Redistribution of Intracellular Ca^{2+} in Mitogen-Stimulated Human Peripheral Blood Lymphocytes

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Abstract. The fluorescent probe chlortetracycline (CTC) was used to investigate redistribution of intracellular Ca^{2+} in concanavalin A (Con A)-stimulated human peripheral blood lymphocytes. The addition of the mitogen to CTC-equilibrated lymphocytes induced(within 10 to 15 minutes) a Con A-concentration dependent decrease in CTC fluorescence indicating the release of membrane-bound Ca^{2+}. The effect was independent of the level of extracellular Ca^{2+} and could be observed in the presence of EGTA; it was supressed by the metabolic inhibitors FCCP, antimycin and sodium cyanide. Analysis of the excitation spectra of CTC fluorescence indicated that the observed effect is caused by redistribution of intracellular Ca^{2+} rather than Mg^{2+}. Thus the lectin interaction with the lymphocyte plasma membrane results in Ca^{2+} release into the cytosol from the intracellular stores.

Key words: Chlortetracycline fluorescence — Intracellular Ca^{2+} — Lymphocyte activation

Introduction

Mitogenic activation of lymphocytes is one of the most valuable models to study regulation of the processes of growth and proliferation in mammalian cells (Hume and Weidemann1980; Sitkovsky 1979). Interaction of mitogens with lymphocyte plasma membrane receptors initiates a chain of biochemical events leading to DNA synthesis and cell division. Of particular interest is the question of the "second messenger" which transmits into the cell a signal generated by mitogen binding to the membrane. The calcium ion is known to play an important role in the regulation of a number of physiological processes such as stimulus-secretion

The abbreviations used: HBL — human peripheral blood lymphocytes; Con A — concanavalin A; CTC — chlortetracycline; EGTA — ethylene glycol bis (β-aminoethyl ether) — N, N′ — tetraacetic acid; FCCP — carbonyl cyanide m-fluorophenylhydrazone.

coupling, hormone action etc. (Rasmussen et al. 1979; Whitfield et al. 1979). In this connection it has been suggested that an increase in intracellular Ca^{2+} may act as a trigger of lymphocyte activation (Hume and Weidemann 1980). The concentration of cytosolic Ca^{2+} may increase in two ways: by an enhanced influx from the medium, or due to the release of intracellular membrane-bound Ca^{2+}. A number of recent results provided evidence against the idea that the only trigger of the lymphocyte activation is a mitogen-induced increase in Ca^{2+} influx (Diamanshtein and Ulmer 1975; Dubois and Crumpton 1980; Hesketh et al. 1977; Kay and Mattson 1979; Larner et al. 1980; Miller and Moticka 1977; Resch et al. 1978). The most important was the finding that lymphocyte activation by mitogens may be initiated in the absence of extracellular Ca^{2+}as well (Diamanshtein and Ulmer 1975; Dubois and Crumpton 1980; Kay and Mattson 1979; Miller and Moticka 1977).

The second way of increasing the cytosolic Ca^{2+} — by its mobilization from intracellular stores — has been given much less attention. Quite recently there have appeared first studies on redistribution of intracellular Ca^{2+} during mitogenic stimulation of lymphocytes. In these works (Mikkelsen and Schmidt–Ulrich 1980; Sergeeva et al. 1980; Shestakova et al. 1982) changes in membrane-bound Ca^{2+} in stimulated lymphoid cells were monitored by means of the fluorescent probe chlortetracycline. It is known that CTC penetrates readily into cells, forming complexes with Ca^{2+} and Mg^{2+}, and that the quantum yield of its fluorescence sharply increases when CTC binds to divalent cations in the membrane or in another hydrophobic environment (Caswell and Hutchison 1971; Caswell 1972). These properties of CTC were used to investigate Ca^{2+} accumulation in isolated mitochondria (Caswell and Hutchison 1971; Caswell 1972; Korkina et al. 1973; Luthra and Olson 1976) and sarcoplasmic reticulum (Caswell and Warren 1972; Jilka and Martonosi 1975), and lately to monitor changes in Ca^{2+} distribution within intact cells (Chandler and Williams 1978a, b; Feinstein 1980; LeBreton 1976; Mikkelsen and Schmidt–Ulrich 1980; Naccache et al. 1979).

In the present work CTC was used to examine changes in intracellular Ca^{2+} evoked by Con A interaction with human peripheral blood lymphocytes (HBL). It is shown that Con A induces a decrease in CTC fluorescence indicating a release of membrane-bound Ca^{2+} into the cytosol.

