Action of a cytotoxin from *Pseudomonas aeruginosa* on human leukemic cell lines

Increase in cell permeability to Ca^{2+} and Mn^{2+} and lack of stimulation of inositol lipid turnover

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Quin2 loaded human leukemic, JURKAT and K562 cells, were exposed to various doses of *Pseudomonas aeruginosa* cytotoxin. This cytotoxin induced an increase in quin2 fluorescence indicating an increase in the cytoplasmic free Ca^{2+} concentration. The rate of the fluorescence increase and the lag time before the response were dependent on the doses of the cytotoxin. Addition of $MnCl_2$ to the cytotoxin-treated cells induced a decrease in the quin2 fluorescence at rates dependent on the doses of the cytotoxin. The cytotoxin did not stimulate the inositol lipid turnover in JURKAT cells, which was determined by the accumulation of [^{3}H]inositol phosphates in *myo*-[2-^{3}H]inositol-prelabeled cells in the presence of LiCl. These results indicate that the cytotoxin increases cell permeability to both Ca^{2+} and Mn^{2+} by direct breakdown of the permeability barrier of the plasma membrane.

Cytotoxin Pseudomonas aeruginosa *Quin2* *Permeability change* *Human leukemic cell line* *Inositol phosphate*

1. INTRODUCTION

Pseudomonas aeruginosa is an important pathogen in medicine. A number of bacterial factors have been implicated to be involved in the pathogenesis of the microbe. One of these is an acidic cytotoxic protein, called cytotoxin, with an M_r of about 25000 [1]. The toxin attacks the plasma membranes of many mammalian cells resulting in an increase in permeability to ions and small molecules within a short time of intoxication [2–4]. In cultured endothelial cells, it has been demonstrated that the toxin stimulates the production of prostacyclin [4].

From studies on rabbit leukocytes, Hirayama and Kato [5,6] reported that pseudomonal leukocidin induced rapid metabolic changes of phosphatidylinositol and polyphosphoinositides to accumulate phosphatidic acid, resulting in the process of leukocyte destruction by an increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Leukocidin and cytotoxin are similar proteins. Both toxins were isolated from an autolysate of *P. aeruginosa* strain 158 [1,7,8]. Amino acid analyses indicate that both toxins have a high content of amino acids with nonpolar side chains [1,8]. Rabbit antiserum against leukocidin inhibited the action of cytotoxin on human granulocytes (Lutz, F., unpublished). However, the molecular mass of leukocidin was estimated to be about 42 kDa, which is 1.7-times larger than that of cytotoxin [8]. In this paper, we studied the effect of cytotoxin on

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Abbreviations: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PHA, phytohemagglutinin

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$[Ca^{2+}]_i$ and on the inositol lipid turnover in JURKAT cells, a human T-cell leukemia line [9], and K562 cells, a human chronic myelogenous leukemia line [10].

2. MATERIALS AND METHODS

2.1. *Cytotoxin from* Pseudomonas aeruginosa

Cytotoxin was prepared from autolysate of *P. aeruginosa* strain 158 as described by Lutz [1] and stored in phosphate-buffered saline (0.01 M sodium phosphate, pH 7.4, 0.14 M NaCl, 3 mM KCl, 0.5 mM $MgCl_2$).

2.2. *Cell culture*

JURKAT-FHCRC and K562 cells were cultured in RPMI-1640 medium supplemented with 6.7% heat-inactivated (56°C for 30 min) fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 300 μg/ml L-glutamine.

2.3. *Quin2 loading and fluorescence measurements*

Quin2 tetraacetoxy methyl ester (Dojin Chemicals, Kumamoto, Japan) was added at 100 μM to the cell suspension in RPMI-1640 medium containing 3×10^7 cells/ml and incubated for 20 min at 37°C. The suspension was then diluted 10-fold with RPMI-1640 medium and incubated for a further 40 min. After loading, the cells were washed once with Tris-buffered saline (containing 0.7 mM phosphate) [11], resuspended in the same saline at 2.5×10^6 cells/ml and kept at room temperature. For measurement of fluorescence, 2 ml of the cell suspension was transferred to a cuvette. Quin2 fluorescence was recorded at 37°C with a Hitachi 650-10S fluorescence spectrophotometer fitted with a magnetic stirrer and a thermostatically controlled cell holder. Excitation and emission wavelengths were 339 and 492 nm with 4 and 10 nm slits, respectively. Fluorescence was calibrated in terms of $[Ca^{2+}]_i$ from the fluorescence value at 0.9 mM Ca^{2+} in the presence of 0.1% Triton X-100 and the fluorescence value at 0.5 mM $MnCl_2$ in the presence of 0.1% Triton X-100 [12–14]. Triton X-100 was added from 10% (v/v) solution. $MnCl_2$ was added from 0.1 M solution.

