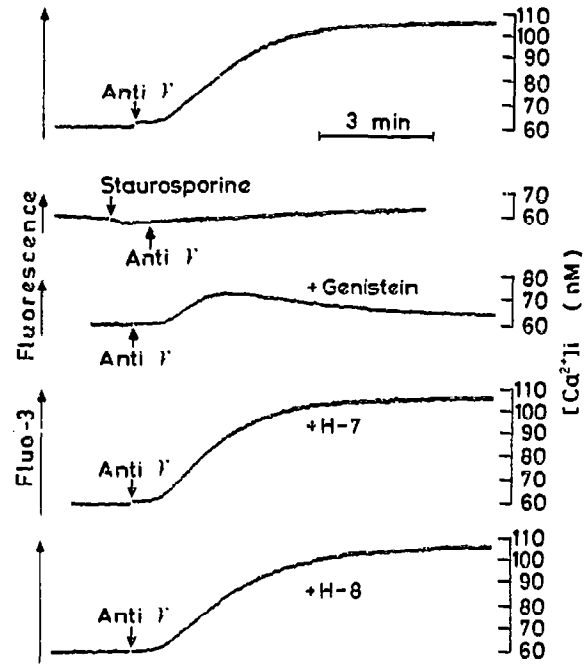

Fig. 4. Effects of staurosporine, genistein, H-7 and H-8 on the anti-human IgG (*anti* γ)-induced Ca^{2+} response in RPMI 8075 cells.

response to a limited extent (Fig. 4). H-7 and H-8 did not have any obvious effect on the Ca^{2+} response of RPMI 8075 cells (Fig. 4). Another human B cell line, Daudi, responded to anti-human IgM with an increase in $[Ca^{2+}]_i$. Daudi cells did not respond to anti-human IgG with an increase in $[Ca^{2+}]_i$, nor RPMI 8075 cells to anti-human IgM. The effects of staurosporine and genistein on the Ca^{2+}-response in Daudi cells were

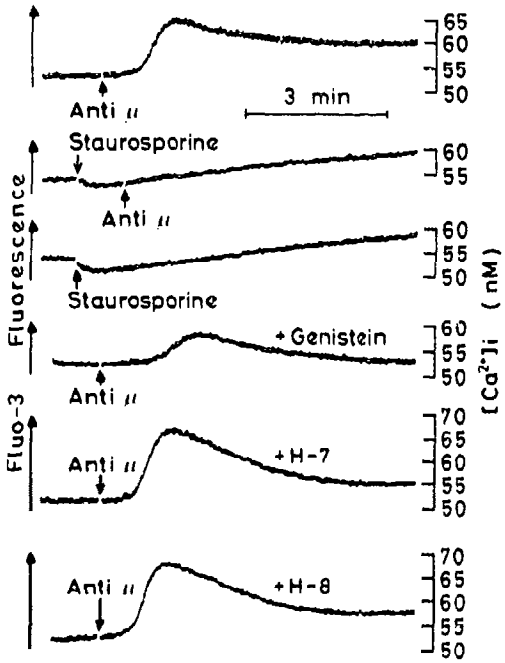

Fig. 5. Effects of staurosporine, genistein, H-7 and H-8 on the anti-human IgM (*anti* μ)-induced Ca^{2+} response in Daudi cells.

almost identical with those found in Jurkat cells and RPMI 8075 cells (Fig. 5). In Daudi cells, a small enhancement of the Ca^{2+} response by H-7 and H-8 was observed (Fig. 5).

4. DISCUSSION

The receptor-stimulated activation of PLC in such ligand-receptor systems as those used in this study [2,4,8,9,11,16,21,22,26,27] can be indirectly monitored by the Ca^{2+} responses, which are by far more sensitive and convenient than the assay of [^{3}H]inositol phosphate release from the cells labeled with *myo*-[^{3}H]inositol. The results presented in this paper indicate that the two types of receptor-stimulated increase in $[Ca^{2+}]_i$ and, by inference, receptor-stimulated activation of PLC can be clearly distinguished by the use of staurosporine: the responses mediated by a PTK are completely inhibited by staurosporine, whereas those mediated by a G-protein are not affected at all. The mechanism of this drug effect can be attributed to its potent inhibitory effect on PTKs: half maximal concentration for the inhibition by staurosporine has been reported to be 6.4 nM for $pp60^{v\text{-}src}$ PTK, while that for protein kinase C and cAMP-dependent protein kinase is 2.7 nM and 8.2 nM, respectively [17–19]. Herbimycin A and genistein, but not staurosporine, have been used by other investigators as PTK inhibitors to examine the participation of a PTK in the receptor-stimulated activation of PLC [10–12,16]. Addition of staurosporine at 1 μM and 1 min before the ligand addition completely inhibited the PTK-dependent Ca^{2+} responses (Figs. 1,3,4 and 5), whereas with herbimycin A an incubation of cells at 1–10 μM for more than 10 h is necessary to suppress the PTK-dependent increase in $[Ca^{2+}]_i$ and activation of PLC [11,12,16]. Herbimycin A may not be a classic competitive inhibitor; the inhibition appears to require PTK degradation [11,28].

It is difficult to obtain enough inhibition by genistein due to a low potency and a limited solubility of the drug; in our optical measurements it was not possible to add genistein at 100 μM or more. The concentration of genistein for half maximal inhibition was 2.5 μM for EGF receptor PTK and 28 μM for $pp60^{v\text{-}src}$ PTK [29,30]; this difference in the inhibitory effect of genistein between receptor PTK and non-receptor PTK may possibly account for complete suppression of the PDGF-triggered Ca^{2+} response by 40 μM genistein (Fig. 1) and for partial suppression of the OKT3-triggered and anti-Ig-triggered Ca^{2+} responses by the same concentration of genistein (Figs. 3,4 and 5).

The results shown in Figs. 3, 4 and 5 demonstrate that the Ca^{2+} responses of human T and B cell lines induced by anti-CD3, anti-IgG and anti-IgM are completely inhibited by staurosporine. By deduction from the results in WFB cells, it is possible to conclude that the PTK activity plays an essential role in the PLC activation through the TCR/CD3 complex and through mIgG and mIgM. H-7 and H-8 had no inhibitory effect on any of the Ca^{2+} responses studied; this is consistent with the assumption that PTKs are the target of the inhibition by staurosporine in the Ca^{2+} responses. The PLC activation through the TCR/CD3 complex and mIg can be grouped into a new type of the receptor-stimulated activation of PLC, which is characterized, presumably, by the participation of nonreceptor PTKs in the coupling of a receptor to PLC. Although the non-receptor PTKs involved in the signal transduction through this PLC pathway are not yet identified, $p59^{fyn}$ and $p56^{lck}$ are possible candidates for the non-receptor PTK mediating the PLC activation through the TCR/CD3 complex [31].

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