Material and Methods

Material. CTC (Serva), Con A, antimycin, FCCP (Sigma), EGTA (Fluka), Ca^{2+}-ionophore A23187 (Eli Lilly), and chemicals of reagent grade have been used.

Cell preparation. Heparinized human venous blood was obtained from healthy donors. Lymphocytes were separated by gradient centrifugation using a modified Ficoll-Hypaque technique (Astashkin et al. 1977; Boyum 1968). Monocytes were removed by adherence to glass, and also using their ability to

adsorb particles of carboxyl iron. Erythrocytes were removed with 0.83% NH_4Cl. The cells incubated in medium 199 at 37 °C were washed prior to use and resuspended in Hanks' balanced salt solution (1.3 mmol.l^{-1} Ca^{2+}, 1 mmol.l^{-1} Mg^{2+}, pH 7.4) or in phosphate-buffered saline (137 mmol.l^{-1} NaCl, 3 mmol.l^{-1} KCl, 5 mmol.l^{-1} Na_2HPO_4, 1.5 mmol.l^{-1} KH_2PO_4, pH 7.4). In experiments with a decreased sodium content Hanks' buffer was diluted 2.7-fold with 0.28 mol.l^{-1} mannitol + 5.4 mmol.l^{-1} KCl. The medium obtained in this way contained (in mmol.l^{-1}): 55 NaCl, 1.5 KCl, and 177 of mannitol. In all experiments cell viability as estimated by tryphan blue exclusion exceeded 95%.

Fluorescence. Lymphocytes were loaded with CTC for not less than an hour by one of the following procedures: (a) cells incubated in 2 ml of Hanks'solution with 1×10^{-4}mol.l^{-1} CTC were centrifuged and subsequently resuspended in 2 ml of the same medium without CTC; (b) cells were equilibrated with 1×10^{-4} mol.l^{-1} CTC in 0.2 ml of Hanks'solution and then diluted to 2 ml with CTC-free buffer; (c) cells incubated with 2×10^{-5} mol.l^{-1} CTC in 2 ml of Hanks' buffer were directly assayed in the fluorometer. In all the three cases the final lymphocyte concentration was 4 to 7×10^6 cells per ml.

Corrected excitation spectra (emission wavelength 529 nm) and difference spectra were measured on a SLM 4800 (Urbana, Ill) spectrofluorometer with a thermostated cuvette holder. The time course of CTC fluorescence was monitored in a special unit with a built-in fluorometer described previously (Kholmukhamedov et al. 1980). The cell suspension in the thermostated cuvette was continuously magnetically stirred. The cuvette temperature was maintained at 37°C. The excitation and emission wavelengths were 405 and 520 nm, respectively.

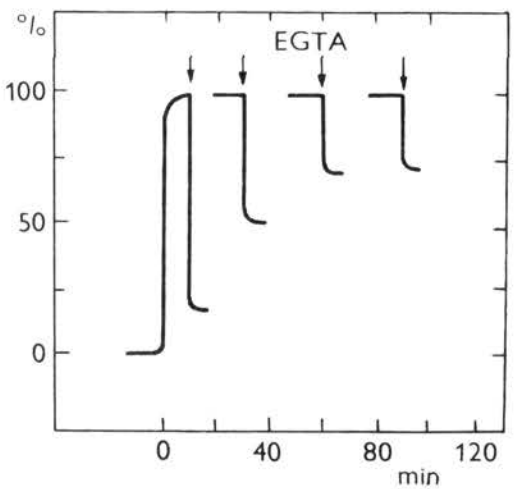

Fig. 1. Changes in CTC fluorescence upon the addition of 2 mmol.l^{-1} EGTA to human peripheral blood lymphocytes equilibrated with CTC for different periods of time (Hanks' solution, pH 7.4). The fluorescence intensity at the moment of EGTA addition was taken for 100%. Lymphocytes were loaded with CTC by the procedure (b) (see Methods). The cell suspension in a thermostated (37°C) cuvette was continuously magnetically stirred. Excitation and emission wavelengths were 405 and 520 nm, respectively. The lymphocyte concentration was 4 to 6×10^6 cells per ml. Arrows indicate the moments of the EGTA addition.

