Interleukin 2 Increases T Lymphocyte Membrane Mobility before the Rise in Cytosolic Calcium Concentration†

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ABSTRACT: Using stopped-flow fluorometry with three different fluorescence probes [2-[(1-pyrenylbutyryl)oxy]stearic acid, chlortetracycline and Quin 2], we have studied initial stages of T lymphocyte activation after interleukin 2 (IL-2) binding to a specific cell-surface receptor. After IL-2 binding to cytotoxic T lymphocyte (IL-2-dependent mouse LC7 and CTLL-2 cells), membrane mobilities of the cells increased first (4.5 ± 0.3 s^{-1} for LC7 and 3.8 ± 0.2 s^{-1} for CTLL-2), then calcium was released from intracellular stores into the cytoplasm (1.6 ± 0.1 s^{-1} for LC7 and 2.1 ± 0.1 s^{-1} for CTLL-2), and lastly, calcium was transported from the external medium into the cytoplasm (1.3 ± 0.1 s^{-1} for LC7 and 1.5 ± 0.1 s^{-1} for CTLL-2). The slowest process, the calcium influx from the external medium, was suppressed in the presence of a calcium channel blocking agent (verapamil). These observations give us a new information to discuss a model in T lymphocyte activation after IL-2 binding to cell-surface receptors.

Interleukin 2 (IL-2) plays an essential role in triggering proliferation of activated T cells. IL-2 is released by T cells in response to two signals (anigen and interleukin 1) provided by antigen-pulsed accessory cells (Farrar et al., 1982; Smith, 1980; Robb et al., 1981). As with polypeptide hormones and other growth factors, the mode of action of IL-2 in promoting T cell proliferation involves binding to a specific cell-surface receptor. However, little is known about the mechanism whereby the signal of ligand binding is transmitted to the cell nucleus (Smith et al., 1983; Robb, 1984).

In several systems, ligand binding is known to lead to an increase in cytosolic calcium concentration. This rise in cytosolic calcium is suggested to follow the breakdown of phosphatidylinositol bisphosphate into diacylglycerol and inositol trisphosphate. The latter is known to cause release of calcium from intracellular stores (Michell, 1982; Nishizuka, 1984; Michell, 1984; Berrigde, 1984). To understand the physical aspects of such kinds of signal transfer, we have studied here early transmembrane events (membrane mobility changes, redistribution of calcium, and calcium influx into the cytoplasm) involved in T lymphocyte activation after IL-2 binding to cell surface receptors, using stopped-flow fluorometry with three different fluorescence probes (Utsunomiya et al., 1985). From these experiments, we discussed the relationship between the above early transmembrane events involved in the cell activation and the second messenger function of inositol trisphosphate and diacylglycerol in signal transduction.

Materials and Methods

Quin 2AM was purchased from Dojin Chemical Ind., A23187 from Calbiochem, and RPMI 1640 from Gibco. Chlortetracycline (CTC) and verapamil were obtained from Sigma. Human IL-2 made by recombinant DNA techniques was given by Yoshitomi Pharmaceutical Co. Its amino acid sequence is identical with that described by Taniguchi et al. (1983). Human IL-2 is known to support the long-term growth of CTL (cytotoxic T lymphocytes) of both human and murine origins in culture (Robb, 1984).

Interleukin 2 dependent mouse CTL (LC7) is a long-term cultured blast-cell line originating from secondary mixed-lymphocyte culture between C57BL/6 (H-2^b, responder) and DBA/2 (H-2^d, stimulator). The cell line (LC7) was grown in cell cultures in RPMI 1640 + 10% FCS (fetal calf serum, with concanavalin A supernatant) (Yamazaki et al., 1982). Another IL-2-dependent mouse CTL (CTLL-2) was generated by the previous procedures (Gillis & Smith, 1977).

Fluorescence compounds were incorporated into LC7 and CTLL-2 cells in PBS (phosphate-buffered saline) by incubation with 10 μM Quin 2AM in PBS for 40 min at 37 °C, with 50 μM CTC in PBS for 40 min at 37 °C, or with 10 μM 2-[(1-pyrenylbutyryl)oxy]stearic acid in PBS for 40 min at 37 °C. Quin 2AM was hydrolyzed into Quin 2 by the cell esterases. These processes were monitored by the shift in the emission spectrum from the peak of Quin 2AM at 430 nm to the Quin 2 peak around 490 nm (Tsien et al., 1982a,b). After labeling, cells were centrifuged for 1 min at 1000 rpm in a Fisher centrifuge Model 59. Then they were washed with PBS and were resuspended in PBS (+1 mM Ca^{2+}) for stopped-flow measurements. Cell viability (more than 98%) was not changed after stopped-flow measurements. It was checked by trypan blue dye exclusion.