2.4. *Analysis of accumulation of [^{3}H]inositol phosphates in JURKAT cells prelabeled with* myo-*[2-^{3}H]inositol in the presence of LiCl*

The experiment was performed in the same manner as described in [15,16].

3. RESULTS AND DISCUSSION

3.1. *Cytotoxin induces an increase in permeability to Ca^{2+} and Mn^{2+} in JURKAT and K-562 cells*

As reported in [14], PHA induces an increase in fluorescence of the quin2 loaded JURKAT cells, indicating an increase in $[Ca^{2+}]_i$ (fig.1). Addition of $MnCl_2$ to PHA-treated JURKAT cells caused an immediate, small drop in fluorescence followed by a small gradual decrease (fig.1), which indicates slow penetration of Mn^{2+} into the cells.

Figs 2 and 3 illustrate the effect of cytotoxin on $[Ca^{2+}]_i$ of quin2-loaded JURKAT cells. On addition of 12.5 μg cytotoxin per ml cell suspension

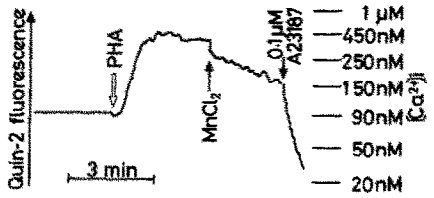

Fig.1. Fluorescence response of quin2-loaded JURKAT cells to PHA and the effect of $MnCl_2$ on the response. Quin2 loading, fluorescence monitoring, and calculation of $[Ca^{2+}]_i$ were done as described in section 2. Traces represent responses of quin2 fluorescence to additions of 20 μg/ml of PHA (leucoagglutinin, Sigma), $MnCl_2$, and A23187. A23187 (Calbiochem-Behring) was added from 100 μM solution in dimethyl sulfoxide.

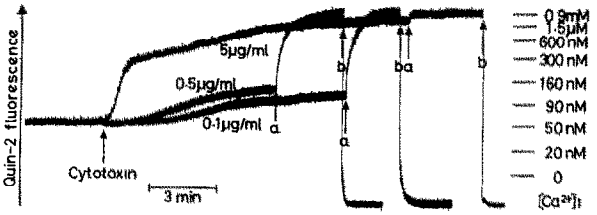

Fig.2. Fluorescence response of quin2 loaded JURKAT cells to cytotoxin. The experiments were done as described in section 2. Traces represent responses of quin2 fluorescence to additions of the indicated doses of cytotoxin, Triton X-100 (arrow a), and $MnCl_2$ (arrow b).

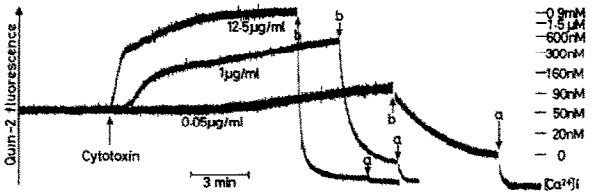

Fig.3. Fluorescence response of quin2 loaded JURKAT cells to cytotoxin and the effect of $MnCl_2$ on the response. The experiments were performed as for fig.2 except that $MnCl_2$ (arrow b) was added before Triton X-100 (arrow a).

(2.5×10^6 cells), there was an immediate increase in quin2 fluorescence (fig.3). The rise in fluorescence was steep for the first 40 s, when the fluorescence intensity represents a 6-fold increase in $[Ca^{2+}]_i$. The increase in cytotoxin-induced $[Ca^{2+}]_i$ continued during the next 5 min and reached a concentration equivalent to that of the medium. The fluorescence response to cytotoxin was dose-dependent. Addition of 0.05 μg cytotoxin per ml cell suspension caused a small increase in $[Ca^{2+}]_i$ after a lag period of 5–6 min (fig.3). Both the lag time and rate of $[Ca^{2+}]_i$ increase are dependent on the doses of cytotoxin (figs 2 and 3). In contrast to the $[Ca^{2+}]_i$ response to PHA and other Ca^{2+}-mobilizing intercellular signals (figs 2 and 3) [14,17,18], cytotoxin-induced $[Ca^{2+}]_i$ did not reach a maximal value within a few minutes of toxin addition but persistently increased toward the concentration in the medium during incubation.

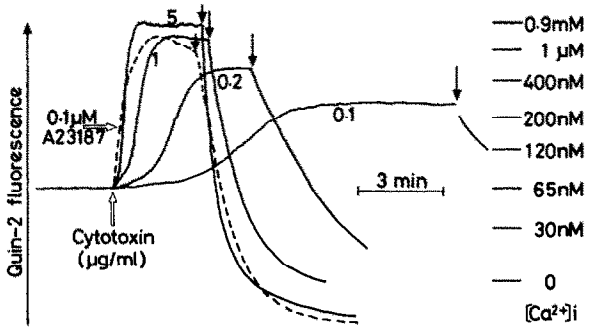

Fig.4. Fluorescence response of quin2 loaded K562 cells to cytotoxin and the effect of $MnCl_2$ on the response. The experiments were performed as for fig.3. $MnCl_2$ was added at the time points indicated by closed arrows.