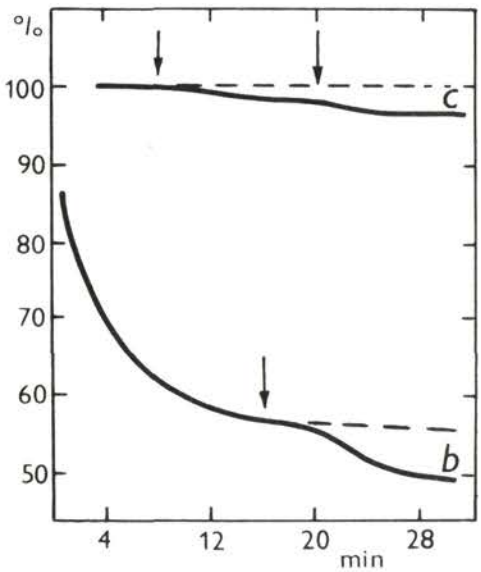

Fig. 2. Changes in CTC fluorescence in lymphocytes equilibrated with CTC by procedures (b) or (c) (see Methods) upon the addition of 20 μg/ml Con A (arrows). Experimental conditions as described in Fig. 1.

Results

To estimate the time required for CTC penetration into lymphocytes and its binding to intracellular membrane-bound cations, experiments were carried out with EGTA addition to lymphocytes pre-equilibrated with CTC for different periods ranging from 5 to 90 min (Fig. 1). With short periods of incubation with CTC the addition of EGTA reduced CTC fluorescence about to zero. Obviously, under these conditions CTC complexes with divalent cations are mainly localized on the external surface of the plasma membrane. The same was observed by Sergeeva et al. (1980), Sitkovsky et al. (1981) and Shestakova et al. (1982). However the addition of EGTA to cells preincubated for one hour with 10^{-4} mol.l^{-1} CTC reduced the fluorescence by 30—40% only (Fig. 1). In this case the major portion of the fluorescence came from CTC complexes with divalent cations bound in membrane structures within the cell, and inaccesible to EGTA.

In our experiments lymphocytes equilibrated with CTC for a period of one hour were either washed and resuspended in CTC-free buffer (method (a)) or diluted into CTC-free medium (b), or the mitogen was added directly to the cell suspension containing CTC in the medium (c) (see Methods). Using the procedures (a) and (b), dilution of CTC-equilibrated cells with CTC-free buffer led at first to a sharp decrease in the fluorescence due to CTC redistribution between the cells

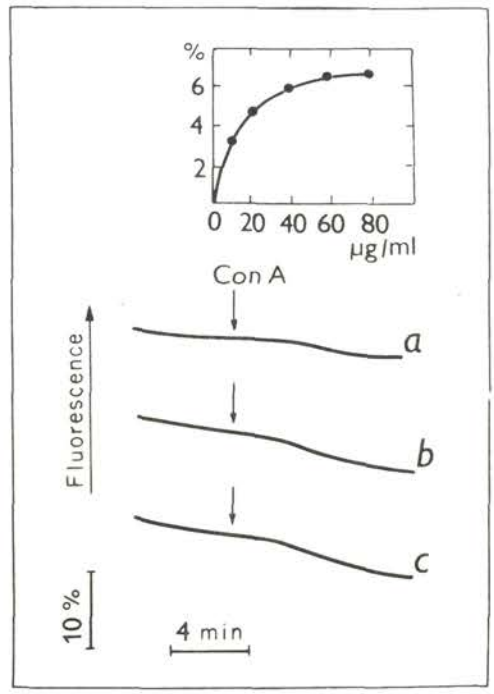

Fig. 3. Time course of CTC fluorescence after the addition of Con A (*a*) 10 μg/ml; (*b*) 20 μg/ml; (*c*) 80 μg/ml. Inset: the relationship between the Con A concentration and the absolute change in fluorescence after a 10 min incubation of cells with Con A. Experimental conditions as described in Fig. 1.

and the medium. In several minutes this rapid non-linear decrease changed to a slow linear one (Fig. 2*b*). With the procedure (c) the fluorescence did not change after an hour of incubation with CTC (Fig. 2*c*), and upon the addition of Con A a decrease in fluorescence was also observed. It is important that the effect of the mitogen was essentially independent of the equilibration procedure: with any of the above mentioned methods, provided a sufficiently long CTC-incubation, the addition of Con A produced an identical effect, i. e. a decrease in CTC fluorescence (Fig. 2). All the data presented below were obtained by the more convenient procedure (b). Double centrifugation used in method (a) diminished the cell viability; as for the method (c) the changes in fluorescence upon addition of Con A were much weaker because of CTC present in the medium.