Fluorescence spectra were observed with a Hitachi fluorescence spectrophotometer Model 650-10S. Stopped-flow fluorescence measurements were taken by a Union Giken stopped-flow spectrophotometer RA-401 in combination with a microcomputer RA-450 system (Nakanishi & Tsuboi, 1978). In our present experiment, the sensitivity of stopped-flow measurements was improved by data accumulation in an on-line computer. That is, 64 successive data points for 20 ms were stored as one averaged data point in a computer. The experimental trace (Figures 1 and 2) was a result of 6–10 accumulated individual stopped-flow measurements, which consisted of a series of averaged data points mentioned above. We used a Hoya Y46 cut filter (which allows emitted light with wavelength longer than 460 nm to enter the detector) in the fluorescence measurements.

Results

Calcium Influx into the Cytoplasm. Quin 2 is known to be a fluorescent indicator of intracellular free calcium. This tetracarboxylate anion binds calcium ions with a 1:1 stoichiometry: binding causes an increase in fluorescence emission (Tsien et al., 1982a,b). After mixing of Quin 2 loaded CTL

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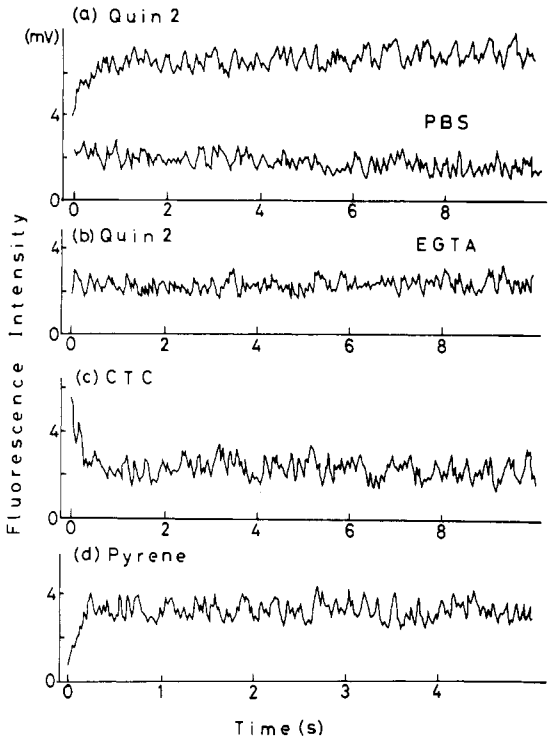

FIGURE 1: Stopped-flow fluorometry traces indicating the effects of IL-2 on mouse CTL (LC7). Fluorescence-labeled CTL (final 1×10^5 cells/mL) was mixed with IL-2 (100 unis/mL) at 25 °C. (a) Effect of IL-2 on fluorescence of Quin 2 loaded CTL (LC7). "PBS" means a control experiment where we mixed PBS alone with LC7 cells containing Quin 2. Excited at 340 nm. (b) Effect of IL-2 on fluorescence of Quin 2 loaded CTL (LC7) where EGTA (2 mM) was present in the external medium. Excited at 340 nm. (c) Effect of IL-2 on fluorescence of CTC-loaded CTL (LC7). Excited at 400 nm. (d) Effect of IL-2 on fluorescence of 2-[(1-pyrenylbutyryl)oxy]stearic acid loaded CTL (LC7). Excited at 340 nm.

(LC7 or CTLL-2) with IL-2 (final 25 ng/mL; 1.6 nM IL-2 = 100 units/mL), the fluorescence of the cells rises within 2 s, indicating a rise of intracellular free calcium concentration to a new steady-state level as shown in Figure 1a (rate constants 1.3 ± 0.1 s^{-1} for LC7 and 1.5 ± 0.1 s^{-1} for CTLL-2). From the concentration used here (1.6 nM IL-2) and the values of the equilibrium dissociation constant between IL-2 and its specific receptor [$K_d = (5\text{–}20) \times 10^{-12}$ M; Smith et al., 1983], it is suggested that IL-2 bound to specific cell surface receptors of LC-7 cells with the diffusion-control reaction. Assuming that all the cells in suspension respond to IL-2 molecules, the total observed fluorescence increase corresponds to a 5–6-fold increase of the intracellular free calcium concentration (initial value 1.0×10^{-7} M), following Tsien's procedure with a fluorescence spectrophotometer (1982b). When EGTA [ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (2 mM) was present in the external medium, no fluorescence occurred in the time range of the experiment as shown in Figure 1b. This indicates that the fluorescence increase in Figure 1a corresponds mostly to calcium influx from the external medium into the CTL (LC7). For a control experiment, we also mixed PBS with Quin 2 loaded CTL (LC7): no flluorescence increase was observed as shown in Figure 1a (lower record).