Addition of $MnCl_2$ to cytotoxin-treated JURKAT cells induced a decrease in quin2 fluorescence at a rate dependent on the doses of cytotoxin (fig.3). The affinity of Mn^{2+} for quin2 is 513-fold greater than that of Ca^{2+} [12]. The fluorescence intensity of Mn^{2+}-quin2 complex is 1% of that of Ca^{2+}-quin2 complex [12]. Therefore, this Mn^{2+}-induced quenching of fluorescence indicates that cytotoxin-treated JURKAT cells have an increased permeability to both Ca^{2+} and Mn^{2+}. At a cytotoxin dose of 12.5 μg/ml, almost all of the quin2 became saturated by the added Mn^{2+} within 1–2 min (fig.3).

Treatment of K562 cells with cytotoxin also increased permeability of the cells to both Ca^{2+} and Mn^{2+} (fig.4). The increase was dependent on the doses of cytotoxin. The effect of cytotoxin on JURKAT and K-562 cells is similar to that of A23187, a divalent cation ionophore (fig.4) [14], and distinctly different from the effect of PHA and other Ca^{2+}-mobilizing intercellular signals (fig.1) [14,17,18].

Table 1

Effects of PHA and cytotoxin on the accumulation of [^{3}H]inositol phosphates in Li^+-treated JURKAT cells prelabeled with *myo*-[2-^{3}H]inositol

Addition	Concentration (μg/ml)	Accumulated [^{3}H]inositol phosphates (dpm/10^6 cells)
None		1385; 1481
PHA	50	6896; 7721
Cytotoxin	2	1604; 1778
Cytotoxin	0.2	1421; 1483

JURKAT cells were labeled with 5 μCi/ml of *myo*-[2-^{3}H]inositol for 20 h. The labeled cells were suspended at 4×10^6 cells/ml in phosphate-buffered saline [26] containing 0.1% glucose in which a portion (5 mM) of NaCl had been replaced with 5 mM LiCl. Portions (0.25 ml) of the cell suspension were preincubated for 15 min and then incubated at 37°C for 15 min in the presence of the indicated additions. *myo*-[2-^{3}H]Inositol phosphates, a sum of inositol tris-, bis- and monophosphates, accumulated in the cells were determined by the method of Berridge et al. [23]. Results are 2 values obtained from duplicate incubations

3.2. *Cytotoxin does not stimulate inositol lipid turnover*

Activation of cell-surface receptors for Ca^{2+}-mobilizing intercellular signals induces hydrolysis of PtdIns(4,5)P_2 resulting in the production of 1,2-diacylglycerol and inositol 1,4,5-trisphosphate [19,20]. Inositol trisphosphate is dephosphorylated stepwise to inositol bisphosphate, inositol monophosphate and then to inositol [21]. Li^+ is a potent inhibitor of inositol-1-phosphatase [22]. Berridge et al. [23] showed that Li^+ could greatly amplify the agonist-dependent accumulation of inositol 1-phosphate in brain slices and salivary glands. Since then LiCl has been used to provide a sensitive method for identifying those receptors that are coupled to the hydrolysis of PtdIns(4,5)P_2 [15–17,19,20].

It has been shown that in JURKAT cells PHA induces the hydrolysis of PtdIns(4,5)P_2, formation of inositol trisphosphate and stimulation of ^{32}P labeling of phosphatidic acid [14,16]. Stimulation of *myo*-[2-^{3}H]inositol-prelabeled JURKAT cells with PHA for 15 min at 37°C in the presence of 5 mM LiCl induced a 5-fold increase in [^{3}H]inositol phosphates (table 1). Formation of inositol phosphates is a direct measure of the hydrolysis of inositol lipids by phospholipase C. Cytotoxin at 0.2 μg/ml did not induce accumulation of [^{3}H]inositol phosphates in JURKAT cells during 15 min incubation at 37°C in the presence of 5 mM LiCl (table 1). However, cytotoxin at 2 μg/ml induced a 1.18-fold accumulation of [^{3}H]inositol phosphates. This stimulation of the accumulation is too small to be produced by direct stimulation of inositol lipid turnover by the toxin. The small stimulation is most likely a secondary effect of cytotoxin due to the elevation of $[Ca^{2+}]_i$ and subsequent activation of Ca^{2+}-dependent, phosphatidylinositol-specific phospholipase C [24,25].

3.3. *Conclusion*

Taken together the above results indicate that cytotoxin does not stimulate lipid turnover and suggest that it damages cells by direct breakdown of the permeability barrier of the plasma membrane.

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