The effect induced by Con A, though small, was observed in all the 18 experiments with HBL obtained from 8 donors. The absolute change in fluorescence intensity as compared with control (ΔF) depended on Con A concentration. Fig. 3 shows the ΔF dependence on the mitogen concentration (10 min after Con

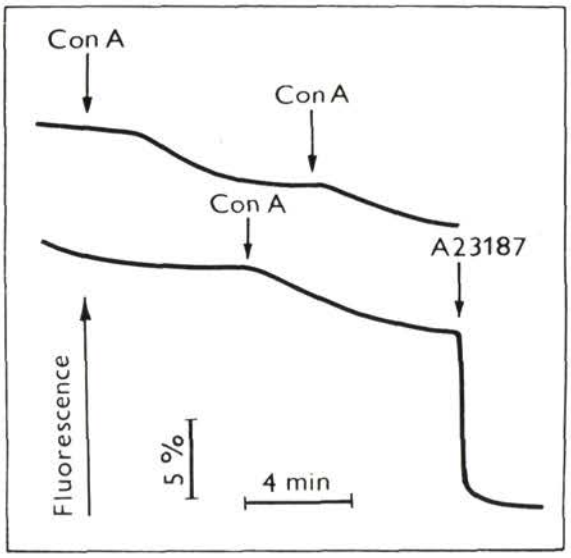

Fig. 4. Changes in CTC fluorescence in human peripheral blood lymphocytes upon the addition of Con A (20 μg/ml) and Ca^{2+}-ionophore A23187 (2×10^{-6} mol.l^{-1}). Experimental conditions as described in Fig. 1.

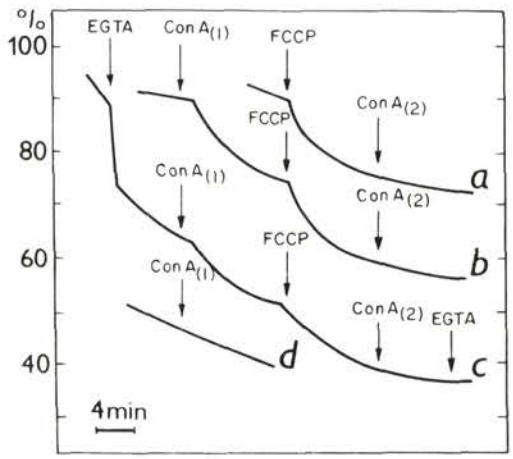

Fig. 5. Changes in CTC fluorescence upon the addition of Con A and metabolic inhibitors to lymphocyte suspensions with different Ca^{2+} concentration in the incubation medium. Arrows: Con $A_{(1)}$ — 20 μg/ml; Con $A_{(2)}$ — 80 μg/ml; FCCP-1 μmol.l^{-1} EGTA — 1 mmol.l^{-1}.(*a*) Hanks' solution, 1.3 mmol.l^{-1} Ca^{2+}; (*b*) and (*c*) phosphate-buffered saline, 0.13 mmol.l^{-1} Ca^{2+}; (*d*) Hanks' solution. 1.3 mmol.l^{-1} Ca^{2+}; cells were pre-incubated with 1 μmol.l^{-1} of antimycin for 15 min. Other conditions as described in Fig. 1.

A addition) obtained with HBL from the same donor. The relationship is described by a sigmoidal curve with the plateau beginning from Con A concentrations of 40 μg/ml. It should be noted that for a given concentration of Con A the ΔF value varied with HBL from different donors. Thus, the ΔF value for a 10-min incubation with 20 μg/ml of Con A varied in five experiments from 3.5 to 5%; in one case this value reached 10%.

The decrease in CTC fluorescence under the action of the mitogen was observed 1 to 3 min following the Con A addition, the lag period being usually shorter for higher concentrations of Con A. In some cases with lower mitogen concentrations (10—20 μg/ml) the lag reached 6 min. The decrease in fluorescence was rather sharp for the first 7 to 10 min with the subsequent recovery of the low linear rate of fluorescence decrease observed before Con A addition. Con A-induced changes in CTC fluorescence were observed for both mitogenic and higher lectin concentrations. The dependence of Con A mitogenic activity on its concentration is known to be represented by a bell-shaped curve with a maximum at the optimum concentrations. In contrast, ΔF dependence on mitogen concentration is described by a curve with saturation (Fig. 3).