Redistribution of Calcium. Next, we measured the effects on CTC fluorescence of IL-2 in CTL (LC7 cells). After IL-2 binding to LC7 cells, the CTC fluorescence decreases with a rate constant of 1.6 ± 0.1 s^{-1}, which is slightly larger than that of the Quin 2 fluorescence increase (1.3 ± 0.1 s^{-1}) (Figure 1c). This is also true for CTLL-2 cells. CTC fluorescence intensity

Table I: Summary of Fluorescence Changes Observed in Early Stages of T Lymphocyte Activations by IL-2 (at 25 °C)[a,b]

		without antagonist (s^{-1})	with verapamil (s^{-1})[c]
LC7	Quin 2	1.3 (0.1)[d]	no change
	CTC	1.6 (0.1)	1.8 (0.2)[d]
	pyrene	4.5 (0.3)	4.6 (0.1)
CTLL-2	Quin 2	1.5 (0.1)	
	CTC	2.1 (0.1)	
	pyrene	3.8 (0.2)	

[a] Cytotoxic T lymphocyte (LC7 or CTLL-2) = 1×10^5 cells/mL. [b] IL-2 = 100 units/mL. [c] Verapamil = 5×10^{-5} M. [d] Standard deviations are in parentheses.

is expected to decrease as calcium is released from internal stores, for example, from endoplasmic reticulum into cytoplasm (Le Breton et al., 1976; Feinstein, 1980). It is difficult to estimate the absolute amount of calcium ions redistributed among the calcium stores and cytoplasm, but it should be much smaller than the amount of calcium ions that are transported from the external medium into cytoplasm. The observed rates (1.6 ± 0.1 s^{-1} for LC7 and 2.1 ± 0.1 s^{-1} for CTLL-2) of these CTC fluorescence changes indicate that the redistribution of calcium from internal stores is more rapid than the overall rate of the calcium influx into cytoplasm (1.3 ± 0.1 s^{-1} for LC7 and 1.5 ± 0.1 s^{-1} for CTLL-2).

Membrane Mobility Change. Further, we measured a time-dependent change of membrane mobility (fluidity) in CTL after IL-2 binding to a specific cell-surface receptor. Membrane mobility (fluidity) can be measured by the use of a fluorescent pyrene derivative that forms excimers in fluid biological membranes (Vanderkool & Callis, 1974; Galla & Sackmann, 1974; Utsunomiya et al., 1985; Ohga et al., 1985). Fluorescence spectra of 2-[(1-pyrenylbutyryl)oxy]stearic acid embedded in CTL membranes showed two monomer fluorescence peaks at 382 and 400 nm and a broad excimer fluorescence peak around 480 nm. After binding of IL-2, the excimer fluorescence in the LC7 cell membranes rises at 4.5 ± 0.3 s^{-1} as shown in Figure 1d (3.8 ± 0.2 s^{-1} for CTLL-2 cells), indicating a rise of membrane mobility. This rate is much faster than that of the release rate of calcium from internal stores into cytoplasm (CTC fluorescence). All the results are summarized in Table I.

Calcium Channel Blocking Agents. In order to examine the effects of calcium channel blocking agents on the calcium influx, CTL (LC7) were loaded with Quin 2 in the presence of verapamil (5×10^{-5} M) (Birx et al., 1984). When LC7 cells were mixed with IL-2, the Quin 2 fluorescence increase largely disappeared as shown in Figure 2a. However, the fluorescence intensities of the CTC-loaded or pyrene-loaded CTL were changed after binding to the target cells, even if the calcium antagonists were present (Figure 2b,c). This fact indicates that the observed Quin 2 fluorescence increase (in Figure 1a) corresponds to calcium influx from the external medium into the cytoplasm through membrane calcium channels.