The decrease in fluorescence was also seen following the addition of several doses of Con A or if Ca^{2+}-ionophore A23187 was used instead of Con A (Fig. 4).

The decrease in CTC fluorescence upon addition of Con A indicates a release of divalent cations from the intracellular membrane compartments. Fig. 5 shows the dependence of the observed effect on the level of extracellular Ca^{2+} and on the addition of the Ca^{2+} chelator, EGTA. The addition of 1 mmol.l^{-1} EGTA resulted in a sharp decrease ($\sim$30%) in CTC fluorescence which gradually changed to a slower linear one. This concentration of EGTA was sufficient to chelate all Ca^{2+} in the medium (0.13 mmol.l^{-1}) since the addition of another mmol.l^{-1} EGTA at the end of the experiment had no effect on the fluorescence. Fluorescence response to Con A was observed irrespective of the level of extracellular Ca^{2+}: in the medium containing 1.3 mmol.l^{-1} (Fig. 2) or 0.13 mmol.l^{-1} Ca^{2+} (Fig. 5,6), and even in the presence of EGTA (Fig. 5*c*). The persistence of the Con A-induced effect in the presence of EGTA provides an evidence for the release of divalent cations from intracellular stores rather than from the external surface of the plasma membrane.

The fluorescence changes may have resulted from a redistribution of CTC complexes with both Ca^{2+} and Mg^{2+}. It may be discriminated between these two possibilities when the spectral parameters of CTC complexes with Ca^{2+} and Mg^{2+} are compared to each other. In organic solvents or in membrane prepararions Ca^{2+}. CTC excitation spectra are usually red-shifted as related to those of the Mg^{2+}. CTC complex (Caswell 1972). As shown in Fig. 6, the same is true for CTC complexes with Ca^{2+} and Mg^{2+} in HBL suspension. The excitation spectra were read 3 to 5 min after the addition of CTC to the cells. As noted above (Fig. 1), under these conditions CTC binds to the plasma membrane and does not penetrate

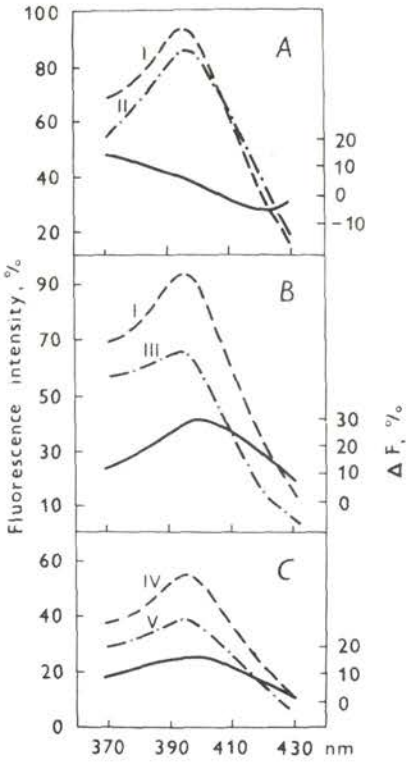

Fig. 6. Excitation CTC spectra (emission wavelength 529 nm) and difference spectra for human peripheral blood lymphocytes (phosphate-buffered saline, pH 7.4).
(*A*) I — 1 mmol.l^{-1} Ca^{2+} + 1 mmol.l^{-1} Mg^{2+}; II — 1 mmol.l^{-1} Ca^{2+}; full line: the difference between spectra I and II. (*B*) I — 1 mmol.l^{-1} Ca^{2+} + 1 mmol.l^{-1} Mg^{2+}; III — 1 mmol.l^{-1} Mg^{2+} + 2 mmol.l^{-1} EGTA; full line: the difference between spectra I and III. (*C*) IV — 0.1 mmol.l^{-1} Ca^{2+}; V — 10 min after the addition of 40 μg/ml Con A; full line: the difference between spectra IV and V. For experimental conditions see text.

into the cell. The CTC excitation maximum for HBL suspension with 1mmol.l^{-1} Ca^{2+} + 1 mmol.l^{-1} Mg^{2+} is at 395 nm (spectrum I), that with 1mmol.l^{-1} Ca^{2+} alone at 396.5 nm (II), and with 1 mmol.l^{-1} Mg^{2+} + 2 mmol.l^{-1} EGTA at 392 nm (III). The distinction between Ca^{2+}. CTC and Mg^{2+}. CTC complexes is particularly clear from the difference excitation spectra. The difference spectrum corresponding to Ca^{2+} removal from the medium, i. e. the difference between the spectrum obtained in the presence of both cations and that in the presence of Mg^{2+} alone (spectrum I less spectrum III) exhibits a maximum at 399 nm (Fig. 6*B*). The maximum of the difference spectrum corresponding to the removal of Mg^{2+} (I less II) is shifted to

substantially shorter wavelengths (~363 nm); in the range 370 to 430 nm (Fig. 6*A*) this spectrum shows a slope.

In HBL suspension equilibrated with CTC for one hour the excitation spectrum was characterized by a maximum at 396 nm; the addition of Con A induced a 3 nm blue shift of the maximum. The spectrum obtained as the difference between CTC excitation spectra before and after Con A addition (Fig. 6*C*) had a maximum at 399 nm and entirely resembled the spectrum resulting from Ca^{2+} release from the membrane (Fig. 6*B*). Thus it is intracelluar Ca^{2+} redistribution that is reflected in the fluorescence changes induced by Con A.

The principal stores of intracellular Ca^{2+} are the mitochondria and the endoplasmic reticulum (Bygrave 1978). It has also been shown that within intact cells CTC accumulates mainly in the mitochondria (Dubuy and Showarge 1961). To reveal the origin of Ca^{2+} release in HBL the effect of respiration inhibitors on Con A-induced changes in CTC fluorescence was examined. Fig. 5 shows that the addition of 1×10^{-6} mol.l^{-1} FCCP an uncoupler of oxidative phosphorylation to CTC-equilibrated lymphocytes, led to a decrease in CTC fluorescence due to the release of the mitochondrial bound Ca^{2+}. With FCCP in the medium, Con A even at high concentrations had no effect on the fluorescence, both in the presence and absence of extracellular Ca^{2+} (Fig. 5). On the other hand, incubation with Con A did not prevent FCCP-induced CTC fluorescence changes. A similar pattern was observed with other inhibitors of respiration. After a 15-min incubation with antimycin (1 μmol.l^{-1}) or with sodium cyanide (2 mmol.l^{-1}) there was no change in CTC fluorescence upon the addition of Con A. The mitochondria may therefore be suggested to be the major source of Con A-induced Ca^{2+} release.

It has been shown recently that a sufficiently high Na^+ concentration in the medium is necessary for the initiation of mitogenic lymphocyte stimulation (Deutch et al. 1981). We have examined whether the fluorescence response to Con A would persist upon a reduction in Na^+ concentration in the medium. A decrease in the extracellular Na^+ to the level supressing lymphocyte activation (60 mmol.l^{-1}, Deutch et al. 1981) had no effect on the value of the Con A-induced fluorescence decrease.

Discussion

Our data indicate that Con A induces Ca^{2+} release from intracellular membrane compartments. The experiments with EGTA (Fig. 5) demonstrated Ca^{2+} release from the intracellular stores and not from the external surface of the plasma membrane. It is known that lymphocyte activation is possible in a Ca^{2+}-free medium (Diamanshtein and Ulmer 1975; Dubois and Crompton 1980; Kay and Mattson 1979; Miller and Moticka 1977). Our data show that, even in a Ca^{2+}-free medium, interaction of a mitogen with plasma membrane receptors may induce an increase in cytosolic Ca^{2+} via a release from the intracellular stores.

Similar results were obtained on Con A-induced redistribution of intracellular Ca^{2+} in rabbit thymocytes (Mikkelsen and Schmidt–Ulrich 1980). In this work, similarly as in our experiments, the cells were equilibrated with CTC for a long periods of time and the addition of Con A to thymocyte suspension led to a decrease in CTC fluorescence. The CTC probe was also used in studies dealing with changes in Ca^{2+} binding to the external surface of the plasma membrane upon mitogenic stimulation of HBL and rat mesenteric lymph node cells (Sitkovsky et al. 1981; Shestakova et al. 1982). In these works low CTC concentrations were used and CTC was not removed from lymphocytes in the medium. Under such conditions the addition of EGTA led to an almost complete disappearance of CTC fluorescence. Upon the addition of Con A a small increase in CTC fluorescence was observed indicating an increase in Ca^{2+} binding to the external surface of the plasma membrane. In contrast to Shestakova et al. (1982) the results obtained by Mikkelsen and Schmidt–Ulrich (1980) and the present work provided evidence for the redistribution of intracellular Ca^{2+}.