Discussion

It is now known that the mode of action of IL-2 in promoting T cell proliferation involves binding to a specific cell surface receptor and a subsequent series of clustering and endocytosis as with polypeptide hormones and growth factors (Robb, 1984). The biological role of each step is not clear. However, IL-2 probably acts at the level of the cell surface to generate a signal that in turn produces a "second messenger" that triggers cell activation (Michell, 1982, 1984; Nishizuka,

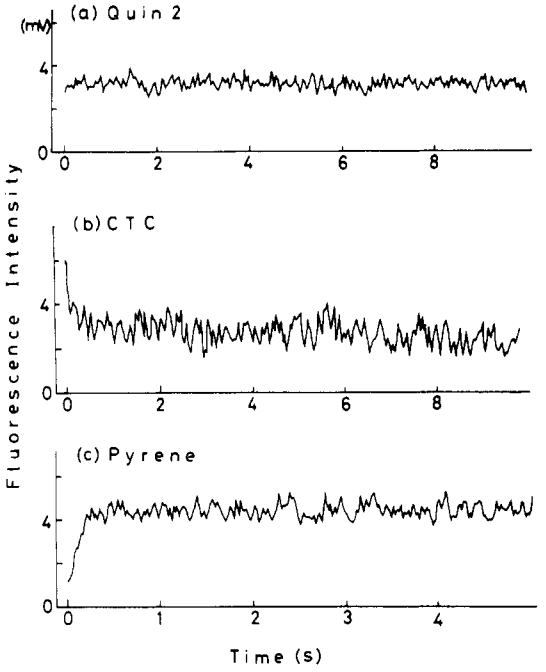

FIGURE 2: Stopped-flow fluorometry traces indicating effects of calcium antagonist (verapamil, 5×10^{-5} M) on CTL (LC7). Fluorescence-labeled T lymphocyte cells (final 1×10^5 cells/mL) were mixed with IL-2 (final 100 units/mL) at 25 °C. Experimental conditions were the same as those in Figure 1. (a) Effect of IL-2 on fluorescence of Quin 2 loaded CTL (LC7). (b) Effect of IL-2 on fluorescence of CTC-loaded CTL (LC7). (c) Effect of IL-2 on fluorescence of 2-[(1-pyrenylbutyryl)oxy]stearic acid loaded CTL (LC7).

1984; Berridge, 1984; Schlessinger, 1983).

Our present results show that IL-2 binding to a specific cell-surface receptor induces a series of early biochemical and biophysical events (increase of membrane mobility, redistribution of calcium, and calcium influx into the cytoplasm), which may contribute to the activation of CTL. It should be pointed out that the early transmembrane events described here are also found in some other systems, including, for example, rabbit blood platelets after binding with thrombin (Ohga et al., 1985) and mouse myeloma cells X5563 (H-2K^k) and mouse lymphoma RDM4 (H-2K^k) after binding of anti H-2K^k monoclonal antibodies (11-4.1) (Utsunomiya et al., 1985). Thus, IL-2 binding to a specific cell-surface receptors, Tac antigen (Uchiyama et al., 1981), seems to generate initial transmembrane signals that are similar to those induced by ligand–receptor interactions in many other systems.

It is suggested now that the key reaction in the signal-transducing mechanism is a receptor-mediated hydrolysis of phosphatidylinositol bisphosphate into inositol trisphosphate and diacylglycerol. Inositol trisphosphate seems to act by releasing calcium from endoplasmic reticulum to the cytoplasm, whereas diacylglycerol stimulates protein phosphorylation (Michell, 1982; Nichizuka, 1984; Berridge, 1984). The receptor-mediated breakdown of the inositol lipids is considered to precede any increase in the intracellular level of calcium (Michell, 1982; Berridge, 1984). These schemes are well consistent with our present stopped-flow experiments. That is, the present results indicate that IL-2 binding to specific cell surface receptors first increases the T-cell membrane mobility, as observed by the pyrene excimer fluorescence. This increase occurs concomitantly with the breakdown of phosphatidylinositol bisphosphate to give the two second messengers (inositol trisphosphate and diacylglycerol). Then, the released inositol trisphosphate may induce a calcium signal by the removal of calcium from an intracellular reservoir (most likely to be the endoplasmic reticulum), which was observed by the CTC fluorescence. This rapid increase in the intracellular level of calcium and/or the released diacylglycerol, which stimulates protein phosphorylation, may mediate the subsequent physiological responses, including the opening of membrane calcium channels. The entry of external calcium, which was observed by Quin 2 fluorescence, must be very important maintain calcium signals in the subsequent cellular responses.

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