The sources of Con A-released Ca^{2+} in lymphocytes remain unknown. The Con A-induced redistribution of intracellular Ca^{2+} is not observed in the presence of the metabolic inhibitors FCCP and antimycin (Fig. 5). This strongly suggests the mitochondria as the major source of the Ca^{2+} release. These data can however be also explained by the release of non-mitochondrial Ca^{2+}, sequestered by ATP-dependent processes, upon ATP depletion under the action of mitochondrial poisons. It is worth mentioning that low concentrations of FCCP (10^{-8} to 10^{-6} $mol.l^{-1}$) enhance the mitogenic activity of lectins (Daniele and Holian 1976). In view of our observations this effect may be attributed to FCCP ability to induce Ca^{2+} release into the cytosol.

Experiments with Ca^{2+}-ionophore A23187 (Fig. 4) serve to confirm the Ca^{2+} release from the mitochondria. As with Con A, the addition of A23187 to CTC-equilibrated lymphocytes results in a decrease in CTC fluorescence. A23187 is known to induce both Ca^{2+} influx from the medium and release of the mitochondria-bound Ca^{2+}, the latter being accompanied by a decrease in CTC fluorescence (Balbock et al. 1976). Evidently, the decrease in fluorescence observed in our experiments upon the addition of A23187 also points to Ca^{2+} release from the mitochondria.

The effect induced by Con A in HBL was about 10%. However it should be noted that the Ca^{2+} concentration in mitochondria ($\sim 10^{-4} mol.l^{-1}$) is much higher than that in cytosol ($\sim 10^{-7}$ $mol.l^{-1}$) (Bygrave 1978; Tsien 1981); the release of even a minor part of the mitochondria-bound Ca^{2+} can thus significantly increase the cytosolic Ca^{2+}. In addition, the contribution of CTC complexes with intracellular Mg^{2+} and extracellular Ca^{2+} and Mg^{2+} into overall CTC fluorescence also reduces the relative magnitude of the fluorescence response to Con A.

It is not clear which of the biochemical events in lymphocyte activation triggers

Ca^{2+} release from the mitochondria. It has been shown for a number of different cells that Ca^{2+} release from isolated mitochondria may be induced by increasing Na^+ concentration in the incubation medium (Crompton et al. 1976). On the other hand, the increase in intracellular Na^+ is one of the early events in activation (Deutch et al. 1981). It seemed probable that the release of mitochondria-bound Ca^{2+} observed in our experiments could have been initiated by an increase in intracellular Na^+. However, as noted above, a 2.5-fold reduction of Na^+ concentration in the medium had no effect on the fluorescence response to Con A. After the completion of our work it has been reported that the mitochondria of pig mesenteric node lymphocytes entirely lack an Na^+, Ca^{2+} exchanger (Dippenaur and Biand 1982). The relationship between intracellular sodium and calcium is likely to be more complex than supposed.

In recent years data have appeared indicating redistribution of intracellular Ca^{2+}, i. e. Ca^{2+} release from intracellular store, in a number of activation processes initiated by the interaction of a stimulus with the cell surface receptors. These include stimulus-secretion coupling in pancreatic acinar cells (Chandler and Williams 1978a, b), chemotaxis of neutrophiles (Naccache et al. 1979), exocytosis in platelets (Feinstein 1980; LeBreton 1976). Striking similarities between the data on the regulatory role of Ca^{2+} in these quite different processes can be noted: (1) Ca^{2+}-ionophore A 23187 can serve as the agent inducing activation. (2) The initiation of the process may be either accompanied or not by an increase in Ca^{2+} influx. (3) The process can be initiated in a Ca^{2+}-free medium. (4) Cytosolic Ca^{2+} increases during the first minutes of activation due to a release from intracellular Ca^{2+} stores.

The effects (1) and (3) have been shown to take place in lymphocyte activation as well (Hume and Weidemann 1980). Our results and the data of Mikkelsen and Schmidt-Ulrich 1980 indicate that intracellular Ca^{2+} redistribution is also seen in mitogenic stimulation of lymphocytes. It is possible that the above effects characterize some general „calcium" response of a cell in transition from resting to active state. Stimulus-induced mobilization of intracellular Ca^{2+} may serve as the main or auxiliary way of increasing the cytosolic Ca^{2+} in activation processes.

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